SECTION 2.3.

DISEASES OF FISH

CHAPTER 2.3.0.

GENERAL INFORMATION

A. SAMPLING

1. Assessing the health status of the epidemiological unit

1.1. Sample material to be used for tests

Sample material depends on the disease or pathogen to be tested for and on both the size of animals and the objective of testing, i.e. diagnosis of overt disease, detection of fish that are subclinical pathogen carriers or sampling for targeted surveillance to demonstrate freedom of a specified disease. See the OIE *Guide for Aquatic Animal Health Surveillance* (2009) and the individual disease chapters in this *Aquatic Manual* for specific details of sample requirements.

1.2. Specifications according to fish size

1.2.1. For the listed viral diseases

Alevin and yolk sac fry: sample the entire fish but remove the yolk sac if present.

Fish 4 to 6 cm: take the entire viscera including the kidney. A piece of encephalon can be obtained after severing the head at the level of the rear edge of the operculum and pressing it laterally.

Fish over 6 cm: take the kidney, spleen, and heart or encephalon and/or tissues appropriate for the specific pathogen being tested for (see individual disease chapter in this *Aquatic Manual* for details.

Adult fish: take the ovarian fluid and/or tissues appropriate for the specific pathogen being tested for (see individual disease chapter in this *Aquatic Manual* for details).

1.2.2. For Epizootic ulcerative syndrome (EUS)

Any size of fish: kidney, liver, muscular tissue (see Chapter 2.3.2 Epizootic ulcerative syndrome for specific details).

1.2.3. For *Gyrodactylus salaris*

Any size of fish: skin and fins (see Chapter 2.3.3. Gyrodactylosis [*Gyrodactylus salaris*] for specific details).

1.2.4. For Koi herpesvirus (KHV)

Fish 4cm to adult: take the gill, kidney, spleen, encephalon and gut tissues depending on test used (see Chapter 2.3.6. Koi herpesvirus disease for specific details).

1.3. Specifications according to fish populations

For general guidelines, see the OIE *Guide for Aquatic Animal Health Surveillance* (2009), and for specific details of sample requirements for a particular listed disease, see the individual disease chapter in this *Aquatic Manual.*

1.4. Specifications according to clinical status

In the case of clinical infection, besides whole fry or entire viscera, organs to be sampled are anterior kidney, spleen and heart or encephalon for virus tests, skin or muscle when sampling for tests for epizootic ulcerative syndrome, and skin and fins for examination for *Gyrodactylus salaries* and gill and gut when sampling for tests for Koi herpesvirus disease. Samples from ten clinically diseased fish should be sufficient for the pathogen test(s) for each epidemiological unit. For detecting subclinical carriers of virus, samples may be combined as pools of no more than ten fish per pool. Pools of ovarian fluid from five broodfish should not exceed a total volume of 5 ml, i.e. 1 ml per broodfish. These ovarian fluid samples are to be taken individually from every sampled female but not following the pooling of ova.

2. General processing of samples

2.1. Macroscopic examination

For the listed diseases, macroscopic examination is mostly used for detecting clinical signs of epizootic ulcerative syndrome or *Gyrodactylus salaris* but this is followed by microscopic examination of histological slides for the former or of wet mounts of skin/fin scrapes for the latter.

2.2. Virological examination

2.2.1. Transportation and antibiotic treatment of samples

Pools of organs or of ovarian fluids are placed in sterile vials and stored at 4°C or on ice until virus extraction is performed in the laboratory. Virus extraction should optimally be carried out within 24 hours after fish sampling, but is still acceptable for up to 48 hours if the temperature is maintained at 0°C–4°C, or for longer periods for clinical disease samples held frozen at –20°C to –80°C. However, freezing of samples for testing for subclinical carriers should be avoided.

