FINAL REPORT

Aquatic Animal Health Subprogram: development of diagnostic tests for the detection of nodavirus

N J Moody, P F Horwood and S McHardy

August 2004

FRDC Project No. 2001/626

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2001/626 Aquatic Animal Health Subprogram: development of diagnostic tests for the detection of nodavirus

PRINCIPAL INVESTIGATOR: Dr N J Moody

ADDRESS: Oonoonba Veterinary Laboratory
Department of Primary Industries and Fisheries
PO Box 1085
Townsville QLD 4810
Telephone: 07 4722 2603 Fax: 07 4778 4307

OBJECTIVES:

1. To optimise and validate a sensitive and specific Nested RT-PCR test for the detection and identification of endemic and exotic nodaviruses from a range of samples and fish species.
2. To establish a cell line which can be used for the isolation, amplification and titration of endemic and exotic nodaviruses from a range fish species.
3. To produce immunodiagnostic tests, capable of localising endemic and exotic nodaviruses in fish tissues and cell cultures.
4. To distribute the above technology and procedures to laboratories as soon as optimisation and validation are complete.
5. To produce an Australian and New Zealand Standard Diagnostic Procedure for the detection of Nodavirus.

NON TECHNICAL SUMMARY:

OUTCOMES ACHIEVED

The molecular and immunodiagnostic procedures produced during the project will enable sensitive and specific detection and monitoring of nodavirus infections. These procedures are applicable to fresh and/or fixed material, from a range of temperate and tropical fish species from both cultured and wild fish populations. The procedures will be made available through the Australian and New Zealand Standard Diagnostic Procedure for the detection of nodavirus, a draft of which is contained in this report. These diagnostic procedures are a significant improvement on the previously available tests and will increase government, industry and community confidence in the advice provided regarding the detection, control and management of this important virus.

Viral Nervous Necrosis (VNN), or Viral Encephalopathy and Retinopathy (VER) is an infectious disease of finfish larvae, fry and juveniles and is caused by a nodavirus. Disease caused by nodavirus has been reported from at least 35 different marine species. More recently the appearance of disease in freshwater fish species has been reported. The only widely used diagnostic test for the diagnosis of nodavirus in Australia has been by microscopic observation of damage in tissue of the brain, spinal cord and retina, using standard histology procedures. However, only fixed tissue samples are appropriate for testing by histology and no standardised procedures exist for the detection of nodavirus in fresh material. Therefore, the development of a range of optimised and validated molecular, cell culture and immunodiagnostic detection procedures was undertaken.

The molecular detection procedure (a Nested RT-PCR) has been routinely used at the Oonoonba Veterinary Laboratory to test unfixed fish tissue samples for over four years. However it was important to ensure this procedure could detect and identify the full range of nodaviruses present in Australia. This procedure is required to screen unfixed fish tissue samples and broodstock spawning and blood samples and will detect nodavirus infections in fish that are infected, but are not showing any signs of disease. Cell culture is considered the gold standard for virus detection and is the only test capable of detecting live, infectious virus. Development of cell lines that grow nodavirus would enable virological screening of fresh fish samples and the ability to further study and characterize the nodaviruses after growth in the cells. The immunodiagnostic procedures would be used to test fixed tissue sections, which is especially important when the damage caused by the nodavirus is unusual or difficult to see. The immunodiagnostic procedures could also be used to confirm the identity of nodaviruses isolated in the cell lines.

Optimisation, validation and standardisation of reagents and diagnostic procedures would ensure harmonisation of test methods nationally. Ensuring that the tests will detect exotic nodavirus isolates would enable rapid diagnostic capability in the event of an exotic nodavirus outbreak.

Molecular Detection Test Development - Nested RT-PCR procedure

A sensitive and specific molecular detection procedure (Nested RT-PCR) was developed. This procedure could successfully detect Australian nodavirus infections in larvae or broodstock samples obtained from Queensland, the Northern Territory and Tasmania and detected two exotic nodavirus isolates tested at the Fish Diseases Laboratory at the Australian Animal Health Laboratory. The molecular detection procedure always detected nodavirus in clinically affected barramundi larvae and fry. Positive results were never produced from negative samples.

While the molecular detection procedure developed was robust and repeatable with larvae, fry and juvenile tissue, material from broodstock was more difficult to test. Addition of another reagent (bovine serum albumin) to the molecular detection procedure for the detection of nodavirus in blood, improved the repeatability and sensitivity. Generally, broodstock blood samples were more appropriate for testing than spawning fluids. One of the major constraints to testing broodstock ovarian and sperm samples was the effective disruption of the samples. Further research involving repeated blood and spawning fluid testing of infected and uninfected broodstock is required to understand the nature of the nodavirus infection in broodstock. This will also the most appropriate broodstock sample collection and processing procedures to be developed.

From the evaluation of different reagents used in the molecular detection test it was observed that not all are appropriate for use. When two of the reagents were evaluated for use, the molecular detection procedure failed. As a consequence, it is strongly recommended that, when different reagents are used, comparative testing with the established procedure described in this report is undertaken to ensure quality is maintained. Sequencing of three Australian nodavirus isolates allowed the relatedness of these endemic isolates and the exotic nodaviruses to be determined. The sequencing molecular procedure will enable nodavirus isolates from new species, or from new geographical locations, to be compared with the known nodavirus isolates for epidemiological studies.

Cell line development

Over 60 primary brain cell lines and 39 cloned brain cell lines were successfully produced. Fifteen primary cell lines and eight cloned cell lines were considered suitable for screening to determine if the nodavirus would grow. Unfortunately, none of these cell lines would support the growth of nodavirus. Dead cells were observed after infected material had been added but when attempts were made to regrow the virus from these cells in fresh cell lines, no cell death occurred. It is unknown why the cell lines made were not susceptible to the virus. The original barramundi brain (LCB) cell line grew nodavirus up to and including passage 35, but from passage 36 and above no virus growth was observed. The only cell line widely used to grow nodavirus overseas (the SSN-1 cell line) was evaluated but this cell line also failed to grow the endemic nodavirus isolates tested. A cell line that supported the growth of nodavirus would have allowed the environmental tolerances of the virus, for example the tolerated temperature range, effect of freezing and thawing, and the effect of storage in different solutions and at different temperatures, to be determined. The cell line would also have allowed development of effective disinfection and control measures.

Immunodiagnostic Tests – Immunohistochemistry test (IHCT) and Indirect Immunofluorescent Antibody Test (IFAT)

The project produced sensitive and specific immunodiagnostic reagents, which were used to develop two immunodiagnostic procedures; an immunohistochemistry test (IHCT) and an indirect immunofluorescent antibody test (IFAT). The immunodiagnostic reagents can be used at a dilution of 1/1000 in these procedures to detect nodavirus in histological sections. Comparative histology and immunodiagnostic test analysis of sections from infected tissue samples showed a 100% agreement between the presence of nodavirus damage and positive immunodiagnostic test results. Testing of juvenile fish surviving experimental infection with nodavirus demonstrated the increased sensitivity of the immunodiagnostic tests, when compared to histology. When the same tissue sections were tested, nodavirus was detected in 77.9% of the sections using the immunohistochemistry test. Only 49.8% of these sections were positive by histology, indicating a greater sensitivity in detecting unapparent carrier fish using the immunodiagnostic procedures. The immunodiagnostic procedures detected exotic nodavirus isolates in fixed material from France, Israel, Norway, Japan and the Philippines. The immunodiagnostic tests could also be used in the future to confirm the identity of nodaviruses isolated in cell cultures, when a cell line that grows nodavirus is available.

Technology transfer

The procedures developed during the project have been distributed to Co-Investigators' laboratories. Over one litre of polyclonal antibody has been produced, evaluated, aliquoted and stored and is available to laboratories requiring nodavirus immunodiagnostic capability. All the procedures developed will be made freely available through the publication of an Australian and New Zealand Standard Diagnostic Procedure for the detection of nodavirus.

KEY WORDS: nodavirus, betanodavirus, viral nervous necrosis, nervous necrosis virus, viral encephalopathy and retinopathy, RT-PCR, Nested RT-PCR, cell culture, immunodiagnostics, IHCT, IFAT, polyclonal antibodies, barramundi, striped trumpeter, larvae, broodstock, virus detection.

2. Background

The disease, Viral Encephalopathy and Retinopathy (VER), or Viral Nervous Necrosis (VNN) is caused by a nodavirus. VNN is a common disease of marine finfish larvae and fry with clinical signs including cessation of feeding, abnormal swimming behaviour, colour changes and mass mortalities in larvae and juveniles (OIE, 2003; Anderson and Moody, 2004). VNN has a worldwide distribution with at least 35 different marine fish species being recorded as having VNN caused by nodavirus (Munday *et al*., 2002; OIE, 2003). More recently, VNN infections have been detected in fresh water fish species (Athanassopoulou *et al*., 2003; Hedge *et al*., 2003; N Moody, pers. obs.).

Piscine nodaviruses (or betanodaviruses) are members of the family *Nodaviridae* and are small (25-30nm), non-enveloped and icosahedral in shape. The virions contain single stranded, bipartite positive sense RNA. The larger RNA segment, RNA1 (3.1 kb), encodes a non-structural protein of approximately 110 kDa while the smaller RNA segment, RNA2 (1.4 kb), encodes the 42 kDa coat protein (Mori *et al*., 1992; Murphy *et al*., 1995; Skliris *et al*., 2001).

A range of diagnostic tests for nodavirus diagnosis have been published by the World Organisation for Animal Health (OIE, 2003). These include:

- Histological examination of brain, spinal cord and retina.
- Isolation in SSN-1 or E-11 cell lines.
- Indirect fluorescent antibody test (IFAT) or immunohistochemistry test (IHCT) staining of the virus in histological sections.
- Detection of viral RNA by reverse transcription followed by one round of amplification by polymerase chain reaction (RT-PCR).

The only widely used method to detect nodavirus infection in Australia is currently restricted to histological examination of fixed tissue. At least two different RT-PCR tests are used laboratories throughout Australia, however, it is not known whether these RT-PCR tests will detect all endemic and exotic nodavirus isolates. There are currently no immunodiagnostic tests available for the detection of nodavirus in Australia. Therefore, there is a requirement for the development, validation and standardisation of a broader range of more sensitive and specific diagnostic tests.

The gold standard diagnostic test for fish virus detection is isolation of the virus in susceptible cell cultures. Unfortunately, the fish nodaviruses do not grow in the more common fish cell lines. There are only a small number of cell lines which support the growth of the piscine nodaviruses (Frerichs *et al*., 1996 – SSN-1; Chew-Lim *et al*., 1996 – a seabass cell line; Chi, *et al*., 1999 - grouper fin cell line; Iwamoto *et al*., 2000 - E-11 clone of SSN-1; Lai *et al*., 2001 - grouper brain cell line; Aranguren *et al*., 2002 – turbot cell line). Unfortunately, the SSN-1, E-11 and seabass cell lines are persistently infected with retroviruses which makes it undesirable to import them into Australia due to quarantine concerns. The other cell lines either demonstrate variable growth of nodaviruses, have only been screened with a small number of isolates, have yet to be characterized or are not widely available. Therefore, there is a critical need for a cell line to be developed that is specific to the nodavirus species/strains that infect fish in Australia. With this objective, Dr N Moody at the Oonoonba Veterinary Laboratory developed a barramundi brain (LCB) cell line that supported the growth of nodaviruses isolated from barramundi and sleepy cod. The causative agent of the cytopathic effect (CPE) was confirmed as a nodavirus using the RT-PCR procedure reported by Nishizawa *et al*. (1994) followed by purification by density gradient ultracentrifugation and

visualisation of nodavirus particles by transmission electron microscopy. Unfortunately, the cell line transformed at approximately passage 30 and no longer expressed CPE after inoculation with nodavirus-infected material. It is unknown if viral replication was continuing without the production of CPE. Establishment of a nodavirus-susceptible cell line is crucial for the rapid identification of infectious nodavirus from fish samples. The ability to isolate and amplify nodavirus in a cell line will produce a broadly practical diagnostic procedure for the detection of infectious virus. A nodavirus susceptible cell line will also create the ability to characterise isolates and determine the environmental and chemical tolerances of nodaviruses.

While cell culture isolation would be the gold standard method of isolating infectious nodavirus, the non-specific nature of cell culture isolation means there is a requirement for an immunodiagnostic confirmatory test to ensure the agent producing the CPE is actually nodavirus. An indirect fluorescent antibody test (IFAT), using rabbit anti-NNV polyclonal antibodies, has been widely used overseas as a confirmatory test for nodavirus in cell culture (Iwamoto *et al*., 1999; Castric *et al*., 2001). An IFAT would be an invaluable tool to rapidly confirm the identity of nodavirus isolated in cell culture in Australia. The immunohistochemistry test (IHCT), also using rabbit anti-NNV polyclonal antibodies, has been used overseas for confirmation of nodavirus as the agent responsible for lesions seen in histological sections (Munday *et al*., 1994; Curtis *et al*., 2001; Grove *et al*., 2003). The typical histological presentation of fish infected with nodavirus is characterised by distinctive lesions in the nervous tissue of the spinal cord, brain and retina. However, due to the large range of fish species that are susceptible to nodavirus infection, the histological presentation can vary somewhat. In addition, sub-clinical infections and the age group of the infected fish may affect the pathologist's ability to identify nodavirus infection in histological sections. Therefore, it is essential to have a sensitive and specific confirmatory test to identify nodavirus as the causative agent of the lesions. Immunodiagnostics used in immunohistochemistry can be used to identify the association of nodavirus with tissue changes and to determine the distribution of the virus in tissue or organ sections. Therefore, IFAT and IHCT can be used to identify nodavirus in cell culture and histological sections.

These immunodiagnostic tests are developed using polyclonal or monoclonal antibodies, which react against a part of the virus. The antibodies required can be produced from purified virus preparations or from recombinant proteins expressed in cell or bacterial cultures. Recombinant proteins have been used to produce immunodiagnostics for diagnostic tests overseas (Sideris, 1997; Tanaka *et al*., 2001). Nodavirus recombinant coat protein has been successfully used as a coating antigen for ELISA detection of antibodies, with no significant difference in activity when compared to results obtained using purified virus (Watanabe *et al*., 2000). These authors commented that it was easier and cheaper to produce and purify expressed recombinant protein than it was to grow and purify virus in cell culture. The barramundi nodavirus coat protein gene has been cloned and expressed at the DPI&F Queensland Agricultural Biotechnology Centre (T Mahoney, pers. com.). This expressed protein will be used as an immunogen for the production of antibodies, against the nodavirus coat protein, in sheep. The method used to produce the antibodies will be standardised. Therefore, polyclonal antibodies developed from the uninfectious expressed nodavirus coat protein will be used to develop immunodiagnostic test procedures for infected cell cultures and histological sections.

Piscine nodaviruses have been studied intensively by Japanese and European researchers due to the importance of marine finfish aquaculture in these areas. A sensitive reverse transcription-polymerase chain reaction (RT-PCR) detection test has been developed

(Nishizawa *et al*., 1994). The primers used in this RT-PCR test recognise almost all sub-groups of the nodavirus from different fish species (Nishizawa *et al*., 1997), including the barramundi and sleepy cod nodaviruses that have been isolated at OVL, however sensitivity has been improved through preliminary optimisation of reagents Unfortunately, the RT-PCR test failed to consistently produce a product of the required size from cell culture material known to be infected with nodavirus. For this reason the use of a nested primer set was adopted using the procedure described by Thiery *et al*. (1999). The Nested RT-PCR (nRT-PCR) consistently resulted in the production of bands of the required size when barramundi and sleepy cod isolates were tested. When applied to barramundi broodstock material, the Nested RT-PCR also demonstrated a significant increase in sensitivity (12/13 positive) when compared to RT-PCR (0/13 positive) from the same samples of RNA (N. Moody, unpubl.). There is one report of the use of a Nested RT-PCR to test broodstock blood and spawning material (Dalla Valle *et al*., 2000), but this procedure used a different RT-PCR primer set and no comparative evaluation was reported to determine the most appropriate broodstock sample to use. However, the results did indicate that broodstock blood samples should be evaluated along with the broodstock spawning material. While the results of Nested RT-PCR for screening broodstock are encouraging, further research needs to be undertaken to determine the minimum sample volume required and optimise sample collection and processing procedures. Optimisation and validation of the Nested RT-PCR with nodaviruses from different geographical regions (around Australia and overseas) and different species is yet to be done but is vital to ensure a standardised test is used nationally. Testing of broodstock spawning fluids and blood samples, taken concurrently, will enable development of a sampling procedure to allow selection of negative broodstock and possibly eliminate the occurrence of nodavirus-infected larvae.

The development of the three validated and standardized diagnostic tests (cell culture, Nested RT-PCR and immunodiagnostics) to detect nodavirus infections in broodstock and fish tissue will significantly improve the sensitivity, specificity and confidence of nodavirus diagnostics in Australia. A range of different diagnostic tests will allow selection of an appropriate test, depending on the type of sample submitted. For example, the Nested RT-PCR and cell culture could be used for primary detection of nodavirus in unfixed broodstock spawning material and fish tissue samples, the IFAT and/or IHCT could be used for primary detection of nodavirus in fixed material and the Nested RT-PCR and IFAT/IHCT could be used confirmation of the identity of nodavirus, isolated in cell culture.

3. Need

Funding to improve the diagnostic capability of Australian laboratories with regard to aquatic diseases was a major component of a Commonwealth Government new budget initiative entitled "Building a National Approach to Animal and Plant Health". After consultation with aquaculture and fishing industries, the AQUAPLAN Business Group determined that viral encephalopathy and retinopathy (VNN/VER), caused by nodavirus, was one of eight pathogens/diseases identified as priorities for diagnostic test development. VNN was identified as a priority by the Aquaculture Association of Queensland, Aquaculture Council of Queensland, Australian Barramundi Farmers Association and the Tasmanian Fish Health Advisory Group. The Tasmanian Fish Health Advisory Group provided a letter of support.

Validate a sensitive and specific Nested RT-PCR test

- There is a need to ensure the Nested RT-PCR test developed at OVL will detect the range of endemic nodaviruses from a variety of finfish species. It is also important to ensure the test will detect exotic nodavirus isolates for use in the event of an exotic nodavirus incursion.
- Standardisation of reagents and sample collection and preservation procedures will enable consistency of test methods between laboratories.
- A test is required to screen broodstock samples to eliminate nodavirus-positive carrier fish from production facilities. Due to the small sample sizes obtainable, the Nested RT-PCR test is the best test option.

Establishment of a cell line

- Cell culture is considered the gold standard for virus detection. The cell culture will enable cost-effective screening of larvae for sale or release and to develop management procedures.
- Cell culture is more tolerant to sample degradation than other detection methods and allows testing of material that is unsuitable for use in other tests.
- The vast majority of cell lines currently available have been produced from temperate species. Availability of cell lines, from tropical fish species, is required.

Immunodiagnostics

- IFAT tests can confirm the identity of viruses isolated in cell culture during diagnostic or surveillance activities. Their use is critical for the identification of viruses.
- Immunohistochemistry/immunofluorescence is a sensitive and specific test that can be used on fixed material. It is not always possible to obtain fresh samples and confirmation of nodavirus in tissue sections can be required. Immunohistochemistry is also a tool to identify the tissues targeted by nodavirus.

4. Objectives

1. To optimise and validate a sensitive and specific Nested RT-PCR test for the detection and identification of endemic and exotic nodaviruses from a range of samples and fish species.

2. To establish a cell line which can be used for the isolation, amplification and titration of endemic and exotic nodaviruses from a range fish species.

3. To produce immunodiagnostic tests, capable of localising endemic and exotic nodaviruses in fish tissues and cell cultures.

4. To distribute the above technology and procedures to laboratories as soon as optimisation and validation are complete.

5. To produce an Australian and New Zealand Standard Diagnostic Procedure for the detection of Nodavirus.

5. Modification of the Nodavirus Nested RT-PCR and development of sample testing procedures

Objective 1: "To optimise and validate a sensitive and specific Nested RT-PCR test for the detection and identification of endemic and exotic nodaviruses from a range of samples and fish tissues".

5.1 Methods

5.1.1 Initial Nested RT-PCR procedure

The NNV Nested RT-PCR test was based on the RT-PCR primers (R3: 5'-CGAGTCAACACGGGTGAAGA-3', F2: 5'-CGTGTCAGTCATGTGTCGCT-3') and cycling conditions described in Nishizawa *et al*. (1994) and the nested primers (NR'3: 5'-GGATTTGACGGGGCTGCTCA-3', NF'2: 5'-GTTCCCTGTACAACGATTCC-3') and cycling conditions described in Thiery *et al*. (1999). This procedure used the modifications described in Anderson and Moody (2004) and is described below.

