

RED SEA BREAM IRIDOVIRAL DISEASE

1. Scope

For the purpose of this chapter, red sea bream iridoviral disease (RSIVD) (8) is caused by infection with red sea bream iridovirus.

RSIVD is a significant cause of mortality in farmed red sea bream (*Pagrus major*) and more than 30 other species of cultured marine fish (15, 21) belonging mainly to the orders Perciformes and Pleuronectiformes. The first outbreak of RSIVD was recorded in cultured red sea bream in Shikoku Island, Japan in 1990 (8). Since then, the disease has caused mass mortalities in cultured fish populations in the western part of Japan, mainly among juvenile red sea bream. Affected fish become lethargic, exhibit severe anaemia, petechiae of the gills, and enlargement of the spleen (8, 12, 24). The disease is characterised by the appearance of enlarged cells stained deeply with Giemsa solution in the histopathological observations of the spleen, heart, kidney, intestine and gill of infected fish (8).

Recently, it has been proved that the disease is caused not only by RSIV (8–10, 18, 20, 29) and its synonyms (2–6, 12, 13, 16, 19, 22, 40), but also by infectious spleen and kidney necrosis virus (ISKNV) (7, 31). The disease is found not only in Japan but also widely in East and South-East Asian countries (2–6, 9, 10, 12, 13, 16, 19, 22, 31, 40).

A monoclonal antibody (MAb) against RSIV (30) can detect both RSIV and ISKNV, whereas it does not recognise fish ranaviruses (family: Iridoviridae) by immunofluorescent antibody tests (IFAT) (28, 31).

A number of useful diagnostic methods are in use, such as the observation of stained impression smears or tissue sections, an IFAT using an MAb, and polymerase chain reactions (PCR) (11, 17, 25, 28, 32, 33).

A formalin-killed vaccine for RSIVD is effective and is commercially available in Japan (26, 27).

2. Disease information

2.1. Agent factors

2.1.1. Aetiological agent, agent strains

The disease is caused by red sea bream iridovirus (RSIV) (8–10, 18, 20, 29) Ehime-1 strain and other genotypes of RSIV, including many viruses that are considered to be synonyms of RSIV (2–6, 12, 13, 16, 22, 40). The disease is also caused by infectious spleen and kidney necrosis virus (ISKNV) (7, 31), which is one of the viruses related to, but distinct from, RSIV. A number of other iridoviruses that cause similar diseases in ornamental freshwater fish have been reported (34, 39). These viruses are difficult to distinguish genetically from ISKNV, and it has not been determined whether these diseases should be included in RSIVD. Recently, turbot reddish body iridovirus (TRBIV) (37) and its probable synonyms (4, 10), which are related to, but distinct from, RSIV and ISKNV, were reported from the People's Republic of China and the Republic of Korea. Further investigation is necessary before it can be determined whether or not the disease caused by TRBIV should also be included in RSIVD. All these agents belong to the fifth genus of the family Iridoviridae, and they are distinguishable genetically and biologically from fish ranaviruses such as epizootic haematopoietic necrosis virus (EHNV), European catfish iridovirus (ECV) and grouper iridovirus (GIV= Singapore grouper iridovirus [SGIV]) (14, 23, 35–38), none of which is pathogenic to the red sea bream (24).

2.1.2. Survival outside the host

Unknown.

2.1.3. Stability of the agent (effective inactivation methods)

Inactivated at 56°C for 30 minutes; sensitive to ether and chloroform; inactivated by formalin (0.1%); stable in tissue at –80°C.

2.1.4. Life cycle

Not applicable.

