Betanodavirus Infections of Finfish

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Part 1 - Diagnostic Overview

Summary

Viral nervous necrosis (VNN), syn. viral encephalopathy and retinopathy (VER), is a serious viral disease of finfish caused by a Betanodavirus, *syn. nervous necrosis virus (NNV). Disease has been reported in over 40 fish species from tropical to temperate climates in most continents around the world, and most reports of disease have been associated with species in aquaculture facilities. Clinical disease is most commonly observed in larval and juvenile finfish, and this is the case in Australia, although reports of clinical disease in adult fish are increasing. Mortality rates of up to 100% are most commonly seen in larval fish and mortality rates tend to decrease as the age of the infected fish increases. Fish surviving infection can become sub-clinical carriers. The most common mode of transmission appears to be vertical, from sub-clinically infected broodstock to progeny during spawning, but horizontal transmission can occur.* Betanodavirus *infection has a significant economic, social and environmental impact in Australia through direct losses, inhibition of trade, restriction on locations suitable for aquaculture expansion and suspension of fish restocking programs due to concerns on the impact of the virus to native fish species due to translocation of infected stock. Tests have been developed to detect the infection in fish and for health certification purposes. As serology testing for the detection of antibodies in finfish is not routinely practiced in diagnostic laboratories, all tests are based on direct detection of the virus. Virus isolation is used to detect infectious virus in fresh unfixed material. Real-time reverse-transcriptase polymerase chain reaction (RT-qPCR) and nested reverse-transcriptase polymerase chain reaction (RT-nPCR) tests are used to detect viral nucleic acid sequences in fresh and/or fixed material, and histological examination is used for disease diagnosis in formalin-fixed material. Immunological tests (immunocytochemical test (ICCT), or indirect immunofluorescent antibody test (IFAT)), RT-qPCR or RT-nPCR are used to confirm the identity of viruses isolated in cell culture as* Betanodavirus, *with sequencing of amplicons produced by conventional RT-PCR used for confirmation of strain identity. The immunohistochemical test (IHCT) or IFAT are used to confirm or exclude nodavirus from fixed histological tissue sections where the nature of the lesions may be ambiguous.*

Aetiology

The virus causing VNN is a member of the genus *Betanodavirus* of the family *Nodaviridae*.[1] Virions are non-enveloped and icosahedral in shape with a diameter of approximately 25 to 30 nm and contain two segments (RNA1 and RNA2) of positive-sense single-stranded RNA (ssRNA) with the RNA2 segment containing the sequence for the viral coat protein.[2] The RNA2 segment is highly conserved among isolates and is the target for the detection of viral RNA by PCR methods. The viral coat protein is the target for immunological methods. There are four recognised genotypes: striped jack nervous necrosis virus (SJNNV), tiger puffer nervous necrosis virus (TPNNV), barfin flounder nervous necrosis virus (BFNNV) and red-spotted grouper nervous necrosis virus (RGNNV).[3] A possible fifth genotype, turbot betanodavirus (TNV), has been proposed.[4] All Australian *Betanodavirus* isolates have been identified as members of the RGNNV genogroup, where they occur in two distinct clusters (RGNNV 1a and RGNNV 1c).[5]

Clinical Signs

Clinical signs are most commonly observed in larvae and fry and are due to damage to the nervous tissue of the spinal cord, brain and retina caused by the viral infection. Typically, affected fish display abnormal behaviour, including spiral swimming and rapid uncoordinated darting movement, with mass mortalities occurring over a short period of time.[6, 7] Changes in surface pigmentation (usually darkening of the skin of diseased fish), cessation of feeding, abnormal inflation of the swim bladder and increased susceptibility to cannibalism may also be observed.

Epidemiology

VNN is an acute infectious disease of primarily larvae and fry of a range of finfish species cultured in seawater. Mortalities of up to 100% are most common in larvae with susceptibility generally decreasing as the age of the fish increases. However, significant mortalities of some fish species at harvest size[8, 9] and of some species cultured in freshwater have been reported.[10] As other finfish species are evaluated for aquaculture potential, the range of species found to be susceptible to infection is likely to increase. Nodaviruses have been detected in juvenile fish surviving experimental and natural infection[7, 11-13] and while the duration of viral persistence is unknown, virus has been detected in halibut and Australian bass surviving acute infection for at least 12 months after the initial disease outbreak.[11, 14] Virus has also been detected in healthy adult fish of known susceptible species,[8, 11-13, 15] and from healthy fish of species in which disease has not been observed previously.[16, 17] Exposure to the virus, or recombinant coat protein of the virus, can induce a protective neutralising antibody response in susceptible fish,[18] although duration of this response is unknown. Antibodies have been detected by enzyme-linked immunosorbent assay (ELISA) in broodstock.[19, 20] As ELISA-positive fish have still produced VNN virus-positive offspring,[21] the relationship between antibody status and presence of infectious virus within the fish remains to be clarified. The most common mode of transmission is thought to be vertical from infected broodstock to progeny;[6, 22] however, horizontal transmission can occur.[6, 23]

Occurrence and Distribution

The disease has been described in more than 40 fish species from all continents. In Australia, *Betanodavirus* has been detected in Australian bass (*Macquaria*

novemaculeata), barramundi (*Lates calcarifer*), barramundi cod (*Cromileptes altilevis*), goldspotted rockcod (*Epinephelus coioides*), flowery cod (*Epinephelus fuscoguttatus*) and striped trumpeter (*Latris lineata*) from marine aquaculture facilities in New South Wales, the Northern Territory, Queensland, South Australia and Tasmania and from sleepy cod (*Oxyeleotris lineolatus*) from a freshwater aquaculture facility in Queensland.[5] The Australian isolates have been identified as members of the RGNNV genotype, where they occur in two distinct clusters (RGNNV 1a and RGNNV 1c).[5] The RGNNV 1a group contains isolates from Queensland, the Northern Territory and Tasmania and the RGNNV 1c group contains isolates from New South Wales and South Australia. As the effect of the isolates on other native finfish species is unknown, strict controls are in place to reduce the risk of translocation of virus with commercial stock or stock used for restocking programs, and to reduce the risk of escape of virus from aquaculture facilities into the environment. Exclusion of the virus from aquaculture premises, good hygiene in these premises and reduced stocking densities have contributed to decreasing the incidence of VNN outbreaks. Screening by nested RT-PCR and use of only NNV-negative broodstock has also reduced the occurrence of disease in larvae.[21, 24, 25] *Betanodavirus* infection has not been reported in New Zealand.

Pathology

Gross lesions are uncommon, but over-inflation of the swim bladder in infected sevenband grouper[8] and red drum[26] has been reported. VNN is characterised pathologically by vacuolating encephalopathy and retinopathy.[27] Lesions are usually less severe in older fish than in juvenile fish and, depending on the age of the fish, severity of the vacuolation can range from one or two affected cells to necrosis of entire regions of the brain and retina. In barramundi, vacuolation occurs more often in the optic tectum and cerebellum than in the telencephalon and medulla oblongata. Focal pyknosis and karyorrhexis of the neural cells, granularity of the neuropil and accumulation of eosinophilic material in macrophages may also be observed.[27] Detailed descriptions of microscopic changes in infected fish species of Australian origin have been published.[6, 7, 27]

Diagnostic Tests

Clinical signs in larvae, fry and juvenile finfish are indicative of nodavirus infection, but definitive diagnosis requires observation of vacuoles in nervous tissue in histological sections combined with detection of the viral antigen in fresh or fixed tissue, detection of viral genome by molecular tests or virus isolation followed by identification of *Betanodavirus* by immunological or molecular methods.

Tests on fixed material

Diagnosis can be made on the observation of vacuoles in the nervous tissue of the spinal cord, brain and retina fixed in formalin or Bouin's fixative, processed and stained with haematoxylin and eosin (H&E), using light microscopy. If clinical signs have been observed, especially in larvae or fry, then observation of vacuoles in nervous tissue is sufficient for diagnosis. However, if there is any doubt, confirmation of the presence of virus in the tissue sections by transmission electron microscopy (TEM), immunohistochemistry test (IHCT) or indirect immunofluorescent antibody test (IFAT) is required. While both the IHCT and IFAT perform equally well when lesions are observed in H&E-stained sections by light microscopy, the IHCT is more sensitive at detecting nodavirus in sub-clinically infected fish than the IFAT, which in turn is more sensitive than histology alone.[13] Comparisons of TEM with the IHCT and IFAT are not available;

however, as TEM requires specialised equipment and expertise that is not always readily available, the IHCT is recommended for routine confirmation of infection in fixed material.