RNA was extracted from a 200µl aliquot of the homogenised, clarified sample using the High Pure Viral RNA Extraction Kit (ROCHE, USA). The extraction was performed according to the manufacturer's instructions and the optional kit step, which involved incubating the binding buffer and sample for 10 minutes prior to loading the spin column, was always undertaken. The sample RNA was heated to 90°C for 5 minutes and cDNA was transcribed in a 20µl reaction mix, containing 9.5µl RNA sample, 1x RT-PCR buffer (QIAGEN, USA), 5U RNasin (Promega, USA), 0.5mM dNTPs (Promega, USA), 1.0µM R3 primer, 4U Omniscript™ RT (QIAGEN, USA) and RNase-free deionised water, at 37°C for 60 minutes. PCR amplification was carried out in a 50µl reaction mix, containing 5µl of the RT reaction, 0.2µM of each primer (R3 and F2), 2.5mM $MgCl_2$, 25µl of HotStarTaq™ Master Mix (QIAGEN, USA) and deionised water, in a Mastercycler thermal cycler (Eppendorf, Germany) programmed with the following cycles; 95°C for 15 minutes, 25 cycles of 95°C for 40 seconds, 50°C for 40 seconds, 72°C for 40 seconds and finally 72°C for 10 minutes. Nested PCR amplification was carried out in a 50µl reaction mix, containing 1µl of the PCR reaction, 1.0µM of each primer (NR'3 and NF'2), 25µl of HotStarTaq™ Master Mix (QIAGEN, USA) and deionised water, in a Mastercycler thermal cycler (Eppendorf, Germany) programmed with the following cycles; 95°C for 15 minutes, 25 cycles of 94°C for 40 seconds, 50°C for 40 seconds, 72°C for 40 seconds and finally 72°C for 10 minutes. Reaction products were analysed following electrophoresis at 60V through a 2% agarose gel using Tris-acetate-EDTA (TAE, 40mM Tris-acetate, 1mM EDTA) electrophoresis buffer. Amplicons were visualised using a UV transilluminator after staining the gel with 10mg/ml ethidium bromide.

For the evaluation of the different RNA extraction techniques, Reverse Transcriptases and *Taq* Master Mixes, the Nested RT-PCR described above was used as the base standard.

5.1.2 Optimisation of NNV Nested RT-PCR reaction conditions

5.1.2.1 Optimisation of Nested PCR magnesium chloride concentration

To optimise the concentration of magnesium chloride in the Nested PCR, concentrations of $MgCl_2$ ranging from 1.5mM to 4.0mM were tested on three replicated template dilutions

(Neat, 10^{-3} and 10^{-5}) of a known BNNV-positive homogenate. RNA extraction and RT-PCR testing were as described in Section 5.1.1. For the Nested PCR step all reaction components and cycling conditions were as described in Section 5.1.1, with the exception of $MgCl_2$ concentration, which was modified according to Table 1.

Table 1: Concentrations of magnesium chloride evaluated.

TEMPLATE DILUTION	FINAL $MgCl_2$ CONCENTRATION					
	A	B	C	D	E	F
Neat	1.5mM	2mM	2.5mM	3mM	3.5mM	4mM
1/1000	1.5mM	2mM	2.5mM	3mM	3.5mM	4mM
1/100000	1.5mM	2mM	2.5mM	3mM	3.5mM	4mM
Negative	2.5mM	2.5mM	2.5mM	2.5mM	2.5mM	2.5mM

5.1.2.2 Optimisation of Nested PCR primer concentration

To optimise the concentration of primer in the Nested PCR, concentrations of primer ranging from 0.25µM to 4.0µM were tested on three replicated template dilutions (Neat, 10^{-3} and 10^{-5}) of a known BNNV-positive homogenate. RNA extraction and RT-PCR testing were as described in Section 5.1.1. For the Nested PCR step all reaction components and cycling conditions were as described in Section 5.1.1, with the exception of primer concentration, which was modified according to Table 2.

Table 2: Concentrations of Nested primer evaluated.

TEMPLATE DILUTION	FINAL PRIMER CONCENTRATION					
	A	B	C	D	E	F
Neat	0.25µM	0.5µM	1.0µM	2.0µM	3.0µM	4.0µM
1/1000	0.25µM	0.5µM	1.0µM	2.0µM	3.0µM	4.0µM
1/100000	0.25µM	0.5µM	1.0µM	2.0µM	3.0µM	4.0µM
Negative	0.25µM	0.5µM	1.0µM	2.0µM	3.0µM	4.0µM

5.1.2.3 Optimisation of Nested RT-PCR cycle number

To optimise the number of cycles for each PCR, either 25 or 30 cycles for each PCR were tested on three replicated template dilutions (Neat, 10^{-3} and 10^{-5}) of a known BNNV-positive homogenate. RNA extraction and RT-PCR testing were as described in Section 5.1.1. For the Nested PCR step all reactions components and cycling conditions were as described in Section 5.1.1, with the exception of cycle number, which was modified according to Table 3.

Table 3: Different cycle combinations for each PCR step evaluated.

	REPLICATE 1		REPLICATE 2		REPLICATE 3		REPLICATE 4	
TEMPLATE DILUTION	RT-PCR	Nested PCR	RT-PCR	Nested PCR	RT-PCR	Nested PCR	RT-PCR	Nested PCR
Neat	25	25	25	30	30	25	30	30
1/1000	25	25	25	30	30	25	30	30
1/100000	25	25	25	30	30	25	30	30
Negative	25	25	25	30	30	25	30	30

5.1.3 Comparison of RNA extraction techniques and variability of Reverse Transcriptase and Hot Start Taq Master Mixes.

RNA extraction reagents, reverse transcriptases and hot start *Taq* polymerases were compared to observe any possible variability between commercial products. The reagents used were those commercially available at the time. As products have changed, and more are becoming available all the time, only those that were incorporated into the Nested RT-PCR procedure have been identified.

5.1.3.1 RNA extraction techniques

To determine the most effective method of RNA extraction, four commercially available kits and reagents were evaluated. Methods were assessed based on their ability to extract nucleic acid from a sample in solution, as this would represent the type of sample most likely to be tested using the Nested RT-PCR (eg. broodstock spawning fluids, cell culture supernatant, homogenised larvae or fish organs). The major factor used to determine the performance of each product was sensitivity, with cost and ease of use secondary considerations. Three procedures used silica-membrane technology while one used precipitation after extraction with an organic solvent.

Larvae from three clinical BNNV submissions (2-41712: 2002, 1-56700; 2001 and 0-48186; 2000) were homogenised and clarified as described in section 5.1.5. Serial, ten-fold dilutions, from 10^{0} to 10^{-9}, were prepared from each sample in viral transport media. Replicate aliquots of each set of serial dilutions were stored at -80°C until required. RNA was extracted from each serial dilution for each of the three samples, according to the manufacturer's instructions, and tested using the Nested RT-PCR procedure described in Section 5.1.1.

5.1.3.2 Variability of Reverse Transcriptases

Four Reverse Transcriptases were compared to determine the most sensitive in the Nested RT-PCR test. The major factor used to determine the performance of each reverse transcriptase was sensitivity, with cost a secondary consideration.

RNA was extracted from the clarified homogenates from the same submissions used above (2-41712: 2002, 1-56700; 2001, and 0-48186; 2000) using the High Pure Viral RNA Kit (ROCHE). Serial, ten-fold dilutions, from 10^{0} to 10^{-9}, were prepared from each RNA sample in nuclease-free deionised water. Replicate aliquots of each set of dilutions were stored at -80°C until required. A replicate of the RNA dilution series from each submission was used to assess each reverse transcriptase, which was used according to the manufacturer's instructions. PCR and Nested PCR were carried out using the procedure described in Section 5.1.1.

5.1.3.3 Variability of *Taq* DNA Polymerase

Four *Taq* Polymerases were compared to determine the most appropriate for use in the Nested RT-PCR test. The major factor used to determine the performance of each polymerase was sensitivity, with cost a secondary consideration.

RNA was extracted from the clarified homogenates from the same submissions used above (2-41712: 2002, 1-56700; 2001, and 0-48186; 2000) using the High Pure Viral RNA Kit (ROCHE). Serial, ten-fold dilutions, from 10^{0} to 10^{-9}, were prepared from each RNA sample

in nuclease-free deionised water. Replicate aliquots of each set of serial dilutions were stored at -80°C until required. Reverse transcription was carried out on the replicates of the dilution series from each submission using the standard procedure (section 5.1.5). Each *Taq* DNA Polymerase was used to test a replicate of the dilution series for each submission in the PCR, according to the manufacturer's instructions. Nested PCR was carried out as described in Section 5.1.1.

5.1.3.4 Nodavirus Nested RT-PCR procedure

The kits and reagents that resulted in the most sensitive Nested RT-PCR test were incorporated into the procedure as follows. RNA was extracted from 200µl of homogenised, clarified sample using the High Pure Viral RNA Kit (ROCHE, USA). The extraction was performed according to the manufacturer's instructions and the optional kit step, which involved incubating the binding buffer and sample for 10 minutes prior to loading the spin column, was always undertaken. Aliquots of sample RNA were heated to 90°C for 5 minutes and cDNA was transcribed in a 20µl reaction mix, containing 9.5µl RNA sample, 1x StrataScript™ buffer (Stratagene, USA), 5U RNasin (Promega, USA), 0.5mM dNTPs (Promega, USA), 1.0µM R3 primer, 20U StrataScript™ RT (Stratagene, USA) and RNase-free water, at 42°C for 60 minutes then 90°C for 5 minutes. PCR amplification was carried out in a 50µl reaction mix, containing 5µl of the RT reaction, 0.2µM of each primer (R3 and F2), 2.5mM $MgCl_2$, 25µl of HotStarTaq™ Master Mix (QIAGEN, USA) and deionised water, in a Mastercycler thermal cycler (Eppendorf, Germany) programmed with the following cycles; 95°C for 15 minutes, 30 cycles of 95°C for 40 seconds, 50°C for 40 seconds, 72°C for 40 seconds and finally 72°C for 10 minutes. Nested PCR amplification was carried out in a 50µl reaction mix, containing 1µl of the PCR reaction, 1.0µM NR'3 and NF'2 primers, 25µl of HotStarTaq™ Master Mix (QIAGEN, USA) and deionised water, in a Mastercycler thermal cycler (Eppendorf, Germany) programmed with the following cycles; 95°C for 15 minutes, 25 cycles of 94°C for 40 seconds, 50°C for 40 seconds, 72°C for 40 seconds and finally 72°C for 10 minutes. Reaction products were analysed after electrophoresis at 60V through a 2% agarose gel using TAE as the gel and electrophoresis buffers. Amplicons were visualised using a UV transilluminator, after staining with 10mg/ml ethidium bromide.

5.1.4 Nested RT-PCR testing of endemic and exotic nodavirus isolates

5.1.4.1 Queensland

Samples submitted to Oonoonba Veterinary Laboratory for routine health testing and diagnostic purposes were tested during the project. Formalin-fixed and frozen material produced during infection experiments, including material generated during FRDC 1999/205 "The effect of barramundi nodavirus on important freshwater fishes" was also available. This material contained of over 470 formalin fixed sections, from five species of fish that had survived the 28-day infection trial. Corresponding fresh material from the same fish had been tested for nodavirus by Nested RT-PCR during FRDC 1999/205. A bag of frozen trevally (*Pseudocaranx dentex*) collected after a fish kill north of Townsville, that had tested positive for nodavirus (L. Owens, pers. com), were obtained from Dr Leigh Owens, Department of Microbiology and Immunology, James Cook University, Townsville.

5.1.4.2 Northern Territory

Dr John Humphrey, Berrimah Veterinary Laboratory, Northern Territory and Dr Colin Shelley, Darwin Aquaculture Centre, Northern Territory provided barramundi larvae and broodstock spawning material and blood samples.

5.1.4.3 Tasmania

Dr David Morehead and Dr Stephen Battaglene, Tasmanian Aquaculture and Fisheries Institute, Tasmania, provided striped trumpeter broodstock spawning material and blood samples

5.1.4.4 Overseas

Nested RT-PCR testing of two exotic nodavirus isolates, striped jack nervous necrosis virus (SJNNV) and barfin flounder nervous necrosis virus (BFNNV), was conducted at the Australian Animal Health Laboratory Fish Diseases Laboratory, Victoria.

5.1.5 Optimisation of fish tissue processing procedures

Samples were stored at 4°C and processed within 24 hours, or stored at -80°C. Fresh or thawed fish or organ samples were homogenised in approximately two volumes of Viral Transport Medium (VTM; Medium 199 supplemented with 1000IU/ml benzylpenicillin, 1mg/ml streptomycin sulphate and 2µg/ml amphotericin B). For small sample volumes (i.e. < 2ml fluid), tissue was homogenised in a stomacher bag using a 2lb hammer. The homogenate was transferred to 1.5ml O-ring, screw-cap microcentrifuge tubes and stored at -80°C until required. For larger sample volumes, tissue was homogenised using a rotor-stator (Heidolph, Germany) fitted with an 18G attachment. The homogenates were clarified by centrifugation at 900 × g for 20 minutes at 5°C and the supernatant stored in 0.9ml aliquots in O-ring screw-cap microcentrifuge tubes at -80°C until required. Immediately prior to use, the sample was thawed, centrifuged at 10,000 × g for 10 minutes and the supernatant used for the analysis.

5.1.6 Broodstock blood and spawning material testing

The first broodstock blood samples tested were three clotted blood samples from barramundi that had been stored at -80°C. The infection status of these samples was unknown. After thawing, RNA extraction and testing of the samples was conducted as described in the Nested RT-PCR procedure (Section 5.1.3.4). After analysis of the amplification products by agarose gel electrophoresis, further test improvements were required.

5.1.6.1 Optimisation of broodstock blood sample processing and testing procedure

Two strategies were employed in an attempt to eliminate the presence of smearing and non-specific bands seen after RT-PCR testing. Samples tested were the same as those used in section 5.1.6. The first strategy was to dilute the extracted RNA template with an equal volume of deionised water and then proceed with the Nested RT-PCR test. The second strategy was to use the High Pure Viral RNA Kit (ROCHE), instead of the High Pure Viral Nucleic Acid Kit (ROCHE). The alternate kit was used, as it was possible that excessive DNA in the sample RNA may have been inhibiting the reaction. Using the kit that was

specific for RNA may help reduce this contaminating DNA and improve the quality of the Nested RT-PCR.

5.1.6.2 Evaluation of bovine serum albumin for testing broodstock blood samples

Due to the continued presence of smearing and non-specific bands, and equivocal results when blood samples were tested, the addition of different concentrations of bovine serum albumin (BSA) was evaluated as BSA can stabilise the PCR (Forbes and Hicks, 1996). As the quantity of known BNNV-infected blood was limited, the RNA template from Sample 2, which had previously tested positive, was used to evaluate the addition of BSA. Sample 2 template RNA, extracted using the High Pure Viral RNA Kit (ROCHE) was reverse transcribed as usual. Nested RT PCR reaction conditions were conducted according to the Nested RT-PCR procedure (Section 5.1.3.4) with the following modification; for the PCR amplification, BSA was added to the master mix at final concentrations of 0, 0.2, 0.4, 0.6, 0.8 and 1.0μg/μl.

After the initial assessment of the effect BSA in the primary PCR, BSA was added to the master mix at final concentrations of 0, 1.0, 1.5 and 2.0μg/μl. Three replicates of each BSA concentration series were prepared and used to test the template RNA from Sample 1, 2, and 3, which had been reverse transcribed as usual. The remaining PCR amplification and Nested PCR reaction conditions were as described in Section 5.1.3.4.

The modifications were evaluated by testing 22 barramundi broodstock blood samples that had been collected using EDTA as the anticoagulant and transported on ice bricks from the Darwin Aquaculture Centre to Oonoonba Veterinary Laboratory. As the samples were fresh, blood cells were lysed by addition of an equal volume of sterile deionised water and incubation at 4°C for 60 minutes. The samples were clarified by centrifugation at 10,000 x *g* for 10 minutes and RNA was extracted using the High Pure Viral RNA Kit (ROCHE). The extracted RNA templates were tested using the Nested RT-PCR procedure (Section 5.1.3.4), with the inclusion of BSA at 1μg/μl in the PCR step of the RT-PCR. All other reaction components and cycling conditions were unchanged.

5.1.6.3 Optimisation of broodstock spawning material processing

Samples obtained from striped trumpeter broodstock (ovarian samples and sperm) were homogenized as follows; the sample was repeatedly drawn back and forth through an 18 Gauge needle until the viscosity had been reduced and an even homogenate was produced. The homogenate was incubated in the extraction kit binding buffer for 10 minutes then the solution was applied to a QIAshredder™ (QIAGEN, USA) and centrifuged at 24,000 × g for 15 minutes at 5°C. The RNA was extracted from 200μl of the lysate using the High Pure Viral RNA Kit (ROCHE) according to the manufacturer's instructions. The High Pure Viral RNA Kit (ROCHE) was used instead of the High Pure Viral Nucleic Acid Kit (ROCHE) based on previous results from testing broodstock blood. Comparative testing had demonstrated there was no significant difference in sensitivity between the two kits when fish tissue homogenates were tested (data not shown). The Nested RT-PCR testing was conducted according to the Nested RT-PCR procedure (Section 5.1.3.4).

After the broodstock spawning material had been tested as described above, it was observed that for some samples incomplete sample homogenisation had occurred and the material was sitting on the top of the spin column. Therefore, seven methods (Table 4) were evaluated to determine the best method for sample homogenisation prior to RNA extraction. The samples

used for the evaluation were replicate aliquots form a 300ml stock of Nested RT-PCR positive, fertilised barramundi eggs. After each different method, the disrupted samples were analysed after electrophoresis through a 1.5% formaldehyde agarose gel prepared according to standard methods (Sambrook and Russell, 2001). Nucleic acid was visualised on a UV-transilluminator after staining with ethidium bromide.

Table 4: Methods evaluated to disrupt the barramundi egg samples, prior to RNA extraction and testing, in an effort to improve the homogenisation procedure.

METHOD	DESCRIPTION
Nothing	A 200µl aliquot of the sample was removed.
QIAshredder (QIAGEN, USA)	The sample was applied to a QIAshredder and centrifuged at 20,000 x *g* for 5 minutes.
Syringe (18G)	The sample was homogenised by repeated filling and purging of a 3ml syringe fitted to an 18-gauge needle.
Syringe (18G) and QIAshredder	The sample was homogenised by repeated filling and purging of a 3ml syringe fitted to an 18-gauge needle followed by centrifugation at 20,000 x *g* for 5 minutes in a QIAshredder.
QIAshredder and centrifuge	The sample was applied to a QIAshredder and centrifuged at 20,000 x *g* for 5 minutes then further clarified by centrifugation at 10,000 x *g* for 10 minutes at 5°C.
Syringe (18G) and centrifuge	The sample was homogenised by repeated filling and purging of a 3ml syringe fitted to an 18-gauge needle then clarified by centrifugation at 10,000 x *g* for 10 minutes at 5°C.
Syringe (18G), QIAshredder and centrifuge	The sample was homogenised by repeated filling and purging of a 3ml syringe fitted to an 18 gauge needle, centrifuged at 20,000 x *g* for 5 minutes in a QIAshredder then further clarified by centrifugation at 10,000 x *g* for 10 minutes at 5°C.
Centrifuge	The sample was clarified by centrifugation at 10,000 x *g* for 10 minutes at 5°C.

5.1.7 Comparative testing of broodstock spawning material and blood samples

To ascertain whether blood or spawning material was the most appropriate sample type to use to detect nodavirus, blood and spawning material samples were collected from captive and wild-caught striped trumpeter in Tasmania (Table 5). Ovarian material was collected by catheter and transferred to sterile 5ml vials and sperm was collected by gentle abdominal pressure into sterile syringes and transferred to sterile 5ml vials (D. Morehead, S. Battaglene, pers. com.). Samples were stored in liquid nitrogen and transported to OVL in dry ice. Blood was collected by needle and syringe and transferred to untreated 5ml vials and allowed to clot. On one occasion blood was also collected into sterile 5ml vials containing lithium heparin as an anticoagulant. Blood samples were stored at 4°C and transported to OVL on ice bricks.

Table 5: Striped trumpeter samples received from Tasmania.

DATE COLLECTED	CLOTTED BLOODS	WHOLE BLOODS	OVARIAN SAMPLES	SPERM SAMPLES
August, 2002	17	17	11	6
November, 2002	21	0	7	12
July, 2003	20	0	0	1
August, 2003	20	0	8	6
December, 2003	26	0	10	12
TOTAL	**104**	**17**	**36**	**37**

The ovarian samples were thawed and homogenised by repeated filling and purging of a 3ml syringe fitted to an 18-gauge needle. Two hundred microlitres of the sample was incubated with 500µl of the binding buffer for 10 minutes and then centrifuged through a QIAshredder

at 20,800 × g for 10 minutes at 5°C. RNA was extracted from the lysate using the High Pure Viral RNA Kit (ROCHE) and tested using the Nested RT-PCR (Section 5.1.3.4).

For the testing of sperm sample, 50µl and 100µl aliquots were diluted with VTM to a final volume of 200µl, to produce three sub-samples containing 50µl, 100µl and 200µl of original broodstock material. This was undertaken to determine whether the volume of sperm tested l was significant and what a minimum starting volume might be. The sperm samples were processed and tested in the same way as the ovarian samples.

Blood samples were lysed by addition of an equal volume of sterile deionised water followed by incubation at 4°C for 60 minutes. After removing cellular debris by centrifugation at 10,000 x *g* for 10 minutes, RNA extraction and testing were conducted as described in the Nested RT-PCR (Section 5.1.3.4) with the inclusion of BSA at 1µg/µl in the PCR step of the RT-PCR.

