CHAPTER 2.3.11.

ONCORHYNCHUS MASOU VIRUS DISEASE

1. Scope[1]

Oncorhynchus masou virus disease (OMVD) is an oncogenic and skin ulcerative condition coupled with hepatitis among salmonid fish in Japan, and probably in the coastal rivers of eastern Asia that harbour Pacific salmon.

2. Disease information

2.1. Agent factors

2.1.1. Aetiological agent, agent strains

The aetiological agent is *Oncorhynchus masou* virus (OMV) in the family *Herpesviridae*, although it has also been given the name nerka virus Towada Lake, Akita and Amori Prefecture (NeVTA), yamame tumour virus (YTV), coho salmon tumour virus (CSTV, COTV), *O. kisutch* virus (OKV), coho salmon herpesvirus (CHV), rainbow trout kidney virus (RKV), and rainbow trout herpesvirus (RHV) (Yoshimizu et al., 1995).

2.1.2. Survival outside the host

A significant reduction in the infectious titre of OMV was observed within 3 and 7 days in environmental water at 15°C and 10°C, respectively. However, the infectivity remained for 7–14 days below 5°C (Yoshimizu et al., 2005) indicating evidence for the presence of bacterial strains in the water with anti-viral activity.

2.1.3. Stability of the agent (describe effective inactivation methods)

Pathogen-free water sources are often essential in aquaculture. Water that comes from rivers or lakes, commonly used in hatcheries, contains fish pathogens. Such open water supplies should not be used without treatment to kill fish pathogens. Fish viruses are divided into two groups based on sensitivity to UV. OMV belongs to a sensitive group and is inactivated by treatment with 10^4 µW second cm^{-2} ultraviolet dose (Yoshimizu et al., 1986). At 15°C for 20 minutes, minimum concentrations showing 100% plaque reduction of OMV by iodophor, sodium hypochlorite solution, benzalkonium chloride solution, saponated cresol solution, formaldehyde solution and potassium permanganate solution were 40, 50, 100, 100, 3500 and 16 mg $litre^{-1}$, respectively (Hatori et al., 2003).

OMV is heat-, ether-, and acid (pH 3)-labile and does not haemagglutinate human O-cells. It is completely inactivated by ultraviolet (UV) irradiation with 3.0×10^3 µW second cm^{-2}. In the presence of 50 µg ml^{-1} of the pyrimidine analogue, 5-Iododeoxyuridine (IUdR), replication is inhibited. Replication of OMV is also inhibited by anti-herpesvirus agents such as phosphonoacetate (PA), acyclovir (ACV), (E)-5-(2-bromovinyl)-2'-deoxyuridine (BVdU), and 1-B-D-arabino- furanosylcytosine (Ara-C), which is caused by the inhibition of DNA polymerase induced by OMV (Kimura et al., 1981a; Kimura et al., 1983a; Kimura et al., 1983b).

2.1.4. Life cycle

Following the septicaemia phase of OMV infection, an immune response takes place that results in the synthesis of neutralising antibodies to OMV. A carrier state frequently occurs that leads to virus shedding via the sexual products at the time of spawning.

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1   NB: Version adopted by the World Assembly of Delegates of the OIE in May 2013. This disease is no longer listed by the OIE.

2.2. Host factors

2.2.1. Susceptible host species (common and Latin names)

Fish species that are susceptible to OMV include: kokanee (sockeye) salmon (*Oncorhynchus nerka*), masu salmon (*O. masou*), chum salmon (*O. keta*), coho salmon (*O. kisutch*) and rainbow trout (*O. mykiss*) (*Kimura et al., 1983c*).

2.2.2. Susceptible stages of the host

The age of the fish is critical and 1-month-old alevins are the most susceptible target for virus infection (Kimura et al., 1981b; Kimura et al., 1983c). The main environmental factor favouring OMV infection is low water temperature, below 15°C (Kumagai et al., 1994).

2.2.3. Species or subpopulation predilection (probability of detection)

Salmonids are the only fish species susceptible to OMV infection, the order of the fish species from the most to the least susceptible is kokanee salmon, chum salmon, masou salmon, coho salmon and rainbow trout (Kimura et al., 1983a).