The real-time reverse-transcriptase polymerase chain reaction (RT-qPCR) is the most appropriate test for molecular detection of viral RNA in tissue fixed in 80-95% analytical grade ethanol or RNAlater™ as this assay is 10-1000-fold more sensitive than the nested reverse-transcriptase polymerase chain reaction (RT-nPCR), depending on the genotypes being tested.[11] Confirmation of RT-qPCR results by conventional RT-nPCR and sequencing of representative amplicons should be undertaken to confirm the positive result. This is especially important when no clinical signs have been observed or the disease is suspected in a new species or in a new geographical location.

Tests on unfixed material

Detection of NNV in unfixed (fresh) nervous tissue is undertaken by RT-qPCR[11] or RT-nPCR,[5, 13, 28] or virus isolation in cultures of susceptible cell lines.[22] Virus isolation can be undertaken in cultures of either the snakehead (SSN-1)[29] or snakehead clone (E-11)[30] cell lines with confirmation of NNV isolation in cell culture undertaken by RT-qPCR, RT-nPCR, ICCT or IFAT.

Testing of broodstock reproductive or spawning material (unfertilised eggs, fertilised eggs or milt) and blood can be undertaken by RT-qPCR or RT-nPCR, although optimal sample type, sample collection method, sample preparation and testing protocols are still to be determined. While positive results of valid tests can be accepted, negative results may be false-negative results.

Summary for recommended tests for surveillance and diagnostic testing

The methods currently available for targeted surveillance and diagnosis of VER/VNN are listed in Table 1. The assigned designations in the table are based on those of the OIE manual,[22] published research and the authors' experience.

Table 1 Methods currently used for surveillance and diagnostic testing for *Betanodavirus*

Method	Targeted surveillance	Presumptive Diagnosis	Confirmatory diagnosis
Histology	d	c	d
Histology followed by IHCT or IFAT	c	a	a
Transmission EM	d	c	b
Virus isolation	c	b	b
Virus isolation followed by IHCT or IFAT or RT-nPCR and sequencing	b	b	a
RT-nPCR	b	a	c
RT-qPCR	a	a	b
RT-nPCR followed by sequencing	b	a	a

The designations used in the table, according to the OIE Manual,[22] indicate: a = the recommended method for reasons of availability, utility, and diagnostic specificity and

sensitivity; b = standard method with good diagnostic sensitivity and specificity, but cost and availability limit its application; c = the method has application in some situations but other factors severely limit its application; d = the method is 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.[22]

Safety and Biosecurity Requirements

Betanodavirus is not known or suspected to cause human infection. Further, while this virus is widespread in the Australian marine environment, laboratory containment is required due to the potential for different genetic variants to be introduced into naïve environments. Laboratory studies involving the handling of infectious material or amplification of virus should preferably be conducted under PC2 or similar containment conditions as described in AS/NZ 2243.3:2010. [31]

Part 2 - Test Methods

The target audience of this ANZSDP is staff working in laboratories performing routine diagnostic testing within an accredited quality system. As such, competencies relating to health and safety requirements for reagents and equipment used, and technical skills required to perform the assays and interpret the results, have been assumed

Storage of tissue specimens

Samples for histology, including immunodiagnostic testing, or TEM testing should be placed in the appropriate fixative (10% formalin or Bouin's) immediately after euthanasia of the fish and processed using standard procedures.

Tissue fixed in 80-95% analytical grade ethanol for PCR tests can be transported and stored at ambient temperature, and tissue fixed in RNAlater™ (or equivalent) for PCR tests can be transported at ambient temperature and stored according to the manufacturer's recommendations.

Unfixed samples should be held at temperatures less than 10°C at all times and transported with wet ice to the laboratory within 24 hours. If samples cannot be transported within this time they should be frozen at -70°C or lower, until transport to the laboratory with dry ice. Samples can be stored at -70°C or lower for at least two years before loss of integrity is observed.

Immunohistochemistry test (IHCT) for detection of *Betanodavirus* in fixed material

Principle of the Test

This test uses polyclonal antibodies raised in sheep against the recombinant coat protein (rCP) of a barramundi (BNNV) or sleepy cod *Betanodavirus* (SCNNV) isolate and an anti-sheep immunoglobulin G (IgG)-horseradish peroxidase conjugated secondary antibody, to localise viral coat protein in histological sections containing neural tissue of finfish. The test is used to confirm or exclude *Betanodavirus* as the agent causing lesions observed in

H&E-stained sections, or to diagnose *Betanodavirus* infection in tissue sections in the absence of histology expertise.

Reagents and Materials

Polyclonal antibody. The primary sheep anti-BNNV rCP or sheep anti-SCNNV rCP polyclonal antibody is available from the suppliers described in Part 3.

Peroxidase-conjugated secondary antibody. The assay described here was validated using rabbit anti-sheep IgG [H+L] horseradish peroxidise (HRP) (Cat No. 313-35-003, Jackson ImmunoResearch, USA). Any commercial anti-sheep IgG [H+L] HRP conjugate can be used. New batches should be tested using positive and negative control slides. Other reagents are:

Tris buffered saline (TBS; 20 mM Tris, 500 mM NaCl)

Tris	9.68 g
NaCl	116.9 g
Deionised water	4 L

0.1% trypsin in TBS

Trypsin (1:250)	0.2 g
TBS	4 L

3% H_2O_2 in methanol

H_2O_2	6.0 mL
Methanol	200 mL

5% bovine serum albumin (BSA) in TBS

BSA	2.5 g
TBS	50 mL

2.5% BSA in TBS

BSA	1.25 g
TBS	50 mL

Metal Enhanced DAB Substrate Kit (Pierce).

Any commercially-available HRP substrate can be used. New batches should be tested using positive and negative control slides.

Deionised water. Any source of deionised water is suitable.

Mayer's haematoxylin

Aluminium ammonium sulphate	10 g
Deionised water	200 mL
Haematoxylin	0.2 g
Sodium iodate	0.04 g
Citric acid	0.2 g
Chloral hydrate	10 g

Dissolve the aluminium sulphate in the distilled water using a magnetic stirrer and large stir bar. Do not heat. When completely dissolved, add the haematoxylin. Once the haematoxylin is completely dissolved, add in the following order: sodium iodate, citric acid and chloral hydrate. Ensure that all chemicals are completely dissolved.

Lithium carbonate

Lithium carbonate	2.8 g
Deionised water	200 mL

Mounting medium: Any standard aqueous mounting medium is suitable.

Deparaffinised, rehydrated tissue sections mounted on positively charged glass histology slides.

Hydrophobic marker.

Humidified 37°C chamber.

Compound light microscope.

Test Procedure

Circle the tissue sections with a hydrophobic marker. Tissue sections should not be allowed to dry at any stage. Add 1 mL 0.1% (w/v) trypsin to each tissue section and incubate at 37°C for 30 minutes in a humidified chamber. Wash three times with TBS. Block endogenous peroxidase by immersing the tissue sections in 3% (v/v) H_2O_2 in methanol at room temperature for 20 minutes. Wash three times with TBS. Block non-specific binding sites by incubating each tissue section with 1 mL 5% (w/v) BSA in TBS in a humidified chamber at 37°C for 20 minutes. Wash three times with TBS. Add 1 mL sheep anti-NNV rCP polyclonal antibody, diluted 1/1000 in 2.5% BSA in TBS, to each tissue section and incubate in a humidified chamber at 37°C for 60 minutes. Wash three times with TBS. Add 1 mL of rabbit anti-sheep IgG [H+L] HRP conjugate, diluted 1/1000 in 2.5% BSA in TBS, to each tissue section and incubate in a humidified chamber at 37°C for 60 minutes. Wash three times with TBS. Prepare the Metal Enhanced DAB Substrate Kit according to the manufacturer's instructions. Add 1 mL to each tissue section and incubate for 10 minutes at room temperature. Stop development by immersing the slides in deionised water. Counterstain tissue sections with Mayer's haematoxylin for 60 seconds, rinse in tap water for 60 seconds, blue in lithium carbonate for 60 seconds and rinse in tap water for 60 seconds. Mount tissue sections under a coverslip using an aqueous mounting medium and examine with a compound microscope.

Positive and negative control slides must be included in every test. Ideally, a positive slide showing a low level of infection should also be included. For the test to be valid, dark brown or black staining of neuronal cells must be observed in the neural tissue of the spinal cord, brain and/or retina of the positive control slides (Figure 1). No specific staining should be seen in the negative control slide. Some non-specific staining may be seen in the stomach.