5.1.8 Production of synthetic nodavirus coat protein RNA for use as a positive control

Maintaining sufficient quantities of infected material for use as positive controls in the Nodavirus Nested RT-PCR test has always been difficult. As positive and negative controls are mandatory requirements for any diagnostic test, synthetic RNA from a portion of the nodavirus coat protein gene was produced. This work was conducted in collaboration with staff working on the FRDC Project "The production of nodavirus-free fish fry and the nodaviruses natural distribution" (FRDC 2002/043). Plasmids from two bacterial colonies, containing the BNNV coat protein gene insert, were obtained from FRDC 2002/043 project staff. The plasmids obtained were known to transcribe coat protein RNA from the vector T7 promoter site using the Promega SP6/T7 Riboprobe® *in vitro* Transcription System (N Levy, pers., com.). Transcription of RNA from the plasmid was conducted as follows. Each colony was cultured overnight in LB broth at 37°C with shaking at 150 rpm. After centrifugation at 900 × g for 10 minutes at 5°C, the plasmids were purified using the Wizard® *Plus* SV Minipreps DNA Purification System (Promega, USA) according to the manufacturer's instructions. Synthetic RNA was transcribed in a 100µl reaction using the SP6/T7 Riboprobe® *in vitro* Transcription System (Promega, USA) according to the manufacturer's instructions, using the T7 RNA polymerase. Serial 10-fold dilutions of the transcribed RNA, from 10^{-2} to 10^{-11}, were prepared in RNase-free deionised water and tested using the Nodavirus Nested RT-PCR (section 5.1.3.4).

5.1.9 Sequencing of the coat protein gene and comparison of isolates

5.1.9.1 Evaluation of primers for sequencing

Different primers, and primer combinations, reported to amplify a segment of the nodavirus coat protein gene were assessed by RT-PCR to determine the most suitable primer pair to use to produce the PCR amplicon for nucleotide sequencing. The primers evaluated are listed in Table 6. The primer combinations used and the expected amplicon sizes are in Table 7. Nodavirus isolates from three different species (sleepy cod, trevally and barramundi) were tested with each combination of primer pairs. Suitability of primers was based on the presence and intensity of an amplicon of the expected size.

For the sequencing RT-PCR, RNA was extracted with the High Pure Viral RNA Kit (ROCHE, USA) according to the manufacturer's instructions. Aliquots of sample RNA were heated to 90°C for 5 minutes and cDNA was transcribed in a 20µl reaction mix, containing

9.5µl RNA sample, 1x StrataScript™ buffer (Stratagene, USA), 5U RNasin (Promega, USA), 0.5mM dNTPs (Promega, USA), 1.0µM reverse primer, 20U StrataScript™ RT (Stratagene, USA) and RNase-free water, at 42°C for 60 minutes followed by 90°C for 5 minutes. PCR amplification was carried out in a 50µl reaction mix, containing 5µl of RT reaction, 1x PCR buffer (Fermentas, USA), 0.4µM forward and reverse primers, 2.5mM $MgCl_2$, 0.2mM dNTPs (Promega, USA), *Taq* DNA Polymerase (Fermentas, USA) and deionised water, in a Mastercycler thermal cycler (Eppendorf, Germany) programmed with the following cycles; 95°C for 15 minutes, 30 cycles of 95°C for 40 seconds, 50°C for 40 seconds, 72°C for 40 seconds and finally 72°C for 10 minutes. Reaction products were analysed after electrophoresis at 100V through a 1.5% agarose gel using TAE as the gel and electrophoresis buffer. Amplicons were visualised using a UV transilluminator, after staining with 10mg/ml ethidium bromide.

Table 6: Previously reported primers that were evaluated for the generation of a PCR product suitable for sequencing

PRIMER	SEQUENCE	REFERENCE
F5	5'- ATGGTACGCAAAGGTGA –3'	Grotmol *et al*, 2000
F1	5'- GGATTTGGACGTGCGACCAA –3'	Grotmol *et al*, 2000
F2	5'- CGTGTCAGTCATGTGTCGCT –3'	Nishizawa *et al*, 1995
F'2	5'- GTTCCCTGTACAACGATTCC -3'	Thiery *et al*, 1999
R3A	5'- GGCCATTTAACCACATG –3'	Grotmol *et al*, 2000
R1	5'- GACAAGACTGGTGAAGCTGG –3'	Nishizawa *et al*, 1995
R3	5'- CGAGTCAACACGGGTGAAGA –3'	Grotmol *et al*, 2000
R'3	5'- GGATTTGACGGGGCTGCTCA -3'	Thiery *et al*, 1999

Table 7: Primer combinations and the expected product sizes

PRIMER SET ID	PRIMER PAIR	EXPECTED AMPLICON SIZE
1	F5 – R'3	950 bp
2	F5 – R3	1000 bp
3	F5 – R1	1300 bp
4	F5 – R3a	1500 bp
5	F1 – R'3	850 bp
6	F1 – R3	900 bp
7	F1 – R1	1200 bp
8	F1 – R3a	1400 bp
9	F2 – R3	450 bp
10	F2 – R1	700 bp
11	F2 – R3a	1000 bp
12	F'2 – R1	600 bp
13	F'2 – R3a	900 bp

5.1.9.2 Cloning of Nodavirus PCR products and sequence analysis

The primer pair F1-R'3, which produced an 832 bp amplicon, was used to generate the amplicons for sequencing. The nodavirus isolates used for sequencing were from juvenile barramundi tissue (BNNV), juvenile barramundi cod tissue (BCNNV), juvenile sleepy cod tissue (SCNNV), broodstock striped trumpeter sperm (STNNV) and juvenile trevally tissue (TNNV). The barramundi, barramundi cod and sleepy cod samples were NNV RT-PCR positive while the striped trumpeter and trevally samples were NNV Nested RT-PCR positive. The reaction mix and cycling parameters were as above (section 5.1.9.1). Following amplification, the presence of the positive product was checked by electrophoresis at 100V

through a 1.5% agarose gel using TAE as the gel and electrophoresis buffer and visualised using a UV transilluminator, after staining with 10mg/ml ethidium bromide. Positive PCR amplicons were purified from the RT-PCR reaction mix using the Wizard® *Plus* SV Minipreps DNA Purification System (Promega, USA). The products were inserted into a p-GEMT vector (Promega, USA) and cloned using JM-109 competent cells and the p-GEMT Vector System 2 (Promega, USA) according to the manufacturer's instructions. Four single colonies of ampicillin resistant clones from each accession were picked off the plates and cultured overnight in Luria-Bertani broth (LB broth, 1% tryptone, 0.5% yeast extract, 1% sodium chloride, pH 7.0) at 37°C with shaking at 150 rpm. After centrifugation at 900 × g for 10 minutes at 5°C, the plasmids were purified using the Wizard® *Plus* SV Minipreps DNA Purification System (Promega, USA) according to the manufacturer's instructions. Plasmids were tested for the target insert using the F1-R'3 RT-PCR outlined above (section 5.1.9.1).

The sequencing reaction was set up as per instructions from the Australian Genome Research Facility (AGRF). Briefly, 400ng of plasmid and 3.2pmol of primer were combined in a final volume of 8µl. All plasmids were sequenced using the forward and reverse primers in duplicate. The nucleotide sequences were analysed using Sequencher V4.0.5 (Gene Codes Corporation, USA) and the consensus sequence for each isolate determined from the replicate plasmid sequences using GeneDoc Multiple Sequence Alignment Editor Version 2.6.002 (Nicholas *et al*., 1997). The exotic nodavirus sequences were obtained from BLASTn searches of the National Centre for Biotechnology Information (NCBI, USA) database (http://www.ncbi.nlm.nih.gov/BLAST/) and are detailed in Table 8 and Table 9. The endemic and exotic nodavirus sequences were aligned using GeneDoc Multiple Sequence Alignment Editor and Shading Utility Version 2.6.002 (Nicholas *et al*., 1997). For the phylogenetic analysis of the endemic and exotic nodavirus sequences, the nucleotide sequences were aligned using the neighbour-joining guide trees method of Clustal-W (Thompson *et al*., 1994), the phylogenetic analysis of the alignment was carried out using PHYLIP and the phylogenetic tree was generated using DRAWGRAM (Felsenstein, 1993). The work was conducted online at the Biology WorkBench 3.2 facility at the San Diego Supercomputer Center (SDSC), USA website (http://seqtool.sdsc.edu/CGI/BW.cgi) on 18th May, 2004.

Table 8: Exotic nodavirus isolates used in nucleotide acid sequence comparisons.

ID USED	COMMON NAME	SPECIES NAME	ACCESSION	REFERENCE
MGNNV	Malabar grouper	Epinephelus malabaricus	AF245003	Lin et al., 2001
SGNNV	Sevenband grouper	*Epinephelus septemfasciatus*	AY324870	Iwamoto et al., 2004
DGNNV	Dragon grouper	*Epinephelus lanceolatus*	AF254004	Lin et al., 2001
EcNNV	Orange spotted grouper	*Epinephelus coioides*	AF534998	Chen et al., (unpubl.)
YGNNV	Yellow grouper	*Epinephelus awoara*	AF283554	Chiou et al., (unpubl.)
GGNNV	Greasy grouper	*Epinephelus tauvina*	AF281657	Hedge et al., (2002)
DlEV	Sea bass	*Dicentrarchus labrax*	Y08700	Sideris (1997)
GNNV	Guppy	*Poicelia reticulata*	AF99774	Hedge et al., (2003)
JFNNV	Japanese flounder	*Paralichthys olivaceus*	AB045980	Suzuki et al., (unpubl.)
AHNNV	Atlantic halibut	*Hippoglossus hippoglossus*	NSP254641	Grotmol et al., (2000)
TNNV	Turbot	*Scophthalmus maxiums*	AJ608266	Johansen et al., (unpubl.)
SJNNV	Striped jack	*Pseudocaranx dentex*	D30814	Nishizawa et al., (1995)

Table 9: Exotic nodavirus isolates used in amino acid sequence comparisons.

ID USED	COMMON NAME	SPECIES NAME	ACCESSION	REFERENCE
MGNNV	Malabar grouper	Epinephelus malabaricus	AAG22495	Lin et al., 2001
SGNNV	Sevenband grouper	*Epinephelus septemfasciatus*	AAQ90063	Iwamoto et al., 2004
DGNNV	Dragon grouper	*Epinephelus lanceolatus*	AAG22496	Lin et al., 2001
EcNNV	Orange spotted grouper	*Epinephelus coioides*	AAQ12273	Huang and He, submitted 26/4/02
YGNNV	Yellow grouper	*Epinephelus awoara*	AAP80590	Chiou et al., (unpubl.)
GGNNV	Greasy grouper	*Epinephelus tauvina*	AAL78765	Hedge et al., (2002)
DlEV	Sea bass	*Dicentrarchus labrax*	CAA69959	Sideris (1997)
GNNV	Guppy	*Poicelia reticulata*	AAQ61198	Hedge et al., (2003)
JFNNV	Japanese flounder	*Paralichthys olivaceus*	BAB00609	Suzuki et al., (unpubl.)
AHNNV	Atlantic halibut	*Hippoglossus hippoglossus*	CAB53257	Grotmol et al., (2000)
TNNV	Turbot	*Scophthalmus maxiums*	CAE55208	Johansen et al., (unpubl.)
SJNNV	Striped jack	*Pseudocaranx dentex*	BAA06491	Nishizawa et al., (1995)

5.2 Results and discussion

5.2.1 Initial Nested RT-PCR

The initial Nested RT-PCR test produced distinct amplicons of approximately 426bp for the RT-PCR and 294bp for the Nested PCR with no non-specific bands observed after testing of fish tissue. The Nested PCR step resulted in a 10^4-fold to 10^5-fold increase in the sensitivity of the test (Figure 1).

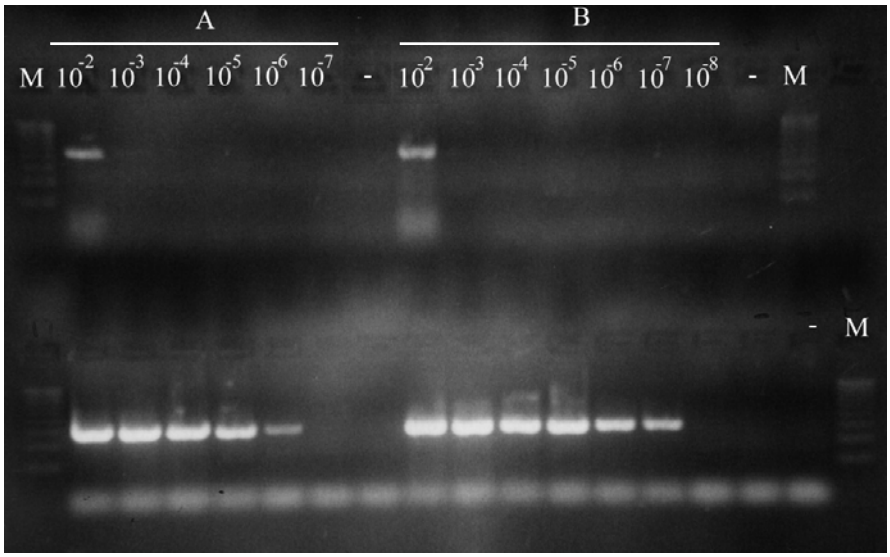


Figure 1: Nested RT-PCR of serial 10-fold dilutions for two sample RNA preparations (A = sample 1, B = sample 2). M; markers[ϕ], -; -ve control.

5.2.2 Optimisation of NNV Nested RT-PCR reaction conditions

5.2.2.1 Optimisation of Nested PCR magnesium chloride concentration

All concentrations of magnesium chloride evaluated produced strong amplicons of the expected size of 294bp (Figure 2). There was little difference between the various concentrations, although template that had been diluted 1/100000 demonstrated an overall decrease in intensity compared to the other two dilutions used. As the different magnesium chloride concentrations produced very similar bands in terms of intensity, a concentration of 1.5mM magnesium chloride was adopted as this represents the most common concentration of magnesium chloride in the Master Mix kits used, including the HotStarTaq™ Master Mix (QIAGEN, USA). A reduction in the number of reagents to be added to the master mix was considered desirable, as this would reduce the potential for reagent contamination and test failure.

[ϕ] While the authors acknowledge the poor resolution of the Marker lanes in this image, and some subsequent images, the experimental analysis and amplicon size determination were obtained from the original gel, viewed on the UV-transilluminator, where the resolution was significantly greater. All results were fully scrutinised to ensure the bands observed were of the required size. While Marker band intensity could obviously be greater, the faintness of individual Marker bands does not detract from the overall result presented on the gel.

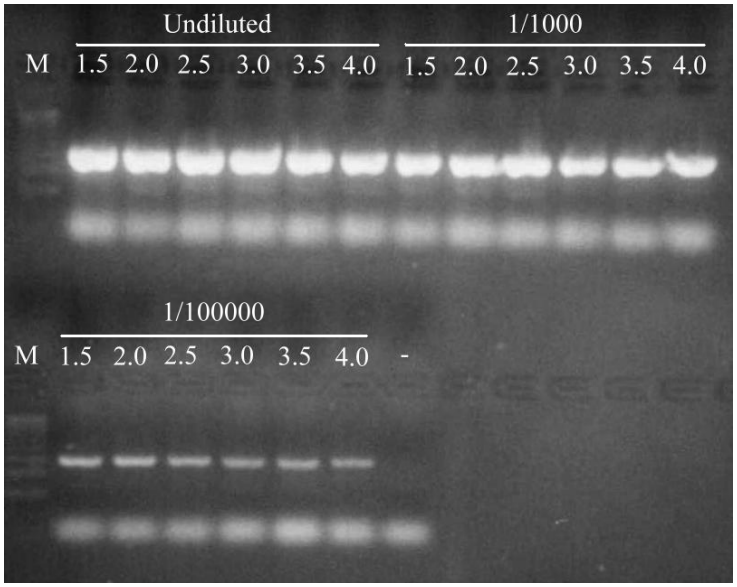


Figure 2: Effect of different concentrations of magnesium chloride with three dilutions of template (Neat, 10^{-3},10^{-5}) in the Nested PCR. M; markers, -; -ve control, magnesium chloride concentrations are in mM.

5.2.2.2 Optimisation of Nested PCR primer concentration

All concentrations of primer evaluated produced a 294bp amplicon (Figure 3). Band intensity was reduced at primer concentrations of 0.25µM but there was no obvious difference between concentrations of 0.5µM or greater. Increasing the final concentration of the nested primers had little effect on the intensity of the bands produced, but did increase the visible primer dimer formation. Therefore, a final concentration of 1.0µM for both the nested primers was retained.

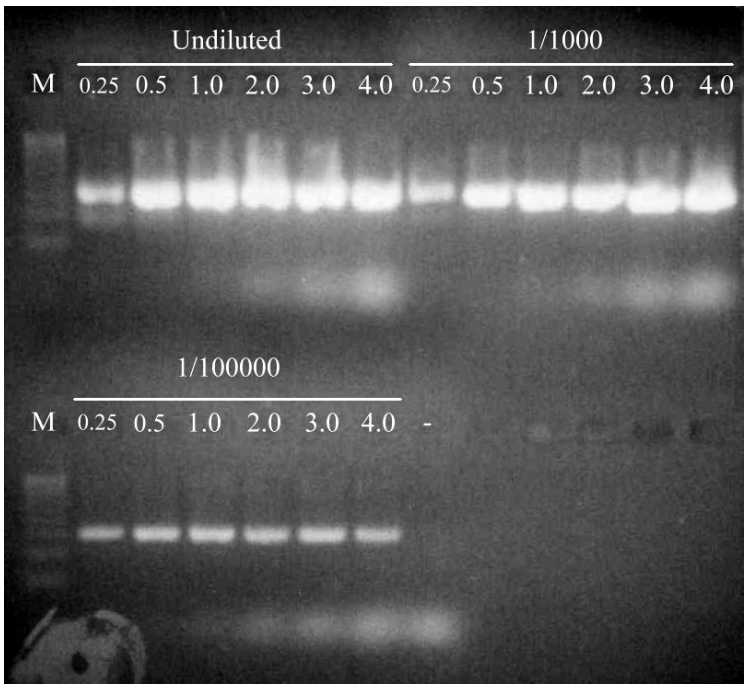


Figure 3: Effect of different concentrations of Nested PCR primer with three dilutions of template (Neat, 10^{-3}, 10^{-5}) in the Nested PCR. M; markers, -; -ve control, primer concentrations are in µM.

5.2.2.3 Optimisation of Nested RT-PCR cycle number

All combinations of cycle number produced bright intense bands, with the exception of the samples diluted 10^{-5} and amplified using 25 cycles for each PCR step (Figure 4). Therefore, the procedure was amended to use 30 cycles in the RT-PCR and 25 cycles in the Nested RT-PCR.

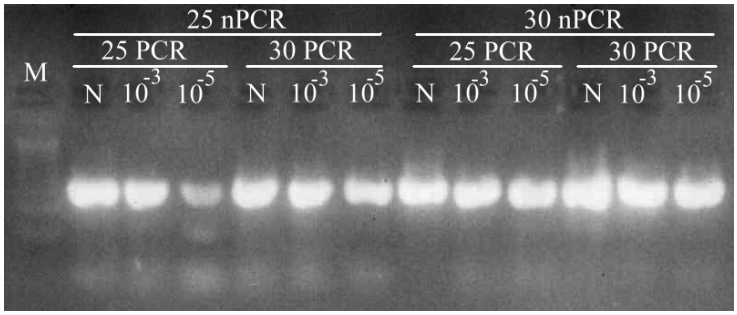


Figure 4: Nested RT-PCR results, after varying cycle number in the primary PCR and Nested PCR, with three dilutions of template (Neat, 10^{-3}, 10^{-5}). M; markers. The negative control was negative (data not shown).

5.2.3 Comparison of RNA extraction techniques and variability of Reverse Transcriptase and Hot Start Taq Master Mixes.

5.2.3.1 RNA extraction techniques

Procedure A (High Pure Viral Nucleic Acid Kit - ROCHE) was the most efficient method for RNA extraction (Table 10, Figure 5). Template extracted using this kit consistently produced positive amplicons at higher dilutions than the other procedures. While all procedures took approximately the same amount of time (1.25 to 2 hours) the spin-column formats were simpler and more user-friendly than the solvent-based precipitation methods. Procedure B failed to produce a positive result for the Nested RT-PCR for sample 0-48168, which is a false-negative result, so this product would be considered unacceptable for use in the Nested RT-PCR test. While most RNA extraction kits and reagents could be used for the Nested RT-PCR test, this work demonstrated that no kits or reagents should be used without adequate validation.

Table 10: RT-PCR and Nested PCR results for the four RNA extraction procedures evaluated. Each extraction procedure was applied to 10-fold dilutions of material, from three different submissions, followed by Nested RT-PCR testing of each dilution.