2.2.4. Target organs and infected tissue

Clinically, the initial infection by OMV appears as a systemic and frequently lethal infection that is associated with oedema and haemorrhages. Virus multiplication in endothelial cells of blood capillaries, haematopoietic tissue and hepatocytes underlies the clinical signs. Four months after this first clinical condition, a varying number of surviving fish exhibit epithelioma occurring mainly around the mouth (upper and lower jaw) and, to a lesser extent, on the caudal fin, operculum and body surface (Kimura et al., 1981a; Yoshimizu et al., 1987). This neoplasia may persist for up to 1 year post-infection. In the case of coho salmon, 1-year-old infected fish in particular show ulcers on the skin, white spots on the liver and neoplastic tissues around the mouth parts or body surface. In rainbow trout, commercial sized fish were infected and the diseased fish exhibit almost no external signs, although some fish manifest ulcerative lesions on the skin. Internally, intestinal haemorrhage and white spots on the liver are observed (Furihata et al., 2003).

2.2.5. Persistent infection with lifelong carriers

Under natural conditions, survivors of OMVD are persistently infected with virus and they shed the virus and fish retain the virus until maturation (Yoshimizu et al., 1993).

2.2.6. Vectors

Water is the major abiotic vector. However, animate vectors, e.g. other fish species, parasitic invertebrates and piscivorous birds and mammals may also be involved in transmission.

2.2.7. Known or suspected wild aquatic animal carriers

Masu salmon that were caught at the river mouth had neoplasia around the mouth and OMV was isolated from tumours. Recently rainbow trout that are living in the river and that may have escaped from farms were infected with OMV and died (Furihata et al., 2003; Yoshimizu & Nomura, 2001)

2.3. Disease pattern

2.3.1. Transmission mechanisms

The reservoirs of OMV are clinically infected fish and covert carriers among groups of cultured, feral or wild fish. Infectious virus is shed via faeces, urine, sexual products and probably skin mucus, while the kidney, spleen, liver and tumours are the sites where virus is the most abundant during the course of overt infection. The transmission of OMV is horizontal and possibly 'egg-surface associated'. Horizontal transmission may be direct or vectorial, water being the major abiotic factor. Disinfection of the eggs just after fertilisation and eyed stage is effective in preventing OMV infection. OMV disease was not reported in alevins originating from disinfected eggs that had been incubated and hatched in virus-free water (Yoshimizu, 2009).

2.3.2. Prevalence

OMV was isolated from masu salmon at all the investigated sites with the exception of one hatchery. Based on our epizootiological study, the roots of OMV was assumed to be along the Japan Sea coast of Hokkaido and presumed original host species was masu salmon. In the 1960s, eggs of masu salmon were collected from the rivers of Japan Sea coast of Hokkaido, and transported to Honshu Island, main land of Japan. With the unrestricted fish movement, the virus spread to several places in Honshu where the first cancer disease of masu salmon was observed (Kimura, 1976). Subsequently, coho salmon and rainbow trout were cultured in the same water systems where masu salmon was cultured. Coho salmon might be infected with OMV at fry stage in fresh water because tumour tissues were found around the mouth of pen cultured coho salmon, the hatchery from where coho salmon was transplanted to pen had a history of OMVD (Furihata et al., 2003; Kumagai et al., 1994; Yoshimizu & Nomura, 2001).

2.3.3. Geographical distribution

Following the first reports of OMVD in Northern Japan (Kimura et al., 1980), the geographical range of the disease has become extensive inside Japan. There are no reports of disease occurrence outside Japan.

2.3.4. Mortality and morbidity

Susceptibility of several salmonid fry to OMV has been studied experimentally by immersion in water containing 100 $TCID_{50}$ (median tissue culture infective dose) ml^{-1} OMV at 10°C for 1 hour. Comparing the five different salmonid fry, at the age of 1 month, kokanee salmon exhibited the greatest sensitivity with 100% mortality. Masu and chum salmon also exhibited high sensitivity at 87% and 83% mortality, respectively. Coho salmon and rainbow trout were shown to be less sensitive to OMV infection at 39% and 29% mortality, respectively. Thus the host range of OMV is wide in salmonid species (Kimura et al., 1983c). Eight age groups of chum salmon, (0, 1, 2, 3, 4, 5, 6, and 7 months old), were immersed under the same conditions. The cumulative mortality of just hatching chum salmon, observed in ensuing 4 months, was 35%, but between 1-month and 5-month-old fry, it was more than 80%. At 6 and 7 months, the fry's susceptibility was reduced and only 7%, and 2% fish had succumbed to the disease. There were no deaths among 8-month-old fingerlings. On the other hand, 1-month-old masu salmon fry was most sensitive and the cumulative mortality reached 87%. In 3- to 5-month-old fry, cumulative mortality decreased from 65% to 24% (Kimura et al., 1983c). Since 1988, herpesvirus had been isolated from the liver, kidney, and developing neoplasm in pond and pen-cultured coho salmon (Kumagai et al., 1994). Affected fish showed the following disease signs, ulcers on their skin, white spots on their liver and neoplastic tissues around their mouth part or body surface. Coho salmon culture is economically damaged by this disease. All of these viruses were neutralised by anti-OMV or NeVTA rabbit serum, and the oncogenicity was confirmed by experimental infection. Isolated virus showed strong pathogenicity to coho salmon. Massive mortality has occurred among 1-year-old rainbow trout in pond cultures since 1992 in Hokkaido. The diseased fish exhibited almost no clinical signs. Some fish did manifest ulcerative lesions on their skin. Internally, intestinal haemorrhage and white spots on the liver were observed. Epizootics occurred in cultured rainbow trout weighing 12 g to 1.5 kg at 18 fish farms from February 2000 to January 2001 in Nagano Prefecture, Japan. High infectivity titres about $10^8 TCID_{50} g^{-1}$ were demonstrated in the main internal organs and multiple necrotic foci were observed in the liver. The virus was identified as OMV using serological tests and polymerase chain reaction (PCR). In more than 80% cases, the outbreaks were linked with introductions of live fish (Furihata et al., 2003).