Interpretation of Results

Any positive staining indicates the presence of *Betanodavirus* coat protein and the fish is considered to be undergoing an active infection. When a single fish from a larger group is positive in an IHCT, it is a strong indication of the presence of the virus and additional sampling and/or testing should be considered.

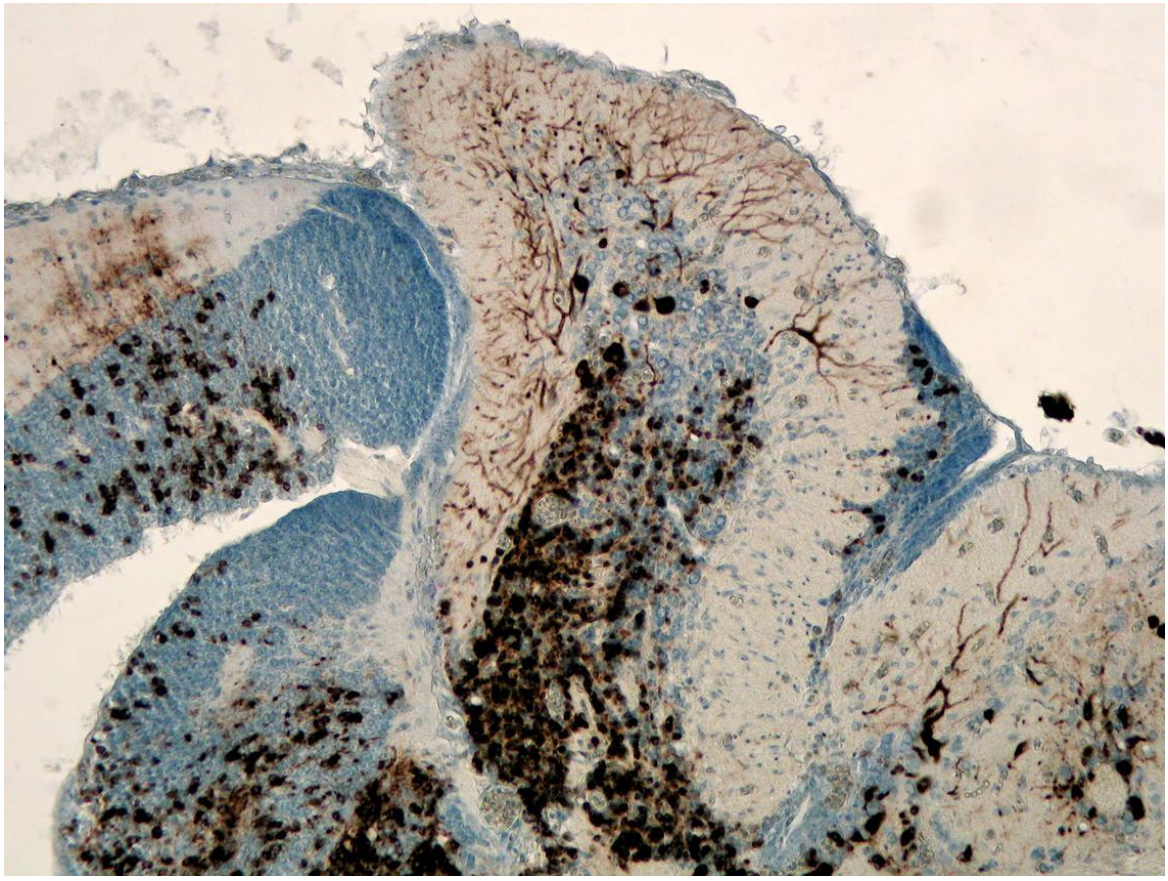


Figure 1 An example of a positive NNV IHCT in the brain of a heavily infected barramundi, indicated by the dark brown/black staining.

Indirect Fluorescent Antibody Test (IFAT) for detection of *Betanodavirus* in fixed material

Principle of the Test

This test uses polyclonal antibodies raised in sheep against the recombinant coat protein of a barramundi or sleepy cod *Betanodavirus* isolate and an anti-sheep IgG fluorescein-conjugated secondary antibody, to localise viral coat protein in histological sections containing neural tissue of finfish. The test is used to confirm or exclude *Betanodavirus* as the agent causing lesions observed in H&E-stained sections, or to diagnose *Betanodavirus* infection in tissue sections in the absence of histology expertise.

Reagents and Materials

Polyclonal antibody. The primary sheep anti-BNNV rCP or sheep anti-SCNNV rCP polyclonal antibody is available from the suppliers, as described in Part 3 (contact details for relevant suppliers are listed in Part 4).

Fluorescent conjugated secondary antibody. The assay described here was validated using rabbit anti-sheep IgG [H+L] Cy2 (No longer available, Jackson ImmunoResearch, USA). Any commercially-available anti-sheep IgG [H+L] fluorescent conjugate can be used. Cyanine 2 (Cy2™) or equivalent is more intense and less prone to photo-bleaching than fluorescein isothiocyanate (FITC). New batches should be tested using positive and negative control slides.

Tris buffered saline (TBS; 20 mM Tris, 500 mM NaCl)

Tris	9.68 g
NaCl	116.9 g
Deionised water	4 L

0.1% trypsin in TBS

Trypsin (1:250)	0.2 g
TBS	4 L

5% BSA in TBS

BSA	2.5 g
TBS	50 mL

2.5% BSA in TBS

BSA	1.25 g
TBS	50 mL

Aqueous mounting medium.

Deparaffinised, rehydrated tissue sections mounted on positively charged glass histology slides.

Hydrophobic marker.

Humidified 37°C chamber.

Fluorescence microscope.

Test Procedure

Circle the tissue sections with a hydrophobic marker. Tissue sections should not be allowed to dry at any stage. Block non-specific binding sites by incubating each tissue section with 1 mL 5% BSA in TBS in a humidified chamber at 37°C for 60 minutes. Wash three times with TBS. Add 1 mL sheep anti-NNV rCP polyclonal antibody, diluted 1/1000 in 2.5% BSA in TBS, to each tissue section and incubate in a humidified chamber at 37°C for 60 minutes. Wash three times with TBS. Add 1 mL of rabbit anti-sheep IgG [H+L]-Cy2™ conjugate, diluted 1/1000 in 2.5% BSA in TBS, to each tissue section and incubate in a humidified chamber at 37°C for 60 minutes. Wash three times with TBS. Mount the tissue sections under a coverslip using an aqueous mounting medium and examine with a fluorescence microscope.

Positive and negative control slides must be included every time the test is performed. Ideally, a positive slide showing a low level of infection should also be included. For the test to be valid, bright green fluorescent staining of neuronal cells must be observed in the neural tissue of the spinal cord, brain and/or retina of the positive control slides (Figure 2). No specific staining should be seen in the negative control slide.

Interpretation of Results

Any positive staining indicates the presence of the *Betanodavirus* coat protein and the fish is considered to be undergoing an active infection. When a single fish from a larger group is positive in an IFAT, it is a strong indication of the presence of the virus and additional sampling and/or testing should be considered.