Organ samples may also be transported to the laboratory by placing them in vials containing cell culture medium or Hanks' balanced salt solution (HBSS) with added antibiotics to suppress the growth of bacterial contaminants (one volume of organ in at least five volumes of transportation fluid). Suitable antibiotic concentrations are: gentamycin (1000 µg ml^{-1}) or penicillin (800 International Units [IU] ml^{-1}) and streptomycin (800 µg ml^{-1}). Antifungal compounds, such as Mycostatin® or Fungizone®, may also be incorporated into the transport medium at a final concentration of 400 IU ml^{-1}. Serum or albumen (5–10%) may be added to stabilise the virus if the transport time will exceed 12 hours.

2.2.2. Virus extraction

This procedure is conducted below 15°C and preferably at between 0 and 10°C.

- Decant antibiotic-supplemented medium from the organ sample.
- Homogenise organ pools in transport medium at a final dilution of 1/10 using a mortar and pestle or electric homogeniser until a paste is obtained.
- Centrifuge the homogenate in a refrigerated centrifuge at 2–5°C at 2000 to 4000 ***g*** for 15 minutes, collect the supernatant and treat for either four hours at 15°C or overnight at 4°C with antibiotics, e.g. gentamicin 1 mg ml^{-1}. If shipment of the sample has been made in a transport medium (i.e. with exposure to antibiotics) the treatment of the supernatant with antibiotics may be omitted. The antibiotic treatment makes filtration through membrane filters unnecessary.
- Likewise, ovarian fluid samples may be treated with antibiotics to control microbial contamination but should not be diluted more than fivefold in the HBSS and antibiotic medium.
- Ovarian fluid samples should be centrifuged in the same way as organ homogenates, and their supernatants used directly in subsequent steps.

2.2.3. Treatment to neutralise enzootic viruses

Fish are often subclinical carriers of endemic viruses, such as birnaviruses (e.g. infectious pancreatic necrosis virus [IPNV]), which induce a cytopathic effect in susceptible cell cultures and thus complicate isolation and identification of target pathogens. In such situations, the infectivity of the enzootic viruses should be neutralised before testing for the viruses listed in the *Aquatic Code*. However, when it is important to determine whether one of the enzootic viruses is present, samples should be tested with and without the presence of neutralising antibodies (NAbs).

To neutralise aquatic birnaviruses, mix equal volumes (200 µl) of a solution of NAbs against the indigenous birnavirus serotypes with the supernatant to be tested. Allow the mixture to react for 1 hour at 15°C or overnight at 4°C prior to inoculation on to susceptible cell monolayers. The titre of the NAb solution used (it may be a multivalent serum) should be at least 2000 in a 50% plaque reduction test versus the viral serotypes present in the given geographical area.

When samples are from a country, region, fish population or production unit considered to be free from enzootic viral infections, this treatment of the organ homogenate should be omitted.

This approach can also be used to neutralise other viruses enzootic to the area being tested.

2.3. Parasitic examination

See Chapter 2.3.3. Gyrodactylosis (*Gyrodactylus salaris*) for specific details.

2.4. Fungal examination

See Chapter 2.3.2. Epizootic ulcerative syndrome for specific details.

B. MATERIALS AND BIOLOGICAL PRODUCTS REQUIRED FOR THE ISOLATION AND IDENTIFICATION OF FISH PATHOGENS

1. Fish viruses

1.1. Fish cell lines

The following fish cell lines will be required to test for the fish pathogens mentioned in the *Aquatic Manual:*
Epithelioma papulosum cyprini (EPC)
Bluegill fry (BF-2)
Fathead minnow (FHM)
Rainbow trout gonad (RTG-2)
Chinook salmon embryo (CHSE-214)
Salmon head kidney (SHK1)
Atlantic salmon kidney (ASK)
Atlantic salmon (TO)
Grunt fin (GF)
Koi fin (KF-1)
Cyprinus carpio brain (CCB)

1.2. Culture media

Traditional Eagle's minimal essential medium (MEM) with Earle's salt supplemented with 10% fetal bovine serum (FBS), antibiotics and 2 mM L-glutamine is the most widely used medium for fish cell culture.

