CHAPTER 2.3.7.

**infection with RED sea bream iridovirus**

**1. Scope**

Infection with red sea bream iridovirus is considered to be infection with the pathogenic agent red sea bream iridovirus (RSIV) of the genus *Megalocytivirus*, Family *Iridoviridae*.

**2. Disease information**

**2.1. Agent factors**

**2.1.1. Aetiological agent**

The pathogen is an icosahedral virion 140−200 nm in diameter consisting of a central electron-dense core (120 nm) and an electron translucent zone (Inouye *et al.,* 1992) with a double-stranded DNA genome approx. 110 kbp in length (Kawato *et al.,* 2017a). The viral genome has a G+V content of 53–55%, containing about 120 potential open reading frames (ORFs).

Phylogenetic analyses using major capsid protein (MCP) and ATPase genes shows that the viruses causing the similar clinical signs can be divided into three different genotypes: RISV, infectious spleen and kidney necrosis virus (ISKNV) (He *et al.,* 2000; 2001), and turbot reddish body iridovirus (TRBIV) genotype (Shi *et al.,* 2004; 2010).

The aetiological agent of infection with RSIV is RSIV (Inouye *et al.,* 1992; Jeong *et al.,* 2003) and other strains belonging in the RSIV genotype (Go *et al.,* 2016; Koda *et al.,* 2018; Kurita & Nakajima, 2012). Similar diseases with the characteristic, enlarged basophilic cells within infected organs, typical of infections with megagalocytiviruses, classified into ISKNV genotype and TRBIV genotypes are excluded in this chapter. RSIV genotypes are differentiated from ISKNV and TRBIV genotypes based on nucleotide sequence analysis which is required for confirmatory diagnosis. Scale drop disease virus is another virus in the genus *Megalocytivirus* causing different clinical signs in Asian seabass, *Lates calcarifer* (Groof *et al.,* 2015).

RSIV was first found in red sea bream, *Pagrus major,* from which the virus name (RSIV) is derived (Inouye *et al.,* 1992). As RSIV has a broad host range as shown in Section 2.2.1. *Susceptible host species*, many viruses that can be classified into RSIV genotype are synonyms of RSIV and defined to be the aetiological agents in this chapter, e.g. rock bream iridovirus (RBIV) (Do *et al.,* 2004; Jung & Oh 2000), Taiwan grouper iridovirus (TGIV) (Chou *et al.,* 1998), large yellow croaker iridovirus (LYCIV) (Chen *et al.,* 2003), orange-spotted grouper iridovirus (OSGIV) (Lu *et al.,* 2005), spotted knifejaw iridovirus (SKIV) (Dong *et al.,* 2010) and giant seaperch iridovirus (GSIV) (Wen & Hong, 2016).

**2.1.2. Survival and stability inside the host tissues**

Unknown

**2.1.3. Survival and stability outside the host**

Unknown

For inactivation methods, see Section 2.4.6.

**2.2. Host factors**

**2.2.1. Susceptible host species**

In the case of RSIV infection:

| **Family** | **Scientific name** | **Common name** |
| --- | --- | --- |
| Carangidae | *Pseudocaranx dentex*  | striped jack |
| *Seriola dumerili*  | greater amberjack |
| *Seriola lalandi*  | yellowtail amberjack |
| *Seriola lalandi* × *Seriola quinqueradiata*  | hybrid of yellowtail amberjack and Japanese amberjack |
| *Seriola quinqueradiata*  | Japanese amberjack |
| *Trachinotus blochii*  | snubnose pompano |
| Centrarchidae | *Micropterus salmoides*  | largemouth bass |
| Centropomidae | *Lates calcarifer*  | barramundi or sea bass |
| Haemulidae | *Parapristipoma trilineatum*  | chicken grunt |
| *Plectorhinchus cinctus*  | crescent sweetlips |
| *Trachurus japonicus*  | Japanese jack mackerel |
| Kyphosidae | *Girella punctata*  | largescale blackfish |
| Lateolabracidae | *Lateolabrax japonicas*  | Japanese sea perch |
| *Lateolabrax* sp.,  |  |
| Lethrinidae | *Lethrinus haematopterus*  | Chinese emperor |
| *Lethrinus nebulosus*  | spangled emperor |
| Moronidae | *Morone saxatilis* × *M. chrysops* | hybrid of striped sea bass and white bass |
| Oplegnathidae | *Oplegnathus fasciatus*  | Japanese parrotfish |
| Paralichthyidae | *Paralichthys olivaceus*  | bastard halibut |
| Pleuronectidae | *Verasper variegatus*  | spotted halibut |
| Rachycentridae | *Rachycentron canadum*  | cobia |
| Sciaenidae | *Pseudosciaena crocea*  | croceine croaker |
| Scombridae | *Scomber japonicus*  | chub mackerel |
| *Scomberomorus niphonius*  | Japanese Spanish mackerel |
| *Thunnus thynnus*  | northern bluefin tuna |
| Sebastidae | *Sebastes schlegeli*  | rockfish |
| Serranidae | *Epinephelus akaara*  | Hong Kong grouper |
| *Epinephelus awoara*  | yellow grouper |
| *Epinephelus bruneus*  | longtooth grouper |
| *Epinephelus coioides*  | orange-spotted grouper |
| *Epinephelus fuscoguttatus*  | brown-marbled grouper |
| *Epinephelus lanceolatus*  | giant grouper |
| *Epinephelus malabaricus*  | Malabar grouper |
| *Epinephelus septemfasciatus*  | convict grouper |
| *Epinephelus tauvina*  | greasy grouper |
| *Oplegnathus punctatus*  | spotted knifejaw |
| Sparidae | *Acanthopagrus latus*  | yellowfin sea bream |
| *Acanthopagrus schlegeli* | black porgy |
| *Evynnis japonica*  | crimson sea bream |
| *Pagrus major*  | red sea bream |
| Tetraodontidae | *Takifugu rubripes*  | torafugu |

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

Species for which there is incomplete evidence to fulfil the criteria for listing as susceptible to infection with (RSIV) according to Chapter 1.5 of the *Aquatic Code* are: Under study.

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

Juvenile through to adult stages are susceptible; however, the susceptibility of juveniles is generally higher than adults. Fish belonging to the genus *Oplegnathus* may be more susceptible than others.

**2.2.4. Distribution of the pathogen in the host**

Infected cells are observed in the spleen, kidney, heart, liver, intestine and gill.

**2.2.5. Aquatic animal reservoirs of infection**

Unknown

**2.2.6. Vectors**

Unknown

**2.3. Disease pattern**

**2.3.1. Mortality, morbidity and prevalence**

Depending on host fish species, fish size, fish age, water temperature, and other culture conditions, mortality rates range between 0% and 100%. Morbidity is unknown.

**2.3.2. Clinical signs, including behavioural changes**

Affected fish become lethargic and show abnormal and conspicuous respiratory movements.

**2.3.3. Gross pathology**

Fish exhibit severe anaemia, petechiae in the gills, and enlargement of the spleen and kidney.

**2.3.4. Modes of transmission and life cycle**

The principal mode of transmission of RSIV is horizontal via the water. Vertical transmission of RSIV has not yet been investigated.

**2.3.5. Environmental factors**

Outbreaks have been seen mostly in the summer season at water temperatures of 25°C and above.

**2.3.6. Geographical distribution**

The first outbreak was recorded in marine cultured red sea bream in Japan in 1990. From then on, further outbreaks and infections have been reported in many marine fish and freshwater fish. The international trade of ornamental fish has contributed significantly to the spread of megalocytiviruses (Johan & Zainathan, 2020).

See WAHIS (<https://wahis.oie.int/#/home>) for recent information on distribution at the country level.

**2.4. Biosecurity and disease control strategies**

**2.4.1. Vaccination**

Effectiveness of a vaccine consisting of formalin-inactivated supernatant from RSIV-infected GF cell culture has been confirmed experimentally and in field trials (Nakajima *et al.,* 1997; 1999). Currently, the formalin-inactivated vaccine for infection with RSIV is commercially available for red sea bream (*Pagrus major*), striped jack (*Pseudocaranx dentex*), Malabar grouper (*Epinephelus malabaricus*), orange-spotted grouper (*Epinephelus coioides*) and other fish species belonging to the genus *Seriola* in Japan. Protection of fish belonging to the genus *Oplegnathus* by vaccination is difficult.