2.2. Host factors

2.2.1. Susceptible host species

In the case of RSIV infection: red sea bream (*Pagrus major*), black porgy (*Acanthopagrus schlegelii*), yellowfin sea bream (*Acanthopagrus latus*), crimson sea bream (*Evynnis japonica*), Japanese amberjack (*Seriola quinqueradiata*), greater amberjack (*Seriola dumerilii*), yellowtail amberjack (*Seriola lalandi*), hybrid of yellowtail amberjack and Japanese amberjack (*S. lalandi* × *S. quinqueradiata*), striped jack (*Pseudocaranx dentex*), northern bluefin tuna (*Thunnus thynnus*), Japanese Spanish mackerel (*Scomberomorus niphonius*), chub mackerel (*Scomber japonicus*), Japanese jack mackerel (*Trachurus japonicus*), Japanese parrotfish (*Oplegnathus fasciatus*), spotted knifejaw (*Oplegnathus punctatus*), cobia (*Rachycentron canadum*), snubnose pompano (*Trachinotus blochii*), chicken grunt (*Parapristipoma trilineatum*), crescent sweetlips (*Plectorhinchus cinctus*), Chinese emperor (*Lethrinus haematopterus*), spangled emperor (*Lethrinus nebulosus*), largescale blackfish (*Girella punctata*), rockfish (*Sebastes schlegelii*), croceine croaker (*Pseudosciaena crocea*), Hong Kong grouper (*Epinephelus akaara*), convict grouper (*Epinephelus septemfasciatus*), Malabar grouper (*Epinephelus malabaricus*), longtooth grouper (*Epinephelus bruneus*), orange-spotted grouper (*Epinephelus coioides*), yellow grouper (*Epinephelus awoara*), greasy grouper (*Epinephelus tauvina*), brown-marbled grouper (*Epinephelus fuscoguttatus*), giant grouper (*Epinephelus lanceolatus*), Japanese sea perch (*Lateolabrax japonicus*), *Lateolabrax* sp., barramundi or sea bass (*Lates calcarifer*), hybrid of striped sea bass and white bass (*Morone saxatilis* × *M. chrysops*), largemouth bass (*Micropterus salmoides*), bastard halibut (*Paralichthys olivaceus*), spotted halibut (*Verasper variegatus*), and torafugu (*Takifugu rubripes*), are known to be susceptible. In the case of ISKNV infection: Chinese perch (*Siniperca chuatsi*), red drum (*Sciaenops ocellatus*), flathead mullet (*Mugil cephalus*), and *Epinephelus* sp. are known to be susceptible.

2.2.2. Susceptible stages of the host

Juvenile to adult (the susceptibility of juveniles is generally higher than adults).

2.2.3. Species or subpopulation predilection (probability of detection)

In the case of RSIV infection, fish belonging to the genus *Oplegnathus* are more sensitive than others.

2.2.4. Target organs and infected tissue

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

2.2.5. Persistent infection with lifelong carriers

Unknown.

2.2.6. Vectors

Unknown.

2.2.7. Known or suspected wild aquatic animal carriers

Unknown.

2.3. Disease pattern

2.3.1. Transmission mechanisms

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

2.3.2. Prevalence

Unknown.

2.3.3. Geographical distribution

RSIVD caused by RSIV and ISKNV has been reported not only from Japan but also widely from other East and South-East Asian countries (Chinese Taipei, China [People's Rep. of], Hong Kong, Korea [Rep. of], Malaysia, Philippines, Singapore, and Thailand) (2–6, 9, 10, 12, 13, 15, 19, 22, 31, 40).

2.3.4. Mortality and morbidity

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

2.3.5. Environmental factors

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

2.4. Control and prevention

2.4.1. Vaccination

Effective formalin-killed commercial vaccines for RSIVD are currently available for red sea bream, striped jack 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

Not available.

2.4.3. Immunostimulation

Under investigation.

2.4.4. Resistance breeding

Under investigation.

2.4.5. Restocking with resistant species

Under investigation.

2.4.6. Blocking agents

Unknown.

2.4.7. Disinfection of eggs and larvae

No data available.

2.4.8. General husbandry practices

A number of general husbandry practices are used to reduce RSIVD-associated losses. These include: introducing pathogen-free fish; implementing hygiene practices on farms; and avoiding practices that can decrease water quality and/or increase stress, such as overcrowding and overfeeding.

3. Sampling

3.1. Selection of individual specimens

Moribund fish should be selected.

3.2. Preservation of samples for submission

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

3.3. Pooling of samples

Tissue samples (spleen and kidney) can be pooled from no more than five juvenile fish (less than 3 cm).

3.4. Best organs or tissues

Although gill and visceral organs such as spleen, heart, kidney, liver and intestine can be used, it is also recommended to sample spleen and/or kidney tissues; spleen especially may be the most appropriate organ for the preparation of smears for use in the IFAT.

3.5. Samples/tissues that are not suitable

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

4. Diagnostic methods

4.1. Field diagnostic methods

4.1.1. Clinical signs

Affected fish become lethargic, exhibit severe anaemia, petechiae in the gills, and enlargement of the spleen.

4.1.2. Behavioural changes

Diseased fish swim inactively and show abnormal and conspicuous respiratory exercises caused by anaemia.

4.2. Clinical methods

4.2.1. Gross pathology

Pale gills and enlarged spleen.