2.3.5. Environmental factors

General sanitation measures are standard practice in hatcheries. Special care must be taken to avoid the movement of equipment from one tank to another and all should be disinfected after use. Methods to sanitise a hatching unit should be carefully developed with respect to chemical toxicity for fish, effects of water temperature and their repeated use. It should be remembered that workers themselves might serve as efficient vectors for pathogens and proper disinfection of hands and boots are required to prevent dissemination of viruses. Although it may be difficult to sanitise hatching and rearing units during use, raceways and ponds should be disinfected with chlorine before and after use (Yoshimizu, 2009).

2.4. Control and prevention

OMV is sensitive to ultraviolet irradiation, ozone or iodophor treatment (Yoshimizu & Kasai, 2011). Since 1983, it has been strongly recommended as a control strategy that inspection of the ovarian fluid from mature fish and the disinfection of collected eggs in all hatcheries in Hokkaido with iodine at the early eyed stage be done. Currently OMV is no longer detected in most of the hatcheries in this area. Nowadays, all eggs and facilities had been disinfected by iodophor just after fertilization and again at the early eyed stage. As a result, OMV cannot be isolated

in Hokkaido and Tohoku area, and could avoid the outbreak of OMVD of masu salmon and coho salmon except rainbow trout (Furihata et al., 2003; Yoshimizu, 2009).

2.4.1. Vaccination

Vaccination of mature rainbow trout with formalin-inactivated OMV could reduce the positive ratio of OMV in ovarian fluid (Yoshimizu & Kasai, 2011). Also vaccination using formalin-inactivated OMV is very effective to protect the OMV infection at the fry stage (Yoshimizu & Kasai, 2011).

2.4.2. Chemotherapy

The therapeutic efficacy of ACV was evaluated using OMV and chum salmon fry. The fish were experimentally infected with OMV, and were treated with ACV either orally or by immersion. Daily immersion of fish into ACV solution (25 µg ml^{-1}, 30 minutes per day, 15 times) reduced mortality of the infected fish. Oral administration of the ACV (25 µg per fish per day, 60 times) did not affect survival of the chum salmon. On the contrary, the group administered IUdR by the oral route showed a higher survival than the ACV-administered group. This suggested that an effective level of ACV was not maintained in fish given the drug by the oral route. Daily immersion of infected fish into ACV solution (25 µg ml^{-1}, 30 minutes per day, 60 times) considerably suppressed the development of tumours induced by OMV (Kimura et al., 1983a; Kimura et al., 1983b).

2.4.3. Immunostimulation

There is currently no published information on the use of immunostimulants to control OMVD in salmonids. However, it is known to be an area of research interest.

2.4.4. Resistance breeding

There is currently no published information on the use of resistance breeding to control OMVD in salmonids.

2.4.5. Restocking with resistant species

Hybrids represent a potential control method to prevent serious losses from OMVD. Studies on a population of triploid hybrid salmonid (tetramer rainbow trout × brown trout) found them to be resistant to OMVD (Kohara & Denda, 2010).

2.4.6. Blocking agents

Not applicable.

2.4.7. Disinfection of eggs and larvae

Disinfection of eggs can be achieved by iodophor treatment. OMV has been shown to be inactivated by iodophor at 50 mg litre^{-1} for 15 minutes at 15°C or 25 mg litre^{-1} for 20 minutes at 15°C (Yoshimizu, 2009).

