Aquatic Birnavirus Infections of Finfish

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Part 1. Diagnostic Overview

Summary

Aquatic birnaviruses (aquabirnaviruses), which are double-stranded, bisegmented RNA viruses belonging to the Family Birnaviridae, include both virulent and avirulent viruses of aquatic animal species. Isolates that are pathogenic for fish species within the Family Salmonidae are known as infectious pancreatic necrosis (IPN) viruses, and they are of particular economic importance to the salmonid industries of Norway and Scotland. Tasmanian aquabirnavirus (TAB), which is the only known aquabirnavirus in Australia, has been found in wild and farmed fish in Macquarie Harbour, Tasmania. It has never been associated with mortalities in freshwater hatcheries.

IPN viruses may cause an acute, contagious disease of farmed salmonids (rainbow trout Oncorhynchus mykiss*; brook trout* Salvelinus fontinalis*; brown trout* Salmo trutta*; Atlantic salmon* Salmo salar*; and several Pacific salmon species* Oncorhynchus spp*) with*

up to 100% mortality in young first-feeding fry. Usually, susceptibility tends to decrease with age but, more recently, mortalities have also been recorded in older Atlantic salmon smolt (after transfer from freshwater to seawater) in some Nordic and Scottish farms. The main microscopic lesions are in the pancreas and intestine. Survivors may become life-long carriers of the virus.

IPN occurs in most of the major salmonid farming countries of the world. Aquatic birnaviruses have been described from most regions of the world, including Australia and New Zealand. However, in both these countries, the viruses are restricted in their distribution to localised marine environments, and there is no evidence of disease at freshwater sites. Consequently, both countries are still regarded as being free from IPN.

*Other pathogenic aquatic birnaviruses related to IPNV have been reported in other farmed marine species, for example, yellowtail (*Seriola quinqueradiata*) and other, seemingly non-pathogenic aquabirnaviruses have been detected in a wide range of estuarine and freshwater fish species.*

Diagnosis of aquabirnaviruses is based on a range of procedures. A presumptive diagnosis of a virulent aquabirnavirus may be made on clinical and gross pathological signs, but there may be no gross evidence of infection with avirulent strains. A definitive diagnosis of any aquabirnavirus depends upon detection of virus and/or viral antigen and/or viral genome in affected tissues or following virus isolation in cell culture.

Aetiology

The genus *Aquabirnavirus* includes icosahedral, double-stranded (ds), bisegmented RNA viruses with a non-enveloped capsid belonging to the Family Birnaviridae. Viruses within this genus are assigned to one of two serogroups: A, within which there are nine serotypes, and B, containing just one serotype.[1] Tasmanian aquabirnavirus (TAB) is included in serogroup A.[2] In addition, six genogroups based on analysis of 28 aquatic birnavirus isolates including the type strains of all nine serotypes of serogroup A have been demonstrated.[3]

The genus includes both virulent and avirulent viruses with the term 'infectious pancreatic necrosis' (IPN) virus being reserved for those isolates that are pathogenic for species within the Family Salmonidae. The term 'marine aquabirnaviruses' (MABV), on the other hand, refers to a group of viruses that has been largely isolated from various (non-salmonid) aquatic species[4] including invertebrates[5] around Japan[6] and Korea[7]. The type strain of MABV is yellowtail ascites virus (YAV), first isolated from diseased yellowtail fingerlings.[8] The taxonomic status of these viruses is not clear but since they appear to be closely related to each other and distinct, serologically and genotypically, from other aquabirnaviruses, it has been proposed that these viruses form a seventh genogroup.[9,10]

Clinical signs

IPN virus may cause an acute disease in young salmonids with clinical signs typical of a systemic infection.[11] These signs include: darkening of the skin; exophthalmia; abdominal swelling (especially in Atlantic salmon); and cutaneous petechiae, especially at the base of the fins. Cast-like pseudofaeces and abnormal behavioural signs may also be seen, the latter ranging from lethargy to short bursts of erratic swimming (for example, 'corkscrewing'). Acute infections may culminate in high mortality (up to 100%). Of further interest, IPN-like viruses have been isolated from non-salmonid fish species in Europe and UK and, in some cases, the fish have demonstrated IPN-like clinical signs and lesions.[12,13,14,15,16]

Clinical signs in marine aquabirnaviruses vary with the virus isolate and the fish species infected.[4] Acute disease in finfish is characterised by abdominal ascites and/or deformity (spinal curvature).[8,9,17,18]

No abnormal clinical signs have been reported in fish infected with TAB.[2]

Epidemiology

Knowledge about the epidemiology of aquatic birnaviruses comes mostly from IPNV, which was first isolated in 1957, but descriptions of IPN may date back to the 1920s.[19] IPN viruses have long been known to cause an acute, contagious disease resulting in up to 100% mortality in very young, first-feeding, salmonid fry.[20] The susceptibility of young fish to IPN declines as they age. Generally, when farmed fish are moved to the marine environment they are no longer susceptible to disease, although subclinical infection with IPNV may still occur. However, recently, mortality due to IPN virus has also become important in Atlantic salmon smolt on some Nordic and Scottish farms.[21,22]

Salmonids differ in their sensitivity to infection with IPN virus with brook trout (*Salmo fontinalis*) being more sensitive than rainbow trout (*O mykiss*) and Atlantic salmon (*S salar*).[23]