Stoker's medium, however, which is a modified form of the above medium comprising a double-strength concentration of certain amino acids and vitamins, is particularly recommended to enhance cell growth, using the same supplementations as above + 10% tryptose phosphate.

These media are buffered with either sodium bicarbonate, 0.16 M tris-hydroxymethyl aminomethane (Tris) HCl, or, preferably, 0.02 M N-2-hydroxyethyl-piperazine-N-2-ethanesulfonic acid (HEPES). The use of sodium bicarbonate alone is restricted to those cell cultures made in tightly closed cell culture vessels.

Alternatively, Leibovitz medium (L15) supplemented with FBS (5% or 10%), L-glutamine (4 mM) and gentamicin (50 µg ml^{-1}) can be used for some cell lines, e.g. SHK1.

For cell growth, the FBS content of the medium is usually 10%, whereas for virus isolation or virus production it may be reduced to 2%. Similarly, the pH of the culture medium for cell growth is 7.3–7.4 and is adjusted to 7.6 for virus production or virus assay.

The composition of the most frequently used antibiotic mixture is penicillin (100 IU ml^{-1}) and dihydrostreptomycin (100 µg ml^{-1}). Mycostatin (50 IU ml^{-1}) may be used if fungal contamination is expected.

Other antibiotics or antibiotic concentrations may be used as convenient for the operator depending on the antibiotic sensitivity of the bacterial strains encountered.

1.3. Virus positive controls and antigen preparation

1.3.1. Virus nomenclature

- Epizootic haematopoietic necrosis virus (EHNV)
- Infectious haematopoietic necrosis virus (IHNV)
- Infectious salmon anaemia virus (ISAV)
- Koi herpesvirus (KHV)
- Red sea bream iridovirus (RSIV)
- Spring viraemia of carp virus (SVCV)
- Viral haemorrhagic septicaemia virus (VHSV)

1.3.2. Virus production

For the production of most of these viruses, the susceptible cell cultures (see relevant sections in this *Aquatic Manual*) should be inoculated with fairly low multiplicities of infection (m.o.i.), i.e. 10^{-2} to 10^{-3} plaque-forming units (PFU) per cell.

The optimal temperatures for virus propagation are:

- 15°C for IHNV, VHSV and ISAV
- 20°C for SVCVand KHV
- 22°C for EHNV
- 25°C for RSIV

1.3.3. Virus preservation and storage

- Centrifuge infected cell cultures at 2–5°C and 2000–4000 ***g*** for 15 minutes then dilute the virus-containing supernatants in order to obtain virus titres averaging 1–2 × 10^6 PFU ml^{-1}.
- Dispense the resulting viral suspensions into sterile vials at volumes of 0.3–0.5 ml each.
- Freeze and store each series of standard virus stocks at –80°C or liquid nitrogen, and check the titre of each virus stock at regular intervals if it has not been used during that time period.

Lyophilisation: long-term storage (decades) of the seeds of standard virus strains is achievable by lyophilisation. For this purpose, viral suspensions in cell culture medium supplemented with 10% fetal calf serum are mixed (v/v) with an equal volume of cryopreservative medium (such as 20% lactalbumin hydrolysate in distilled water) before processing. Seal or plug under vacuum and store at 4°C, in the dark.

3. Techniques

3.1. Serology

3.1.1. Production of rabbit antisera and polyclonal antibodies to fish viruses

There are various ways in which antibodies against fish viruses can be raised in rabbits. Titre and specificity are influenced, however, by the inoculation programme used. The following immunisation protocols may be used to produce antisera for use in the virus isolation and/or identification procedures described later.

3.1.1.1 Antisera to infectious pancreatic necrosis virus

Intravenous injection with 50–100 µg of purified virus on day 0, followed by an identical booster on day 21, and bleeding 5–7 days later. Rabbits may be reused if not bled completely.