2.4.8. General husbandry practices

Biosecurity measures should include ensuring that new introductions of fish are from disease-free sources and installation of a quarantine system where new fish can be held with sentinel fish at permissive temperatures for OMVD. The fish are then quarantined for a minimum of 4 weeks to 2 months before transfer to the main site and mixing with naïve fish. Hygiene measures on site should be similar to those recommended for IHN and include disinfection of eggs, regular disinfection of ponds, chemical disinfection of farm equipment, careful handling of fish to avoid stress and safe disposal of dead fish.

3. Sampling

3.1. Selection of individual specimens

3.1.1. Clinically affected fish

Whole alevin (body length ≤ 4 cm), viscera including kidney (4 cm ≤ body length ≤ 6 cm) or, for larger size fish, skin ulcerative lesions or neoplastic tissues, and kidney, spleen, liver and encephalon.

3.1.2. Apparently healthy fish

Kidney, spleen and encephalon (any size fish) and/or ovarian fluid from broodfish at spawning time.

3.2. Preservation of samples for submission

Whole fish should be sent to the laboratory alive or killed and packed separately in sealed aseptic containers. However, it is highly preferable and recommended to collect organ samples from the fish immediately after they have been selected at the fish production site. Whole fish or selected organ samples should be sent to the laboratory in refrigerated containers (+0°C to 5°C) with ice. The freezing of collected fish or dissected organs should be avoided.

3.3. Pooling of samples

When testing clinically affected fish by cell culture method or PCR-based methods, pooling of samples should be avoided or restricted to a maximum of five fish per pool. For health surveillance testing by cell culture methods, samples should be tested in a maximum of five fish per pool.

3.4. Best organs or tissues

3.4.1. Clinically affected fish

Whole alevin (body length ≤ 4 cm), viscera including liver or kidney (4 cm ≤ body length ≤ 6 cm) or, for larger size fish, skin ulcerative lesions or neoplastic tissues, and liver or kidney.

3.4.2. Apparently healthy fish

Liver, kidney, spleen and encephalon (any size fish) and/or ovarian fluid from brood fish at spawning time.

3.5. Samples/tissues that are not suitable

Fish carcasses showing very advanced signs of tissue decomposition may not be suitable for testing by any methods.

4. Diagnostic methods

4.1. Field diagnostic methods

4.1.1. Clinical signs

Clinically, the initial infection by OMV appears as a systemic and frequently lethal infection that is associated with oedema and haemorrhages. Virus multiplication in endothelial cells of blood capillaries, haematopoietic tissue and hepatocytes underlies the clinical signs (Kimura et al., 1981b). Four months after this first clinical condition, a varying number of surviving fish exhibit epithelioma occurring mainly around the mouth (upper and lower jaw) and, to a lesser extent, on the caudal fin, operculum and body surface (Kimura et al., 1981a). This neoplasia may persist for up to 1 year post-infection. In the case of coho salmon, 1-year-old infected fish in particular show ulcers on the skin, white spots on the liver and neoplastic tissues around the mouth parts or body surface. In rainbow trout, the diseased fish exhibit almost no external signs, although some fish manifest ulcerative lesions on the skin. Internally, intestinal haemorrhage and white spots on the liver are observed (Yoshimizu & Kasai, 2011).

4.1.2. Behavioural changes

Fish become lethargic, gather at the water outlet or sides of a pond. Some fish may experience loss of equilibrium and disorientation.

4.2. Clinical methods

4.2.1. Gross pathology

Gross signs in infected fish are inappetence and exophthalmia, and petechiae on the body surface, especially beneath the lower jaw. Agonal or abnormal swimming behaviour has not been observed. Internally, the liver

shows white spots lesions, and in advanced cases the whole liver becomes pearly white. In some cases the spleen is found to be swollen. The digestive tract is devoid of food (Kimura et al., 1981b).

4.2.2. Clinical chemistry

No published information available.