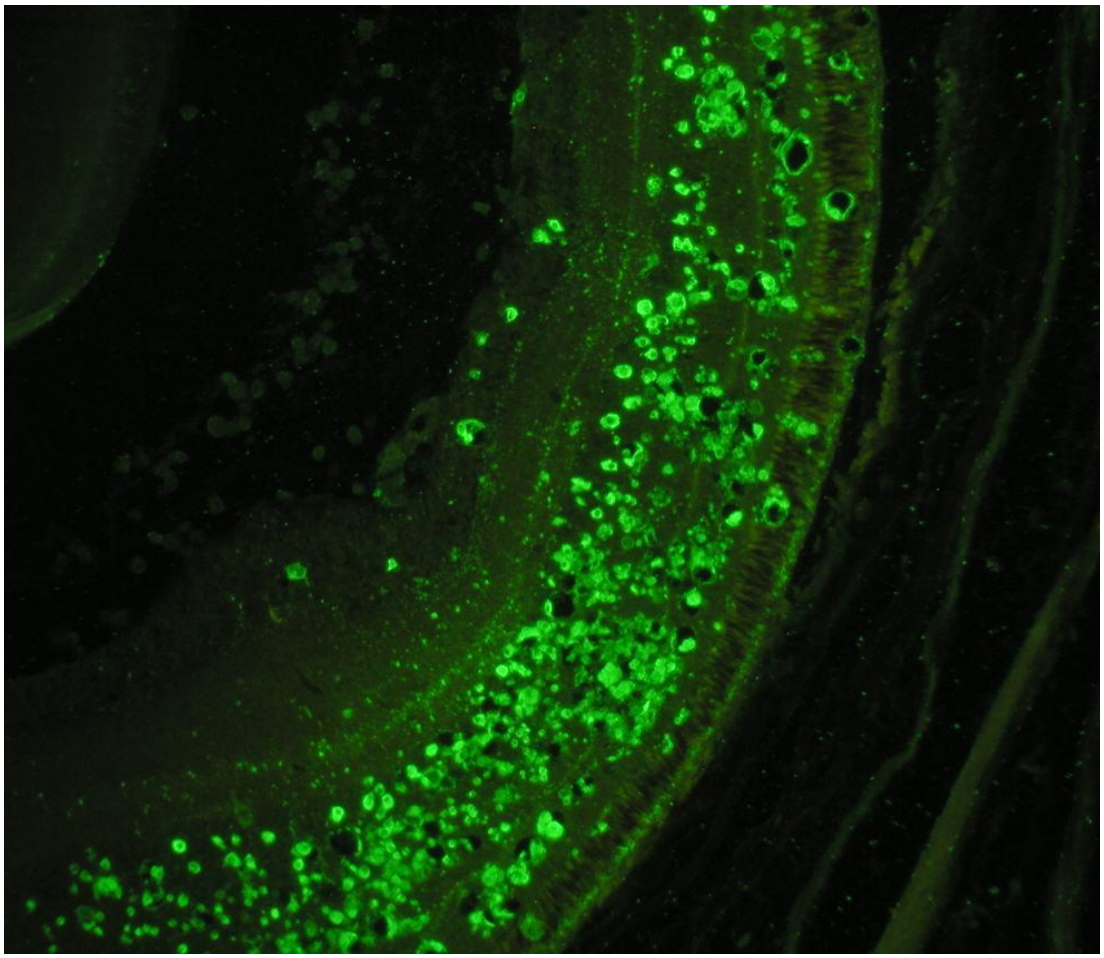


Figure 2 An example of a positive NNV IFAT in the retina of a heavily infected barramundi, indicated by the bright green staining.

Real-time RT-PCR (RT-qPCR)

Principle of the Test

RT-qPCR is used to amplify a specific sequence from an RNA target. Firstly, reverse transcription (RT) converts the specific target sequence of viral RNA into complementary DNA (cDNA). Incorporation of a sequence-specific probe with a fluorescent dye at the 5' end and a quencher at the 3' end increases the specificity of the assay, as this probe must also bind to the target sequence with the primers during the annealing stage. As the PCR amplifies the specific sequence from this cDNA to produce multiple copies, the 5' to 3' exonuclease activity of the *Taq* polymerase releases the fluorophore from the bound probe. As the effect of the quencher has been eliminated, the fluorescence emission is detected in real time. As the amount of target sequence is increased by the PCR, the amount of fluorescence increases as the PCR continues. The primers, probe and cycling conditions used in these procedures are fully described.[11] The protocol described here is the modification validated for use through equivalence testing at the Commonwealth Scientific Industrial Research Organisation (CSIRO) Australian Animal Health Laboratory (AAHL) Fish Diseases Laboratory (AFDL).

Table 2 Primer and probe sequences used in the NNV RT-qPCR test

Primer	Sequence
QR2T-F	5'- CTT CCT GCC TGA TCC AAC TG -3'
QR2T-R	5'- GTT CTG CTT TCC CAC CAT TTG -3'
Probe	
QR2Tprobe	5'- **6FAM** CAA CGA CTG CAC CAC GAG TTG **TAMRA** -3'

Due to the highly sensitive nature of PCR tests, highly developed technical skills as well as quality control procedures and separate work areas for the different components of the PCR test are required to avoid cross-contamination and production of false-positive results.

Reagents and Materials

Transportation Medium (TM). Hank's balanced salt solution (Life Technologies) containing 200 IU/mL penicillin, 200 µg/mL streptomycin sulphate and 2% (v/v) foetal bovine serum. Any transportation medium can be used after validation for use.

Fixatives. Ethanol (80-95% analytical grade) or RNAlater

QIAamp Viral RNA Mini Kit (Qiagen) or MagMAX™-96 Viral RNA Isolation Kit (Life Technologies). Both kits have been validated for use by AFDL for RNA and DNA extractions. Alternative kits should be validated before use.

AgPath-ID One-Step RT-PCR Kit (Life Technologies). The AgPath-ID One-Step RT-PCR Kit is specifically mentioned as this reagent is routinely used for RT-qPCR assays by AFDL. The QuantiTect Probe RT-PCR Kit (Qiagen) has also been evaluated during equivalence testing. Alternative kits should be validated for use.

Primers and probe (Table 2)

RNase-free deionised water

Real-time PCR instrument (Life Technologies 7500 Fast). Any real-time PCR instrument can be used after validation.

Test Procedure

Whole larvae/fry or organ samples are homogenised in buffer (e.g. TM or homogenisation buffers provided with extraction kits). The tissue sample required is dependent on the size of the fish (Table 3) with a 1:10 (w/v) tissue:TM ratio required for subsequent analysis. Homogenisation can be achieved using a mortar and pestle, bead beater[11] or stomacher bag and 2 lb hammer.[13]

Table 3 Tissue samples required

Fish size (length)	Tissue sample
<1 cm	Whole fish
1–5 cm	Whole head
5–8 cm	Trimmed head
>8 cm	Dissected brain and eye

For smaller sample volumes, tissue is homogenised in a mortar and pestle. The homogenate is clarified by centrifugation at 3000 ***g*** for 15 minutes at 4°C. An aliquot of the clarified supernatant is used for RNA extraction.

Samples obtained from broodstock (eggs and sperm) are homogenised by bead-beating or drawing the sample repeatedly back and forth through an 18 gauge needle in homogenization buffer, until the viscosity is reduced and an even homogenate is produced. Preparation of broodstock spawning material has not been optimized or validated for testing by RT-qPCR.

Blood samples are lysed by addition of an equal volume of sterile deionised water and incubated at 4°C for 60 minutes. Cellular debris is removed by centrifugation at 10,000 ***g*** at 5°C for 10 minutes and the supernatant used for RNA extraction.

Cell-free supernatants from cell cultures can be used directly for RNA extraction.

RNA is extracted according to the manufacturer's instructions. Two µL of template is added to replicate wells of Master Mix containing 12.5 µL 2× AgPath-ID One-step RT-PCR Buffer, 1 µL 25× RT-PCR Enzyme Mix, 0.9 µM each primer, 0.25 µM probe and water to 23 µL. Negative (e.g. no template control (NTC), extraction negative control), positive and internal controls must be included with each test run. Reverse transcription and PCR amplification are conducted in a thermal cycler programmed as follows: 1 cycle of 50°C for 30 minutes, 1 cycle of 95°C for 10 minutes, 45 cycles of 95°C for 15 seconds and 60°C for 60 seconds. Results are recorded and analysed using the software provided by the manufacturer of the real-time PCR instrument.

For the assay and test results to be accepted the following criteria must be fulfilled: The NTC and/or negative sample control must have no evidence of typical amplification curves. Each positive control must yield a typical amplification curve and mean C_T values within the acceptable range according to quality control data accumulated by the National Association of Testing Authorities Australia (NATA)-accredited diagnostic laboratory. Test samples with typical amplification curves are considered positive**.** At the AFDL any sample producing a typical amplification curve is considered positive and additional verification techniques are undertaken. These include conventional RT-nPCR and sequencing of amplicons. This is an acceptable alternative to using a C_T cut-off to determine positive or negative status of a test sample[32]. Each laboratory will need to determine their own appropriate cut-off values according to results of testing during implementation of the assays.

Interpretation of Results

A positive RT-qPCR result is indicative of the presence of *Betanodavirus* RNA in the sample. However, a positive RT-qPCR does not indicate whether the sample contains infectious virus. Follow-up testing with conventional RT-nPCR and sequencing of amplicons should be undertaken where samples are test-positive from facilities, species, or geographical locations in which *Betanodavirus* infections have not been reported previously. This is especially important when no other diagnostic test has been used. A negative result from finfish tissue is indicative of the absence of nodavirus RNA in the sample.