SAMPLE	DILUTION	PROCEDURE A		PROCEDURE B		PROCEDURE C		PROCEDURE D	
		PCR	nPCR	PCR	nPCR	PCR	nPCR	PCR	nPCR
2-41712	N	+	+	+	+	+	+	+	+
	10^{-1}	+	+	+	+		+	+	+
	10^{-2}		+		+		+		+
	10^{-3}		+				+		
	10^{-4}		+						
	10^{-5}								
1-56700	N	+	+	+	+	+	+	+	+
	10^{-1}	+	+		+	+	+	+	+
	10^{-2}		+				+		+
	10^{-3}		+						+
	10^{-4}		+						+
	10^{-5}		+						
	10^{-6}								
0-48186	N		+				+		+
	10^{-1}		+						+
	10^{-2}		+						
	10^{-3}								
Product		High Pure Viral Nucleic Acid Kit		Supplier B		Supplier C		Supplier D	

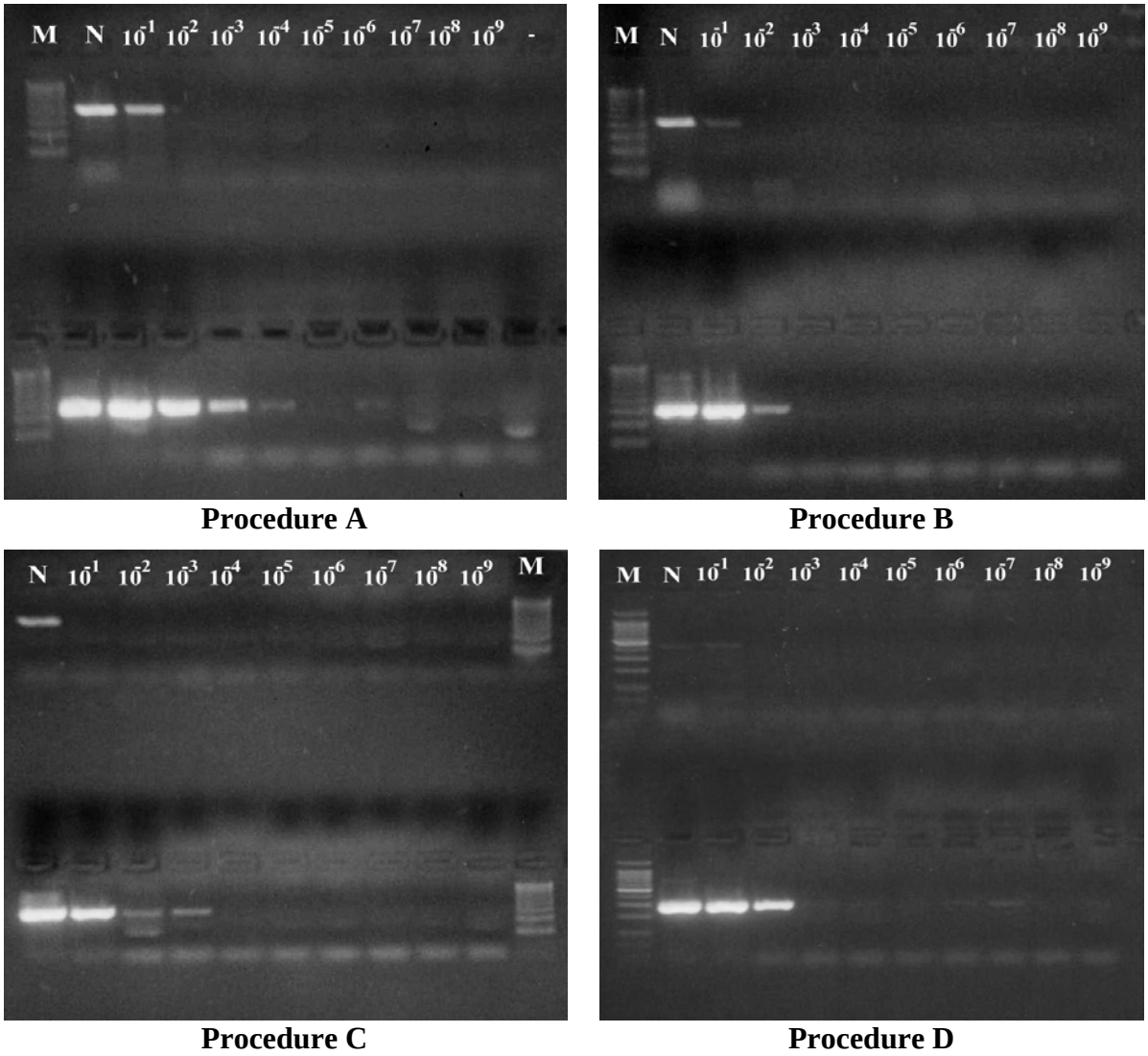


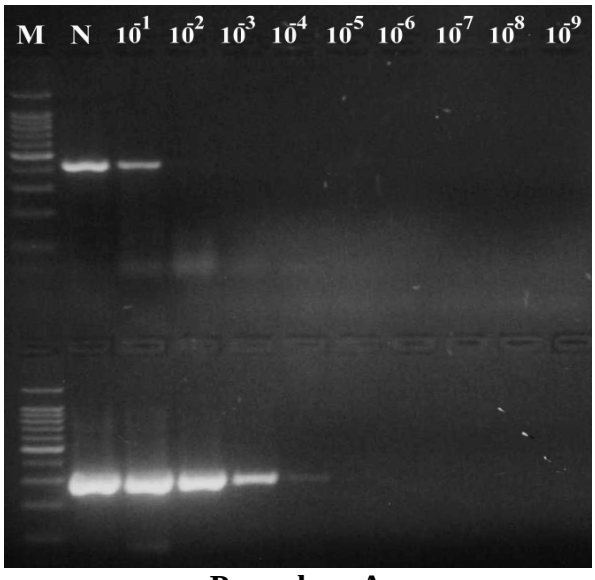
Figure 5: Nested RT-PCR results after the different RNA extraction methods were applied to replicated 10-fold dilutions of sample 2-41712. The top of the gel contains the RT-PCR results and the bottom the Nested PCR results. M; markers, -; -ve control.

5.2.3.2 Variability of Reverse Transcriptases

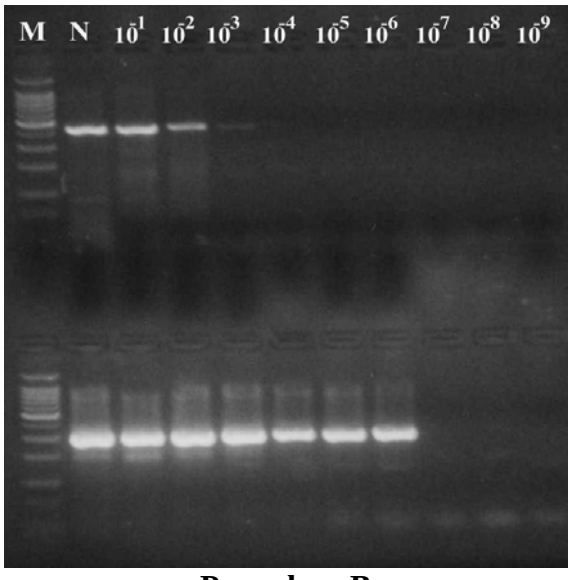
Procedure B (StrataScript, Stratagene) and Procedure C were the most suitable for reverse transcription (Table 11, Figure 6). These two procedures produced very similar results, however Procedure B was the least expensive at the time of testing. Procedure C was the only reverse transcriptase to produce a positive RT-PCR result for the oldest sample, 0-48186, although the Nested PCR results are the same for this sample using Procedure B and Procedure C. As it is strongly recommended that all samples are tested using the complete Nested RT-PCR, both of these reverse transcriptases are appropriate. Therefore, it was decided to adopt StrataScript (Stratagene, USA) for the reverse transcription primarily due to the lower cost of this reagent. While all Reverse Transcriptase enzymes could be used for the Nested RT-PCR test, none should be used without adequate validation.

Table 11: RT-PCR and Nested PCR results for the four Reverse Transcriptase enzymes evaluated. Each Reverse Transcriptase was applied to 10-fold dilutions of template RNA, from three submissions, followed by Nested RT-PCR testing of each dilution.

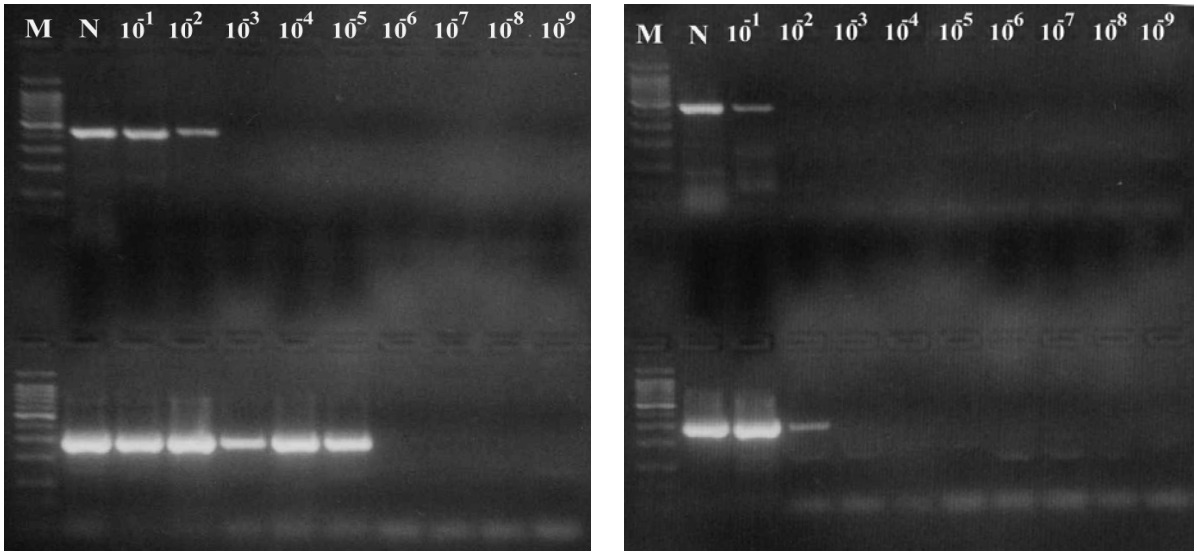
SAMPLE	DILUTION	PROCEDURE A		PROCEDURE B		PROCEDURE C		PROCEDURE D	
		PCR	nPCR	PCR	nPCR	PCR	nPCR	PCR	nPCR
2-41712	N	+	+	+	+	+	+	+	+
	10^{-1}	+	+	+	+	+	+	+	+
	10^{-2}		+	+	+	+	+		+
	10^{-3}		+	+	+		+		
	10^{-4}		+		+		+		
	10^{-5}				+		+		
	10^{-6}				+				
	10^{-7}								
1-56700	N	+	+	+	+	+	+	+	+
	10^{-1}	+	+	+	+	+	+	+	+
	10^{-2}		+	+	+	+	+		+
	10^{-3}		+	+	+		+		
	10^{-4}		+		+		+		
	10^{-5}				+		+		
	10^{-6}				+		+		
	10^{-7}								
0-48186	N		+		+	+	+		+
	10^{-1}		+		+		+		+
	10^{-2}				+		+		
	10^{-3}				+		+		
	10^{-4}								
Product		Supplier E		StrataScript		Supplier F		Supplier G	



Procedure A



Procedure B



Procedure C **Procedure D**

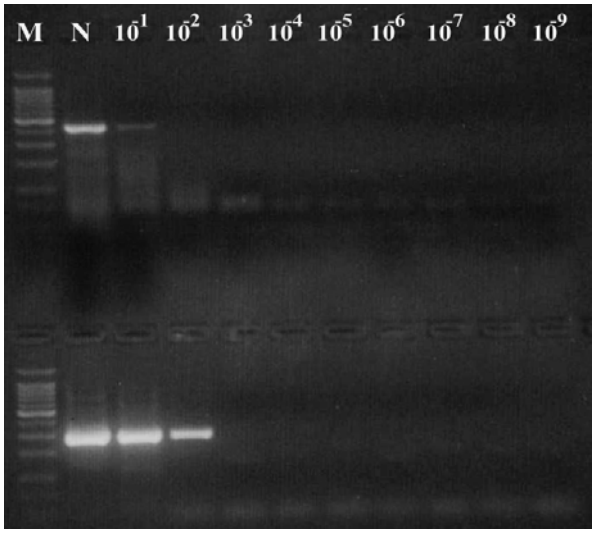
Figure 6: Nested RT-PCR results after the different Reverse Transcriptase enzymes were used in testing replicated 10-fold dilutions of template RNA from submission 2-41712. The top of each gel contains the RT-PCR results and the bottom the Nested PCR results. M; markers. The negative control was negative (data not shown).

5.2.3.3 Variability of Taq DNA Polymerase

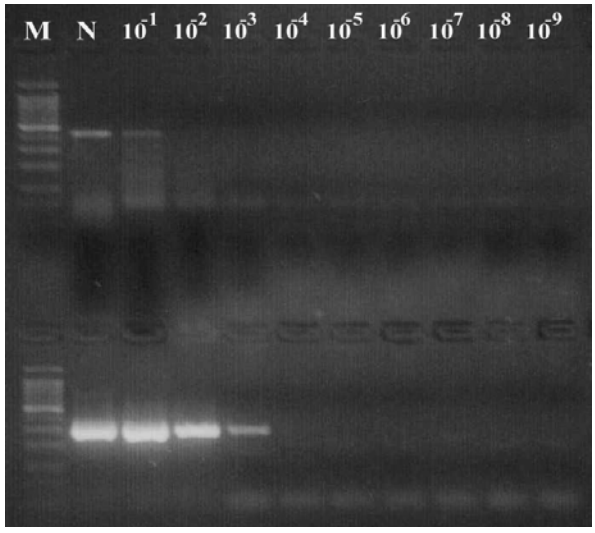
Procedure B and Procedure D (HotStarTaq Master Mix Kit, QIAGEN, USA) were the most sensitive for PCR amplification (Table 12, Figure 7). Procedure C failed to produce a positive result for the Nested RT-PCR for sample 0-48168, which is a false-negative result, so this product would be considered unacceptable for use in the Nested RT-PCR test. While Procedure B was more sensitive than Procedure D for the older sample (0-48186), both produced positive Nested PCR amplicons and the Master Mix format of Procedure D made reaction set-up much quicker, simpler and reduced the risk of introducing contaminants into the reagents and reactions. Therefore, the HotStarTaq Master Mix Kit (QIAGEN, USA) was retained as the DNA polymerase for the test. While most DNA Polymerase enzymes could be used for the Nested RT-PCR test, this work demonstrated that no polymerase should be used without adequate validation.

Table 12: RT-PCR and Nested PCR results for the four DNA Polymerase enzymes evaluated. Each DNA Polymerase was applied to 10-fold dilutions of cDNA, from three submissions, followed by standard Nested RT-PCR testing of each dilution.

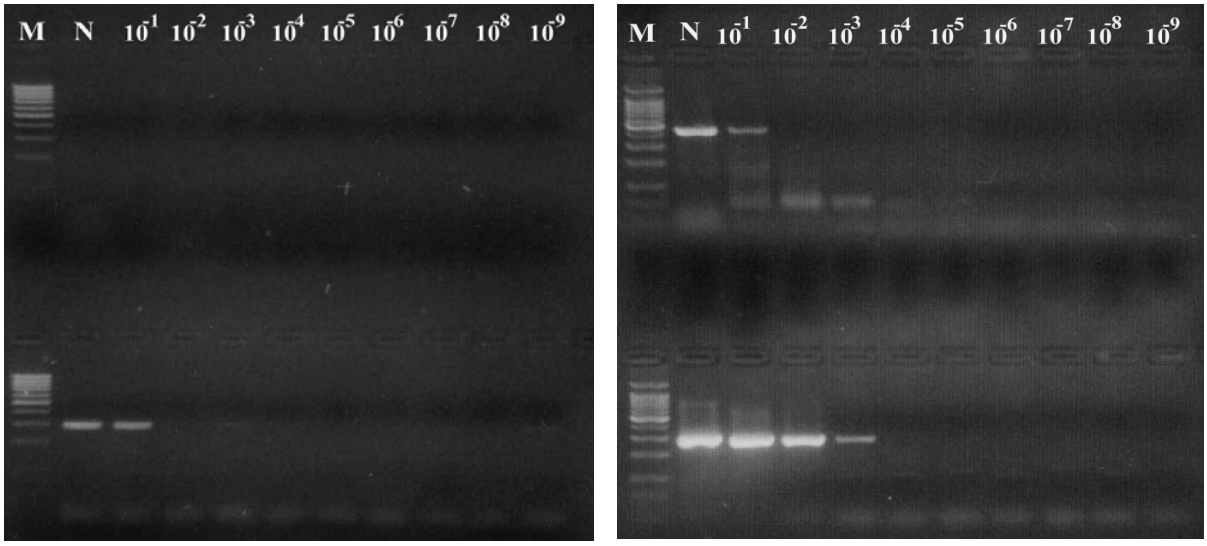
SAMPLE	DILUTION	PROCEDURE A		PROCEDURE B		PROCEDURE C		PROCEDURE D	
		PCR	nPCR	PCR	nPCR	PCR	nPCR	PCR	nPCR
2-41712	N	+	+	+	+		+	+	+
	10^{-1}	+	+	+	+		+	+	+
	10^{-2}		+		+				+
	10^{-3}				+				+
	10^{-4}								
1-56700	N	+	+	+	+		+	+	+
	10^{-1}	+	+	+	+		+	+	+
	10^{-2}		+		+				+
	10^{-3}				+				+
	10^{-4}								
0-48186	N		+	+	+				+
	10^{-1}		+		+				+
	10^{-2}				+				
	10^{-3}								
Product		Supplier H		Supplier I		Supplier J		HotStarTaq Master Mix Kit	



Procedure A



Procedure B



Procedure C **Procedure D**

Figure 7: Nested RT-PCR results after the different DNA Polymerase enzymes were used in testing replicated 10-fold dilutions of cDNA from submission 2-41712. The top of each gel contains the RT-PCR results and the bottom the Nested PCR results. M; markers. The negative control was negative (data not shown).

Most of the different kits or reagents evaluated were successful in producing an amplicon of the required size in either the RT-PCR or Nested RT-PCR. However, two products (an RNA extraction kit and a DNA Polymerase) did produce false-negative results for the Nested RT-PCR and these products would be inappropriate for use in the Nested RT-PCR. The reagents described in NNV Nested RT-PCR in this report are the choice of the authors' and other reagents could be used without compromising test quality. However, this work has highlighted the requirement to fully validate any alternate kits and reagents prior to use.

5.2.3.4 Nodavirus Nested RT-PCR procedure

The results of the optimisation and evaluation experiments were incorporated into the NNV Nested RT-PCR procedure. Typical examples of the results produced by this procedure when fish tissue is tested appear in Figure 8. These samples were obtained after sequential daily sampling of experimentally infected barramundi and each sample corresponds to a pool of five fish, with the sample number corresponding to the day of collection. From Day 12 (Sample 12) onwards, all samples were consistently RT-PCR positive. This was similar to previous results where fish exhibiting clinical signs consistently produced a positive RT-PCR result. However, there was inconsistency with fish tested by RT-PCR alone from Day 5 to 12. Testing with the Nested PCR resolved the inconsistency of these results and especially the negative RT-PCR result for Sample 10, where this would be considered a false-negative result if only the RT-PCR test had been conducted. The failure of the RT-PCR test to produce an amplicon of the required size may be due to operator error, or excessive pooling of the samples. Regardless of the cause, testing these samples with the Nested RT-PCR eliminated any ambiguity, which is highly desirable, given the absence of any other confirmatory diagnostic test.

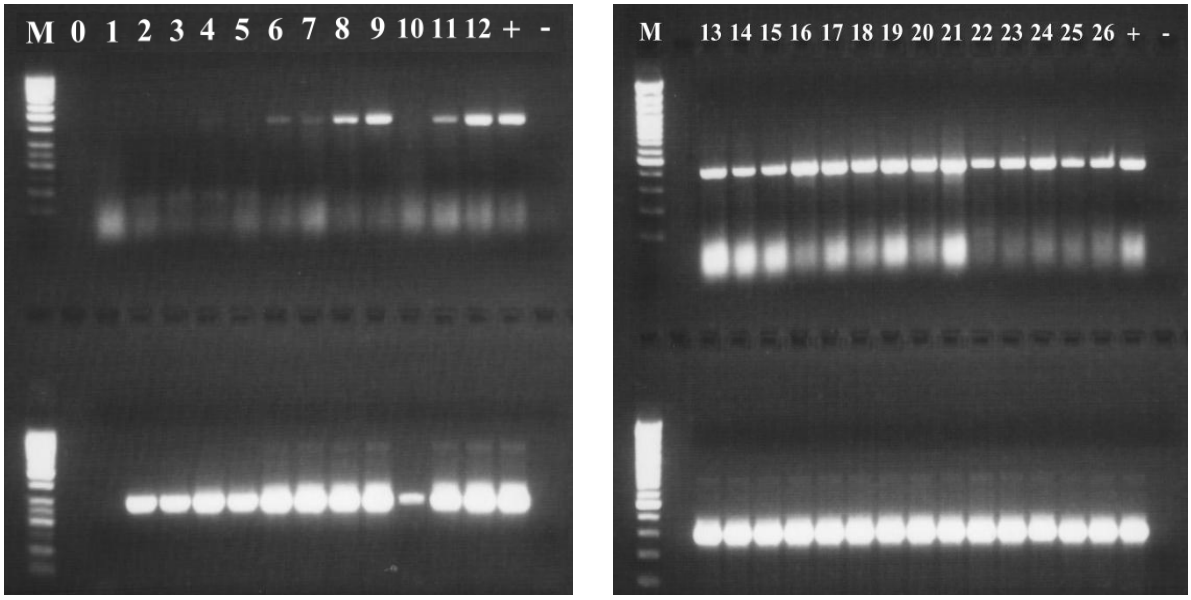


Figure 8: Example of results obtained using the Nested RT-PCR procedure after incorporation of the improvements identified during the optimisation reactions conditions and evaluation of the different kits and reagents. Lanes 0-26; pools of five experimentally infected fish, harvested daily post infection, pooled and tested by Nested RT-PCR. The top of the gel contains the RT-PCR results and the bottom the Nested PCR results. Numbers correspond to the day post infection the samples were collected. M; markers, +; +ve control, -; -ve control.