4.2.3. Microscopic pathology

The kidney of OMV-infected 1- and 3-month old masu salmon, 1-month-old coho salmon and 2-month-old chum salmon is the principal target organ for the virus as judged by the severity of histopathological changes found in infected 1-month-old masu salmon. Necrosis of epithelial cells and kidney were observed in the early moribund specimens while partial necrosis of the liver, spleen and pancreas was seen in later moribund specimens from this group. Necrosis of the kidney haematopoietic tissue was observed in infected 3-month-old masu salmon. While, the kidney was considered to be the early target organ for OMV, it gradually became resistant to OMV infection. For this reason, it was considered that the principal target organ moved from the kidney to the liver and marked histopathological changes were observed in the later stages. Foci of necrosis in the liver tended to become more severe with a longer incubation period. Hepatocytes showing margination of chromatin were present. Cell degeneration in the spleen, pancreas, cardiac muscle and brain was also observed (Kimura et al., 1983c). Histopathological changes observed in coho salmon and chum salmon were the same as those of masu salmon (Tanaka et al., 1984). In the case of rainbow trout, high infectivity titres were demonstrated in the main internal organs and multiple necrotic foci were observed in the liver. The definite change was necrosis of OMV-infected cells, which were observed in the spleen, haematopoietic tissues in the kidney, liver, intestine, heart, gill filaments, epidermis and lateral musculature. In particular, the intestines showed severe necrosis and haemorrhage in the epithelium and underlying tissues, which is the new description of rainbow trout OMVD (Furihata et al., 2003).

4.2.4. Wet mounts

OMV has been identified in touch imprints of kidney by indirect fluorescence antibody test (IFAT).

4.2.5. Smears

No published information available.

4.2.6. Electron microscopy/cytopathology

Viral particles have been detected by transmission electron microscopy (TEM) examination of liver tissues from clinically infected chum salmon, masu salmon, coho salmon and rainbow trout (Kimura et al., 1980; Tanaka et al., 1984). Electron microscopy of infected cells reveals that the intra nuclear hexagonal capsids have a diameter of 115 nm. Abundance of budding, enveloped virions, 200 × 240 nm in diameter, are also observed on the surface and inside cytoplasmic vesicles. The calculated number of capsomeres of negatively stained virions is 162. These features confirm that OMV is a herpesvirus.

4.3. Agent detection and identification methods

The agent's infectivity remains unchanged for at least 2 weeks at 0°C to 5°C, but at –20°C, 99.9% of the infectivity is lost within 17 days. Virus isolation should be carried out using fish, transported on ice to the laboratory. For filtration of the OMV, a 0.40 µm nucleopore filter (polycarbonate) is recommended because cellulose acetate membrane filter traps virus particles. For the purpose of a virological survey of mature salmonid, ovary fluid is collected by the method described by Yoshimizu et al., 1985, with the addition of the same volume of antibiotic and reacted at 5°C, overnight. In the case of the tumour tissue, tissue is cut and disinfected with iodophor, then washed with Hank's BSS and transported with antibiotic solution to the laboratory. Tumour tissues must be prepared for the primary culture or co-culture with RTG-2 cells. After the one subculture of primary culture cells, the virus inspection of the culture medium should be carried out. Usually RTG-2 cells are harvested and inoculated, suitable incubation temperature is 15°C. In the laboratory, rabbit serum or monoclonal antibody against OMV was used for a fluorescent antibody test (Hayashi et al., 1993), and also DNA probe was used for detection of virus genome (Gou et al., 1991). PCR using a F10 primer and R05 primer (Aso et al., 2001) amplified a 439 base-pair segment of DNA from OMV strains isolated from masu salmon, coho salmon and rainbow trout, and liver, kidney, brain and nervous tissues. Agarose gel profile of amplified DNA was able to distinguish OMV and *H. salmonis* (Aso et al., 2001).

4.3.1. Direct detection methods

OMV has been identified in touch imprints of kidney by IFAT. The most commonly used method for detection of OMV directly in fish tissues is using PCR method specific for OMV.

4.3.1.1. Microscopic methods

4.3.1.1.1. Wet mounts

Virus antigens have been detected in infected tissues by IFAT. In the case of coho salmon, pond cultured fish are transplanted to net pens in the sea. Kidney tissues were pressured hard to adapt to the marine environment. During this period, OMV replicate and OMV antigen appeared in kidney tissues. Indirect fluorescent antibody method is useful and effective to detect the OMV infected fish (Kumagai et al., 1994).

4.3.1.1.2. Smears

No published information available.

4.3.1.1.3. Fixed sections

The method detailed in Section 4.3.1.1.1above is also suitable for detection of OMV antigen in paraffin wax tissue sections fixed in 10% neutral buffered formalin (NBF). A common treatment is incubation of the sections with 0.1% trypsin in PBS at 37°C for 30 minutes. The sections are then washed in cold PBS.

4.3.1.2. Agent isolation and identification

4.3.1.2.1. Cell culture/artificial media

Cell line to be used: *RTG-2* or *CHSE-214*.