**2.4.2. Chemotherapy including blocking agents**

Not available.

**2.4.3. Immunostimulation**

Not applicable.

**2.4.4. Breeding resistant strains**

An RSIV-resistant strain of red sea bream (*Pagrus major*) has been developed using marker-assisted selection combined with DNA-based family selection (Sawayama *et al.*, 2019).

**2.4.5. Inactivation methods**

RSIV is inactivated at 56°C for 30 minutes and by treatment with either ether, chloroform or formalin (0.1%), and by exposure to pH 3.0. The virus is stable in tissue at –80°C and at pH 7.0 and pH 11.0 (Nakajima & Sorimachi, 1994).

**2.4.6. Disinfection of eggs and larvae**

Unknown

**2.4.7. General husbandry**

Not available.

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

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

**3.1. Selection of populations and individual specimens**

Clinical inspections should be carried out during a period when water temperature is conducive to development of clinical disease (see Section 2.3.5). All production units (ponds, tanks, etc.) should be inspected for the presence of dead, weak or abnormally behaving fish. For the purposes of disease surveillance, fish to be sampled are selected as follows:

i) The most susceptible species should be sampled preferentially (see Section 2.2.3). Other susceptible species listed in Section 2.2.1 should be sampled proportionally.

ii) Risk-based criteria should be employed to preferentially sample lots or populations with a history of abnormal mortality, potential exposure events or where there is evidence of poor water quality or husbandry. If more than one water source is used for fish production, fish from all water sources should be included in the sample.

iii) If weak, abnormally behaving or freshly dead fish are present, such fish should be selected. If such fish are not present (e.g. during surveillance of apparently healthy populations), the fish selected should include normal appearing, healthy fish collected in such a way that all parts of the farm as well as all year classes are proportionally represented in the sample. Smaller fish may be more appropriate because infection with RSIV can cause higher mortality in juvenile or yearling fish. However, adult fish are also susceptible to RSIV infection as the viral genome has been detected from apparently healthy broodstock. Infection with RSIV has not been reported in hatchery fish.

For disease outbreak investigations, moribund fish or fish exhibiting clinical signs of infection with RSIV should be collected. Ideally fish should be collected while alive, however recently dead fish can also be selected for diagnostic testing. It should be noted however, that there will be a significant risk of contamination with environmental bacteria if the animals have been dead for some time.

**3.2. Selection of organs or tissues**

Although gill and visceral organs such as spleen, heart, kidney, liver and intestine can be used, it is recommended to sample spleen or kidney tissues; spleen is the most appropriate organ for the preparation of imprints for use in the IFAT. For surveillance of apparently healthy populations, spleen or kidney should be sampled.

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

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

Use of inappropriate fixatives (where required), poor sample quality, inappropriate tissues and lack of information provided with the submission may render samples unsuitable for testing.

**3.4. Non-lethal sampling**

Not available.

**3.5. Preservation of samples for submission**

Store fish at 4°C for use within 24 hours (or at –80°C for longer periods [up to a few years]).

For guidance on sample preservation methods for the intended test methods, see Chapter 2.3.0.

**3.5.1. Samples for pathogen isolation**

The success of pathogen isolation depends strongly on the quality of samples (which will be affected by time since collection and time in storage). Fresh specimens should be kept on ice and preferably sent to the laboratory within 24 hours of collection. To avoid degradation of samples, use alternate storage methods only after consultation with the receiving laboratory.

**3.5.2. Preservation of samples for molecular detection**

Tissue samples for PCR testing should be preserved in 70–90% (v/v) analytical/reagent-grade (undenatured) ethanol. The recommended ratio of ethanol to tissue is 10:1 based on studies in terrestrial animal and human health. The use of lower grade (laboratory or industrial grade) ethanol is not recommended. If material cannot be fixed it may be frozen.

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

Tissue samples for histopathology should be fixed immediately after collection. The recommended ratio of fixative to tissue is 10:1.

**3.5.4. Samples for other tests**

Not applicable.