Survivors of infection with IPN virus may become life-long latent carriers of the virus.[24] Carriers and acutely-infected fish, may transmit the virus both horizontally or vertically.[25,26,27] It is also postulated that aquabirnaviruses may be transmitted to fish, directly or indirectly, by fish-eating birds and by many invertebrates.[28,29]

IPN virus is a relatively stable virus under environmental conditions, for example, storage at approximately 18°C for 4 weeks has little effect on virus titre.[30,31] The virus also appears to be resistant to heat and changes in pH.[30]

Occurrence and Distribution

Aquatic birnaviruses (aquabirnaviruses) are the most widespread pathogenic organisms in aquatic animal species, having been identified in most parts of the world, and in 32

families of fish, 11 species of mollusc and 4 species of crustacean.[1] Aquabirnaviruses are of particular economic importance to the salmonid industries of Norway and
140 Scotland.[21,22]

Aquabirnaviruses have been isolated in Thailand, the People's Republic of China, Taiwan, Korea, Japan, and New Zealand.[20,30,32] None was isolated in Australia until 1998 when Tasmanian aquabirnavirus (TAB) was isolated from wild and farmed fish in
145 Macquarie Harbour, Tasmania.[2] Since then, there have been several further isolations from fish in Tasmania, but always restricted to Macquarie Harbour, and never from freshwater hatcheries (Crane et al, unpublished).

Pathology

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Fish may become infected with IPN virus by ingestion, or via the gills.[33] In acute infections, virus can be isolated from many tissues including kidney, spleen, pancreas, pyloric caecae, liver and gonads.[34,35] By comparison, in chronic infections, virus is generally restricted to pancreas, pyloric caecae, intestine, anterior kidney and gonads of
155 most fish.[35]

Compromised function of the pancreas and intestine in infected fish may account for a poorer food conversion rate in these fish compared with uninfected fish.[36] Infection with IPN virus may predispose fish to other infectious diseases.[36]

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Gross lesions associated with IPN include darkening of the skin, exophthalmia, abdominal swelling, and cutaneous petechiae. In addition, petechiae may be present throughout the viscera, and the stomach and anterior intestine may contain a clear-milky mucus, which is said to be pathognomonic.[36]

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Microscopically, the most severe lesions occur in the pancreas and the intestine. Pancreatic necrosis may range from multifocal to extensive, and may affect both acinar and islet cells. Surrounding adipose tissue may also be affected, possibly due to release of pancreatic enzymes. An acute, catarrhal enteritis is characterised by necrosis and
170 sloughing of the intestinal mucosa, which is considered to be the most likely cause of mortality in acute cases.[36]

Pancreatic lesions, consistent with IPN virus infection, have been noted in some fish naturally infected with TAB (Figure 1).[2] However, virus was also isolated from some fish
175 that had no histological lesions.

Lesions found in non-salmonid fish species infected with IPNV include necrosis of the haematopoietic tissues of the kidney and spleen.[12,13,14,15,16]

180 Lesions associated with one virus belonging to the marine aquabirnaviruses (AY-98) included pancreatic necrosis and body deformities.[37] The most common lesions associated with YAV are severe ascites and haemorrhage in the liver.[8,9,17,18]