4.3.1.2.1.1. Inoculation of cell monolayers

i) Make an additional tenfold dilution of the 1/10 organ homogenate supernatants and transfer an appropriate volume of each of the two dilutions on to 24-hour-old cell monolayers. Inoculate at least 2 cm^2 of drained cell monolayer with 100 µl of each dilution.

ii) Allow to adsorb for 1 hour at 15°C and, without withdrawing the inoculate, add cell culture medium buffered at pH 7.4 and supplemented with 2% fetal bovine serum (FBS) (1 ml/well for 24-well cell culture plates), and incubate at 15°C.

4.3.1.2.1.2. Monitoring incubation

i) Follow the course of infection in positive controls and other inoculated cell cultures by microscopic examination at ×4 or 10 magnification for 14 days. The use of a phase-contrast microscope is recommended.

ii) Maintain the pH of the cell culture medium at between 7.2 and 7.4 during incubation. This can be achieved by the addition of sterile bicarbonate buffer (for tightly closed cell culture flasks) or Tris buffer solution (for cell culture plates) to the inoculated cell culture medium or, even better, by using HEPES-buffered medium (HEPES = N-2-hydroxyethyl-piperazine-N-2-ethanesulfonic acid).

iii) If a cytopathic effect (CPE) appears in those cell cultures inoculated with the dilutions of the tested homogenate supernatants, identification procedures have to be undertaken immediately (see Neutralisation test 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 or the zone (if it was approved previously) from which the virus-positive sample originated. The suspension of approved status will be maintained until it is demonstrated that the virus in question is not OMV.

iv) If no CPE develops in the inoculated cultures (despite normal progression of CPE in the virus controls), the inoculated cultures should be subcultured for a further 7 days. Should the virus controls fail to develop CPE, the process should be repeated with fresh susceptible cells and new batches of samples.

4.3.1.2.1.3. Subcultivation procedures

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

ii) If required, repeat the neutralisation test to infectious pancreatic necrosis virus (IPNV) and/or infectious haematopoietic necrosis virus (IHNV) as previously described (see Chapter 2.3.0., Section 2.2.3.), with a dilution of the above supernatant (1/1 to 1/100).

iii) Inoculate cell monolayers as described above.

iv) Incubate and monitor as described above.

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

4.3.1.2.1.4. Isolation of OMV from cultures of neoplastic cells

i) Collect neoplastic tissues, disinfect with iodophor, 50 parts per million for 20 minutes, and wash three times with Hanks' balanced salt solution.

ii) The tissues are left overnight in 0.25% trypsin in phosphate buffered saline (PBS) at 5°C. Then, 3.5×10^5 neoplastic cells/ml are seeded in a tissue culture flask and incubated with culture medium containing 20% fetal bovine serum (FBS).

iii) Harvest the primary neoplastic cell culture and co-cultivate with RTG-2 or CHSE-214 cells.

iv) Incubate and monitor as described above.

4.3.1.2.2. Antibody-based antigen detection methods

Neutralisation test

i) Collect the culture medium of the cell monolayer exhibiting CPE and centrifuge at 2000 ***g*** for 15 minutes at 4°C to remove cell debris.

ii) Dilute the virus-containing medium from 10^2 to 10^4 ml^{-1}.

iii) Mix aliquots (for example 200 µl) of each virus dilution with equal volumes of an antibody solution specific for OMV, and similarly treat aliquots of each virus dilution with cell culture medium.

   (The neutralising antibody [NAb] solution must have a 50% plaque reduction titre of at least 2000.)

iv) In parallel, other neutralisation tests must be performed against:

   • a homologous virus strain (positive neutralisation test),

   • a heterologous virus strain (negative neutralisation test).

v) If required, a similar neutralisation test may be performed using antibodies to IPNV, to ensure that no IPNV contaminant has escaped the first anti-IPNV test.

vi) Incubate all the mixtures at 15°C for 1 hour.

vii) Transfer aliquots of each of the above mixtures on to cell monolayers (inoculate two cell cultures per dilution) and allow adsorption to occur for 0.5~1 hour at 15°C; 24- or 12-well cell culture plates are suitable for this purpose, using a 50 µl inoculum.

viii) When adsorption is completed, add cell culture medium supplemented with 2% FCS and buffered at pH 7.4~7.6 into each well and incubate at 10~15°C.

ix) Check the cell cultures for the onset of CPE and read the results as soon as it occurs in non-neutralised controls (cell monolayers being protected in positive neutralisation controls). Results are recorded either after a simple microscopic examination (phase-contrast preferable) or after discarding the cell culture medium and staining the cell monolayers with a solution of 1% crystal violet in 20% ethanol.

x) The tested virus is identified as OMV when CPE is prevented or noticeably delayed in the cell cultures that had received the virus suspension treated with the OMV-specific antibody, whereas CPE is evident in all other cell cultures.

xi) In the absence of any neutralisation by NAb to OMV, it is mandatory to conduct an IFAT with the suspect sample.