3.1.1.2 Antisera to other viruses

The immunisation protocols alternate an intramuscular or intradermal injection with further intravenous boosters:

Day 0: primary injection, 500–1000 µg of purified virus is mixed (v/v) with adjuvant (Freund's incomplete or other[1]) giving a total volume of 1.2 ml. This antigen is delivered to the rabbit as multipoint intradermal injections (20 points on each side) after the animal has been shaved.

Day 21: collect about 20 ml of blood and check for reactivity (neutralisation, fluorescence); boost intravenously with the same amount of purified virus as in the primary injection, but without adjuvant. Prior to the intravenous booster injection, the rabbit should be treated with promethazine (12 mg intramuscularly) to prevent a possible anaphylactic response.

Day 28: sample the blood, check the serum reactivity and bleed or boost according to the results.

For rhabdoviruses, this immunisation procedure is well suited to production of antisera to be used in immunofluorescence and the enzyme-linked immunosorbent assay. However, a more efficient method for production of neutralising antisera is regular intravenous injection without adjuvant (0.2 ml) every 3–4 days (twice a week). As many as 15 injections may be necessary; 1 week after the last injection, a serum sample should be collected and tested.

3.1.3. Processing and storage of immune sera

After blood clotting, collect and centrifuge the serum at 20°C and heat it for 30 minutes at 56°C. Filter the resulting heat-inactivated serum through a membrane filter (450 nm pore size) and temporarily store it at 4°C for the time necessary for the screening of its reactivity and specificity and for checking that these properties are not affected by preservation conditions (e.g. freezing or lyophilisation). Sterile rabbit sera can be kept for at least 2 months at 4°C without any change in their properties. Dispense (usually as small volumes) and freeze at –20°C or lyophilise.

Immunoglobulins (Ig) may be extracted from antisera using conventional methods suitable for Ig purification. Selective attachment to protein A constitutes a reliable and effective method. The concentration of Ig solutions is adjusted to the values required for further conjugate preparation or storage.

Preservation of Ig: Mix a solution of Ig of concentration 2 mg litre^{-1} with sterile pure glycerol (v/v) and keep at –20°C. Solutions of Ig with a higher concentration may also be prepared in glycerol.

3.1.4. Mouse monoclonal antibodies

Monoclonal antibodies (MAbs) to most of the fish viruses have been raised over the past years. Some of them, singly or as two or three associated MAbs, have given rise to biological reagents suitable for the identification of virus groups (IPN, VHS, IHN). Other MAbs, taken individually or as components of Ab panels, allow accurate typing of VHSV and IHNV. These MAbs can be obtained from the Reference Laboratories listed at the end of this *Aquatic Manual*.

In theory, mouse monoclonal IgGs can be processed and stored as for polyclonal IgGs. However, the reactivity of certain MAbs may be impaired by processes such as enzymatic- or radio-labelling or lyophilisation. It is thus necessary to test various MAbs for the conditions under which they will be used.

3.2. Direct microscopy

Samples for direct microscopic examination of smears or tissue imprints should be examined as soon as possible after collection. Live specimens should be used whenever possible, or fresh, chilled, or 10% buffered formalin-fixed specimens used when live specimens are not practical. If an adequate field laboratory is available, it should be used to process and examine samples near the site of collection.

3.3. Histological techniques

3.3.1. Tissue fixation and embedding

Only live or moribund specimens of fish with clinical lesions should be sampled for histology. The removed tissues should be fixed immediately in 10% buffered formalin. Use at least ten volumes of

1 Use of Freund's complete adjuvants may be restricted on animal welfare grounds. Alternative synthetic adjuvants include trehalose dimycolate and monophosphate lipid A.

fixative for each volume of tissue sample and allow to fix for at least 24 hours. After removal from the fixative, tissue samples are then dehydrated in ascending ethanol concentrations, cleared in a wax miscible agent such as xylene and then embedded in paraffin using standard protocols.

3.3.2 Tissue sectioning and staining

Cut sections of approximately 5 μm thickness from the block. Mount each section on a glass slide, de-wax in a wax-miscible agent, such as xylene or 'Clearene', and rehydrate.