Nested RT-PCR (RT-nPCR)

Principle of the Test

RT-nPCR is used to amplify a specific sequence from an RNA target. Firstly, reverse transcription (RT) converts the specific target on the viral RNA into cDNA. Then the primary (first-step) PCR amplifies the specific sequence from this cDNA to produce multiple copies. To achieve even greater sensitivity a second, or 'nested', PCR, which

targets a specific DNA sequence within the primary PCR amplicon, is used. The NNV RT-nPCR test is based on the R3-F2 primers (Table 4) and RT-PCR cycling conditions to amplify a 426bp primary sequence and the nested primers NR'3-NF'2 (Table 4) and nested PCR cycling conditions to amplify a 294bp secondary sequence from the T4 region of the *Betanodavirus* coat protein gene. The primers and cycling conditions used in these procedures,[33, 34] with modifications, are fully described.[5, 13] One significant modification exists for testing of blood, where BSA is added at a concentration of 1 µg/µL to the primary PCR. StrataScript™ RT was originally used for the optimisation and validation work for the NNV RT-nPCR. However, this reagent is no longer available and the one-step RT-nPCR procedure described below is in use at the AFDL after comparative testing.

Table 4 Primers used in the NNV RT-nPCR test

Primer	Sequence
R3:	5'- CGA GTC AAC ACG GGT GAA GA -3'
F2:	5'- CGT GTC AGT CAT GTG TCG CT -3'
NR'3:	5'- GGA TTT GAC GGG GCT GCT CA -3'
NF'2:	5'- GTT CCC TGT ACA ACG ATT CC -3'

Due to the highly sensitive nature of the PCR, highly developed technical skills as well as quality control procedures and separate work areas for the different components of the PCR test are essential to avoid contamination and production of false positive results.

Reagents and Materials

Transport Medium (TM). Hank's balanced salt solution (Life Technologies) containing 200 IU/mL penicillin, 200 µg/mL streptomycin sulphate and 2% (v/v) foetal bovine serum.

Fixatives. Ethanol (80-95% analytical grade) or RNAlater

QIAamp Viral RNA Mini Kit (Qiagen, USA) or MagMAX™-96 Viral RNA Isolation Kit (Life Technologies, USA). Both kits have been validated for use by AFDL for RNA and DNA extractions. Alternative kits should be validated for use.

SuperScript III One Step RT-PCR with Platinum Taq (Life Technologies). Any RT could be used; however, not all enzymes perform to the same standard, and comparative testing should be undertaken if reagents are changed.

Primers (see Table 4).

RNase-free deionised water.

HotStarTaq™ Master Mix Kit (QIAGEN). Any *Taq* could be used; however, not all enzymes perform to the same standard,[13] and comparative testing should be undertaken if reagents are changed. The HotStarTaq™ Master Mix Kit is specifically mentioned as this reagent was used for the optimisation and validation work for the NNV RT-nPCR and the hot start format reduces the risk of reagent degradation due to temperature fluctuations, and of contamination due to a reduction in the number of components the operator must add.

10 mg/mL BSA, for testing blood.

DNA ladder and loading dye. Any commercially available DNA ladder that contains bands which enable easy confirmation of the size of the amplicons (~430bp and 295bp) can be used.

Agarose.

Dye (e.g. SYBRSafe gel stain).

Thermal Cycler (Eppendorf MasterCycler).

Gel electrophoresis system.

Gel documentation system.

Test Procedure

Whole larvae/fry or organ samples are homogenised in buffer (e.g. TM or homogenisation buffers provided with extraction kits). The tissue sample required is dependent on the size of the fish (Table 5) with a 1:10 (w/v) tissue:TM ratio required for subsequent analysis. Homogenisation can be achieved using a mortar and pestle, bead beater[11] or stomacher bag and 2 lb hammer.[13]

Table 5 Tissue samples required

Fish size (length)	**Tissue sample**
<1 cm	Whole fish
1 – 5 cm	Whole head
5 – 8 cm	Trimmed head
>8 cm	Dissected brain and eye

For smaller sample volumes, tissue is homogenised in a mortar and pestle. The homogenate is clarified by centrifugation at 3000 ***g*** for 15 minutes at 4°C. An aliquot of the clarified supernatant is used for RNA extraction.

Samples obtained from broodstock (eggs and sperm) are homogenised by bead-beating or drawing the sample repeatedly back and forth through an 18 gauge needle in homogenization buffer, until the viscosity is reduced and an even homogenate is produced. Preparation of broodstock spawning material has not been optimized or validated for testing by RT-nPCR.

Blood samples are lysed by addition of an equal volume of sterile deionised water and incubated at 4°C for 60 minutes. Cellular debris is removed by centrifugation at 10,000 ***g*** at 5°C for 10 minutes and the supernatant used for RNA extraction.

Cell-free supernatants from cell cultures can be used directly for RNA extraction.

RNA is extracted according to the manufacturer's instructions.

RT-PCR is performed in a single step 25 µL reaction mix, containing 2 µL RNA sample, 2 x Reaction Mix, 0.18 µM of each primer (F2 and R3), 1 µL SuperScript III/Platinum Taq Mix and DEPC-treated water.

RT-PCR amplification is carried out in a thermal cycler programmed as follows: 50°C for 30 minutes 94°C for 2 minutes, then 35 cycles of 95°C for 45 seconds, 50°C for 45 seconds and 68°C for 45 seconds with a final extension at 68°C for 7 minutes.

Nested PCR amplification is carried out in a 25 µL reaction mix, containing 2 µL of the primary PCR reaction, 0.36 µM of each primer (NR'3 and NF'2), 12.5 µL HotStarTaq™ Master Mix and deionised water. Reactions are conducted in a thermal cycler programmed as follows: 95°C for 15 minutes, then 30 cycles of 94°C for 45 seconds, 50°C for 45 seconds and 72°C for 45 seconds with a final extension at 72°C for 7 minutes.

Reaction products are analysed after electrophoresis through a 1.5% to 2% agarose gel (using standard procedures) and amplicons are visualised with SYBRSafe gel stain according to the manufacturer's instructions.

The NTC and/or negative sample control must have no evidence of specific amplicons. A positive reaction for the primary RT-PCR results in the production of a discrete 426bp amplicon and for the nested RT-PCR the production of a discrete 294bp amplicon (Figure 3a). Amplicons should be sequenced for confirmation of the presence of nodavirus.

Some non-specific banding is observed after primary RT-PCR testing of broodstock spawning fluids and blood but this is not seen after nested RT-PCR testing and does not affect the quality of the test.

A non-specific band of approximately 900bp is sometimes observed when testing barramundi and Australian bass samples infected with RGNNV 1c isolates (Figure 3b).

Interpretation of Results

Positive RT-nPCR results from finfish tissue are indicative of the presence of *Betanodavirus* RNA in the sample. However, a positive RT-nPCR does not indicate whether the sample contains infectious virus. Amplicons from positive RT-nPCR results from facilities, species or geographical locations where *Betanodavirus* infections have not been reported previously should be sequenced and the sequence compared with known *Betanodavirus* sequences to confirm the result. This is especially important when no other diagnostic test has been used. A negative result from finfish tissue is indicative of the absence of *Betanodavirus* RNA in the sample. When testing broodstock, blood or spawning material, a positive RT-nPCR is indicative of the presence of *Betanodavirus* RNA in the sample. However, a positive RT-nPCR does not indicate whether the sample contains infectious virus. A negative result when testing broodstock blood or spawning material is indicative of the absence of nodavirus RNA in the sample. The role of *Betanodavirus* infections in broodstock, in particular barramundi, is not well understood and a negative RT-nPCR result may be a false-negative result.