**3.6. Pooling of samples**

Pooling of samples from more than one individual animal for a given purpose should only be recommended where robust supporting data on diagnostic sensitivity and diagnostic specificity have been evaluated and found to be suitable. If the effect of pooling on diagnostic sensitivity has not been thoroughly evaluated, larger fish should be processed and tested individually. Small life stages such as fry or specimens can be pooled to provide the minimum amount of material needed for testing. If pooling is used, it is recommended to pool organ pieces from a maximum of five fish.

**4. Diagnostic methods**

The methods currently available for identifying infection that can be used in i) surveillance of apparently healthy populations), ii) presumptive and iii) confirmatory diagnostic purposes are listed in Table 4.1. by life stage. The designations used in the Table indicate:

Key:

+++ = Recommended method(s) validated for the purpose shown and usually to stage 3 of the OIE Validation Pathway;

++ = Suitable method(s) but may need further validation;

+ = May be used in some situations, but cost, reliability, lack of validation or other factors severely limits its application;

Shaded boxes = Not appropriate for this purpose.

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

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

| **Method** | 1. **Surveillance of apparently healthy animals**
 | 1. **Presumptive diagnosis of clinically affected animals**
 | 1. **Confirmatory diagnosis1 of a suspect result from surveillance or presumptive diagnosis**
 |
| --- | --- | --- | --- |
| **Early life stages2** | **Juveniles2** | **Adults** | **LV** | **Early life stages2** | **Juveniles2** | **Adults** | **LV** | **Early life stages2** | **Juveniles2** | **Adults** | **LV** |
| **Tissue imprints** | + | + | + | 1 | + | + | + | 1 |  |  |  |  |
| **Histopathology3** | + | + | + | 1 | ++ | ++ | ++ | 1 |  |  |  |  |
| **IFAT or ICC** | + | + | + | 1 | ++ | ++ | ++ | 1 |  |  |  |  |
| **Cytopathology3** |  |  |  |  |  |  |  |  |  |  |  |  |
| **Cell culture** | + | + | + | 1 | ++ | ++ | ++ | 1 |  |  |  |  |
| **Real-time PCR** | ++ | ++ | ++ | 2 | ++ | ++ | ++ | 2 |  |  |  |  |
| **Conventional PCR** | ++ | ++ | ++ | 2 | ++ | ++ | ++ | 2 | +++ | +++ | +++ |  |
| **Amplicon sequencing4** |  |  |  |  |  |  |  |  | +++ | +++ | +++ |  |
| ***In-situ* hybridisation** |  |  |  |  |  |  |  |  |  |  |  |  |
| **Bioassay** |  |  |  |  |  |  |  |  |  |  |  |  |
| **LAMP** |  |  |  |  |  |  |  |  |  |  |  |  |
| **Ab-ELISA** |  |  |  |  |  |  |  |  |  |  |  |  |
| **Ag-ELISA** |  |  |  |  |  |  |  |  |  |  |  |  |
| **Other antigen detection methods5** |  |  |  |  |  |  |  |  |  |  |  |  |
| **Other serological method5** |  |  |  |  |  |  |  |  |  |  |  |  |

LV = level of validation, refers to the stage of validation in the OIE Pathway (chapter 1.1.2); IFAT = Indirect fluorescent antibody test. ICC = Immunocytochemistry PCR = polymerase chain reaction; LAMP = loop-mediated isothermal amplification; Ab- or Ag-ELISA = antibody or antigen enzyme-linked immunosorbent assay, respectively;
1For confirmatory diagnoses, methods need to be carried out in combination (see Section 6). 2Susceptibility of early and juvenile life stages is described in Section 2.2.3.
3Histopathology and cytopathology can be validated if the results from different operators have been statistically compared. 4Sequencing of the PCR product.
5Specify the test used. Shading indicates the test is inappropriate or should not be used for this purpose.

**4.1. Tissue imprints**

Samples to be tested include tissue imprints of spleen from affected fish. Use a tissue imprint of spleen from known uninfected fish as a negative control and if possible, use a tissue imprint of spleen from confirmed RSIV-infected fish as a positive control.

i) Bleed the fish thoroughly.

ii) Make spleen imprints on cleaned glass microscope slides.

iii) Store the spleen pieces at 4°C together with the other organs that may be required for virus isolation or PCR tests later.