5.2.4 Nested RT-PCR testing of endemic and exotic nodavirus isolates

5.2.4.1 Australia

Positive Nested RT-PCR results were obtained from barramundi (*Lates calcarifer*) larvae and broodstock spawning material and blood samples, juvenile trevally (*Pseudocaranx dentex*) and barramundi cod (*Cromileptes altivelis*) from Queensland, from barramundi larvae and broodstock spawning material and blood samples from the Northern Territory and from spawning material and blood samples collected from striped trumpeter (*Latris lineata*) from Tasmania. Positive results were detected in cultured and wild fish from all states where samples were obtained, indicating the ability of the Nested RT-PCR to detect all known endemic nodavirus isolates.

5.2.4.4 Overseas

One endemic (positive control) and two exotic isolates were tested. All produced amplicons of the required size after Nested RT-PCR testing, although one isolate, Striped Jack nodavirus, resulted in very weak amplification. It should be noted that PCR procedures at AAHL were conducted in the dedicated PCR Suite. As generic procedures have been adopted for use in this Suite, it was not possible to replicate the optimised conditions that were determined at the Oonoonba Veterinary Laboratory.

5.2.5 Optimisation of fish tissue processing procedures

The tissue processing procedure consistently resulted in the production of a single amplicon of the required size after RT-PCR and Nested PCR testing when BNNV-infected fish homogenates were tested. Over 20 nodavirus positive barramundi diagnostic submissions

have been tested. Nested RT-PCR testing of material generated during experimental infections produced similar results (Anderson and Moody, 2004). No non-specific amplicons were produced and positive amplicons were clear and unambiguous. No non-specific amplicons were produced when negative samples were tested and no further optimisation was required.

5.2.6 Broodstock blood and spawning material testing

5.2.6.1 Optimisation of broodstock blood sample processing and testing procedure

Initial testing of broodstock blood by Nested RT-PCR produced a positive result for one sample, Sample 1 (Figure 9). A "smear" was observed after analysis of the RT-PCR reaction. It was possible the "smear" was caused by excessive nucleic acids in the samples as the High Pure Viral Nucleic Acid Kit (ROCHE, USA) would extract both RNA and DNA.

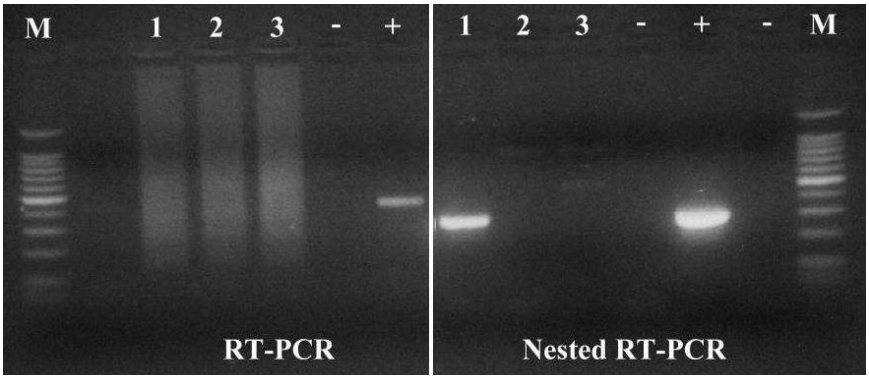


Figure 9: Initial Nested RT-PCR results from blood testing. M; markers, Lane 1-3; clotted blood from three adult barramundi, +; +ve control, -; -ve control. An additional second negative control was used for the Nested PCR.

Dilution of the samples after RNA extraction reduced the intensity of the smear originally seen but also led to the production of four non-specific bands after RT-PCR testing (Figure 10). The test also failed to produce an amplicon for Sample 1, which had previously produced a positive result with undiluted template. It is possible dilution of the sample RNA reduced the concentration of viral RNA to a level below the detection limit of the test. As there is no alternate test to measure the viral load in the samples and determine whether this is actually the case, template RNA will not be diluted prior to testing.

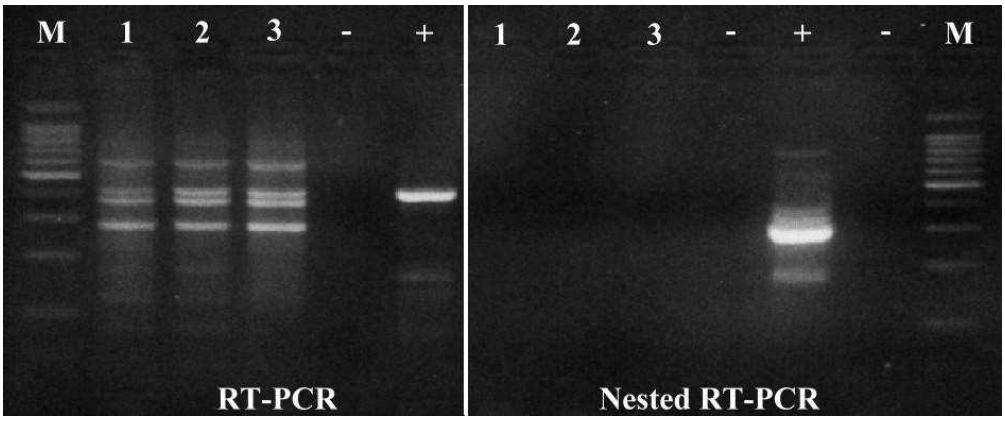


Figure 10: Effect of dilution of template RNA on Nested RT-PCR results for blood testing. M; markers, Lane 1-3; clotted blood samples from three adult barramundi, +; +ve control, -; -ve control. An additional second negative control was used for the Nested PCR.

Replacing the High Pure Viral Nucleic Acid Kit (ROCHE, USA) with the High Pure Viral RNA Kit (ROCHE, USA) reduced the bands and smearing seen with previous testing (Figure 11). Again, there were no positive amplicons produced after RT-PCR testing. Interestingly, Sample 1, which previously gave a positive result, was negative and Sample 2, which was previously negative, was now positive. Obviously, the inconsistency with results using the current procedure needs to be addressed.

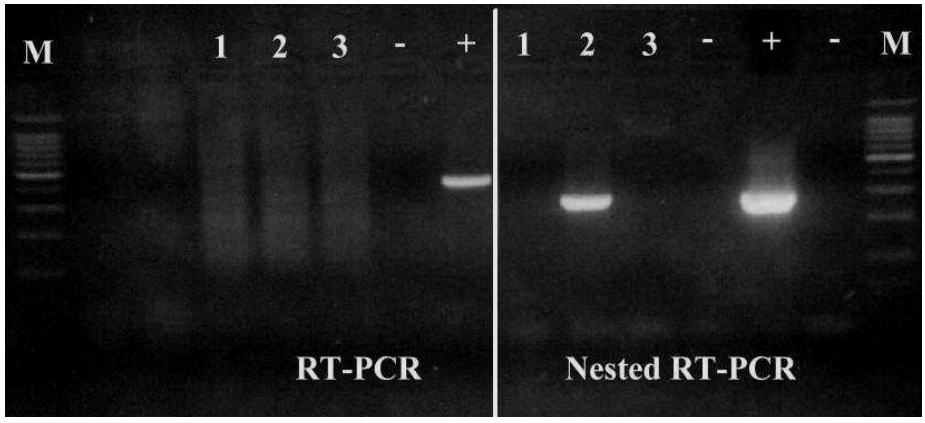


Figure 11: Effect of changing the extraction kit on Nested RT-PCR results for blood testing. M; markers, Lane 1-3; clotted blood from three adult barramundi, +; +ve control, -; -ve control. An additional second negative control was used for the Nested PCR.

5.2.6.2 Evaluation of bovine serum albumin for testing broodstock blood samples

The use of BSA in the PCR step of the RT-PCR, at a concentration of at least 1.0µg/µl, produced an amplicon after Nested RT-PCR testing (Figure 12). All other concentrations failed to produce an amplicon in either the RT-PCR or the Nested PCR. The "smearing" observed previously was also reduced indicating that addition of BSA had improved the PCR.

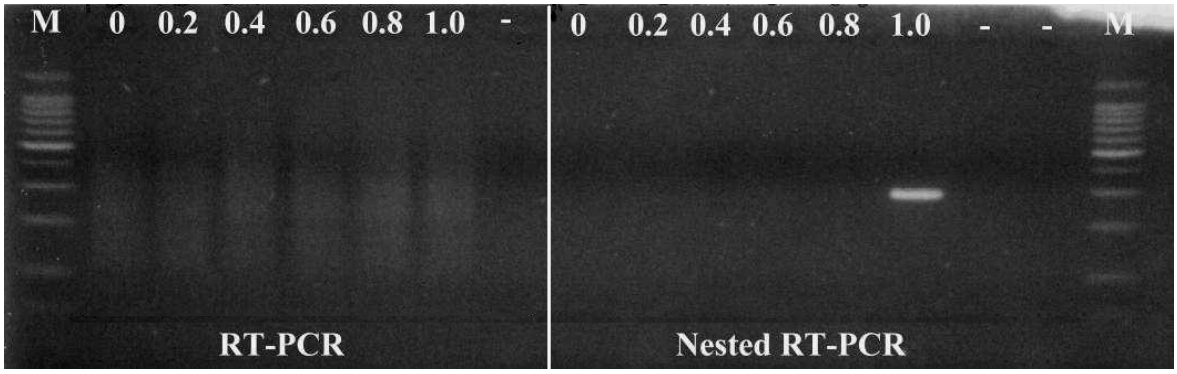


Figure 12: Effect of adding different concentrations of BSA to the primary PCR of the Nested RT-PCR for testing of clotted blood. M; markers, Lane numbers; concentration of BSA added in the primary PCR step in µg/µl, -; -ve control. An additional second negative control was used for the Nested PCR step.

Retesting, with the addition of BSA at 1.0µg/µl, resulted in the production of positive bands for Sample 1 and Sample 2 after Nested RT-PCR testing (Figure 13). For Sample 1 a positive result was produced with no BSA added, and BSA added at 1.0µg/µl and 1.5µg/µl. This again highlights the potentially low level of virus in these samples. The positive result for Sample 2 suggested that the addition of the BSA had stabilised the primary PCR. Therefore, addition of BSA will be included in the Nested RT-PCR procedure for testing of blood.

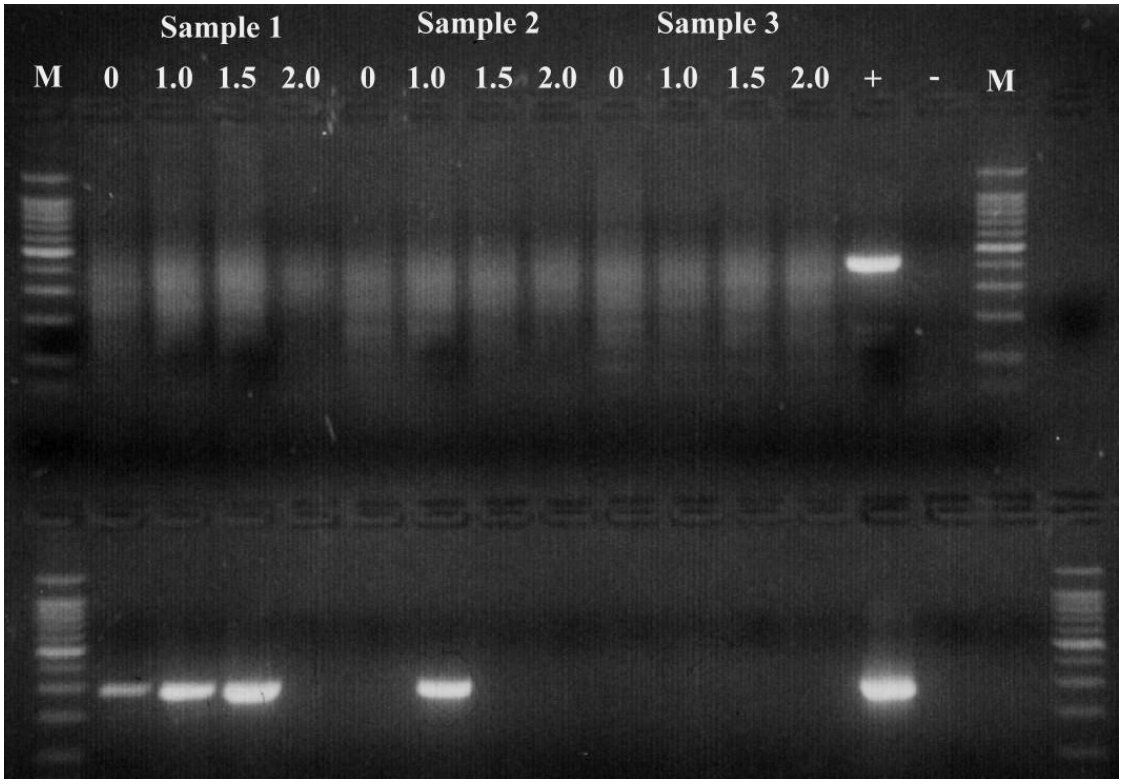


Figure 13: Effect of addition of 1.0µg/µl of BSA to the PCR step of the RT-PCR for Nested RT-PCR testing of three clotted blood samples. The top of the gel contains the RT-PCR results and the bottom the Nested PCR results. M; markers, Lane numbers; concentration of BSA added in the PCR step in µg/µl, +; +ve control, -; -ve control.

Nested RT-PCR testing of 22 barramundi broodstock samples, with the addition of BSA to the primary PCR resulted in the production of intense clear amplicons for 2 samples (Figure 14). There was some non-specific amplification in the RT-PCR step, although the amplicons were markedly fainter than the positive control, and there appeared to be no detrimental effect to the Nested PCR. No non-specific amplicons were observed in the Nested PCR reaction. Again no positive samples were detected after using the RT-PCR test alone, reinforcing the necessity for the Nested PCR step.

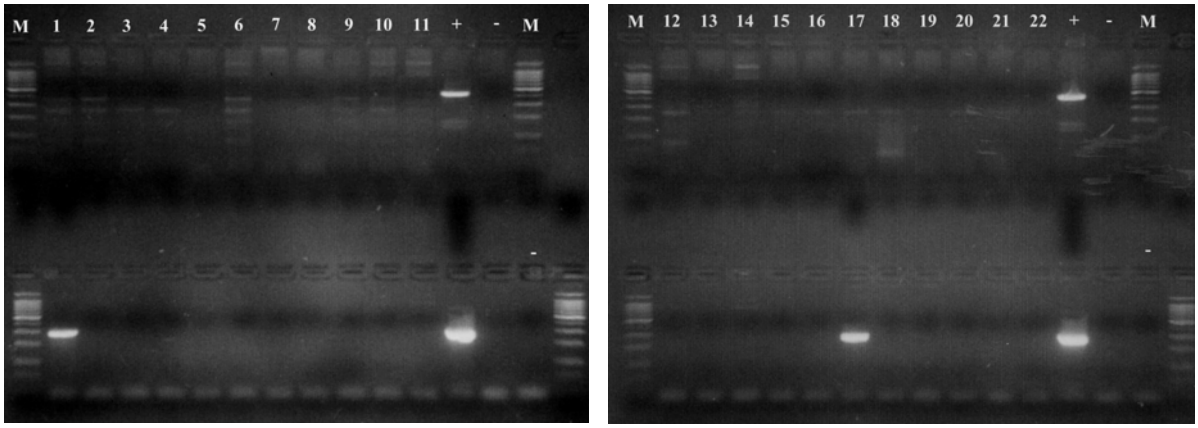


Figure 14: Nested RT-PCR results for 22 barramundi broodstock blood samples, collected into EDTA. The top of the gel contains the RT-PCR results and the bottom the Nested PCR results. M; markers, Lane 1-22; blood samples, +; +ve control, -; -ve control. An additional second negative control was used for the Nested PCR.

5.2.6.3 Optimisation of broodstock spawning material processing

As no viral RNA would be detected in the gel, due to the low concentration on the sample, the yield of total RNA was used as an indicator of extraction procedure efficiency. The most efficient method for the release of nucleic acid from barramundi eggs was to simply proceed with the extraction or homogenise the sample by repeated filling and purging of a 3ml syringe fitted to an 18 gauge needle followed by the standard extraction (Figure 15, Lane 1 and 3). Clarifying the sample after homogenisation reduced the yield (Figure 15, Lane 6). Due to the small volumes of broodstock spawning material obtained and the small number of positive samples detected, no homogenisation trials could be conducted using spawning material. However, as the eggs samples exhibited the same sample preparation issues as the spawning material, these results are applicable to the preparation of broodstock material. Further research is required to optimise the sample preparation procedure for fertilised eggs and broodstock spawning material.

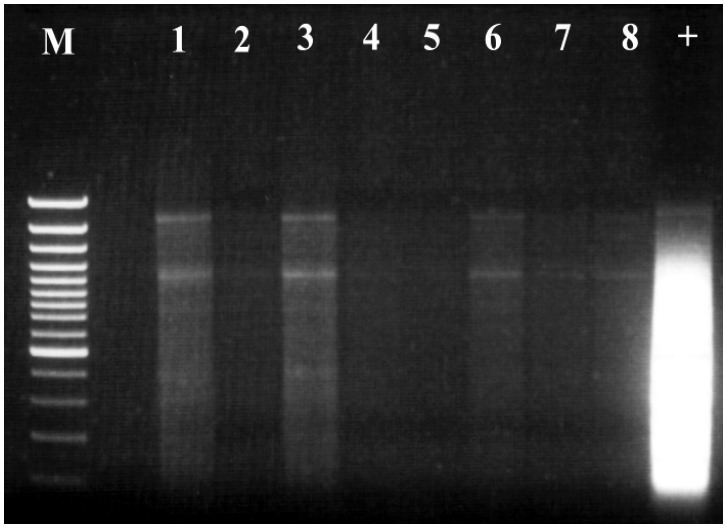


Figure 15: Quality of RNA from BNNV-infected eggs using different homogenisation methods (see Table 4).
M; marker
Lane 1; No treatment
Lane 2; QIAshredder
Lane 3; Syringe (18G)
Lane 4; Syringe (18G) and QIAshredder
Lane 5; QIAshredder + centrifuge
Lane 6; Syringe (18G) + centrifuge
Lane 7; Syringe (18G), QIAshredder + centrifuge
Lane 8; Centrifuge
+ ; +ve control

5.2.7 Comparative testing of broodstock spawning material and blood samples

Comparative testing was first undertaken on whole blood, clotted blood and then the corresponding male and female spawning material, from 17 striped trumpeter broodstock. Positive amplicons were produced from 7/17 of the blood samples. However, the Nested RT-PCR failed to produce consistent results with any of the different sample types (Figures 16 and 17). While similar proportions were positive for each blood sample type (4/17 clotted blood, 3/17 whole blood), no fish showed a positive result for both blood samples. Non-specific amplicons were observed after the RT-PCR and were thought to be due to the high nucleic acid content of the broodstock samples. These non-specific amplicons were not of concern as they were eliminated after dilution of the RT-PCR reaction, during the Nested PCR step. No non-specific amplicons were observed after Nested RT-PCR testing.

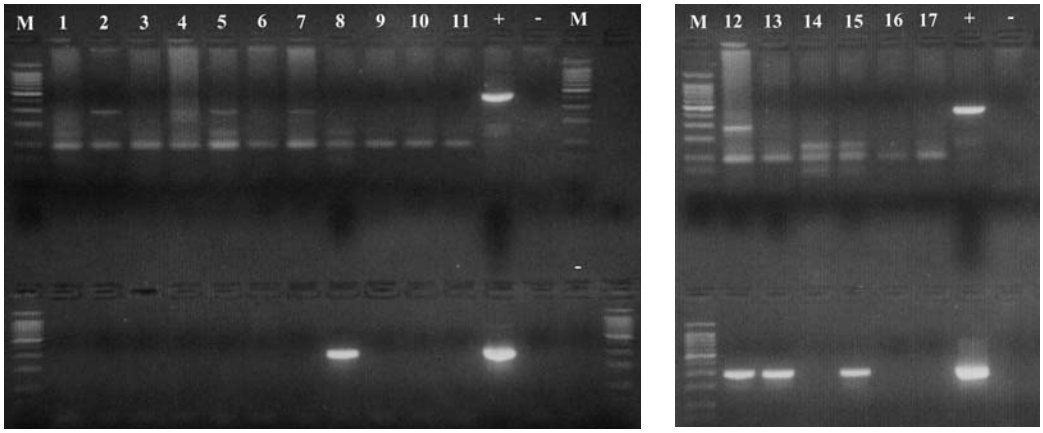


Figure 16: Nested RT-PCR results for 17 striped trumpeter broodstock clotted blood samples. The top of the gel contains the RT-PCR results and the bottom the Nested PCR results. M; markers, Lanes 1-17; individual clotted blood samples, +; +ve control, -; -ve control. An additional second negative control was used for the Nested PCR.