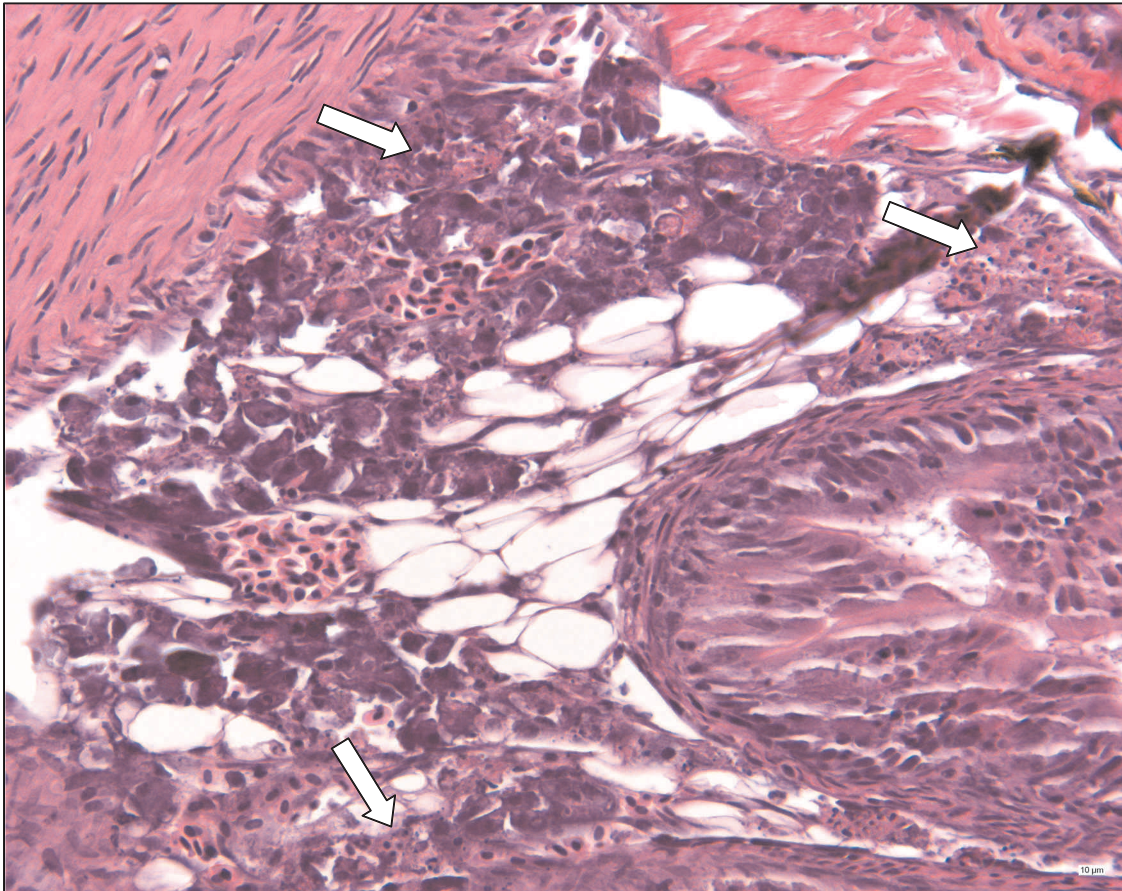
185 

Figure 1. Pancreas from a TAB-infected brook trout. There is extensive necrosis of acinar cells in the pancreas (see arrows). Scale bar represents 10 uM.

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Diagnostic Tests

Limitation statement

195 Because aquabirnaviruses are among the most common pathogens of aquatic organisms, claims about the validity of the following tests must necessarily be restricted to those groups of viruses represented by the isolates that have been tested at the AAHL Fish Diseases Laboratory (AFDL). These include the six genogroups of serogroup A. However, there is only limited access to serogroup B viruses, and no marine 200 aquabirnaviruses have been examined.

The clinical signs associated with infection by aquabirnaviruses are non-specific. While histological lesions (Figure 1) may be suggestive of infection, a definitive diagnosis depends upon detection of virus, or viral antigen, or viral genome in affected tissues or 205 following virus isolation in cell culture.

General comments

This document describes the methods currently used at the AFDL for the isolation and
210 identification of aquabirnaviruses. The methods are based on those recommended for IPN virus in the OIE *Manual of Diagnostic Tests for Aquatic Animals*.[38] Virus isolation is still considered to be the most sensitive technique for the detection of viral infections of fish, and its use is of fundamental importance in the management of disease outbreaks and the control of disease spread.[39] The procedures used for sampling fish have been documented
215 in other ANZSDPs and will not be discussed here.[40,41] Preparation of samples for virus isolation has also been documented previously.[42]

Storage of samples

220 Samples should not be frozen before testing but should be maintained between 4-10°C (shipping on wet ice in a styrofoam shipping container is appropriate). To maximise sensitivity, samples for virus isolation and reverse transcriptase-polymerase chain reaction (RT-PCR) should be prepared and assayed within 24 h of sampling, but, when this is not possible, they must be prepared within 72 h of sampling. Samples for
225 immunohistochemical tests should be prepared within 2 h, and samples for histopathological or electron microscopic examination should be placed in the appropriate fixative immediately after euthanasia of fish.

Tests available

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When an aquabirnavirus infection is suspected, virus isolation should be attempted from the whole viscera, including kidney, or, for larger fish, liver, kidney and spleen. Virus isolation should be attempted by inoculation of cultures of any of a number of fish cell lines, for example, CHSE-214 (chinook salmon embryo; ATCC CRL 1681), or EPC
235 (*epithelioma papulosum cyprini*[43]) cell lines. The BF-2 (bluegill fry) and RTG-2 (rainbow trout gonad; ATCC CCL 55) cell lines are also recommended.[38]

Cultures are incubated at 15°C. At 7 days post-inoculation (pi), material from cultures displaying cytopathic effect (CPE) may be passaged on to fresh cell cultures. When CPE
240 is evident in the second passage, at about 2-3 days pi, cultures can be used for an immunocytochemical test. Tissue culture supernatant fluid from the second passage may also be used for electron microscopic examination.

Where a tentative diagnosis of aquabirnavirus infection is made on the basis of CPE in
245 cell culture, a PCR-based procedure has been developed, firstly, to confirm the diagnosis (generic aquabirnavirus PCR), and then to differentiate, tentatively, a TAB infection from other aquabirnaviruses.

Depending on the size of the affected fish, either a range of tissues, or the entire fish,
250 should be fixed in 10% buffered formalin for histopathological examination. An immunohistochemical test may also be conducted on unstained sections of formalin-fixed tissues.

Acknowledgments

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260

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Part 2. Test Methods

1. Virus isolation

Equipment

380 Equipment required for the establishment and maintenance of a fish cell culture laboratory has been discussed previously.[42]

Reagents

385 Cell culture media:

i) Eagle's minimum essential medium containing Earle's salts and supplemented with 2% (v/v) foetal bovine serum, and 100 IU per mL penicillin and 100 ug per mL streptomycin for CHSE-214 cells
ii) Leibovitz's L-15 medium supplemented with 2% foetal bovine serum and 100 IU
390 per mL penicillin and 100 ug per mL streptomycin for EPC cells.

Procedure

i) Inoculation and monitoring cultures

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a) Prepare tissue homogenate(s), and apply the appropriate volume to a cell monolayer established on the previous day.[42]
b) Allow to adsorb at 10-15°C for 30 min to 1 h, and, without removing the inoculum, add the appropriate cell culture medium. Incubate at 15°C.
400 c) Microscopic examination of the cultures is undertaken daily for 7 days using an inverted light microscope with 4X, 10X and 40X objectives. The cultures are examined for viral cytopathic affect (CPE) or abnormalities such as sample cytotoxicity or contamination by reference to positive and negative control cultures.
405 d) If CPE appears in cell cultures inoculated with the tissue homogenate, aquabirnavirus identification procedures must be undertaken immediately (see below).
e) If no CPE occurs after 7 days of incubation (except in the positive control cultures), the inoculated cultures may be passaged.