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

v) Fix the imprints with cold acetone.

vi) Stain with Giemsa or Diff-Quik.

vii) Mount the microscope slides with cover-slips using a drop of mounting fluid.

viii) Examine under light microscopy using ×40–100 magnification.

A presumptive positive result is indicated by the presence of abnormally enlarged cells. Negative control slides should not exhibit any abnormally enlarged cells. If enlarged cells are observed in the test samples, identification procedures PCR followed by amplicon sequencing must be undertaken immediately.

**4.2. Histopathology and cytopathology**

Examination of histological sections from diseased fish may reveal abnormally enlarged cells from the spleen, heart, kidney, liver, intestine or gill. These enlarged cells react to anti-RSIV MAb M10 (4.9.1.) using an immunohistochemistry test (Bermudez *et al.,* 2018). However, this method is not yet fully validated.

**4.3. Cell culture for isolation**

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

Isolation of RSIV (and ISKNV) is undertaken using the Grunt fin (GF) cell line[[1]](#footnote-1) or SKF-9 cell line (Kawato *et al.,* 2017b); isolation of the viruses from freshwater fish such as gourami is difficult. Spleen and/or kidney tissues from diseased fish are suitable samples. Cells should be grown in Eagle’s basal medium (BME) for GF cell line and Hank’s minimum essential medium (HMEM) for SKF-9 cell line, supplemented with 10% fetal bovine serum (FBS) at 25°C in a temperature-controlled incubator. A virus isolate to be used as positive control can be obtained from the OIE Reference Laboratory for RSIV. Use uninfected cells as negative control. Following development of viral cytopathic effect (CPE), virus identification would be undertaken using conventional PCR and sequencing. SKF-9 cell line can be obtained from the OIE Reference Laboratory for RSIV.

**4.3.1. Virus isolation in cell cultures**

**4.3.1.1. Inoculation of cell monolayers**

i) Cell cultures (GF or SKF-9) maintained at 25°C and passaged 7-14 day intervals should be used for virus isolation to ensure virus susceptibility. Prepare cell monolayers in 25 cm2 flask, 6-well, 24-well, or 96-well plates according to the purpose and sample size on the day before sample inoculation.

ii) Following the virus isolation procedure described in Chapter 2.3.0 *General information* (on diseases of fish), Section A.2.3.2, make an additional tenfold dilution of the 1/10 spleen homogenate supernatants and transfer an appropriate volume of each of the two dilutions onto the cell monolayers. To avoid cytotoxic effect (CTE), final concentration of the organ in the cultured medium should be less than 1% w/v.

iii) Without withdrawing the inoculum, incubate at 25°C.

**4.3.1.2. Monitoring incubation**

i) Follow the course of infection in positive controls and other inoculated cell cultures by daily microscopic examination at ×40–100 magnification for 10 days. The use of a phase-contrast microscope is recommended.

ii) If CPE appears in those cell cultures inoculated with dilutions of the test homogenates, identification procedures by PCR followed by amplicon sequence analysis must be undertaken immediately.

iii) If no CPE develops in the inoculated cultures (despite normal progression of CPE in the positive controls cultures) after 10 days incubation, the inoculated cultures should be subcultured and incubated 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.

**4.3.1.3. Subcultivation procedure**

i) Collect aliquots of cell culture medium from all monolayers inoculated with dilutions of each supernatant of the test homogenates.

ii) Inoculate cell monolayers as described above (Section 4.3.1.1 Inoculation of cell monolayers, steps i and ii).

iii) Incubate and monitor as described above (Section 4.3.1.1 Inoculation of cell monolayers, steps ii and iii and Section 4.3.1.2 Monitoring incubation steps i and ii).

If no CPE occurs, the test may be declared negative.

**4.4. Nucleic acid amplification**

See Chapter 2.3.0 *General information* (on diseases of fish), Section B.2.5 for information on the use of molecular techniques for virus identification. Both real-time PCR and conventional PCR tests are available for RSIV identification. Samples to be tested include spleen from affected fish or supernatants from cell cultures that have developed CPE. The following controls should be run with each assay: negative extraction control; positive control; no template control; internal PCR control. Use extracted DNA from the spleen and kidney of uninfected fish or extracted DNA from the supernatant of an uninfected cell culture as the negative control. Use extracted DNA from the spleen of confirmed RSIV-infected fish or extracted DNA from the supernatant of an infected cell culture or Viral DNA or plasmid in which target sequence is inserted as the positive control. Select controls depending on the kinds of samples to be tested.