4.2.2. Clinical chemistry

Low haematocrit value.

4.2.3. Microscopic pathology

See methods for smears (Section 4.2.5) and electron microscopy/cytopathology (Section 4.2.6). Microscopic pathology should confirm the presence of abnormally enlarged cells in tissues such as the spleen, heart, kidney, intestine or gill.

4.2.4. Wet mounts

None.

4.2.5. Smears

Confirm presence of abnormally enlarged cells in Giemsa-stained stamp-smear of the spleen.

4.2.6. Electron microscopy/cytopathology

Confirm presence of virions (200–240 nm in diameter) in the enlarged cells.

4.3. Agent detection and identification methods

4.3.1. Direct detection methods

4.3.1.1. Microscopic methods

4.3.1.1.1. Wet mounts

None.

4.3.1.1.2. Smears

Examination of acetone-fixed stamp-smears from diseased fish may reveal abnormally enlarged cells from spleen, heart or kidney. These enlarged cells react to an anti-RSIV MAb (M10) by the antibody-based antigen detection (IFAT) test (26).

4.3.1.1.3. Fixed sections

Examination of histological sections from diseased fish may reveal abnormally enlarged cells from the spleen, heart, kidney, liver, intestine or gill. These enlarged cells may react to anti-RSIV MAb by the immunohistochemistry test. However, this method is not yet fully validated.

4.3.1.2. Agent isolation and identification

Isolation of RSIV and ISKNV is undertaken using the Grunt fin (GF) cell line. Spleen and/or kidney tissues from diseased fish are suitable samples. Cells should be grown in L-15 medium supplemented with 10% fetal bovine serum (FBS) at 25°C in a temperature-controlled incubator to ensure subsequent success in the isolation of RSIV. Viruses to be used as positive controls can be obtained from the OIE Reference Laboratory for RSIV. Use uninfected cells as negative controls. Following development of viral cytopathic effect (CPE), virus identification would be undertaken using either antibody-based antigen detection (IFAT) and/or nucleic acid-based methods (PCR).

4.3.1.2.1. Cell culture/artificial media

4.3.1.2.1.1 Isolation of RSIV and ISKNV in cell culture

Inoculation of cell monolayers

- i) Following the virus extraction procedure described in Chapter 2.3.0. Section A.2.2.2, make an additional tenfold dilution of the 1/10 spleen homogenate supernatants and transfer an appropriate volume of each of the two dilutions on to 24 hour-old cell monolayers. Inoculate at least 2 cm² of the drained cell monolayer with 100 µl of each dilution.
- ii) Allow to adsorb for 0.5–1 hour at 25°C and, without withdrawing the inoculum, add cell culture medium buffered at pH 7.6 and supplemented with 2% FBS (1 ml/well for 24-well cell culture plates), and incubate at 25°C using a temperature-controlled incubator.

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 a CPE appears in those cell cultures inoculated with dilutions of the test homogenates, identification procedures have to be undertaken immediately (see below).

If a fish health surveillance/control programme is being implemented, provisions may have to be taken to suspend the approved health status of the production unit and/or the zone (if it was approved previously) from which the suspected virus-positive sample originated. The suspension of approved status will be maintained until it is demonstrated that the virus in question is not RSIV or ISKNV.

- iii) If no CPE develops in the inoculated cultures (despite normal progression of CPE in the virus controls) after 10 days incubation, 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

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

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

4.3.1.2.1.2 Identification of RSIV and ISKNV

RSIV (probably ISKNV also) cannot be identified by neutralisation tests as the antisera generated by the immunisation of rabbits have few neutralising antibodies.

4.3.1.2.1.2.1 Indirect fluorescent antibody test

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 use a RSIV-infected cell monolayer as a positive control.

The IFAT is conducted directly after virus isolation in cell culture.