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ii) Passaging cultures

a) Subject cell culture monolayers to one freeze-thaw cycle. Pool samples of the supernatants from all cell monolayers inoculated with dilutions of organ
415 homogenates.
b) Dilute 1/20 and 1/100, and inoculate new cell monolayers (established on the previous day) as described previously.
c) Incubate at 15°C, and monitor as already described.

Interpretation

The test is valid if the negative control cell cultures retain normal cellular morphology for the full period of incubation and the positive cell cultures show characteristic CPE.

The test sample is negative if the inoculated cell cultures retain normal cellular morphology similar to the negative control cultures, that is, do not demonstrate viral CPE.

If any of the cell cultures inoculated with test samples demonstrate CPE, further investigations, such as an immunocytochemical test, PCR or examination by electron microscopy, are required.

2. Immunocytochemical test

Equipment

Humid chamber, for example, a plastic container with an airtight lid
Acetone (80% v/v)-fixed cell cultures in multi-well plates
Plate shaker
Refrigerator
Inverted light microscope fitted with 4X and 10X objectives
Pipettes capable of dispensing 25 µL to 500 µL
Wash bottles or plate washer

Reagents

Phosphate-buffered saline without Ca^{2+} and Mg^{2+} ions, pH 7.4 (PBSA)
16% (v/v) formalin in PBSA
PBST 0.05% (v/v) Tween 20 in PBSA (Tween-20: Sigma-Aldrich P1379)
1% (w/v) skim milk powder solution in PBSA
0.1% (w/v) skim milk powder solution in PBSA
Polyclonal sheep antiserum raised against IPN virus (serotype N1; Microtek International Ltd, Saanichton, Canada, # SIPN010)
Normal sheep serum
Biotinylated donkey anti-sheep Ig (Amersham Biosciences RPN1025)
Streptavidin-horseradish peroxidase conjugate (Amersham Biosciences RPN1231)
9-ethylcarbazol-3-amine (AEC) Sigma-Aldrich 20 mg tablets (A6926)
N, N-dimethylformamide (DMF), Sigma-Aldrich (D8654)
Acetate buffer 0.05 M, pH 5.0
Hydrogen peroxide H_2O_2 Sigma-Aldrich (H1009)
Deionised water
Mayer's haematoxylin (Lillie's modification) DAKO (S3309)
Scott's tap water (Drury RAB, Wallington EA. *Carleton's Histological Technique.* 5th Ed. 1980)

Procedure

a) Fix cells by adding formalin solution to existing medium in a volume that gives a final concentration of 4% (v/v) formalin. Incubate in a humidified chamber in the refrigerator for at least 1 h (but for no more than one day) to fix the cells. If the immunocytochemical test cannot be performed within one day, remove the formalin solution, wash twice with PBSA. Add PBSA and return the plates to the refrigerator until the test can be performed.
b) Dilute anti-IPNV serum and normal sheep serum to working strength in 1% skim milk.
c) Dilute biotinylated donkey anti-sheep antibody and streptavidin-horseradish peroxidase conjugate to working strength in 0.1% skim milk.
d) Remove supernatant fluid from the wells of the cell culture, and wash the wells twice with 0.05% PBST.
e) Add 50 µL (96-well plate) or 200 µL (24-well plate) of primary antibody to each well. Incubate on a plate shaker set at low speed at room temperature for 1 h.
f) Remove the anti-IPNV and normal sheep serum solutions, and wash the wells 3 times with PBST. Add the biotinylated donkey anti-sheep antibody solution to all wells. Incubate on a plate shaker set at low speed at room temperature for 30 min.
g) Remove the biotinylated donkey anti-sheep antibody, and wash the wells 3 times with PBST. Add streptavidin-horseradish peroxidase conjugate to each well. Incubate on a plate shaker set at low speed at room temperature for 30 min.
h) Prepare the substrate just before use. Add one AEC tablet to 2.5 mL of dimethlyformamide. Once dissolved add this solution to 47.5 mL of 0.05 M acetate buffer, then add 50 µL of 30% [v/v] hydrogen peroxide to activate the solution.
i) Remove the streptavidin-horseradish peroxidase conjugate from the wells, and wash the wells 3 times with PBST.
j) Add 50 µL (96-well plate) or 200 µL (24-well plate) of active substrate solution and incubate at room temperature for 20 min. Use of a plate shaker is optional.
k) Discard the substrate and wash the cells with deionised water to stop the reaction.
l) Counterstain with Mayer's haematoxylin 50 µL (96-well plate) or 200 µL (24-well plate). Incubate at room temperature for 60-90 sec, then rinse twice with water.
m) Add Scott's tap water for 90-120 sec to develop background blue colour.
n) Rinse with tap water and allow to air dry.
o) Examine processed wells by inverted light microscopy.

Interpretation

Positive reaction: grainy, focal, brick-red staining of cells indicates the presence of an aquabirnavirus identified by the diagnostic antibody (Figure 2).

Negative reaction: no red staining apparent. All cells should be stained pale blue due to the counterstain (Figure 3).

