CHAPTER 2.1.17.

WHITE STURGEON IRIDO VIRAL DISEASE

SUMMARY

The white sturgeon iridoviral disease (WSIVD) is a significant cause of mortality among farm-raised juvenile white sturgeon (Acipenser transmontanus) in North America and among Russian sturgeon (A. guldenstadi) in Europe (1, 2, 4). White sturgeon are the host from which the causative agent, the white sturgeon iridovirus (WSIV) was first isolated. Lake sturgeon (A. fluvescens) have been experimentally infected with the WSIV, but the susceptibility of other sturgeon is currently unknown (3).

The original description of white sturgeon iridovirus was among the first hatchery-raised white sturgeon in North America. The source of the virus was assumed to originate from captive wild sturgeon adults collected from the Sacramento River in California, United States of America (USA) for use as broodstock (2). The WSIV has also been detected in cultured white sturgeon from the lower Columbia River in Oregon and Washington states of the USA, the Snake River in the southern region of the State of Idaho and the Kootenai River in northern Idaho, USA (4, 5). These cultured white sturgeon were all progeny originating from captured wild adult white sturgeon. The virus has been detected among wild juvenile white sturgeon collected from the lower Columbia River and the virus is potentially enzootic in wild white sturgeon populations throughout the Pacific Northwest of North America (4). An iridovirus similar to WSIV has been identified in Russian sturgeon from Northern Europe where it may be enzootic among cultured populations of several species of sturgeon (1).

The WSIV is an epitheliotropic virus infecting the skin, gills, and upper alimentary tract. Infections of the oral mucosa and olfactory organ epithelium are presumed causes of the cessation of feeding that leads to a progressive emaciation or starvation of the fish - the principal external sign of the disease (6). Cumulative mortality of up to 95% has been reported among groups of infected fish in the hatchery and secondary infections with external protozoa or bacteria often contribute to the overall mortality (2). Infected fish with moderate to severe emaciation began dying 2-3 weeks following exposure to the virus at water temperatures of 17-19°C (7). Haemorrhages on the abdomen and the ventral scuta may be present, but these are not specific for WSIVD. There are no specific internal signs of infection as the virus does not invade systemically. Viral infection is evident on microscopic observation of stained tissue sections from infected fish. Areas of the integument and particularly the skin may show a focal to diffuse hyperplasia with characteristic amphiphilic to basophilic enlarged Malpighian cells (6). These cells are filled with virus particles as demonstrated by electron microscopy. The virus can be isolated, but with some difficulty, from infected fish using sturgeon cell lines.

The modes of transmission of WSIV are not completely understood but horizontal transmission via the water has been demonstrated in the hatchery and experimentally in the laboratory (3). There is strong circumstantial evidence from epidemiological investigations at the hatchery that the virus is transmitted vertically from adult broodstock, but the virus has never been isolated or observed in adult fish.

There appears to be little antigenic relationship of WSIV to the systemic iridoviral agents represented by epizootic haematopoietic necrosis virus or the red sea bream iridovirus. The larger size and inner membrane structure of WSIV virion, host cell-line specificity, type of cytopathic effect, and location of target host cells (epitheliotropic and not systemic) distinguish the agent from other groups of fish iridoviruses. Although lymphocystivirus (LCDV) has a similar virion morphology, LCDV infects fibroblasts and not Malpighian cells as in WSIV infections, and the cell line specificity and types of cytopathic effects are clearly different between the two agents.

The principal diagnostic methods for WSIV include microscopic observation of characteristic infected cells in stained tissue sections of the oral mucosa, gills and skin, or isolation of the virus in sturgeon cell lines (3). Neutralising polyclonal antibodies and binding monoclonal antibodies recognise WSIV, but not the systemic iridoviral agents. These antibodies can also be used in indirect immunofluorescence tests or for immunohistochemical staining of infected cells in tissue culture and sections of infected tissues.
Control methods currently rely on avoidance of the agent where possible. Because there are currently no methods for detecting the virus in adult broodstocks, quarantine and investigation of juveniles suffering mortality are the principal means to detect WSIV in young fish. Methods to detect the virus in broodstock are currently under development.

DIAGNOSTIC PROCEDURES

The diagnosis of white sturgeon iridovirus (WSIV) is based on the observation of pathognomonic infected cells in stained tissue sections from infected fish and isolation of the virus using sturgeon cell lines. Confirmation of WSIV infection relies on neutralisation of the isolated virus with polyclonal antibodies. The specific binding of monoclonal antibodies (MABs) to viral antigens in infected cell cultures or impression smears from infected fish tissues are under investigation as alternatives to cell culture isolation and virus neutralisation for identification of WSIV.