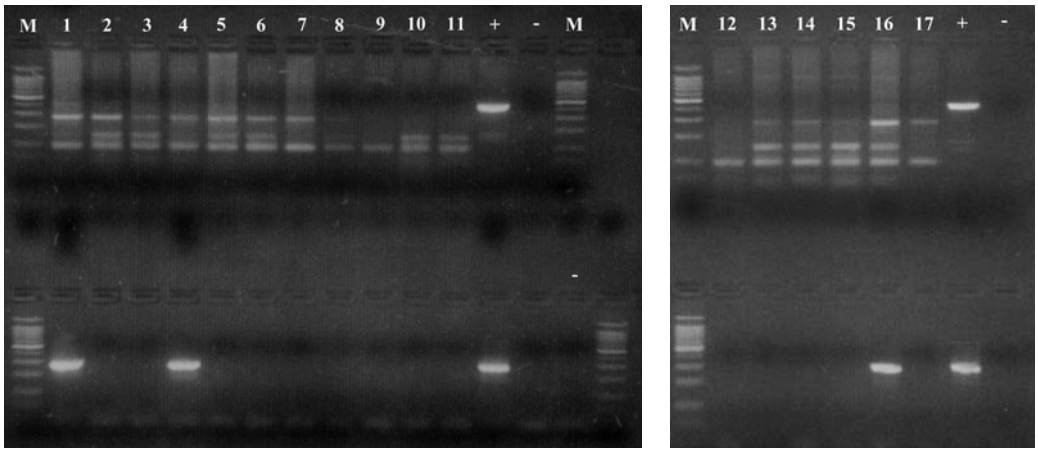


Figure 17: Nested RT-PCR results for 17 striped trumpeter broodstock whole blood samples. The top of the gel contains the RT-PCR results and the bottom the Nested PCR results. M; markers, Lanes 1-17; individual whole blood samples. The samples are from the same fish as those in Figure 16. +; +ve control, -; -ve control. An additional second negative control was used for the Nested PCR.

Positive amplicons were produced from 7/17 of the spawning material samples (Figure 18, 19 and 20), with a higher proportion of positive male samples (4/6) than positive female samples (3/11). There was no volume of sperm sample that consistently resulted in a positive product, although more samples were positive when an initial volume of 100µl (3/6) was used, compared to an initial volume of either 200µl (1/6) or 50µl (2/6). Again, non-specific amplicons were observed after the RT-PCR. However, no non-specific amplicons were observed after Nested RT-PCR testing and these non-specific bands have no effect on the quality of the Nested RT-PCR.

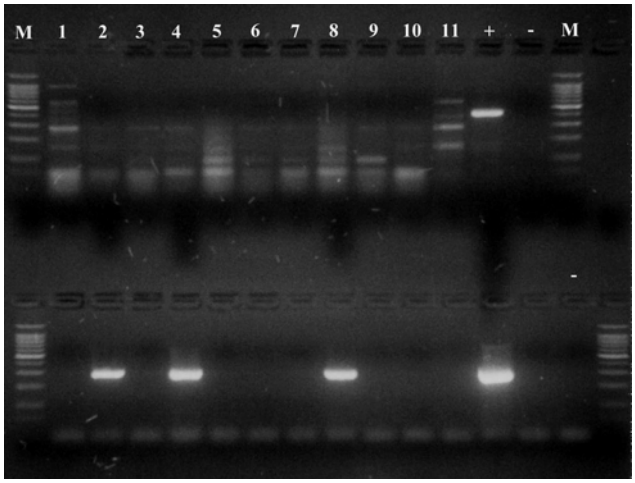


Figure 18: Nested RT-PCR of eleven striped trumpeter broodstock ovarian samples. The top of the gel contains the RT-PCR results and the bottom the Nested PCR results. M; markers, Lanes 1-11; ovarian samples, +; +ve control, -; -ve control. An additional second negative control was used for the Nested PCR.

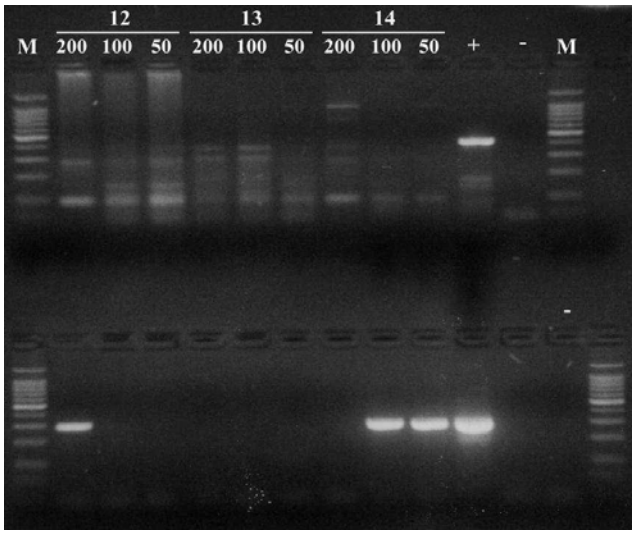


Figure 19: Nested RT-PCR of three striped trumpeter broodstock sperm samples. The top of the gel contains the RT-PCR results and the bottom the Nested PCR results. M; markers, Lanes 12, 13 and 14; sperm samples (200, 100 and 50 refer to the original volume of material used for each sample, in µl). +; +ve control, -; -ve control. An additional second negative control was used for the Nested PCR.

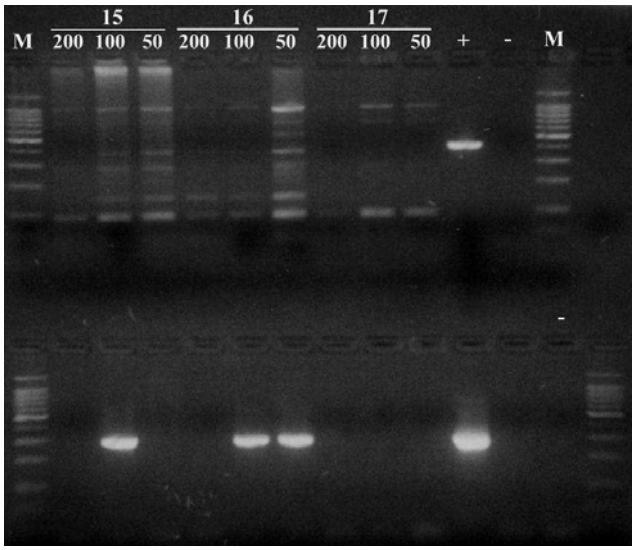


Figure 20: Nested RT-PCR of three additional striped trumpeter broodstock sperm samples. The top of the gel contains the RT-PCR results and the bottom the Nested PCR results. M; markers, Lanes 15-17; sperm samples (200, 100 and 50 refer to the original volume of material used for each sample, in µl). +; +ve control, -; -ve control. An additional second negative control was used for the Nested PCR.

Only 4/17 samples were positive for both spawning material and blood sample (clotted and whole blood). The differences between whole and clotted blood could be attributed to the anticoagulant used in whole blood, but this is unlikely as some samples were positive for whole blood but not clotted blood. Similarly, the differences between blood and spawning material may be due to inhibitors in blood, but this is also unlikely as some samples were positive for blood but not spawning material. It is postulated that the nodavirus levels in the

samples tested were at the lower limits of the test sensitivity. The variability of male spawning material results, where there was no consistency with one starting volume of material, may also be due to inadequate sample homogenisation to release of virus in the tissue.

An additional 144 striped trumpeter broodstock samples (87 clotted blood samples, 26 female samples, 31 male samples), collected on four different occasions (Table 5) were tested. In contrast to the results presented above, while positive results were observed from clotted blood samples (13/87, 14.9%) none of the 57 male and female spawning material samples tested positive. This indicated blood was a more suitable sample for broodstock testing.

Combining all the striped trumpeter broodstock test results, a higher proportion of positive results were obtained from clotted blood (17.0%) or whole blood samples (17.6%) than from ovarian (8.3%) or sperm samples (10.8%) (Table 13). The proportions of positive blood samples were similar for both male (17.0%) and female (17.6%) broodstock as were the proportions of positive ovarian (8.3%) and sperm (10.8) samples. This indicated that no one sex could be targeted as a carrier and both sexes needed to be tested in an effort to reduce vertical transmission of nodavirus the larvae. Table 13 also highlights the requirement to optimise sample preparation for sperm samples. The ambiguous results are attributable to incomplete sample homogenisation combined with low levels of virus in the samples. The fact that no broodstock samples produced positive RT-PCR amplicons reinforces the requirement to use the Nested RT-PCR test for broodstock material. Blood was the most appropriate sample type to determine the infectivity status of the striped trumpeter broodstock by Nested RT-PCR. Ongoing comparative testing of broodstock spawning material and blood samples is required to optimise nodavirus testing of broodstock to ensure repeatability of the test. While positive results were valid, it is unknown what proportion of negative results were false-negatives.

Table 13: Results for comparative testing of striped trumpeter broodstock blood and spawning fluid samples by Nested RT-PCR.

SAMPLES	BLOOD		OVARIAN TISSUE	SPERM SAMPLES			
	Clot	Whole		TOTAL	200µl	100µl	50µl
43	7/43	2/11	3/36	-	-	-	-
57	10/57	1/6	-	4/37	1/37	3/37	2/37
TOTAL	**17/100**	**3/17**	**3/36**	**4/37**	**1/37**	**3/37**	**2/37**
POSITIVE	**17.0%**	**17.6%**	**8.3%**	**10.8%**	**2.7%**	**8.1%**	**5.4%**

5.2.8 Production of synthetic nodavirus coat protein RNA for use as a positive control

Two plasmids were successfully transcribed to produce a significant quantity of synthetic coat protein RNA for use as a positive control (Figure 21). The Nested RT-PCR detected as little as 6fg of synthetic RNA prepared from Plasmid 1 and 30fg prepared from Plasmid 2. The RNA has been stored at -80°C and can be used as positive control, from the initial RNA extraction, in the Nested RT-PCR. The production of the synthetic nodavirus coat protein RNA has significantly reduced the requirement to continually collect clinical material for use as a positive control. Difficulties maintaining adequate stocks of infectious material have always been encountered and amplification of nodavirus in larvae is time consuming, expensive and has animal welfare implications. The use of synthetic RNA could also

significantly reduced the need for interstate transport of infectious nodavirus material and would allow the safe implementation of the Nested RT-PCR in laboratories in areas where nodavirus has not been detected. The synthetic RNA could also be used in proficiency testing programs as part of a laboratory's quality assurance program.

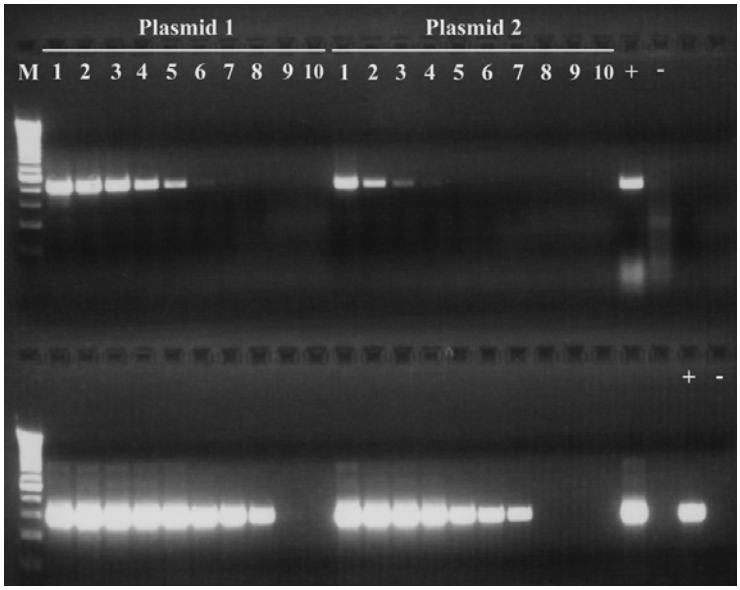


Figure 21: Nested RT-PCR results for serial 10-fold dilutions of two batches of synthetic nodavirus coat protein RNA. The top of the gel contains the RT-PCR results and the bottom the Nested PCR results. M; marker, Lanes 1 to 10; 10-fold dilutions from 10^{-2} to 10^{-11}, +; +ve control, -; -ve control. An additional second negative control was used for the Nested PCR.

5.2.9 *Sequencing of the coat protein gene and comparison of isolates*

5.2.9.1 Evaluation of primers for sequencing

The majority of the primer combinations produced amplicons of the expected size, although intensity of the amplicon varied (Figure 22). It is interesting to note that PCR products were produced for all of the primer sets except those that contained either the primers F5 or R1. This suggests that there are sequence differences between the temperate nodavirus species from which the primers were designed and the tropical species that were tested from Australia. The primer combination regarded as the most suitable for use in generating a DNA fragment for sequencing was F1 and R'3 (Figure 22, Lane 5). This would produce a product of approximately 850bp. This decision was based on product size and band intensity and all subsequent sequencing experiments will use this primer set. The nodavirus isolate from trevally failed to produce a detectable amplicon from any of the primer sets. This accession was positive only using the NNV Nested RT-PCR but not by RT-PCR. The failure of the PCR primers to produce a detectable amplicon was most likely due to the virus being in low concentration in the sample and beyond the limits of detection of the sequencing RT-PCR test.

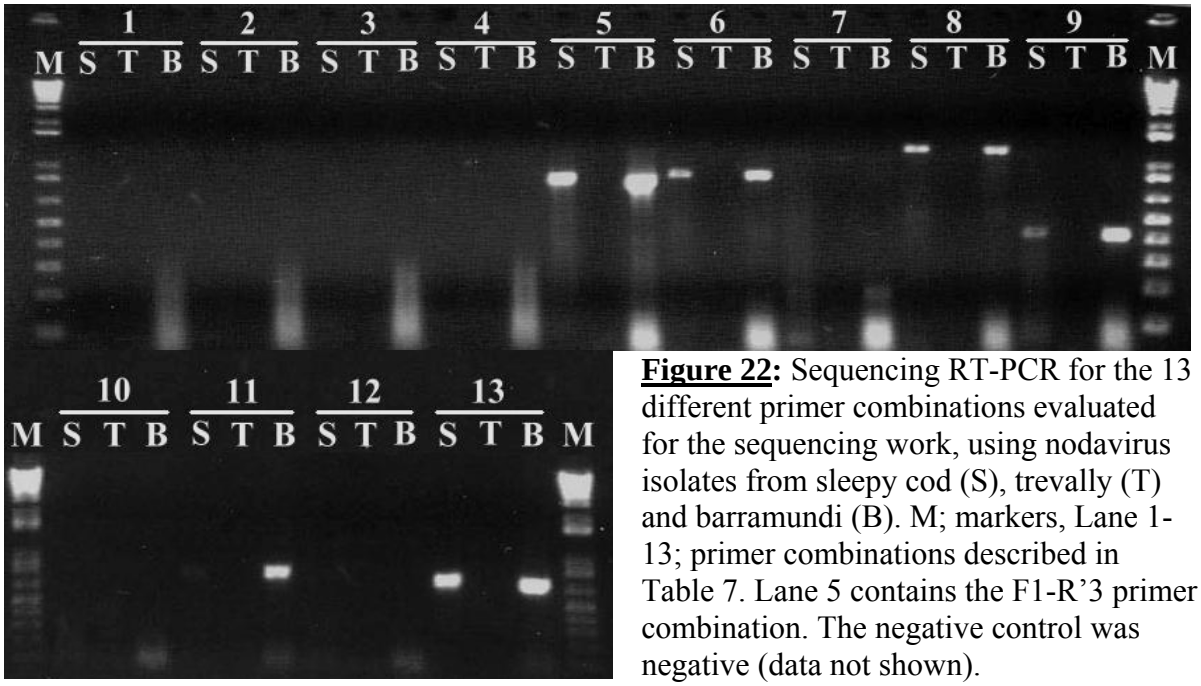


Figure 22: Sequencing RT-PCR for the 13 different primer combinations evaluated for the sequencing work, using nodavirus isolates from sleepy cod (S), trevally (T) and barramundi (B). M; markers, Lane 1-13; primer combinations described in Table 7. Lane 5 contains the F1-R'3 primer combination. The negative control was negative (data not shown).

After sequencing RT-PCR testing of the different nodavirus samples (juvenile barramundi tissue, juvenile barramundi cod tissue, juvenile sleepy cod tissue, broodstock striped trumpeter sperm and juvenile trevally tissue) amplicons of approximately 850bp were produced for the nodavirus isolates from barramundi cod, barramundi and sleepy cod. However, the striped trumpeter sample (Figure 23) and trevally sample (data not shown) failed to produce a specific sequencing RT-PCR amplicon. As mentioned previously, this was most likely due to the virus present in low concentrations in the samples and beyond the limits of detection for the RT-PCR. These samples had only produced a positive amplicon when tested by the NNV Nested RT-PCR, so while the failure to produce an amplicon was disappointing, it was not surprising.

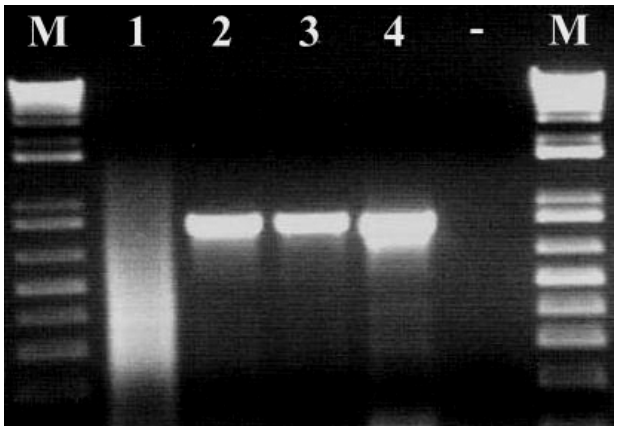


Figure 23: Sequencing RT-PCR results for four nodavirus isolates using the F1 and R'3 primer combination to produce an 850bp product for sequencing. M; markers,
Lane 1; striped trumpeter
Lane 2; barramundi cod
Lane 3; sleepy cod
Lane 4; barramundi
-; -ve control.

5.2.9.2 Cloning of Nodavirus PCR products and sequence analysis

The PCR products from the barramundi cod, sleepy cod and barramundi were successfully cloned and purified. Of the four plasmids, from the four clones produced from each isolate, four of the barramundi plasmids, two of the barramundi cod plasmids and four of the sleepy cod plasmids contained the PCR product insert after confirmatory testing by PCR using the F1 and R'3 primer set (Figure 24). Plasmids from an additional two barramundi cod isolate

clones were prepared and the presence of the insert of interest confirmed by RT-PCR testing using the F1 and R'3 primer combination (data not shown).

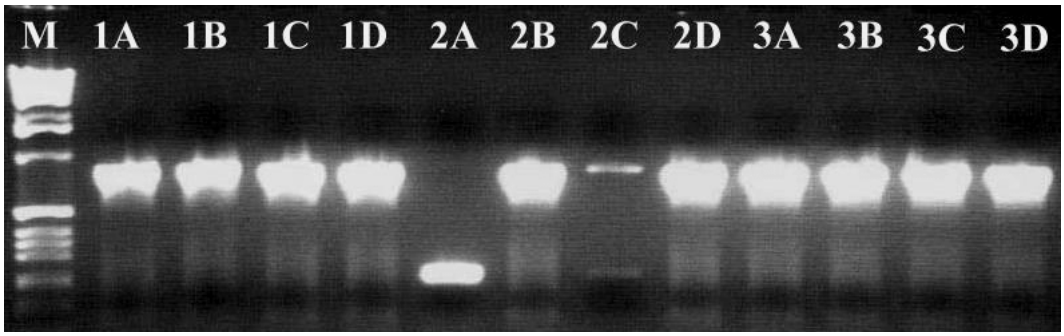


Figure 24: Sequencing PCR of the purified plasmids from the four clones developed from the different nodavirus isolates, using the F1 and R'3 primer combination. M; markers, Lane1A-1D; barramundi isolate (BNNV) plasmids, Lane 2A-2D; barramundi cod isolate (BCNNV) plasmids, Lane 3A-3D; sleepy cod isolate (SCNNV) plasmids. The negative control was negative (data not shown).

A total of four plasmids containing the coat protein gene insert from each isolate were sequenced. An 832 bp gene fragment was sequenced for all four clones, using reverse and forward primers in duplicate, for each isolate from barramundi (BNNV), barramundi cod (BCNNV) and sleepy cod (SCNNV). Comparison of the 16 sequences generated for each clone revealed that there were minor sequence differences between clones from the same isolate. These minor differences were most likely due to *Taq* mismatches during RT-PCR amplification. Comparison of the 16 sequences from each isolate with the computer programs GeneDoc and Sequencher, enabled the identification and elimination of ambiguous bases in the individual sequences (Appendix 1) so a consensus sequence for each isolate could be determined (Figure 25). The nucleotide sequence alignment revealed that the Australian nodavirus isolates were highly homologous with each other. The marine nodavirus isolates, BNNV and BCNNV, differed from each other by only two bases out of the 832 bases sequenced. These isolates both differed from the freshwater nodavirus isolate, SCNNV, by 16 bases out of the 832 bases sequenced.