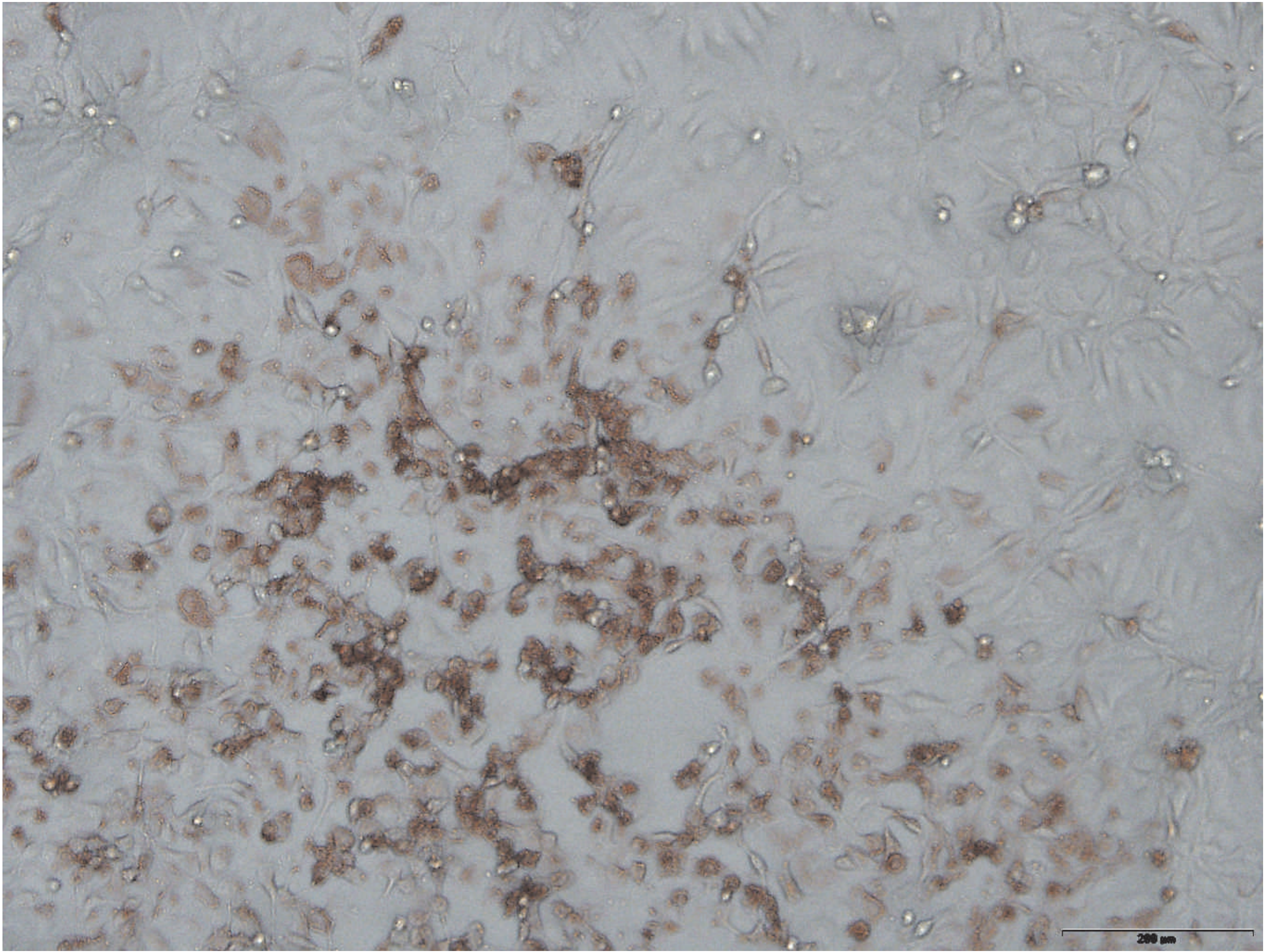

Figure 2. An example of positive staining of IPNV-infected CHSE-214 cells 2-3 days
515 post-infection. Scale bar represents 200 uM.