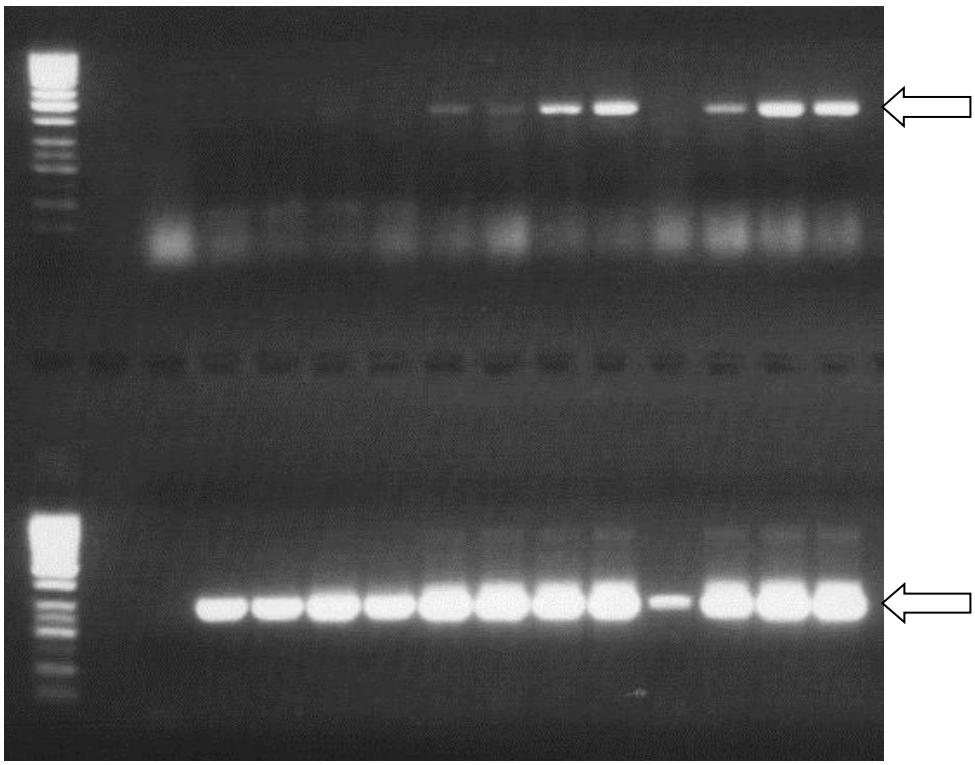


Figure 3a An example of positive RT-nPCR results for Australian RGNNV 1a isolates. Upper gel: Primary RT-PCR – positive amplicon of 426bp (arrowed). Lower gel: RT-nPCR – positive amplicon of 294bp (arrowed).

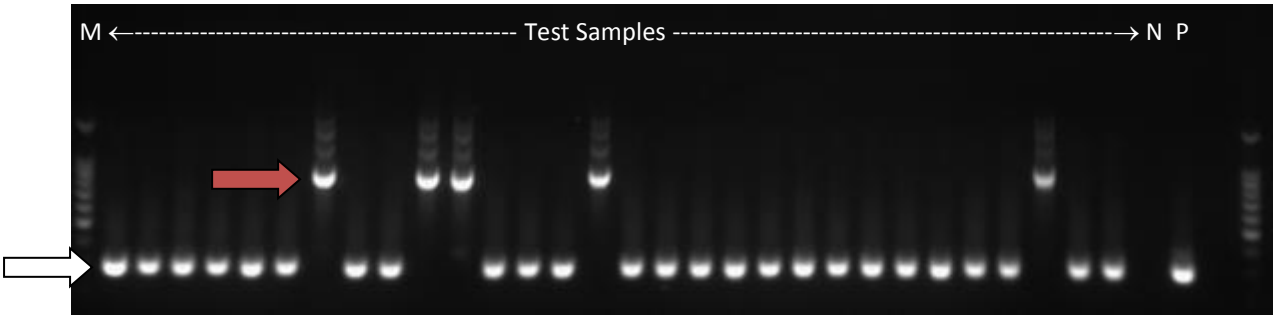


Figure 3b An example of positive RT-nPCR results for Australian RGNNV 1c isolates obtained from barramundi. RT-nPCR – positive amplicon of 294bp (white arrow) and non-specific amplicon of ~900bp (red arrow). M: molecular weight marker, N: no template control, P: positive control

Virus Isolation

Principle of the Test

Virus isolation in monolayer cultures of susceptible cell lines is the "gold standard" for determining the presence of infectious virus. For tissue samples submitted in appropriate condition (unfixed, stored under correct temperature and transportation conditions), processed correctly for virus isolation at a NATA-accredited diagnostic laboratory and inoculated onto cultures of susceptible cell lines, then incubated under appropriate conditions, the development of viral cytopathic effect (CPE) indicates the presence of infectious virus.

Reagents and Materials

SSN-1 and/or E11 cell line(s) (American Type Culture Collection or European Collection of Animal Cell Cultures).

Growth medium: Leibovitz's L-15 medium supplemented with 10% (v/v) foetal bovine serum, 2 mM glutamine, 100 IU/mL penicillin and 100 µg/mL streptomycin sulphate.

Maintenance medium: Leibovitz's L-15 medium supplemented with 2% (v/v) foetal bovine serum, 2 mM glutamine, 100 IU/mL penicillin and 100 µg/mL streptomycin sulphate.

Equipment required for the establishment and maintenance of a fish cell culture laboratory has been discussed previously.[35]

Test Procedure

Whole larvae/fry or organ samples are homogenised in TM and should be kept chilled (4°C to 10°C) during processing. Sample preparation and virus isolation are ideally undertaken on the day of sample receipt. If this is not possible, samples should be stored at -80°C. All manipulations are undertaken in a Class II Biological Safety Cabinet using aseptic technique and sterile equipment and reagents. The sample/tissue required is dependent on the size of the fish (Table 6).

Table 6 Tissue samples required

Fish size (length)	Tissue sample
<1 cm	Whole fish
1–5 cm	Whole head
5–8 cm	Trimmed head
>8 cm	Dissected brain and eye

Prepare two sets of sterile centrifuge tubes labelled with the submission identification (ID) number, sample number and dilution. One set is used for the 1/10 sample dilution and the other set for the 1/100 sample dilution. Add 4.5 mL TM to each 1/100 tube. A clarified 1:10 (w/v) tissue suspension in TM is required after homogenisation. Therefore, weigh a sample container with a tissue sample and subtract the weight of an empty sample container to obtain an estimate of the weight of the tissue sample. Operating within a Class II Biological Safety Cabinet and using 10 mL sterile pipettes, dispense 4.5 mL TM into each 1/100 tube. Place all tubes in a test tube rack in an ice slurry. Homogenise the tissue sample and resuspend in extra TM to achieve a 1:10 (w/v) tissue homogenate. Transfer to the tubes labelled 1/10 and clarify the homogenate by centrifugation at 3000 × $\boldsymbol{g}$ for 15 minutes at 4°C. Pipette 0.5 mL of the supernatant from each 1/10 tissue sample dilution into the corresponding 1/100 test tubes containing 4.5 mL TM.

The procedure described is for virus isolation in cultures of the SSN-1 or E11 cell line established in 24-well tissue culture plates. If different culture vessels are used (e.g. 96-well plates, 25 cm² tissue culture flasks), volumes are adjusted proportionally. Cells are seeded at a density of 1.7×10^5 cells/mL with 1.5 mL of cell suspension (in L-15 medium complete with 10% FBS and antibiotics) and incubated at 25°C overnight. Cultures should be less than 24 hours old when inoculated with the diagnostic sample. On the day of sample inoculation, examine cell cultures to be used by inverted light microscopy. Ensure that they are approximately 70% to 80% confluent, free from overt microbial contamination, and that mitotic figures are visible (that is, the cultures contain actively dividing cells). Any problems should be noted and, if necessary, fresh cultures prepared for use on the next day. Discard the cell culture medium from the SSN-1 or E-11 cultures. Inoculate duplicate cultures with 150 µL of each sample dilution (1/10 and 1/100). One set (column) of four well-cultures on each 24-well tissue culture plate should be used as negative controls, which are inoculated with 150 µL TM only. Incubate the cultures at 25°C for one hour to allow adsorption of any virus present. Following adsorption, add 1.5 mL maintenance medium to each culture (negative controls first) yielding final sample dilutions of 1/100 and 1/1000. Place culture plates in a 25°C incubator. On the day following inoculation, and every 1-3 days thereafter, examine the cultures by inverted light microscopy for any microbial contamination, tissue sample (non-specific) cytotoxicity and viral cytopathic effect (CPE).