- i) Prepare monolayers of cells on cover-slips to reach approximately 80% confluence, which is usually achieved within 24 hours of incubation at 25°C. The FBS content of cell culture medium is reduced to 2%.
- ii) When the cell monolayers are ready, inoculate the virus suspension to be identified at tenfold dilution steps by adding directly into the cell culture wells or flasks.
- iii) Incubate at 25°C for 24–72 hours.
- iv) When CPE appears, remove the cell culture medium, rinse three times with phosphate-buffered saline (PBS). Air-dry the infected cells and fix with cold acetone (stored at –20°C) for 10 minutes.
- v) Allow the cell monolayers to air-dry for at least 30 minutes and process immediately or freeze at –20°C.
- vi) Prepare a solution of MAb M10 at the previously determined dilution.
- vii) Treat the cell monolayers with the MAb solution for 30 minutes at 37°C in a humid chamber.
- viii) Rinse the cells three times for 5 minutes with PBS.
- ix) Incubate with a specific anti-mouse fluorescein isothiocyanate (FITC)-conjugated antibody (prepared according to the supplier's instructions) for 30 minutes at 37°C in darkness in a humid chamber.
- x) Rinse three times for 5 minutes with PBS.
- xi) Examine the treated cell monolayers on plastic plates immediately, or mount the cover-slips using glycerol saline at pH 8.5 prior to microscopic observation.
- xii) Examine under incident UV light using a microscope with ×10 eye pieces and ×20–40 objective lenses. Positive and negative controls must be found to give the expected results prior to any other observation. Positive results are indicated by diffuse fluorescence throughout the cytoplasm.

Levels of validation:

- Specificity and sensitivity: MAb M10 can detect both RSIV and ISKNV (31); it does not detect ranaviruses. The reactivity of the MAb against TRBIV has not yet been confirmed.
- 'Gold' standard: the CPE is characterised by many enlarged cells and virus is confirmed in the CPE by IFAT.

Interpretation of results:

- The virus isolated is RSIV or ISKNV and the disease is RSIVD.

Availability of test: Reagents and biological materials are available from the OIE Reference Laboratory for RSIVD or commercial sources. MAb M10 can be supplied from the same OIE Reference Laboratory.

4.3.1.2.2. Antibody-based antigen detection methods (IFAT) without cell culture

Samples to be tested include stamp-smear of spleen from affected fish.

Use a stamp-smear of spleen from uninfected fish as a negative control, and use a stamp-smear of spleen from confirmed RSIV-infected fish as a positive control.

Positive controls (air-dried and fixed stamp-smear of spleen from infected fish) can be obtained from the OIE Reference Laboratory. Use imprints of healthy fish spleen as negative controls.

Test procedure

- 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 required for virus isolation in case this becomes necessary later.
- iv) Allow the imprints to air-dry for 20 minutes.
- v) Fix the imprints with cold acetone.

- vi) Treat the imprints with the MAb (M10) solution for 30 minutes at 37°C in a humid chamber.
- vii) Rinse three times with PBS.
- viii) 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 instructions of the supplier. These FITC antibodies are most often rabbit or goat antibodies.
- ix) Rinse three times with PBS.
- x) Mount the microscope slides with cover-slips using glycerol saline prior to microscopic observation.
- xi) Examine under indirect UV light using a microscope with ×10 eye pieces and ×20–40 objective lenses. Positive and negative controls must be found to give the expected results prior to any other observation.

Levels of validation:

- Specificity and sensitivity: MAb M10 can detect both RSIV and ISKNV (31) but it does not detect ranaviruses. The reactivity of the MAb against TRBIV has not yet been confirmed.
- 'Gold' standard: abnormal enlarged cell with strong fluorescence is confirmed by IFAT.

Interpretation of results:

- If the test is positive, the fish from which the samples were obtained is considered infected with RSIV or ISKNV and the disease is RSIVD. If the test is negative, process the organ samples stored at 4°C for virus isolation in cell cultures as described above.

Availability of test: Reagents are available from the OIE Reference Laboratory or commercial sources. MAb M10 can be supplied from the OIE Reference Laboratory.

4.3.1.2.3. Molecular techniques

4.3.1.2.3.1 Polymerase chain reaction

Samples to be tested include spleen from affected fish or supernatants from cell cultures that have developed CPE.

Use extracted DNA from the spleen 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 as the positive control. Select controls depending on the kinds of samples to be tested.

DNA extraction from organ sample or cell culture supernatant of isolated virus

Fish samples or cell culture supernatants are prepared as described in Kurita *et al.* (17) for DNA extraction. Use a pre-confirmed RSIV (or ISKNV)-affected organ or supernatant from RSIV (or ISKNV)-infected cell cultures as positive controls. Use organs from healthy fish or supernatants from non-infected cell cultures as negative controls.