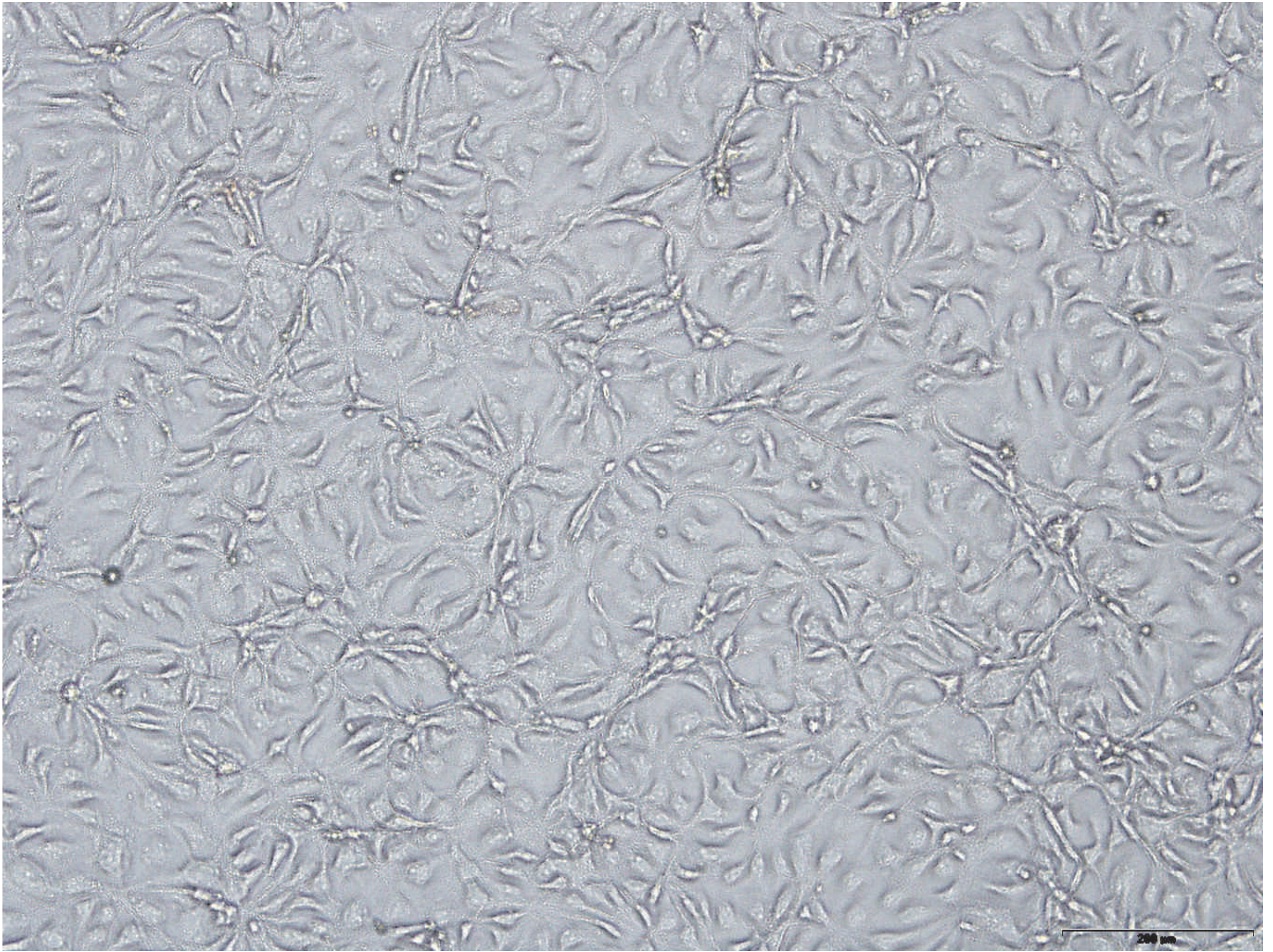

Figure 3. Uninfected control culture of CHSE-214 cell line treated in the same way as the culture in Figure 2. Note that there is no positive reaction. Scale bar represents 200 uM.

3. PCR-based tests

Reagents

525 i) Reagents stored at –20°C

Deionised formamide
Bovine serum albumin (BSA; 1 mg/mL)
100% Ethanol AR grade
530 SuperScript III One-Step RT-PCR with Platinum Taq DNA polymerase (Invitrogen Cat # 12574-026)
Primers (18 µM)
100 bp DNA ladder & loading dye (Promega Cat # G2101)
QIAGEN RNA-AVE buffer mix
535 Cla I restriction enzyme

ii) Reagents stored at room temperature

QIAamp viral RNA Mini Kit
540 QIAGEN Buffer AW2
QIAGEN Buffer AW1
QIAGEN Buffer AVE
QIAGEN Buffer AVL
Agarose (BIORAD Cat # 162-0134)
545 Ethidium bromide (BIORAD Cat #161-0430) or SYBR Safe™ (Invitrogen)
40 x TAE Buffer (Promega Cat # V4281)

Equipment

550 Apart from the normal range of equipment required in the standard diagnostic laboratory (for example, refrigerators, freezers, mixers, micropipettes, biological safety cabinets, centrifuges, balances, microwave oven, thermometers), specialised equipment required to undertake diagnostic PCR may include dry heat blocks, thermocycler, gel electrophoresis equipment, UV transilluminator, camera system and sequencer.
555

Quality control

Molecular diagnosis should be operated under an ISO 17025 accredited and audited quality assurance program. Thus, such a program would include initial evaluation of kits
560 and validation of performance; ongoing internal evaluation through mandatory use of appropriate quality control samples where available; and performance monitoring through quality assessment or proficiency programs.

External quality control samples over the appropriate range of testing must be obtained or manufactured wherever possible. Wherever possible, quality control samples should be included in every assay run and the data presented so that run-to-run performance can be monitored. Positive, negative and reagent controls should be conducted as specified in the protocol. As a norm, formalin-fixed controls would be conducted with formalin-fixed test samples, and appropriate unfixed controls would be conducted with fresh tissue or tissue culture supernatant samples. Stocks of controls should be established. These controls should be evaluated prior to storage and used in a check-testing regimen and as controls for the conduct of disease investigations.

Procedure

i) Sample preparation

Due to the sensitivity of PCR tests, care at every step of sample preparation must be taken to ensure that cross-contamination of diagnostic samples does not occur. Thus all instruments and sample containers must be clean and uncontaminated, that is, not pre-exposed to aquatic pathogens. Wherever possible it is recommended that disposable reagents and plastic-ware are used. At AAHL, samples would be handled and prepared using sterile disposable single use containers, instruments and reagents to minimise the risks of contamination of the samples.

As a general principle, samples to be used in the PCR suite at AAHL for molecular diagnosis will be inactivated by an approved method prior to movement to the PCR suite.