For most disease examinations, the sections can then be stained with haematoxylin and eosin (H&E), by the following procedure:

Taking the slides to water

1. Place slides in xylene or 'Clearene' to remove wax for a minimum of 2 minutes.
2. Repeat step 1 in fresh xylene or 'Clearene'.
3. Place in 100% alcohol to remove the solvent for a minimum of 2 minutes.
4. Repeat step 3 in fresh 100% alcohol.

Staining

5. Wash in running tap water (RTW) for 2–5 minutes. Slides should be clear, not cloudy.
6. Place in haematoxylin solution for 3 minutes
7. Turn blue in RTW for 5–10 minutes (or saturated lithium carbonate); cannot over blue.
8. Differentiate in acid/alcohol for a maximum of 10 seconds.
9. Rinse in RTW (or lithium carbonate) until blue.
10. Microscope check for clear cytoplasm and blue nuclei.
11. Aqueous eosin for 3 minutes.
12. Good wash in RTW to differentiate eosin.

Dehydration, clearing and mounting

13. Rinse well in 70% alcohol but not for too long as it removes eosin.
14. Place in 100% alcohol for 1–2 minutes.
15. Repeat step 14 in fresh alcohol.
16. Place in 50/50 alcohol/Clearene for 1–2 minutes.
17. Place into Clearene.
18. Repeat with fresh Clearene bath, slides should be clear.
19. Mount in D.P.X. and leave to dry.

For observing granulomas and fungal hyphae as occur in epizootic ulcerative syndrome, a general fungal stain such as Grocott-Gomori may be used instead of H&E.

3.4. Electron microscopy

Electron microscopy (transmission or scanning) is a valuable research tool for the study of aquatic animal diseases. However, these methods are not normally used for the routine diagnosis of the fish diseases listed by the OIE so are not described in this *Aquatic Manual*.

3.5. Use of molecular techniques for confirmatory testing and diagnosis

Molecular techniques, including nucleic acid probes and the polymerase chain reaction (PCR), have been developed for the identification of many pathogens of aquatic animals. However, as is the case with several other diagnostic techniques, an advantage in sensitivity is frequently offset by problems in interpretation or susceptibility to technical problems. Whereas methods based on direct culture or serology are relatively robust, PCR can be quite dependent on the conditions under which it is run and can be highly subject to laboratory contamination by previous PCR products, yielding false-positive results. Thus, while several nucleic acid probe

and PCR protocols are included in this version of this *Aquatic Manual* as diagnostic or confirmatory methods for fish, where possible, well established techniques (e.g. virus isolation) are specified as standard screening methods. Whenever these newer molecular techniques are used, they should be performed with caution and with special attention to the inclusion of adequate positive and negative controls.

3.5.1. Sample preparation and types

For these techniques, samples should be prepared to preserve the nucleic acid of the pathogen. Likewise, samples intended for testing with antibody-based methods should be preserved to retain the reactive antigenic sites for the antibodies used.

Samples selected for nucleic acid-based or antibody-based diagnostic tests should be handled and packaged with the greatest care to minimise the potential for cross contamination among the samples or target degradation before the assay can be performed. To prevent contamination, new containers (plastic sample bags or bottles) should be used. A water-resistant label, with the appropriate data filled out, should be placed within each package or container for each sample set.

Some suitable methods for preservation and transport of samples taken for molecular or antibody-based tests are:

- Live iced specimens or chilled specimens: for specimens that can be rapidly transported to the laboratory for testing within 24 hours, pack samples in sample bags surrounded by an adequate quantity of wet ice around the bagged samples in an insulated box and ship to the laboratory.
- Frozen whole specimens: select live specimens according to the purpose of sampling, quick freeze in the field using crushed dry-ice, or freeze in a field laboratory using a mechanical freezer at –20°C or lower temperature. Prepare and insert the label into the container with the samples, pack samples with an adequate quantity of dry-ice in an insulated box, and ship to the laboratory.
- Alcohol-preserved samples: in regions where the storage and shipment of frozen samples is problematic, 90–95% ethanol may be used to preserve, store, and transport certain types of samples. Pack for shipment according to the methods described above.
- Fixed tissues for *in-situ* hybridisation and immuno-histochemistry: for this purpose, classic methods for preservation of the tissues are adequate. Buffered formalin is usually a good choice for later use of molecular probes. For DNA, specifically, over-fixation (over 24–48 hours) should be avoided.