Polymerase chain reaction amplification

RSIV and ISKNV have large double-stranded DNA genomes. One primer set, a forward primer 1-F (5'-CTC-AAA-CAC-TCT-GGC-TCA-TC-3') and a reverse primer 1-R (5'-GCA-CCA-ACA-CAT-CTC-CTA-TC-3') is used for the amplification of the gene sequence (570 bases) of both RSIV DNA and ISKNV DNA by PCR (17). (Please note that the previous RSIV-specific OIE primers 4-F [5'-CGG-GGG-CAA-TGA-CGA-CTA-CA-3'] and 4-R [5'-CCG-CCT-GTG-CCT-TTT-CTG-GA-3'; expected product size is 568 bp] do not amplify ISKNV DNA [31]). Both primer sets have been described by Kurita *et al.* (17).

Extracted DNA (1 µl) is added to Taq polymerase buffer containing 1 mM of each primer, 200 mM of deoxynucleotide triphosphate, and 1.25 U of ExTaq DNA polymerase in 20 mM Mg²⁺ PCR buffer. The mixture is incubated in an automatic thermal cycler programmed for 30 cycles at 94°C for 30 seconds, 58°C for 60 seconds, and 72°C for 60 seconds, and finally held at 72°C for 5 minutes. Amplified DNA (570 bp) is analysed by agarose gel electrophoresis.

Levels of validation:

- Specificity and sensitivity: primer set 1-F and 1-R can amplify both RSIV and ISKNV DNA with adequate sensitivity. The previous primer set 4-F and 4-R also has adequate sensitivity for RSIV,

but it cannot be used to amplify ISKNV DNA. The reactivity of these primer sets against TRBIV has not yet been confirmed.

- 'Gold' standard: PCR product of expected size is clearly confirmed by electrophoresis when primer set 1-F and 1-R are used.

Interpretation of results:

- A positive result by PCR using the primer set 1-F and 1-R, and confirmed specificity by sequencing, indicates the presence of RSIV or ISKNV and that the disease is RSIVD. A positive result by the optional PCR using the previous primer set 4-F and 4-R and run in conjunction with the previous PCR indicates that the virus is RSIV and that the disease is RSIVD caused by RSIV. A negative result with this optional, secondary PCR indicates that the virus is ISKNV and the disease is RSIVD caused by ISKNV.

Availability of test: Reagents are available from the OIE Reference Laboratory or commercial sources.

4.3.1.2.4. Agent purification

By ultracentrifugation in CsCl gradient (10–35% w/w). The gravity of the resultant virion band is approximately 1.25–1.3 g ml⁻¹.

4.3.2. Serological methods

Serological methods using serum of affected fish have not yet been developed.

5. Rating of tests against purpose of use

As an example, the methods currently available for targeted surveillance and diagnosis of RSIVD 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
	Larvae	PLs	Juveniles	Adults		
Gross signs	d	d	d	d	b	d
Bioassay (virus isolation in cell culture) and identification by IFAT or PCR	c	c	c	c	a	a
Direct LM or stamp smear	d	d	c	d	b	d
Histopathology	d	d	d	d	b	d
Transmission EM	d	d	d	d	b	d
Antibody-based assays (IFAT) of isolated virus or stamp-smear	c	c	c	c	a	a/b
DNA probes – <i>in situ</i>	d	d	d	d	c	c
PCR	c	c	c	c	a	a
Sequence	d	d	d	d	a	a

PLs = postlarvae; IFAT = immunofluorescent antibody test; PCR = polymerase chain reaction; LM = light microscopy; EM = electron microscopy.

6. Test(s) recommended for targeted surveillance to declare freedom from Red sea bream iridoviral disease

There is no established detection method for surveillance, because the carrier state of the agents has not yet been investigated. Tentative methods would be virus isolation followed by IFAT or the nested PCR (1).

7. Corroborative diagnostic criteria

7.1. Definition of suspect case

The presence of RSIV or ISKNV should be suspected if at least one of the following criteria is met:

- 1) Presence of typical clinical signs and confirmation of abnormally enlarged cells on stamp-smear or tissue sections.
- 2) Presence of typical clinical signs and confirmation of the presence of virions in abnormally enlarged cells by electron microscopy.
- 3) Virus isolation with specific CPE.
- 4) Presence of IFAT positive cells on stamp-smear.

7.2. Definition of confirmed case

The presence of RSIV or ISKNV should be considered as confirmed if, in addition to the criteria in 7.1, one or more of the following criteria is met:

- 1) Virus isolation with specific CPE and positive result of IFAT using infected cell cultures.
- 2) Virus isolation with specific CPE and positive for PCR using extracted DNA from isolated virus as template.
- 3) Positive for PCR using extracted DNA from affected organs as template.
- 4) Presence of typical abnormally enlarged cells showing positive IFAT results on stamp-smear.

8. References

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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: www.oie.int).