4.3.1.2.3. Molecular techniques

PCR (Aso et al., 2001).

i) Extract nucleic acid from cells infected with OMV strain OO-7812 and *H. salmonis* using the InstGene Matrix (Biorad[2]).

ii) Pellet the virus-infected tissues or infected cultured cells by centrifugation at 19,000 *g* for 15 minutes.

iii) Wash the pellets twice with 1 ml PBS and mix with 200 µl of chelating resin (Sigma).

iv) Incubate the mixture at 56°C for 20 minutes in a water bath, vortex it, and then place it in a boiling water bath for 8 minutes.

v) Vortex the samples and centrifuge at 8200 *g* (10,000 rpm) for 90 seconds.

vi) Subject the supernatant to PCR.

vii) The forward primer (F10) is 5'-GTA-CCG-AAA-CTC-CCG-AGT-C-3', and the reverse primer (R5) is 5'-AAC-TTG-AAC-TAC-TCC-GGG-G-3'.

viii) Incubate the specimens, primer sets and reaction mixtures for 30 cycles in an automatic thermal cycler (GeneAmp PCR 9700, Applied Biosystems), with each cycle consisting of denaturation at 94°C for 30 seconds, annealing at 56°C for 30 seconds and extension at 72°C for 30 seconds.

ix) Analyse the amplified product for size and purity by electrophoresis (100 V for 30 minutes) in 2% agarose gel and stain with ethidium bromide.

x) A PCR using these primer sets amplified a 439 base-pair segment of DNA from OMV strains isolated from masu salmon, coho salmon and rainbow trout, and liver, kidney, brain and nervous tissues, and an 800 base-pair segment of DNA from SalHV-1. SalHV-1 and SalHV-2 could be distinguished by agarose gel profile of this amplified DNA (Aso et al., 2001).

4.3.2. Serological methods

4.3.2.1. Indirect fluorescent antibody test

i) Prepare monolayers of cells in 2 cm² wells of cell culture plastic plates or on cover-slips in order to reach around 80% confluency, which is usually achieved within 4 hours of incubation at 22°C (seed six cell monolayers per virus isolate to be identified, plus two for positive and two for negative controls). The FBS content of the cell culture medium can be reduced to 2–4%. If numerous virus isolates have to be identified, the use of Terasaki plates is strongly recommended.

ii) When the cell monolayers are ready for infection, i.e. on the same day or on the day after seeding, inoculate the virus suspensions to be identified by making tenfold dilution steps directly in the cell culture wells or flasks.

iii) Dilute the control virus suspension of OMV in a similar way, in order to obtain a virus titre of about 5000-10,000 plaque-forming units (PFU) ml^{-1} in the cell culture medium.

iv) Incubate at 15°C for 48 hours.

v) Remove the cell culture medium, rinse once with 0.01 M PBS, pH 7.2, then three times briefly with cold acetone (stored at –20°C) for cover-slips or a mixture of acetone 30%/ethanol 70%, also at –20°C, for plastic wells.

vi) Let the fixative act for 15 minutes. A volume of 0.5 ml is adequate for 2 cm² of cell monolayer.

vii) Allow the cell monolayers to air-dry for at least 30 minutes and process immediately or freeze at –20°C.

viii) Prepare a solution of purified antibody or serum to OMV in 0.01 M PBS, pH 7.2, containing 0.05% Tween 80 (PBST), at the appropriate dilution (which has been established previously or is given by the reagent supplier).

ix) Rehydrate the cell monolayers by four rinsing steps with the PBST solution, and remove this buffer completely after the last rinsing.

x) Treat the cell monolayers with the antibody solution for 1 hour at 37°C in a humid chamber and do not allow evaporation to occur. The volume of solution to be used is 0.25 ml per 2 cm² well.

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2 Reference to specific commercial products as examples does not imply their endorsement by the OIE. This applies to all commercial products referred to in this *Aquatic Manual*.

xi) Rinse four times with PBST as above.

xii) Treat the cell monolayers for 1 hour at 37°C with a solution of fluorescein isothiocyanate (FITC)-conjugated antibody to the immunoglobulin used in the first layer and prepared according to the instructions of the supplier. These FITC antibodies are most often rabbit or goat antibodies.

xiii) Rinse four times with PBST.

xiv) 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.

xv) Examine under incident UV light using a microscope with ×10 eye pieces and ×20–40 objective lens having numerical aperture > 0.65 and > 1.3, respectively. Positive and negative controls must be found to give the expected results prior to any other observation.