3.5.2. Preservation of RNA and DNA in tissues

Tissue is cut to be less than 0.5 cm in one dimension and submerged in 10 volumes of RNAlater (e.g. a 0.5 g sample requires about 5 ml of RNAlater). Small organs such as kidney, liver and spleen can be stored whole in RNAlater. These samples can be stored at 4°C for one month, at 25°C for 1 week or at –20°C indefinitely. Archive RNAlater-treated tissues at –20°C.

3.5.3. DNA extraction

For DNA extraction, grind the preserved tissues to powder. Around 10 volumes of extraction buffer (NaCl [100 mM], ethylene diamine tetra-acetic acid [EDTA, 25 mM], pH 8, and sodium dodecyl sulphate [SDS, 0.5%]) are added with proteinase K (100 µg ml^{-1}). Following overnight incubation at 50°C, DNA is extracted using a standard phenol/chloroform protocol, and precipitated with ethanol. To isolate DNA from tissues preserved in RNAlater, simply remove the tissue from RNAlater and treat it as though it was just harvested. Most tissues can be homogenised directly in lysis or extraction buffer.

Considering time constraints and risks for laboratory staff, commercially available kits may provide satisfactory technical alternatives. Use of commercial kits should be validated by comparison with a standard phenol/chloroform protocol prior to their routine use in diagnostic laboratories.

3.5.4. RNA extraction

To isolate RNA from tissues preserved in RNAlater, simply remove the tissue from RNAlater and treat it as though it was just harvested. Most tissues can be homogenised directly in lysis or extraction buffer.

Considering time constraints and risks for laboratory staff, commercially available kits may provide satisfactory technical alternatives. Use of commercial kits should be validated by comparison with a standard phenol/chloroform protocol prior to their routine use in diagnostic laboratories.

3.5.5. Preparation of slides for *in-situ* hybridisation

For *in-situ* hybridisation (ISH), fish tissues should be fixed in buffered formalin for approximately 24 hours and then embedded in paraffin according to standard histological methods, as described under section 3.3. Sections are cut at a thickness of 5 µm and placed on aminoalkylsilane-coated slides, which are then baked overnight in an oven at 40°C. The sections are de-waxed by immersing in xylene for 10 minutes. This step is repeated once and then the solvent is eliminated by immersion in two successive absolute ethanol baths for 10 minutes each. The sections are then rehydrated by immersion in an ethanol series. The protocol may require a step of membrane permeabilisation enabling access to the target DNA. For this purpose, sections are treated with proteinase K (100 µg ml^{-1}) in TE buffer (Tris [50 mM], EDTA [10 mM]), at 37°C for 30 minutes. For ISH tests, it is essential that both a known positive and a known negative slide be stained to eliminate false positive results due to non-specific staining/stain dropout, and false negative results due to errors in the staining protocol.

4. Additional information to be collected

The geographical origin of samples should be defined by the name of the sampling site associated with either its geographical co-ordinates or its location along a river course or body of water. Other minimum sample information should include the collector's name, organisation, date, time, water-body name, and description of location. There should also be records that provide information to allow trace-backs on the sample movement from the sample site to the storage facility or laboratory and within those facilities. Storage facilities should record information on the preservation method, storage location, and date and time of storage at each storage locker or freezer along with information on the storage temperature (continuously monitored is preferable). This information should be tracked with a unique sample code for all samples. For laboratories, the date of receipt, storage location information, date of analysis, analysis notes, and report date should be maintained for all uniquely coded samples. These data will greatly facilitate the tracking of sample problems and provide assurance that the samples were properly handled.

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