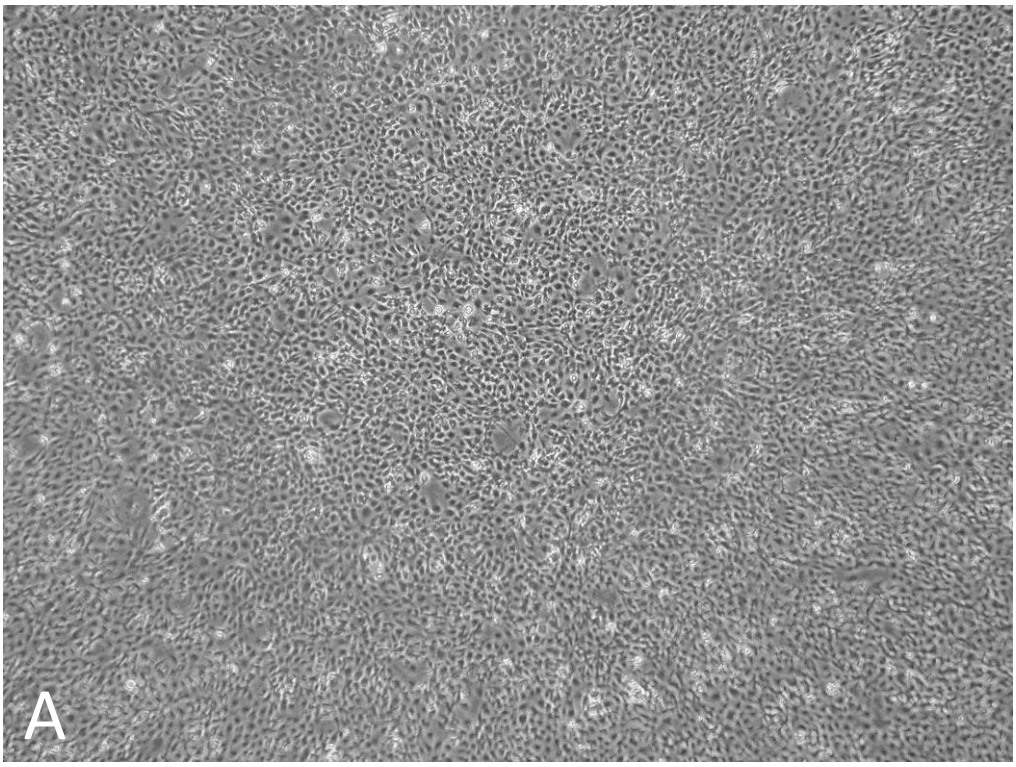
Subculturing

At 7-12 days after inoculation, test cultures not showing CPE should be passaged. For each tissue sample, the contents (medium and cells scraped from the substrate) of each of the four replicate cultures, irrespective of dilution, are pooled into sterile centrifuge tubes. If tissue sample cytotoxicity or bacterial/fungal contamination has been observed during the initial culture period for each pool, the pooled contents should be filtered (0.45 µm) into the sterile centrifuge tube. Alternatively, samples can be centrifuged at 10 000 $\boldsymbol{g}$ for 10 minutes at 5°C. Each pooled sample is diluted (1/10) by pipetting 0.5 mL of the supernatant into a test tube containing 4.5 mL TM. Without decanting the cell culture medium, inoculate duplicate fresh cell cultures in 24-well culture plates, prepared as described above, with 150 µL of the pooled undiluted supernatant and a further duplicate set of wells with the 150 µL of the 1/10 dilution of the pooled supernatant. Place culture plates in a 25°C incubator. Observe these cultures and record observations. Irrespective of the time at which the passage occurred, cell cultures should be observed for at least 21 days for completion of the assay. The test is valid if the negative control cultures retain normal cellular morphology for the full period of incubation in the absence of bacterial

contamination. To ensure cell lines, used on a routine basis, are susceptible to the viruses of concern, titrations of a positive control viral stock on each of the cell lines should be carried out on a regular basis (e.g. 6 monthly).

Interpretation of Results

The test sample is negative if the inoculated cell cultures retain normal cellular morphology similar to the negative control cultures and the cellular monolayer retains normal integrity. If virus isolation is the only test performed, confirmation by ICCT, IFAT or RT-qPCR should be undertaken on a sample of the cell culture to avoid false-negative results. If any of the cell cultures inoculated with test samples demonstrate any abnormalities, such as increased intracellular vacuolation, or monolayer disruption (Figure 4), further investigation is required, such as examination by electron microscopy, further sub-culturing or confirmation of the presence of *Betanodavirus* by molecular methods as described above, or by ICCT or IFAT (see below).



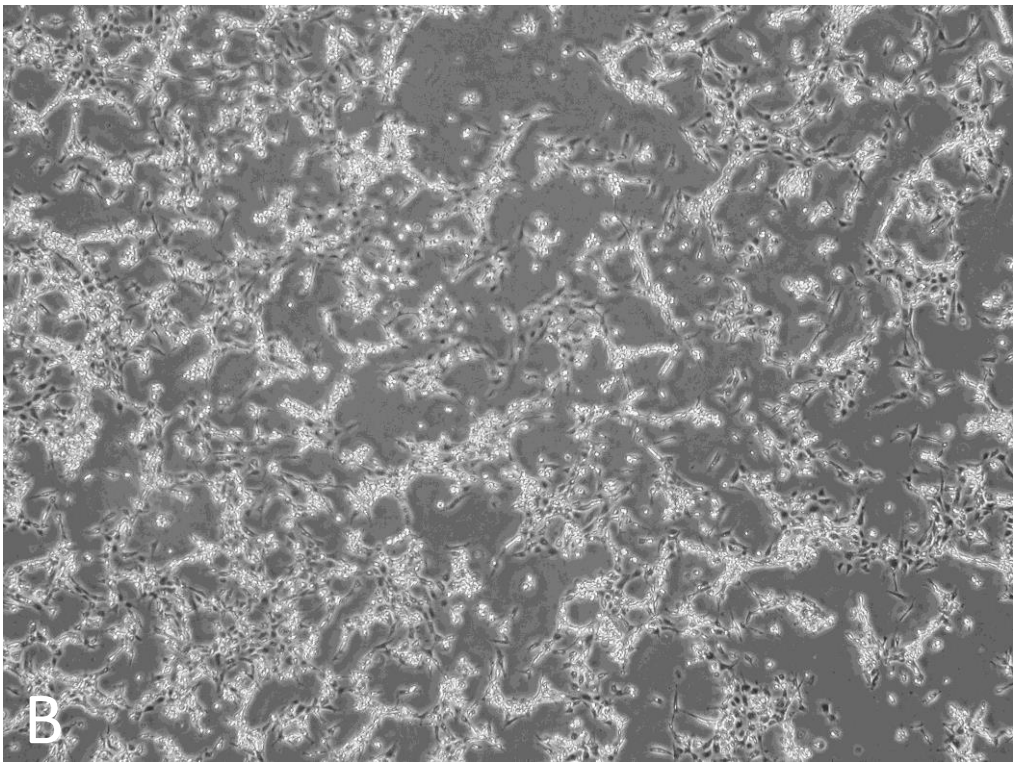


Figure 4 Examples of an uninfected SSN-1 cell culture, 4 days post-seeding (A) and of a BNNV-infected SSN-1 cell culture, 4 days post-seeding, 3 days post-inoculation (B)

Immunocytochemistry Test (ICCT) and Indirect Immunofluorescent Antibody Test (IFAT) of cell cultures

Principle of the Test

This test uses polyclonal antibodies raised in sheep against the recombinant coat protein of a barramundi or sleepy cod *Betanodavirus* isolate and an anti-sheep IgG horseradish peroxidise or Cy2™-conjugated secondary antibody, to localise viral coat protein in fixed cell cultures used in an attempt to isolate NNV from fish tissues. The test is used to confirm or exclude *Betanodavirus* as the agent causing observed CPE or to exclude *Betanodavirus* infection in cell cultures not showing CPE.

Reagents and Materials

As previously stated.

Test Procedure

Fixation

Drain culture medium from the cell cultures. Dispose of the supernatant according to standard procedures. Rinse the cell cultures gently in TBS. Fix the cell cultures in 50% acetone/50% methanol. Add 1 mL per well for 24-well plates (or 3 mL per 25 cm² flask). Incubate for 5 minutes at room temperature, with gentle agitation. Remove fixative and rinse fixed cultures with TBS.

ICCT

Block endogenous peroxidase by adding 3% H_2O_2 in methanol. Add 1 mL per well for 24-well plates (or 3 mL per 25 cm² flask). Incubate for 20 minutes at room temperature. Rinse three times with TBS and block non-specific binding sites with 5% BSA in TBS by adding 1 mL per well for 24-well plates (or 3 mL per 25 cm² flask) and incubate for 30 minutes at 37°C. Rinse each well three times with TBS. Immediately before use dilute sheep anti-NNV rCP polyclonal antibody (1° Ab) 1/1000 in 2.5% BSA in TBS and add to the cell culture: add 500 µL to each well for 24-well plates (or 3 mL per 25 cm² flask). Incubate at 37°C for 60 minutes. Rinse each well three times with TBS. Immediately before use dilute rabbit anti-sheep IgG [H+L] HRP conjugate 1/1000 in 2.5% BSA in TBS and add to the cell culture: Add 500 µL to each well for 24-well plates (or 3 mL per 25 cm² flask). Incubate at 37°C for 60 minutes. Prepare the HRP Chromogen, according to the manufacturer's instructions and add 1 mL per well for 24-well plates (or 3 mL per 25 cm^2 flask). Stop development after 10 minutes by aspirating the HRP Chromogen and replacing with Milli-Q water. Counterstain in Mayer's haematoxylin for 60 seconds, rinse in Milli-Q water for 60 seconds and 'blue' with lithium carbonate for 60 seconds By adding 1 mL per well for 24-well plates (or 3 mL per 25 cm² flask). Rinse in Milli-Q water, rinse each well three times with TBS and observe under an inverted light microscope.