Tissue samples can be homogenised by manual pestle grinding or by bead-beating. Commercially available nucleic acid extraction kits may be used to extract DNA directly from tissues, from tissue homogenates and cell culture supernatants according to the manufacturer’s instructions. A negative extraction control, consisting of extraction reagents only, is included when test samples are extracted. Use a pre-confirmed RSIV-affected organ or supernatant from RSIV-infected cell cultures as positive controls. Use organs from healthy fish or supernatants from non-infected cell cultures as negative controls

N.B. Viral DNA or a plasmid in which the PCR target sequence is inserted that can be used as the positive control can be obtained from the OIE Reference Laboratory for RSIV.

**4.4.1. Real-time PCR**

Several real-time PCR assays available for detection of RSIV have been evaluated (Kawato *et al*., 2021). Two probe-based real-time PCR assays, designated as the Mohr et al. assay (Mohr *et al*., 2015) and the Cummins assay, were deemed equivalent to each other and superior to the other tests evaluated in this study. The primer sets and probes of each of these two assays are designed to detect a major capsid protein (MCP) gene sequence and are as follows:

i) Mohr real-time PCR

RSIV RT F: 5’-TGA-CCA-GCG-AGT-TCC-TTG-ACT-T-3’

RSIV RT R: 5’-CAT-AGT-CTG-ACC-GTT-GGT-GAT-ACC-3’

RSIV Probe: 5’-FAM-AAC-GCC-TGC-ATG-ATG-CCT-GGC-TAMRA-3’

ii) Cummins real-time PCR

AFDL Megalo F: 5’-GGC-GAC-TAC-CTC-ATT-AAT-GTG-3’

AFDL Megalo R: 5’-CAC-CAG-GTC-GTT-AAA-TGA-CA-3’

AFDL Megalo Pr: 5’-FAM-CTG-CGT-GTT-AAG-ATC-CCC-TCC-A-TAMRA-3’

The protocol in use at the OIE Reference Laboratory for RSIV is as follows: Template (2 μl) is added to 23 μl reaction mixture containing 12.5 μl TaqMan Universal PCR Master Mix (Applied Biosystems), 900 nM for each primer, 250 nM for probe, and molecular grade water. After 1 cycle of 50°C for 2 minutes and 95°C for 10 minutes, amplification consists of 45 cycles of 95°C for 15 seconds, 60°C for 60 seconds.

The detection sensitivity limits of both real-time PCRs are approximately 1-10 copies/μl template DNA which is higher than that of conventional PCR. However, since the real-time PCRs have cross-reactivity to the ISKNV and TRBIV genotypes, conventional PCR followed by amplicon sequence analysis (see Section 4.5.) is required for confirmatory diagnosis.

**4.4.2. Conventional PCR**

The conventional PCR primer set consisting of the forward primer 1-F (5’-CTC-AAA-CAC-TCT-GGC-TCA-TC-3’) and reverse primer 1-R (5’-GCA-CCA-ACA-CAT-CTC-CTA-TC-3’) is used for amplification of the genome sequence across two ORFs (Kurita *et al.,* 1998).

The protocol in use at the OIE Reference Laboratory for RSIV, based on Kurita *et al.*, (1998), is as follows: Template (1 μl) is added to 19 μl reaction mixture containing 2 μl 10× reaction buffer, 1.6 μl dNTP mixture (2.5 mM each), 0.2 μl *TaKaRa ExTaq* HS (5 U/μl) (TaKaRa), 1 μM each primer, and molecular grade water. After 1 cycle of 94°C for 2 minutes, PCR amplification consists of 40 cycles of 94°C for 30 seconds, 60°C for 30 seconds, and 72°C for 30 seconds, followed by final extension at 72°C for 2 minutes. Amplified DNA (567 or 570 bp) is analysed by agarose gel electrophoresis using a 1.5% agarose/TAE (Tris-acetate-EDTA) gel containing SYBRTM Safe (Thermo Fisher Scientific) or equivalent.