Infected fish material suitable for virological examination is:

- Clinically affected fish: Fish <6 cm: 1) for virus isolation - gill arches and skin from the fleshy portions of the mouth (oral flap) and fins; 2) for histology - a sagittal section to include gills and the epidermis of the head region, also include larger fins. For larger fish - portions of the gill and fleshy part of the oral flap and punches or portions from fleshy part of larger fins. These tissues can be used for both virus isolation and histological examinations.

- Asymptomatic fish (apparently healthy fish): As above.

1. STANDARD SCREENING METHOD FOR WSIV

1.1. Isolation of WSIV in cell culture

Cell lines to be used: WSS-2 (white sturgeon spleen) or WSSK-1 (white sturgeon skin) cells

Cells should be grown at 20°C in a temperature-controlled incubator using standard minimal essential medium (MEM) supplemented with 10% fetal calf serum (FCS) that can be reduced to 5% after virus inoculation.

a) Inoculation of cell monolayers

i) Remove a portion of the gill, oral flap, and abdomen and prepare a supernatant from a homogenate to yield a final tissue dilution of 1/50. Prepare a second dilution representing 1/100 (w/v). Transfer an appropriate volume of each of the two dilutions on to 24-hour-old cell monolayers. Inoculate at least 2 cm² of drained cell monolayer with 100 µl of each dilution.

ii) Allow to adsorb for 0.5–1 hour at 20°C and, without withdrawing the inoculate, add the cell culture medium buffered at pH 7.6 and supplemented with 5% FCS (2 ml/well for 12-well cell culture plates), and incubate at 20°C using a temperature-controlled refrigerated incubator to ensure successful isolation.
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b) Monitoring incubation

i) Follow the course of infection in inoculated and control cell cultures by daily microscopic examination at ×40 to ×100 magnification for 30 days.

ii) If a cytopathic effect (CPE) appears in those cell cultures inoculated with the dilutions of tested homogenate supernatants, identification procedures have to be undertaken immediately (see Section 1.2. 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 virus positive sample originated. The suspension of approved status will be maintained until it is demonstrated that the virus in question is not WSIV.

iii) 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 15 days. Should the virus control fail to develop CPE, the process should be repeated with fresh susceptible cells and new batches of samples.

c) Subcultivation procedures

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

ii) Inoculate cell monolayers as described above in Section 1.1.a.

iii) Incubate and monitor as described above in Section 1.1.b.

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

1.2. Identification of WSIV

a) Neutralisation test

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

ii) Dilute the virus-containing medium from 10⁻¹ to 10⁻³.

iii) Mix aliquots (for example 200 µl) of each virus dilution with equal volumes of rabbit anti-WSIV serum diluted 1/100, and similarly treat aliquots of each virus dilution with cell culture medium.

iv) Incubate all mixtures at 20°C for 1 hour.

v) 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 20°C; 24- or 12-well culture plates are suitable for this purpose, using a 50 µl inoculum.

vi) When adsorption is completed, add cell culture medium supplemented with 5% FCS and buffered to pH 7.4–7.6 into each well and incubate at 20°C.

b) Indirect fluorescent antibody test

This indirect fluorescent antibody test (IFAT) is to be conducted directly after virus isolation in cell culture.

i) Prepare monolayers of WSS-2 cells in 2 cm² wells of cell culture plastic plates or on cover-slips in order to reach around 80% confluency. The FCS content of the cell culture medium can be reduced to 5%.

ii) When the cell monolayers are ready for infection, 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 WSIV in a similar way, in order to obtain a virus titre of about 10⁰ TCID₅₀ (50% tissue culture infective dose) per ml in the cell culture medium.

iv) Incubate at 20°C for 7 days
v) Remove the cell culture medium, rinse once with 0.01 M phosphate buffered saline (PBS), pH 7.2, then three times briefly with cold fixative. This fixative will be acetone (stored at -20°C) for cover-slips or a mixture of acetone 30%/ethanol 70% (v/v) for plastic, also stored at -20°C.