Inactivation will be carried out by the following procedures by staff approved to work with the categories of agents

1. Cell-free samples, for example, tissue culture supernatants at room temperature are added to an appropriate commercially prepared buffer (for example, Qiagen AVL buffer) containing guanidinium isothiocyanate.

2. Tissue samples (including blood) and other specimens containing cells are homogenised at approximately 10% w/v in water, and then frozen and thawed. Samples are then microfuged (approximately 13000 *g* for 20-30 sec), and the cell-free supernatant fluid collected. An appropriate volume of this is then mixed with an appropriate volume of a commercially prepared buffer (for example, Qiagen AVL buffer).

Nucleic acids are extracted from submitted samples in the Biological Safety Cabinet Class II in the PCR suite. All samples handled in the PCR suite will be less than 5 mL and considered to be of low or intermediate titre (or inactivated if required as above).

ii) Nucleic acid extraction, cDNA preparation and generic aquabirnavirus PCR

Nucleic acid (including aquabirnavirus dsRNA) is obtained from cell-free samples using the QIAamp Viral RNA extraction kit (Qiagen Cat. No. 52904). cDNA is prepared in the one-step RT-PCR. However, because aquabirnaviruses are dsRNA viruses, viral genomic

610 RNA must first be denatured prior to its incorporation into the RT-PCR. 2 uL of extracted nucleic acid (including RNA) is mixed with 1.0 µL of deionized formamide, and incubated at 100°C for 40 sec and then chilled on ice. 2 uL of this preparation is then added to the one-step PCR master mix.

615 The PCR master mixture for a single sample consists of the following reagents: 9.0 µL of water; 12.5 µL of 2x reaction buffer; 1.0 uL of the SuperScript III / Platinum Taq mixture; 0.25 µL of the forward primer (18 µM); and, 0.25 µL of the reverse primer (18 µM). For multiple samples, the volumes are multiplied appropriately. The mixture is incubated in an automatic thermal cycler (Perkin Elmer GeneAmp 2400) that is
620 programmed for: one cycle at 55°C for 30 min, followed by 94°C for 2 min; then 35 cycles at 94°C for 45 sec, 45°C for 45 sec and 68°C for 2 min; and, finally, one cycle at 68°C for 7 min. Amplified DNA (775 bp) is detected by agarose gel electrophoresis.

The two primers that are used in the PCR are:

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Generic forward primer: 5'-ACGAACCCTCAGGACAA-3'
Generic reverse primer[3]: 5'-CACAGGATCATCTTGGCATAGT-3'

iii) Restriction enzyme digestion of a specific aquabirnavirus PCR product

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If a PCR product of the correct size is amplified with the aquabirnavirus primers, the product is purified from the agarose gel (for example, Qiagen Gel Extraction kit, Cat No. 28704). To 10 uL of the eluate is added: 2 uL of restriction enzyme 10X reaction buffer, 2 uL of BSA (1 mg/mL), 10 IU of the restriction enzyme Cla I, and water to a final
635 volume of 20 uL. Digestions are performed at 37°C for 1-2 h, followed by incubation at 65°C for 15 min to inactivate the enzyme. Digested and undigested samples are then examined on an agarose gel containing ethidium bromide, and examined under UV illumination.

640 *Interpretation*

At the completion of the PCR, specific PCR fragments of the correct size are identified by agarose gel electrophoresis:

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- The negative control sample must have no evidence of specific amplified products.
-
- A positive control sample must yield a specific aquabirnavirus fragment (775 bp).
-
- Amplified fragments of the correct size are then eluted from the gel, and a Cla I
650 restriction enzyme digest is performed. If the PCR product has been amplified from TAB, two digestion products will be seen (652 and 123 bp). No digestion will occur with other aquabirnaviruses because the specific Cla I cleavage site is only present in TAB (Figure 4).

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- The remainder of the eluate is used to determine the DNA sequence (by using the PCR primers as sequencing primers). Note that an apparently specific PCR product, and the reactivity of the restriction enzyme with the PCR product, only provide a tentative diagnosis. *The PCR product must be sequenced to make a definitive*
660 *diagnosis*.
-
- Sequence identity and genotype are determined by a Blast search of the GenBank database.