The detection sensitivity limit of the PCR is approximately 10-100 copies/μl template DNA. However, the primer set 1-F and 1-R is confirmed to amplify both RSIV and ISKNV DNA, and hence, amplicon sequencing is required for confirmatory diagnosis. The cross reactivity of these primer sets against TRBIV has not yet been validated.

**4.4.3. Other nucleic acid amplification methods**

Not applicable

**4.5. Amplicon sequencing of the amplicon**

The primer set 1-F and 1-R can amplify both RSIV and ISKNV DNA and sequencing of the amplicon is required for virus identification. Amplicons should be gel-purified and sequenced using both the forward and reverse primer. Consensus sequence, generated after analysis of the quality of the sequence chromatograms, can then be compared to reference sequences, for example by BlastN search of the NCBI database.

**4.6. *In-situ* hybridisation**

Not applicable

**4.7. Histoimmunochemistry**

Not applicable

**4.8. Bioassay**

Not applicable

**4.9. Antibody-based or antigen detection methods**

**4.9.1. Antibody-based antigen detection methods: indirect fluorescent antibody test (IFAT) or immunocytochemistry (ICC)**

Reagent and protocols for detecting RSIV proteins with a monoclonal antibody (MAb) M10 have been published (Kawato *et al.*, 2017b; 2020; Nakajima & Sorimachi, 1995). The MAb M10-reactive epitope has been demonstrated to be a 7 amino acid sequence (EYDCPEY) of a non-structural protein encoded by the laminin-type epidermal growth factor-like domain gene in RSIV and ISKNV (Takano *et al.,* 2019). MAb M10 detects both RSIV- and ISKNV-infected cells (Kawato *et al.*, 2020) but it does not detect ranaviruses (Nakajima & Sorimachi, 1995). The reactivity of MAb against TRBIV has not yet been confirmed. MAb M10 can be obtained from the OIE Reference Laboratory for RSIV.

Samples to be tested include tissue imprints of spleen from affected fish. Use a tissue imprint of spleen from uninfected fish as a negative control and if possible, use a tissue imprint of spleen from confirmed RSIV-infected fish as a positive control. Similarly, IFAT can be conducted directly after virus isolation in cell culture. Samples to be taken for testing include acetone-fixed infected cell monolayers that have developed CPE. Use an uninfected cell monolayer as a negative control and if possible, use a confirmed RSIV-infected cell monolayer as a positive control. The protocol for tissue imprints is as follows and can be adapted for IFAT on cell cultures.

i) Bleed the fish thoroughly.

ii) Make spleen imprints on cleaned glass microscope slides.

iii) Store the spleen pieces at 4°C together with the other organs that may be required for virus isolation.

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

v) Fix the imprints with cold acetone.

vi) Prepare a diluted solution of MAb M10 in PBS (1/100)

vii) Treat the imprints with the MAb M10 solution for 30 minutes at 37°C in a humid chamber.

viii) Rinse three times with PBS.

ix) Incubate the imprints for 30 minutes at 37°C in a humid chamber with a solution of a specific anti-mouse FITC-conjugated antibody prepared according to the supplier’s instructions.

x) Rinse three times with PBS.

xi) Mount the microscope slides with cover-slips using glycerol saline prior to microscopic observation.

xii) Examine using a fluorescence microscope. Positive and negative controls must be found to give the expected results prior to any other observation.

A positive result is indicated by the presence of abnormally enlarged cells with strong fluorescence. Negative control slides should not exhibit any strong fluorescence.

If the test is positive, the fish from which the samples were obtained is considered infected with RSIV or ISKNV.

Alternatively a peroxide-conjugated second antibody could be used rather than fluorescence conjugate.

**4.10. Other methods**

RSIV cannot be identified by neutralisation tests as the antisera generated by the immunisation of rabbits have few neutralising antibodies.

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

As indicated in Table 4.1, real-time PCR is the most appropriate method of screening healthy fish populations for RSIV; however, the available methods are not specific for RSIV. Any real-time positive samples should be tested by conventional PCR followed by amplicon sequence analysis to distinguish megalocytiviruses.