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 Mabs to WSIV in 0.01M PBS, pH 7.2, containing 2% skim milk, at the appropriate dilution.

ix) Rehydrate the dried cell monolayers by four rinsing steps with PBS 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. The volume of solution to be used is 0.25 ml/2 cm² well.

xi) Rinse four times with PBS as above.

xii) Incubate the cell monolayers in appropriately diluted biotinylated anti-mouse antibody (in 2% skim milk in PBS) at 37°C for 1 hour.

xiii) Rinse four times with PBS as above.

xiv) Treat the cell monolayers for 1 hour at 37°C with a solution of FITC conjugate (FITC = fluorescein isothiocyanate).

xv) Rinse four times with PBS.

xvi) Treat the cell monolayers on plastic plates immediately, or mount with cover-slips using glycerol saline at pH 8.5 prior to microscopic observation.

xvii) 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. Positive results are indicated by diffuse fluorescence throughout the cytoplasm.

2. Diagnostic Methods for WSIV

Confirmation of WSIV outbreaks depends on the observation of clinical signs of disease among affected sturgeon, presence of pathognomonic infected cells in stained tissue sections or isolation of the virus in cell culture followed by its identification in a neutralisation test. Immunostaining of cells in target tissues with antibodies to WSIV can replace the need to conduct electron microscopy to view the virions of WSIV.

2.1. Virus isolation and subsequent identification

As in Sections 11 and 12.

2.2. Observation of infected cells in stained tissue sections

a) For fish <6 cm in length, preserve the entire fish in histological fixative

i) Prepare sagittal sections (off midline to include gills) of entire fish for hematoxylin and eosin staining.

ii) Observe the epithelium of the gills, fins (pectoral and caudal) and body including the mouth and esophagus, for evidence of enlarged cells with an amphophilic to basophilic staining cytoplasm and a hypertrophic irregular nucleus.

b) For fish >6 cm in length, remove one gill arch and a 1 cm² portion of the fleshy section of mouth (oral flap), and a punch sample and a similar sized piece of skin from the abdomen.

Fix tissues and prepare stained tissue sections for observation of characteristic infected cells as described above.

2.3. Immunohistochemical test

Begin with tissues fixed in 10% neutral buffered formalin used for histological examinations above.

i) Deparaffinise sections and rehydrate
a. Xylene-1 for 5 minutes
b. Xylene-2 for 5 minutes

c. 100% EtOH for 3 minutes
Ring tissue area with a PAP pen

d. 95% EtOH for 3 minutes

e. 70% EtOH for 3 minutes

f. Rinse in deionised water for 3 minutes

ii) Inactivate endogenous peroxidase by soaking slides in 0.3% H₂O₂ in methanol for 30 minutes at room temperature.

iii) Rinse slide with water then gently wash with PBS.

iv) Block for 30 minutes in PBS with 10% goat serum.

v) Shake off block solution and add 20 µl of biotinylated IIIA11 or IIC7 mouse anti-WSIV MAb in PBS (3 µg/ml) to the section and incubate for 60 minutes at room temperature.

vi) Wash three times.

vii) Add peroxidase-streptavidin (1:1000) in PBS for 10 minutes.

viii) Wash three times.

ix) Stain with AEC (aminoethyl carbazole) for 5-15 minutes at 25°C. Monitor the level of development by viewing the slide on a microscope. Stop development by immersing in PBS.

x) Wash three times.

xi) Counterstain with Mayer’s haematoxylin for 5 minutes at room temperature.

xii) Wash three times.

xiii) Mount slide while wet with Crystal/Mount™ aqueous/dry mounting medium. (AEC substrate is soluble with toluene or organic based mounting media.) After drying overnight, a cover-slip may be mounted on the section using an organic solvent-based mounting media such as Krystalon Mounting Media.
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**PBS – 2 litres of x 10**

1. **80 g. NaCl (0.137 M)**
2. **4.0 g. KCl (0.003 M)**
3. **28.8 g. Na2HPO4 (0.01 M)**
4. **4.8 g. KH2PO4 (0.002 M)**
5. pH to 7.2–7.4 and Q.S. to 2 litres with d.d. H2O.


**Tris buffered saline (TBS)**

<table>
<thead>
<tr>
<th>1 litre</th>
<th>2 litres</th>
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<tbody>
<tr>
<td>50 mM Tris base</td>
<td>6.07 g</td>
</tr>
<tr>
<td>1 mM EDTA</td>
<td>0.409 g</td>
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<tr>
<td>150 mM NaCl</td>
<td>8.7 g</td>
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<tr>
<td>pH to 8.0 with HCl</td>
<td>~2.5 ml conc.</td>
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**TTBS**

To make TTBS add 0.1% Tween-20 (1 ml/litre).

**REFERENCES**