665 An assay is valid only when all controls yield the expected results.

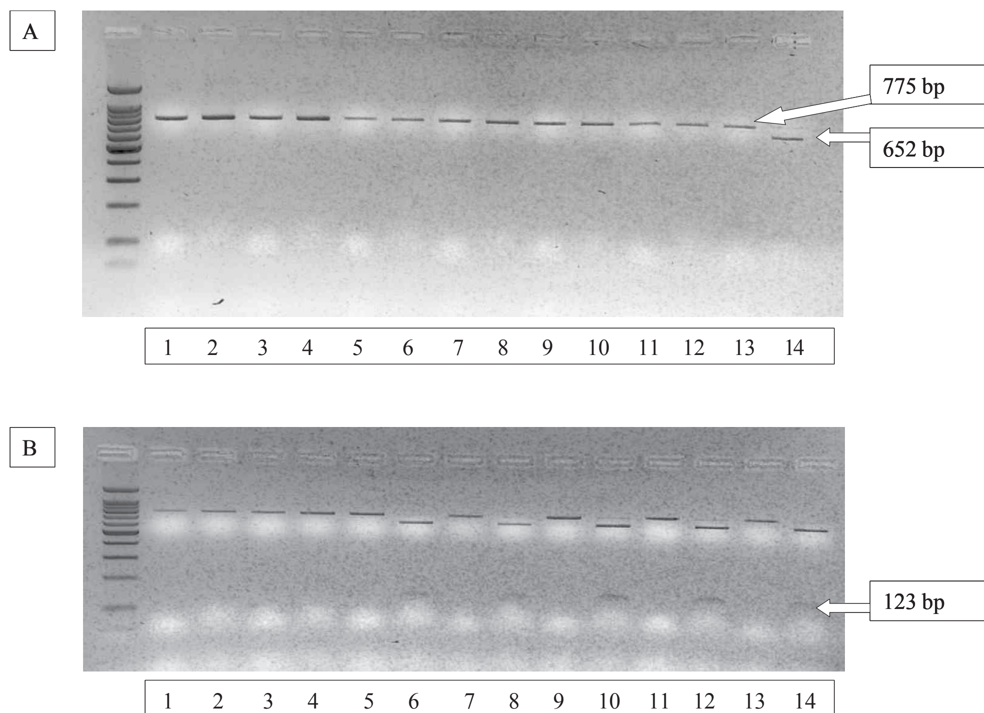

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Figure 4. Generic aquabirnavirus PCR, and Cla I digestion of the PCR products from selected aquabirnaviruses. Viral nucleic acid was extracted, and cDNA prepared. The generic aquabirnavirus PCR was conducted, and specific products from each virus were purified, and digested with the restriction enzyme Cla I. (A) Lanes 1,2: IPN (Canada 3);
675 3,4: IPN (Canada 1); 5,6: IPN (Te); 7,8: IPN (Ab); 9,10: NZ isolate (NZ6); 11,12: NZ isolate (NZ10); 13,14: TAB02 isolate (2002 AS). (B) Lanes 1,2: IPN (Sp); 3,4: IPN (DPL); 5,6: TAB12 (2001 RT); 7,8: TAB11 (1998 AS); 9,10: TAB6 (1998 AS); 11,12: TAB 23 (1998 RT); 13,14: TAB16 (2001 RT). Even-numbered lanes: undigested PCR product; odd-numbered lanes: Cla I digestion of PCR product.

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4. Immunohistochemistry

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Reagents

10% formalin
Trypsin solution (1 mg per mL trypsin; 0.1 M Tris/HCl; 1 mg per mL $CaCl_2$; pH 8.0)
690 Phosphate-buffered saline without Ca^{2+} and Mg^{2+} ions, pH 7.4 (PBSA)
0.1% (w/v) skim milk powder solution in PBSA
Primary antibody: polyclonal sheep antiserum raised against IPN virus (serotype N1; Microtek International Ltd, Saanichton, Canada, # SIPN010)
Normal sheep serum
695 Secondary antibody: biotinylated donkey anti-sheep Ig
3% (v/v) peroxide solution in methanol
Streptavidin-horseradish peroxidase conjugate
AEC (3 amino-9-ethyl carboxyzole) substrate (2 mg AEC, 200 uL dimethylformamide, 10 mL 0.05 M acetate buffer, pH 5.0, 5 uL 30% (v/v) H_2O_2)
700 Deionised water
Mayer's haematoxylin (Lillie's modification)
Scott's tap water

705 *Equipment*

Poly-L-lysine coated glass slides
Humid chamber, for example, a plastic container with an airtight lid
Light microscope fitted with 4X, 10X and 40X objectives

710

Procedure

a) The tissues for examination have to be fixed in aldehyde fixatives, for example, 10% formaldehyde, for 24 h or in absolute alcohol or 96% alcohol. Presumably frozen
715 sections could also be used, but these have not be tried at AFDL.
b) After fixation, the sample is prepared according to normal histological procedures, and then embedded in liquid paraffin.
c) Histological sections are mounted on silanised slides.
d) Sections are then deparaffinised, using standard procedures, followed by incubation
720 with a trypsin solution at 37°C for 20 min (to 'unmask' epitopes that have been cross-linked during formalin-fixation of tissue samples. To facilitate unmasking, tissues should be fixed in formalin for no more than 24 h). The reaction is stopped using cold (4°C) PBSA.
e) Sections are then incubated at 37°C for 1 h with polyclonal sheep antiserum raised
725 against IPN virus or with normal sheep serum diluted to its optimal concentration in 0.1% (w/v) skim milk in PBSA.

f) The sections are then rinsed in PBSA, and incubated at 37°C for 1 h with biotinylated donkey anti-sheep Ig diluted to optimal concentration in 0.1% (w/v) skim milk in PBSA.
730 g) After a further rinse in PBSA, endogenous peroxidase activity can be blocked by immersion of the sections in 3% (v/v) peroxide solution in methanol at room temperature for 20 min.
h) Following a rinse in PBSA, the sections are incubated at 37°C for 1 h with streptavidin-horseradish peroxidase diluted to optimal concentration in 0.1% (w/v)
735 skim milk in PBSA.
i) After another rinse in PBSA, the sections are incubated with freshly prepared AEC substrate solution at room temperature for 20 min.
j) The sections are rinsed in tap-water, counterstained with Mayer's haematoxylin and mounted in mounting medium (Quickmount, Daido Sangyo Co Ltd, Japan) for
740 microscopic examination.

Interpretation

Positive reaction: grainy, focal, brick-red staining of cells indicates the presence of an
745 aquabirnavirus identified by the diagnostic antibody.

Negative reaction: no red staining apparent. All cells should be stained pale blue due to the counterstain.

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