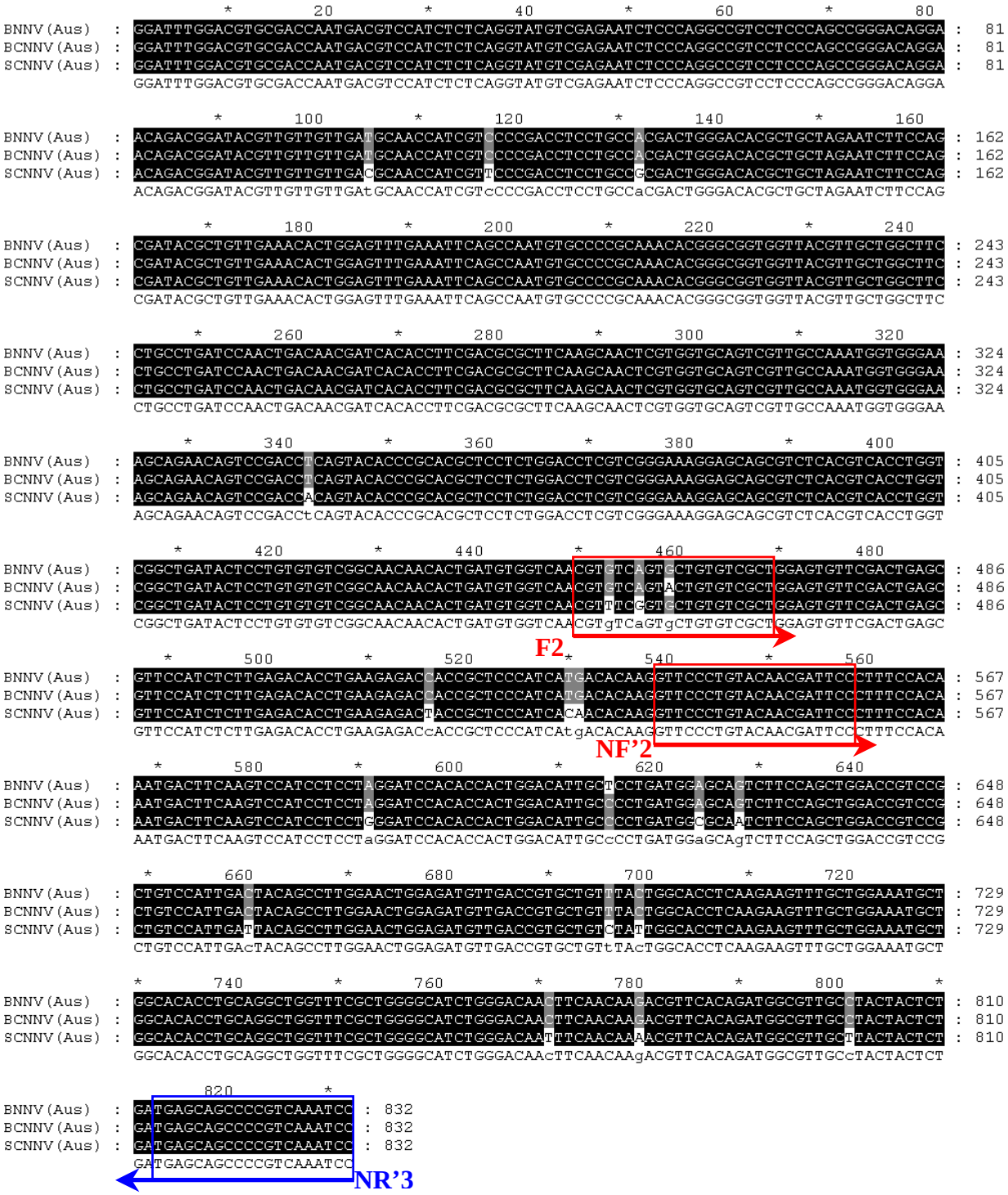


Figure 25: Comparison of the partial 832 bp coat protein gene sequences for Australian nodavirus isolates from barramundi (BNNV), barramundi cod (BCNNV) and sleepy cod (SCNNV). F2, NF'2 and NR'3 refer to three of the NNV Nested RT-PCR primer binding sites.

Comparison of the sequences from the three endemic isolates with exotic nodavirus coat protein sequences from NCBI GenBank demonstrated that the barramundi (BNNV), barramundi cod (BCNNV) and sleepy cod (SCNNV) isolates were highly homologous with several other nodavirus isolates (Appendix 2, Table 14 and Table 15). The barramundi and barramundi cod isolates shared a higher homology with the nodavirus isolates from grouper species (MGNNV, SGNNV, DGNNV, EcNNV, YGNNV and GGNNV), sea bass (DlEV) and

guppy (GNNV) (nucleotide: 98.6-99.4%; amino acid: 97.5-100%) than with the sleepy cod nodavirus isolate (nucleotide: 98.1%; amino acid: 99.6%). All of the Australian isolates displayed the least sequence homology with the striped jack (SJNNV) isolate (nucleotide: 79.1-79.8%; amino acid: 83.4%) and the turbot (TNNV) isolate (nucleotide: 76.9-77.6%; amino acid: 79.8-80.1%).

Table 14: Relative differences between the nucleotide acid alignments of the Australian and exotic nodavirus sequences (see Table 8 isolate details).

NODAVIRUS STRAINS	BNNV (AUS)	BCNNV (AUS)	SCNNV (AUS)
BNNV (Aus)		99.8%	98.1%
BCNNV (Aus)	99.8%		98.1%
SCNNV (Aus)	97.7%	97.7%	
MGNNV	99.4%	99.4%	97.4%
SGNNV	99.3%	99.3%	97.7%
DGNNV	99.2%	99.2%	97.4%
EcNNV	99.0%	99.0%	97.5%
YGNNV	99.0%	99.0%	97.7%
GGNNV	98.8%	98.8%	97.4%
DlEV	99.0%	99.0%	97.7%
GNNV	98.6%	98.6%	97.0%
JFNNV	92.1%	91.9%	91.5%
AHNNV	83.2%	83.2%	82.5%
SJNNV	79.8%	79.7%	79.1%
TNNV	77.6%	77.5%	76.9%

Table 15: Relative differences between the amino acid alignments of the Australian and exotic nodavirus sequences (see Table 9 isolate details).

NODAVIRUS STRAINS	BNNV (AUS)	BCNNV (AUS)	SCNNV (AUS)
BNNV (Aus)		100%	99.6%
BCNNV (Aus)	100.0%		99.6%
SCNNV (Aus)	99.3%	99.3%	
MGNNV	98.9%	98.9%	98.2%
SGNNV	99.3%	99.3%	98.6%
DGNNV	99.6%	99.6%	98.9%
EcNNV	100.0%	100.0%	99.3%
YGNNV	99.6%	99.6%	98.9%
GGNNV	98.9%	98.9%	98.2%
DlEV	99.3%	99.3%	98.6%
GNNV	97.5%	97.5%	96.8%
JFNNV	94.6%	94.6%	94.6%
AHNNV	87.7%	87.7%	87.4%
SJNNV	83.4%	83.4%	83.4%
TNNV	79.8%	79.8%	80.1%

The observation that the barramundi and barramundi cod were more closely related to the exotic grouper isolates than to the endemic sleepy cod isolate was confirmed by phylogenetic analysis of all the sequences (Figure 26). Interestingly, of the three Australian isolates, the barramundi and barramundi cod isolates were obtained from marine fishes, whereas the sleepy cod isolate was obtained from freshwater fish.

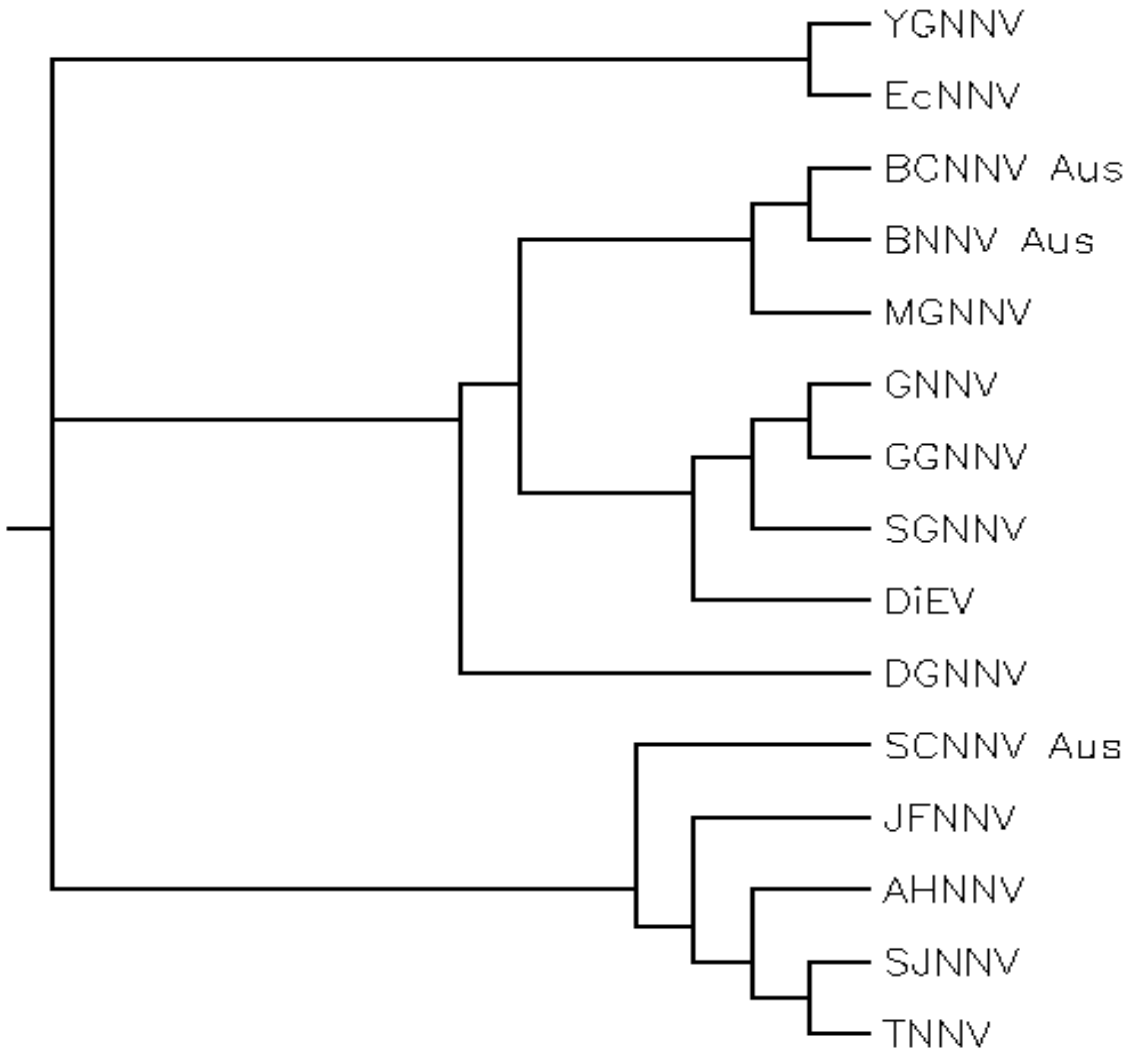


Figure 26: Rooted phylogenetic tree determined from the multiple sequence alignment of the nodavirus coat protein gene sequences of the Australian and exotic nodavirus isolates (from Table 8), showing the relatedness of the isolates.

5.3 Conclusion

The NNV Nested RT-PCR test, optimised and modified during the project, detected all endemic and exotic nodavirus isolates tested. No samples produced a negative result, or false negative result, where there was an indication that nodavirus was responsible for the disease observed. Over 600 larvae, fry, and juvenile fish samples have been testing using the Nested RT-PCR procedure described in this report, or the previous version described in Anderson and Moody (2004). As the procedure described herein contains modifications that improved the quality of the procedure it is still valid to incorporate results obtained using the previous unmodified Nested RT-PCR. Consistent results were obtained from all testing, demonstrating the sensitive, specific, repeatable and robust nature of the procedure for these sample types. No non-specific amplicons, or false positive results, have been observed when negative

samples were tested. Generally, clinically infected barramundi produced RT-PCR positive and Nested PCR positive amplicons. However, results from fish surviving experimental infection with BNNV were more variable with regard to RT-PCR results. Broodstock material had never tested positive by RT-PCR. Therefore, unless the NNV Nested RT-PCR is being used to test fish that are clinically infected with nodavirus, use of the RT-PCR test alone could result in an unacceptably high number of false negative results. Hence, the recommendation to test all samples by Nested RT-PCR

A modification was essential to increase confidence in Nested RT-PCR results when the test was applied to broodstock blood. Blood was a more suitable sample type for broodstock testing, with a higher proportion of broodstock testing positive for nodavirus with this sample compared to spawning material. However, further ongoing testing is required to determine the relationship between presence of nodavirus in the blood and in spawning material from the same broodstock fish, and what implications this has for vertical transmission to larvae. For broodstock blood and spawning material, it was postulated that the levels of infection were approaching the limits of detection of the test. The lack of repeatability when broodstock spawning material was tested was exacerbated by a lack of complete confidence in the homogenisation method used. Using broodstock sperm as an example, where no one sample volume appeared to more appropriate than another, it is believed that the methods used to disrupt the sample, releasing the virus for subsequent RNA extraction, were inadequate. Sample preparation of broodstock ovarian and sperm samples requires further research before a standard method can be developed and more accurate comparisons can be made. While complete confidence could be given to positive results, it is unknown what proportion of negative results are really false negative results.

Evaluation of different commercially available RNA reagents and kits, and reverse transcriptase enzymes and DNA polymerase enzymes found that while most could be used in the Nested RT-PCR, one reverse transcriptase and one DNA polymerase failed to detect nodavirus in a known BNNV-positive sample, which was a false negative result. Therefore, when alternatives to the reagents described for the NNV Nested RT-PCR procedure in this report are used, comparative testing and validation are critical to ensure that any alternative reagents do not compromise the quality of the test. This is a standard Quality Assurance requirement for any alternate reagent for any test and would be expected in any laboratory with a Quality Assurance program.

Synthetic RNA was produced to resolve the issue of a continually available source of material for use as a positive control. The use of synthetic RNA as a positive control has the following advantages; 1) elimination on the reliance of nodavirus outbreaks to obtain clinical material, 2) elimination of the requirement to regularly conduct *in vivo* amplification work in barramundi to generate clinically infected material, 3) elimination of the requirement for interstate transport of infectious material and 4) relatively large volumes of RNA of consistent quality can be produced *in vitro*.

A sequencing RT-PCR was developed and implemented. This enabled the comparison of endemic nodavirus isolates with exotic nodavirus isolates. Sequencing demonstrated that the barramundi (BNNV) and barramundi cod (BCNNV) isolates, obtained from the marine environment, were most closely related to the overseas grouper isolates, while the freshwater sleepy cod isolate (SCNNV) was more closely related to the other, non-grouper isolates.

6. Development of a nodavirus susceptible cell line

Objective 2: To establish a cell line which can be used for the isolation, amplification and titration of endemic and exotic nodaviruses from a range of fish species.

6.1 Methods

6.1.1 Establishment of new brain cell lines

Establishment of new brain cell lines were attempted from barramundi and sleepy cod.

To establish cell lines whole brains or different brain sections were aseptically removed from the fish, after euthanasia by cervical dislocation. The tissue was placed into a petri dish containing antibiotic enriched Earles' Minimum Essential Medium (AeEMEM; EMEM supplemented with 1000IU/ml penicillin G, 1mg/ml streptomycin sulphate and 2mg/ml amphotericin B) pre-warmed to 25°C. In a Biohazard Cabinet, fatty tissue and blood vessels were removed and the tissue was chopped into small (<1mm^3) pieces. The tissue was washed three times in AeEMEM by pelleting, at 100 × g for 5 minutes, and resuspension of the tissue pieces in fresh AeEMEM. Following the final wash and removal of the supernatant, the tissue fragments was resuspended in 2ml of 0.05% trypsin solution, pre-warmed to 25°C, and transferred to a sterile universal bottle containing a small magnetic flea. A further 10ml of 0.05% trypsin solution was added and the tissue was incubated on a magnetic stirrer for 45 minutes at 25°C. The cells were pelleted by centrifugation at 100 × g for 10 minutes and resuspended in 10ml EMEM supplemented with 20% foetal bovine serum (FBS). The cell suspension was then diluted into either 100ml EMEM supplemented with 20% FBS, or 50ml EMEM supplemented with 20% FBS and 50ml EMEM supplemented with 10% FBS. Ten millilitres of the diluted cell suspension was added to a 25cm² tissue culture flask (Iwaki), incubated at 25°C and monitored daily for cell adherence, proliferation and absence of microbial contamination. When adherent cells were present in the tissue culture flasks the growth medium was replaced with fresh EMEM and FBS. Proliferating cell lines were passaged as described below (section 6.1.2). Table 15 details the different occasions brain cell lines were initiated.

Table 15: Cell line initiation dates, species, tissue used and cell line identification.

DATE	SPECIES	AREA	Cell Line ID
28/05/02	Barramundi	Whole brain	LCB-A
04/06/02	Barramundi	Whole brain	LCB-B
03/09/02	Barramundi	Whole brain	LCB-C
21/03/03	Barramundi	Whole brain	LCB-N
13/06/03	Barramundi	Optic tectum Hypothalamus Cerebellum	LCB-O LCB-H LCB-Cer
23/03/04	Sleepy Cod	Whole brain	SCB

6.1.2 Passaging and maintenance of cell lines

The following procedure was used to passage all cell lines including the original LCB cell line, thawed from storage in liquid nitrogen the primary brain cell lines, the clones and the SSN-1 cell line, obtained from James Cook University.

Standard cell passaging was conducted as follows. The growth medium was poured off and the monolayer rinsed in phosphate buffered saline containing 0.0002% phenol red (PBS-PR). Cells were removed from the substrate by addition of 10ml of 0.05% trypsin solution, pre-warmed to 37°C, followed by incubation at room temperature. The flask was shaken to remove any strongly adherent cells. After repeated pipetting of the trypsinised cells to form a single cell suspension, all but 1ml of the cell suspension was removed from the flask. Cell lines were initially cultured in 25cm² tissue culture flask (Iwaki) but when growth rates were sufficient, cells were cultured into two 150cm² stock tissue culture flasks (Iwaki). Until cell lines had demonstrated growth in the 150cm² tissue culture flasks, cell lines were also maintained in the smaller 25cm² tissue culture flasks. After addition of trypsinised cells to the 150cm² tissue culture flasks, 40ml of EMEM supplemented with the required concentration of FBS (either 10% or 20%) was added and the tissue culture flasks were incubated at 25°C and monitored daily for cell adherence, proliferation and absence of microbial contamination. The remaining 9ml of trypsinised cells were discarded, used to seed further 150cm² tissue culture flasks or passaged into 25cm² tissue culture flasks for nodavirus susceptibility screening.

6.1.3 Cloning of LCB and LCB-O cell lines

A vial of the original BNNV and SCNNV susceptible LCB cell line, stored in liquid nitrogen at passage 18, was rapidly thawed in a 37°C waterbath and the contents added to a 150cm² tissue culture flask containing 40ml EMEM supplemented with 20% FBS. The cells were cultured until the growth rate was acceptable for nodavirus growth (twice weekly passaging was required and a confluent monolayer would develop within 48 hours). This growth rate was achieved at passage 22 and at passage 24, when the cell monolayer was 100% confluent, the cells were passaged as described above (section 6.1.2). The trypsinised cells were counted using a haemocytometer and a suspension containing 10 cells/ml was prepared in EMEM supplemented with 20% FBS. One hundred microlitres was added to each well of ten 96-well tissue culture plates and the plates were incubated at 25°C with a 2.5% CO_2 atmosphere. After three days wells containing individual adherent cells were recorded and growth was monitored over time. When there was significant growth in a well, the cells were passaged into 24 well plates. For passaging the wells of the 96-well plate, the cells were rinsed with 150µl PBS-PR and 100µl pre-warmed 0.05% trypsin solution was added. After gentle pipetting the trypsinised cells were removed and added to a well of a 24-well plate containing EMEM supplemented with 20% FBS. These plates were then incubated at 25°C with a 2.5% CO_2 atmosphere. When monolayer formation had occurred in a well of a 24-well plate the cells were trypsinised into 25cm² tissue culture flasks in the same way as passaging of the 96-well plates, using 1ml of PBS-PR and trypsin. The 25cm² tissue culture flasks were incubated at 25°C and when the monolayer was confluent the cells were passaged into 150cm² tissue culture flasks. The LCB-O cell line, initiated from the optic tectum region of a barramundi brain was also cloned as described above.