4.3.2.2. Enzyme-linked immunosorbent assay

i) Coat the wells of microplates designed for enzyme-linked immunosorbent assays (ELISAs) with appropriate dilutions of monoclonal antibody or purified immunoglobulins (Ig) specific for OMV, in 0.01 M PBS, pH 7.2 (200 µl/well).

ii) Incubate overnight at 4°C.

iii) Rinse four times with 0.01 M PBS containing 0.05% Tween 20 (PBST).

iv) Block with skim milk (5% in PBST) or other blocking solution for 1 hour at 37°C (200 µl/well).

v) Rinse four times with PBST.

vi) Add 2% Triton X-100 to the virus suspension to be identified.

vii) Dispense 100 µl/well of a two- or four-step dilution of the virus to be identified and of OMV control virus, and allow to react with the coated antibody to OMV for 1 hour at 20°C.

viii) Rinse four times with PBST.

ix) Add to the wells, biotinylated polyclonal antibody to OMV.

x) Incubate for 1 hour at 37°C.

xi) Rinse four times with PBST.

xii) Add streptavidin-conjugated horseradish peroxidase to those wells that have received the biotin-conjugated antibody, and incubate for 1 hour at 20°C.

xiii) Rinse four times with PBST.

xiv) Add the substrate and chromogen. Stop the course of the test when positive controls react, and monitor the results.

5. Rating of tests against purpose of use

As an example, the methods currently available for targeted surveillance and diagnosis of OMVD 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	b	b	b	b	c
Cell culture	b	b	b	b	b	a
Direct LM	d	d	d	d	d	d

Table 5.1. *Methods for targeted surveillance and diagnosis*

Method	Targeted surveillance				Presumptive diagnosis	Confirmatory diagnosis
	Larvae	PLs	Juveniles	Adults		
Hispathology	b	b	b	b	b	a
Transmission EM	c	c	c	c	c	c
Antibody-based assays	b	b	b	b	b	a
DNA Probes *in situ*	c	c	c	c	c	c
PCR	b	b	b	b	a	a
Sequence	d	d	d	d	d	d

PLs = postlarvae; LM = light microscopy; EM = electron microscopy; PCR = polymerase chain reaction.

6.   Test(s) recommended for targeted surveillance to declare freedom from *Oncorhynchus masou* virus disease

Information on the distribution and incidence of OMV is important for the prevention of transmission to the progeny from mature salmonids. Therefore, study of the occurrence of OMV among mature salmonid fish is important. Sixty fish were sampled and specimens were collected individually. Ovarian fluid specimens were collected according to the method of Yoshimizu et al., 1985. A sterilised automatic pipette tip was inserted into the urogenital opening of the mature fish. One milliliter of ovarian fluid taken from the fish was treated by the antibiotic treatment method. Antibiotic treated specimens were transported to the laboratory with ice.

7.   Corroborative diagnostic criteria

7.1. Definition of suspect case

OMV shall be suspected if at least one of the following criteria is met:

i)   The presence of typical clinical signs of OMVD in a population of susceptible fish.

ii)   Presentation of typical histopathology in liver tissue sections consistent with OMVD.

iii)   A single positive result from one of the diagnostic assays such as IFAT on liver or kidney tissue imprints or PCR.

iv)   Transfer of live fish from a site where presence of OMV has been confirmed, or is suspected, because of the presence of clinical disease, to sites without suspicion of OMV.

v)   Antibodies to OMV have been detected.

7.2. Definition of confirmed case

The following criteria should be met for confirmation of OMV:

i)   Mortality, clinical signs and pathological changes consistent with OMV disease and detection of OMV by one or more of the following methods:

a)   Isolation and identification of OMV in cell culture from at least one sample from any fish on the site as described in Section 4.3.1.2.1;

b)   Detection of OMV by PCR by the methods described in Section 4.3.1.2.3;

c)   Detection of OMV in tissue preparations by means of specific antibodies against OMV (e.g. IFAT on tissue imprints as described in Section 4.3.2.

ii)   In the absence of mortality or clinical signs by one or more of the following methods:

a)   Detection and confirmation of OMV by PCR by the methods described in Section 4.3.1.2.3;

b)   Positive results from two separate and different diagnostic assays described above.

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