IFAT

Block non-specific binding sites by adding 1 mL 5% (w/v) BSA in TBS to each well and incubate for 30 minutes at 37°C. Rinse each well three times with TBS. Immediately before use dilute sheep anti-NNV rCP polyclonal antibody (1° Ab) 1/1000 in 2.5% BSA (w/v in TBS) and add to the cell culture. Use 500 µL to each well for 24-well plates (or 3 mL per 25 cm^2 flask). Incubate at 37°C for 60 minutes. Rinse each well three times with TBS. Immediately before use dilute rabbit anti-sheep IgG [H+L] Cy2™ conjugate 1/1000 in 2.5% BSA (w/v in TBS) and add to the cell culture. Add 500 µL to each well for 24-well plates (or 3 mL per 25 cm^2 flask). Incubate at 37°C for 60 minutes. Turn on the mercury lamp of a fluorescence microscope 20 minutes prior to use. Wash each well three times with TBS and observe under the fluorescence microscope.

Quality Control Aspects

Positive and negative control cell cultures must be included in every test. Ideally, a positive cell culture exhibiting a low level of infection should also be included. For the ICCT to be valid, dark brown or black staining cytoplasm of cells must be observed in the positive control cultures (Figure 5a). For the IFAT to be valid, bright green fluorescence in the cytoplasm must be observed in the positive control cultures (Figure 5b). No specific staining should be seen in the negative control cultures.

Interpretation of Results

Any positive staining indicates the presence of the *Betanodavirus* coat protein and the culture is considered to be positive for *Betanodavirus* infection.

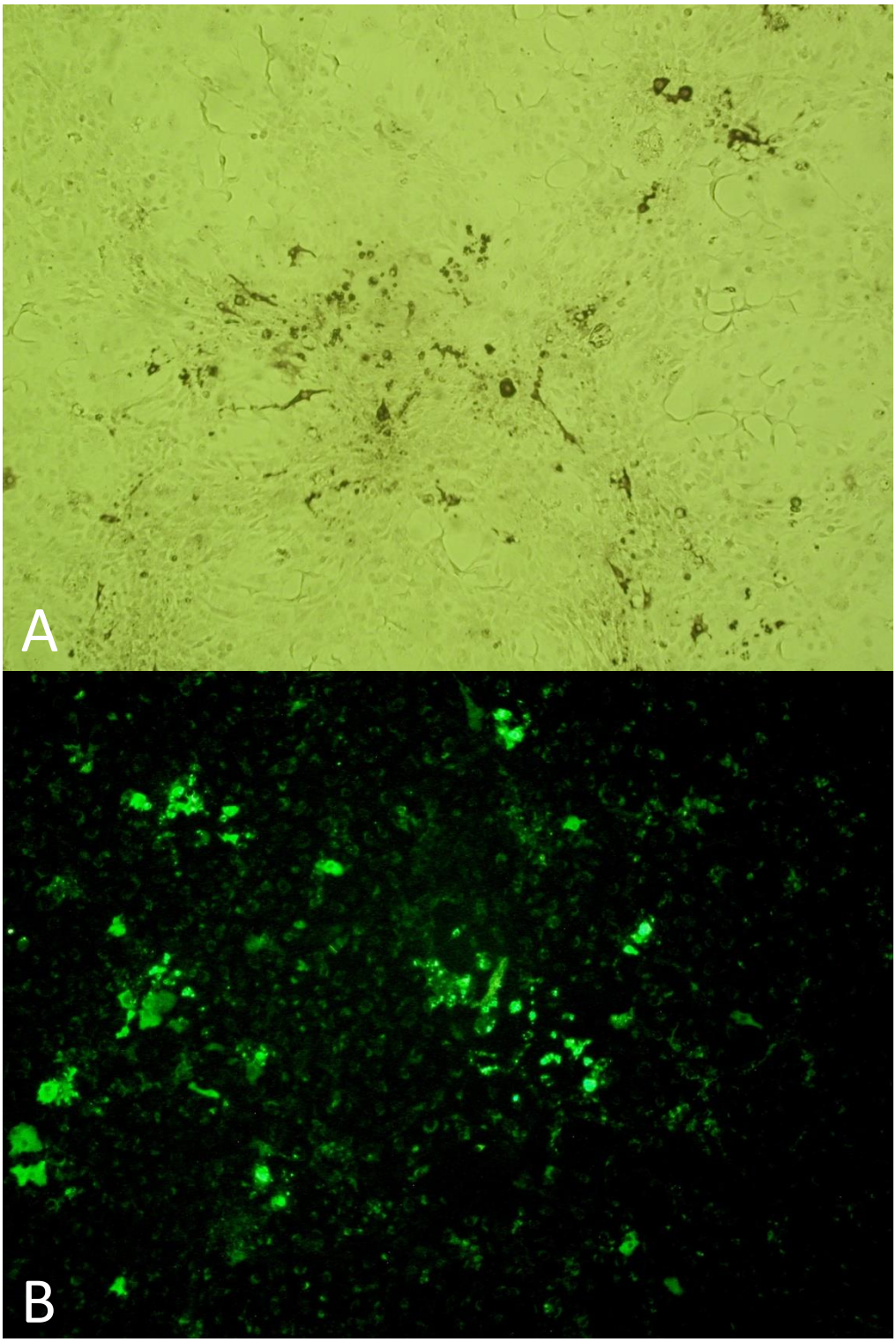


Figure 5 Examples of an ICCT positive BNNV-infected SSN-1 cell culture (A) and of an IFAT positive BNNV-infected SSN-1 cell culture (B)

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Part 3 - Reagents and Kits

Betanodavirus-specific polyclonal antibody

Polyclonal antibodies, raised in sheep against the recombinant coat proteins from barramundi and sleepy cod *Betanodavirus* isolates, are available from the Queensland Government Department of Agriculture, Fisheries and Forestry, Biosecurity Sciences Laboratory (BSL), Brisbane, Queensland.

Betanodavirus-infected finfish for positive controls for IHCT

Tissue from *Betanodavirus*-infected finfish on histological slides is available from the Queensland Government Department of Agriculture, Fisheries and Forestry Biosecurity Sciences Laboratory (BSL), Brisbane, Queensland.

Betanodavirus-infected finfish homogenate and *Betanodavirus*-infected SSN-1 cell culture supernatant for positive controls for RT-qPCR and RT-nPCR

Homogenates from *Betanodavirus* infected finfish and *Betanodavirus*-infected cell culture supernatant are available from the authors.

Suppliers of Reagents and Kits

In Australia:

AAHL Fish Diseases Laboratory
CSIRO Australian Animal Health Laboratory
5 Portarlington Rd
East Geelong VIC 3219
Ph: + 61 3 5227 5000
Fax: +61 3 5227 5555
Email: afdl@csiro.au

Biosecurity Sciences Laboratory (BSL)
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Block 12, 39 Kessels Road,
Coopers Plains QLD 4108
Ph: +61 7 3276 6062
Fax +61 7 3216 6620
Email: bslclo@daff.qld.gov.au

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Tel: 1800 222 287
Fax: 1800 287 222

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Scoresby VIC 3179
Email: InfoScientificAU@thermofisher.com
Tel: 1300 735 292

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Chadstone Centre VIC 3148
Email: orders-au@qiagen.com
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Fax: 03-9840-9888

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Mulgrave VIC 3170
Email: orders.anz@lifetech.com
Tel: 1800 636 327
Fax: 1800 143 363

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Abacus ALS New Zealand
PO Box 97-923
South Auckland Mail Center
Auckland
E-Mail: info@abacus-als.co.nz
Tel:0800 222 170
Fax: 0800 222 180

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P.O. Box 9092
Scoresby VIC 3179
Email: InfoScientificAU@thermofisher.com
Tel: 1300 735 292

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www.qiagen.com
Bio-Strategy Ltd
www.bio-strategy.com
PO Box 303 385
Auckland 0751
E-mail: info@bio-strategy.com
Tel: 0800 34 24 66

Life Technologies
www.Life Technologies.com.au
Life Technologies New Zealand Limited
18-24 Botha Road
Penrose
Auckland 1006
Email: orders.anz@lifetech.com
Tel: 0800 636 327