**6. Corroborative diagnostic criteria**

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

The case definitions for a suspect and confirmed case have been developed to support decision making related to trade and confirmation of disease status at the country, zone or compartment level. Case definitions for disease confirmation in endemically affected areas may be less stringent. It is recommended that all samples that yield suspect positive test results in an otherwise pathogen-free country or zone or compartment should be referred immediately to the OIE Reference Laboratory for confirmation, whether or not clinical signs are associated with the case. If a laboratory does not have the capacity to undertake the necessary diagnostic tests it should seek advice from the appropriate OIE reference laboratory.

**6.1. Apparently healthy animals or animals of unknown health status[[2]](#footnote-2)**

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

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

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

i) Positive result by real-time or conventional PCR;

ii) Cyto- or histopathological changes consistent with RSIV infection or disease;

iii) Cytopathic effect in cell culture;

iv) Positive result from IFAT or ICC.

**6.1.2. Definition of confirmed case in apparently healthy animals**

The presence of infection with RSIV is considered to be confirmed if at least one of the following criteria is met:

i) Positive result by a recommended molecular or antigen detection test with confirmation by conventional PCR and sequence analysis, with sequence consistent with RSIV;

ii) Cyto- or histopathological changes consistent with the presence of the pathogen or the disease with confirmation by conventional PCR and sequence analysis, with sequence consistent with RSIV;

iii) Cytopathic effect in cell culture with confirmation by conventional PCR and sequence analysis, with sequence consistent with RSIV.

Reference Laboratories should be contacted for specimen referral when testing laboratories cannot undertake any of the recommended test methods. Positive test results will result in notification to the OIE.

**6.2 Clinically affected animals**

Clinical signs (see Section 2.3.2) are not pathognomonic for a single disease; however they may narrow the range of possible diagnoses.

**6.2.1. Definition of suspect case in clinically affected animals**

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

i) Presence of gross pathology or clinical signs associated with RSIV disease as described in this chapter, with or without elevated mortality;

ii) Cyto- or histopathological changes consistent with the presence of the pathogen or the disease;

iii) Positive result from IFAT or ICC;

iv) Cytopathic effect typical for RSIV infectin in cell culture;

v) Positive result by real-time PCR or conventional PCR.

**6.2.2. Definition of confirmed case in clinically affected animals**

The presence of infection with RSIV is considered to be confirmed if at least one of the following criteria is met:

i) Positive result by a recommended antigen detection test with confirmation by conventional PCR and sequence analysis, with sequence consistent with RSIV;

ii) Cyto- or histopathological changes consistent with the presence of the pathogen or the disease with confirmation by conventional PCR and sequence analysis, with sequence consistent with RSIV;

iii) Cytopathic effect in cell culture with confirmation by conventional PCR and sequence analysis, with sequence consistent with RSIV;

iv) Positive real-time PCR test with confirmation by conventional PCR and sequence analysis, with sequence consistent with RSIV.

Reference Laboratories should be contacted for specimen referral when testing laboratories cannot undertake any of the recommended test methods. Positive test results will result in notification to the OIE.

**6.3. Diagnostic sensitivity and specificity for diagnostic tests [under study]**

**6.3.1. For presumptive diagnosis of clinically affected animals**

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

DSe: = diagnostic sensitivity, DSp = diagnostic specificity, qPCR: = real-time polymerase chain reaction.

**6.3.2. For surveillance of apparently healthy animals**

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

DSe: = diagnostic sensitivity, DSp = diagnostic specificity, qPCR: = real-time polymerase chain reaction.

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**NB:** There is an OIE Reference Laboratory for red sea bream iridoviral disease
(see Table at the end of this *Aquatic Manual* or consult the OIE web site for the most up-to-date list:
<https://www.oie.int/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3> ).
Please contact the OIE Reference Laboratories for any further information on
infection red sea bream iridoviral disease.

**NB:** First adopted in 2000); Most recent updates adopted in 20XX.

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1. European Collection of Authenticated Cell Cultures (ECACC) Catalogue No. 88010601; [www.phe-culturecollections.org.uk/products/celllines/generalcell/detail.jsp?refId=88010601&collection=ecacc\_gc](http://www.phe-culturecollections.org.uk/products/celllines/generalcell/detail.jsp?refId=88010601&collection=ecacc_gc) [↑](#footnote-ref-1)
2. For example transboundary commodities. [↑](#footnote-ref-2)