6.1.4 Screening of cell lines

The following procedure was used to screen all cell lines for susceptibility to infection with nodavirus. Cells were screened f when the growth of the cells was similar to the growth of the original LCB cell line. The LCB cell line had been in routine use for approximately twelve months before loss of susceptibility to nodavirus infection occurred. The best expression of cytopathic effect (CPE) and amplification of nodavirus occurred when the cells were rapidly growing (required twice weekly passaging and a confluent monolayer would develop within 48 hours) and were 50-60% confluent when inoculated. Using these criteria, repeatable isolations were routinely made from submissions of barramundi exhibiting either clinical

signs, lesions after examination by histology or positive RT-PCR test results (N Moody, pers. obs.). All isolations in the original LCB cells were conducted in 25cm² tissue culture flasks, as it was very difficult to produce a 50-60% confluent monolayer in the well of a 24-well plate with these cells. Therefore, all screening of newly initiated cell lines and the SSN-1 cells was conducted in 25cm² tissue culture flasks when cell monolayers were 50-60% confluent. At least two different BNNV-positive larval submissions were used for screening of each cell line. These submissions were known to be nodavirus positive based on clinical signs, histological analysis and Nested RT-PCR testing. BNNV and SCNNV infected LCB cell culture supernatants, stored at -80°C, was also used for screening the cell lines. As an additional check, all samples used for screening were retested by Nested RT-PCR at the time of screening and were positive by RT-PCR, indicating a relatively high viral load.

Cells were screened for susceptibility to nodavirus as follows. The growth medium was removed the cell monolayers and 0.9ml of inoculum was added. Samples used as a source of infectious nodavirus included clarified BNNV-positive larval homogenate and BNNV-infected LCB cell culture supernatants. The cells were incubated at 25°C for 60 minutes and 10ml of maintenance medium (EMEM supplemented with either 10% or 5% FBS) was added. The amount of FBS in the maintenance medium was half that used in the growth medium. Tissue culture flasks were returned to the 25°C incubator and monitored daily for seven days for the appearance of CPE. After seven days, the flasks were shaken and 0.9ml of the suspended cells from the passage one material was used as the inoculum for a further inoculation of the cell line as described above. The tissue culture flasks were returned to the 25°C incubator and monitored daily for seven days for the appearance of CPE. After seven days the passage two cell cultures were used for a further third infection of the cell line. If no CPE was produced after three passages the cell line was considered to be unsusceptible to nodavirus infection and was discarded. While it would have been desirable to store the cells in liquid nitrogen for further analysis at a later date, this was not possible due to resources constraints.

6.2 Results and discussion

6.2.1 Establishment of brain cell lines

Cell lines were established after each of the initiation attempts from barramundi (Table 15) but no passageable cell line was produced after attempts were made using sleepy cod brain tissue. The cell lines had varying growth rates and while growing cell cultures were established in each of the flasks seeded, only fifteen of these cultures had growth rates that resulted in them being screened for susceptibility. Examples of cell lines initiated on three different occasions (LCB-A, LCB-B and LCB-C) are shown below (Figure 27, 28 and 29). The fastest growth of cell cultures was achieved when an FBS concentration of 20% was used. Some cultures failed to adhere and grow after the first, second or third passage and were discarded. Others had growth rates too slow to be considered useful and in many instances confluent cell monolayers took weeks to develop. As previous work had demonstrated that rapidly growing cells were required for the expression of BNNV CPE (N Moody, pers. obs.), these cell lines were not considered viable candidates for screening. Passaging continued and any change in the growth characteristics noted, however growth rates never improved and after approximately 30 passages, if no improvements in growth were observed, these cell lines were discarded.

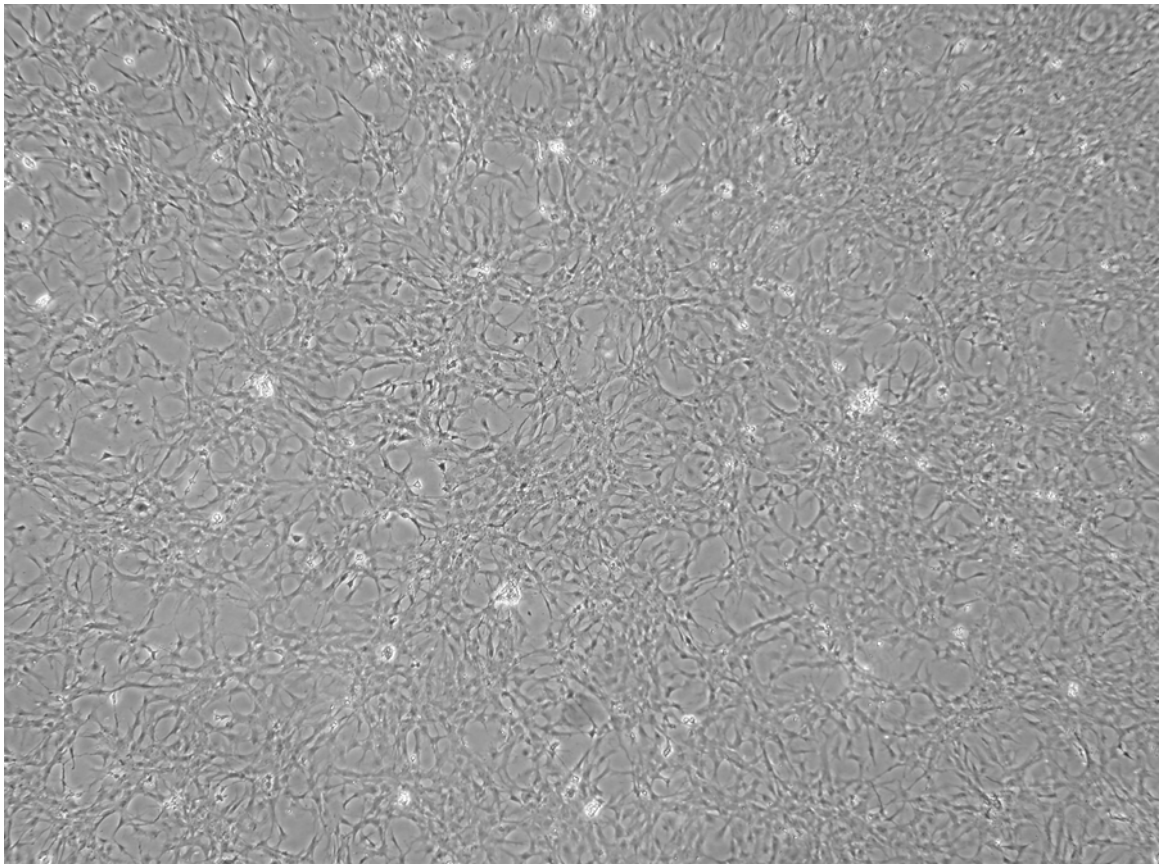


Figure 27: Passage 23 LCB-A cell culture, two days after passage into the flask. Phase contrast, x 40

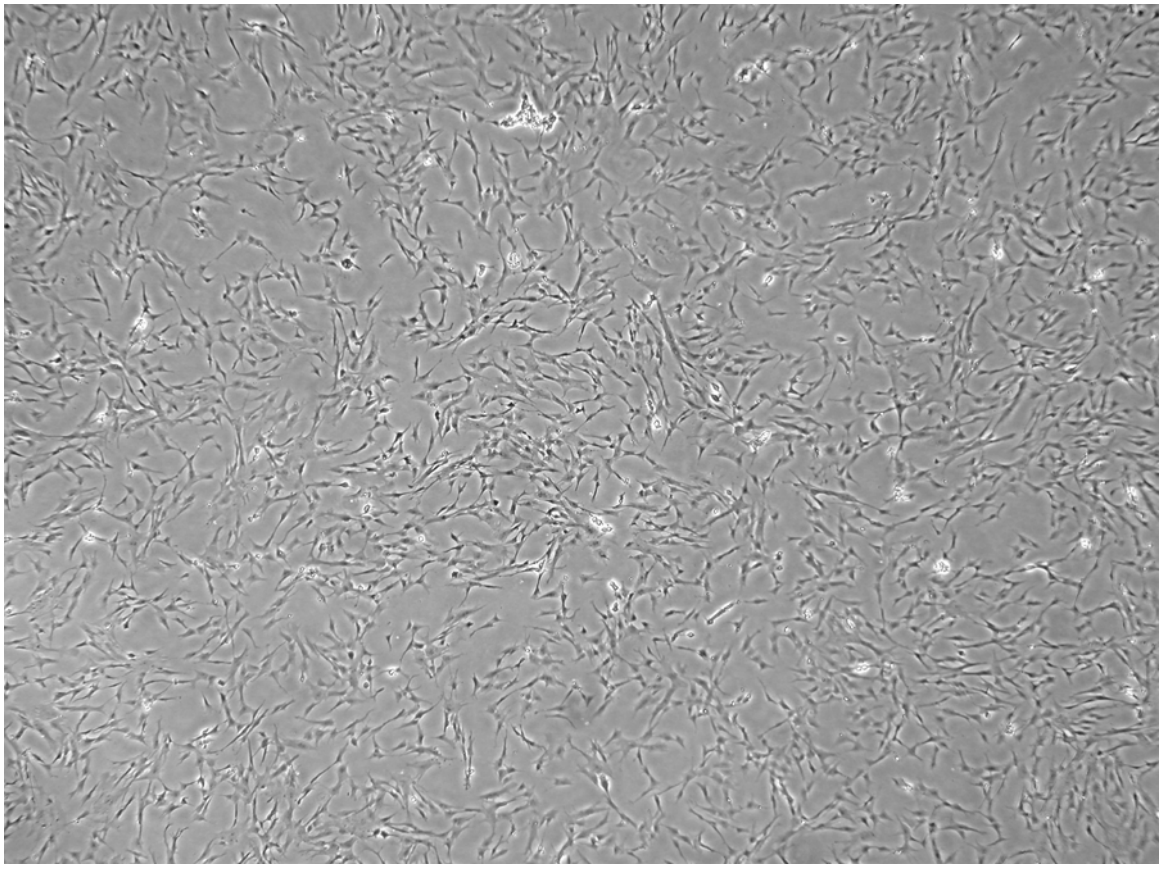


Figure 28: Passage 19 LCB-B cell culture, two days after passage into the flask. Phase contrast, x 40

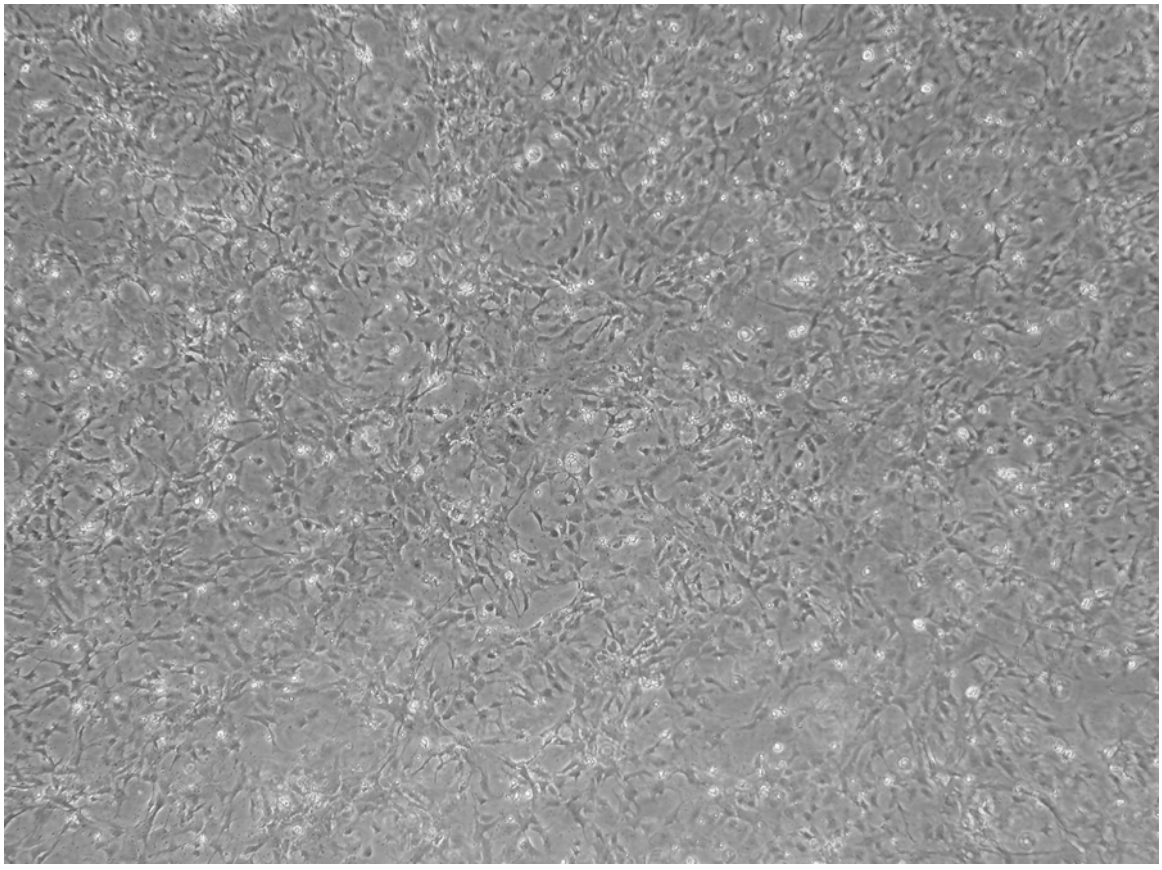


Figure 29: Passage 12 LCB-C cell culture, two days after passage into the flask. Phase contrast, x 40

6.2.2 Passaging and maintenance of cell lines

After passaging of all established cell lines, clones and most newly initiated cell lines adherence and growth of the cells was consistent and repeatable, the cultures maintained growth rates and were simple to maintain. No newly initiated cell lines failed to continue to grow if three passages could be undertaken. During the first three passages, a small number of the cell lines failed to continue to grow although these were usually the slower growing cell lines. All rapidly growing cultures were successfully passaged at least 30 times with no change in the growth characteristics.

6.2.3 Cloning of LCB and LCB-O cell lines

From LCB cells seeded into the wells of ten 96 well plates, single cells were detected in 122 wells. Monolayers developed in 14 of these wells, which were successfully passaged into wells of 24 well plates. Eleven of these cell lines were successfully cultured into 150cm² tissue culture flasks. Five of these cloned cell lines, designated LCB-WT1, LCB-WT2, LCB-WT3, LCB-WT4 and LCB-WT5, could be routinely passaged twice weekly in the 150cm² tissue culture flasks and were considered viable candidates for susceptibility screening. The growth rates of the other cell lines were very slow (3 to 8 weeks between passages). Maintenance of these cell lines continued, however growth rates never improved and these cell lines were eventually discarded. The cloning of the LCB-O cell line resulted in the development of 28 cloned cell lines cultured in 150cm² tissue culture flasks. Three of these cell lines (LCB-O-1, LCB-O-2 and LCB-O-3) were screened for susceptibility to nodavirus.

6.2.4 Screening of cell lines

6.2.4.1 Screening of new cell lines

Fifteen newly initiated cell lines were screened for susceptibility to nodavirus infection. However, CPE did not develop in any cell lines after three passages of BNNV-infected material (Table 16**)**. Some cellular changes were noticed after the first passage of the inoculum in several cell lines, however these changes never progressed to passage two. The changes consisted of rounding up and death of cells from 24 to 48 hours post infection. From 48 hours post infection the changes would either continue to develop and result in complete destruction of the monolayer, or the remaining viable cells would grow and a confluent monolayer would develop after four to five days.

No contaminants were evident when the cultures were examined microscopically. Cellular changes may have been due to toxicity of the inoculum although inoculation of cells with 10-fold dilutions of the original inoculum still produced similar changes for the first passage. No uninfected samples were used during the viral screening work so the sensitivity of the cells to the sample itself is unknown. A barramundi kidney (LCK) cell line, made at the same time as the original LCB cell line, has been in routine use in the Virology section of OVL for seven years and is more sensitive to sample toxicity (including benzocaine, when used for euthanasia prior to virological analysis) than the more well known piscine (BF2, RTG and FHM) and mammalian (VERO, BHK, MDBK, BSR, RK-13) cell lines also used (N Moody, pers. obs.). Cell lines derived from barramundi tissue may have these characteristics. Unfortunately, the NNV immunodiagnostics (Chapter 7) were not available when this screening was being conducted so confirmation of whether nodavirus was the agent

responsible for the changes could not be undertaken. Therefore, although new barramundi brain cell lines were produced, none were susceptible to infection with nodavirus.

Table 16: Susceptibility of newly established brain cell lines to infection with nodavirus.

CELL LINE	PASSAGE	CPE	COMMENT
LCB-A-1	8	P1	No cellular changes past passage 1
LCB-A-2	20	P1	No cellular changes past passage 1
LCB-B-1	21	-	No cellular changes
LCB-B-2	12	P1	No cellular changes past passage 1
LCB-C-1	13	-	No cellular changes
LCB-C-2	22	P1	No cellular changes past passage 1
LCB-C-3	25	-	No cellular changes
LCB-C-4	7	-	No cellular changes
LCB-C-5	7	-	No cellular changes
LCB-D-1	12	-	No cellular changes
LCB-D-2	12	-	No cellular changes
LCB-D-3	12	-	No cellular changes
LCB-H-2	7	P1	No cellular changes past passage 1
LCB-O	8	P1	No cellular changes past passage 1
LCB-H-1	13	P1	No cellular changes past passage 1

6.2.4.2 Screening of the original LCB cells

The LCB cells were susceptible to infection with BNNV up to passage 35 of the cell cultures. Initial CPE was observed 12 to 18 hours post infection and consisted of generalised rounding up and detachment of individual cells. By 24 hours post infection the CPE was obvious throughout the cell monolayer (Figure 30 and 31). The cell monolayer was completely destroyed by 72 hours post infection. This pattern of CPE development was consistently observed after inoculation of pre-passage 35 LCB cultures with over 15 different submissions of clinically infected barramundi homogenate and passaging of nodavirus-infected cell culture supernatant (N Moody pers. obs.). No cellular changes were observed after inoculation of the cells with uninfected homogenates. However, while there were no changes in morphology of the passage 36 LCB cells, at passage 36 and above, no CPE was observed after inoculation of the LCB cell cultures with BNNV. Exposure of the LCB cell cultures to infected material at passage 37, 38 and 39 also failed to result in the appearance of any cellular changes. Previous work with the LCB cells from passage 50 to 75 also failed to result in the appearance of CPE. Why the cells transformed is unknown.

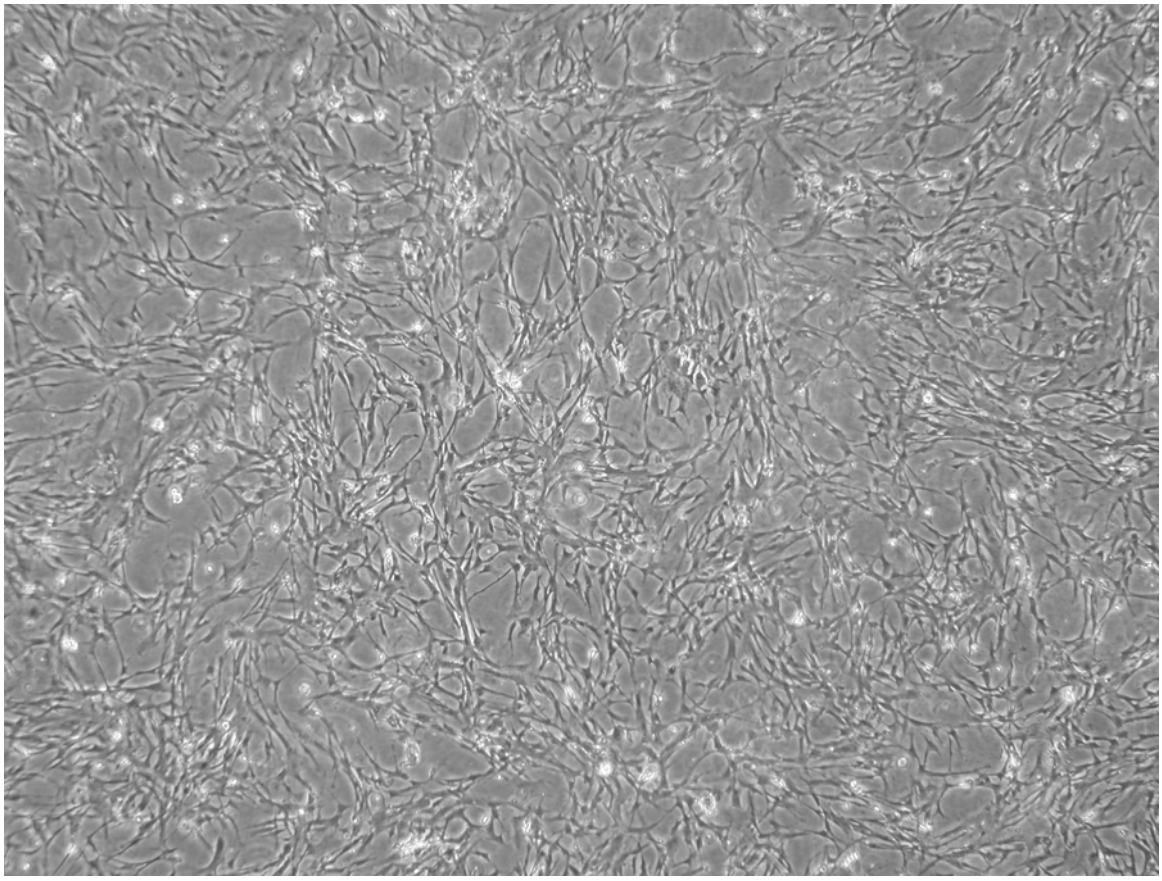


Figure 30a: Passage 29 LCB cells, 2 days old. Phase contrast, x40

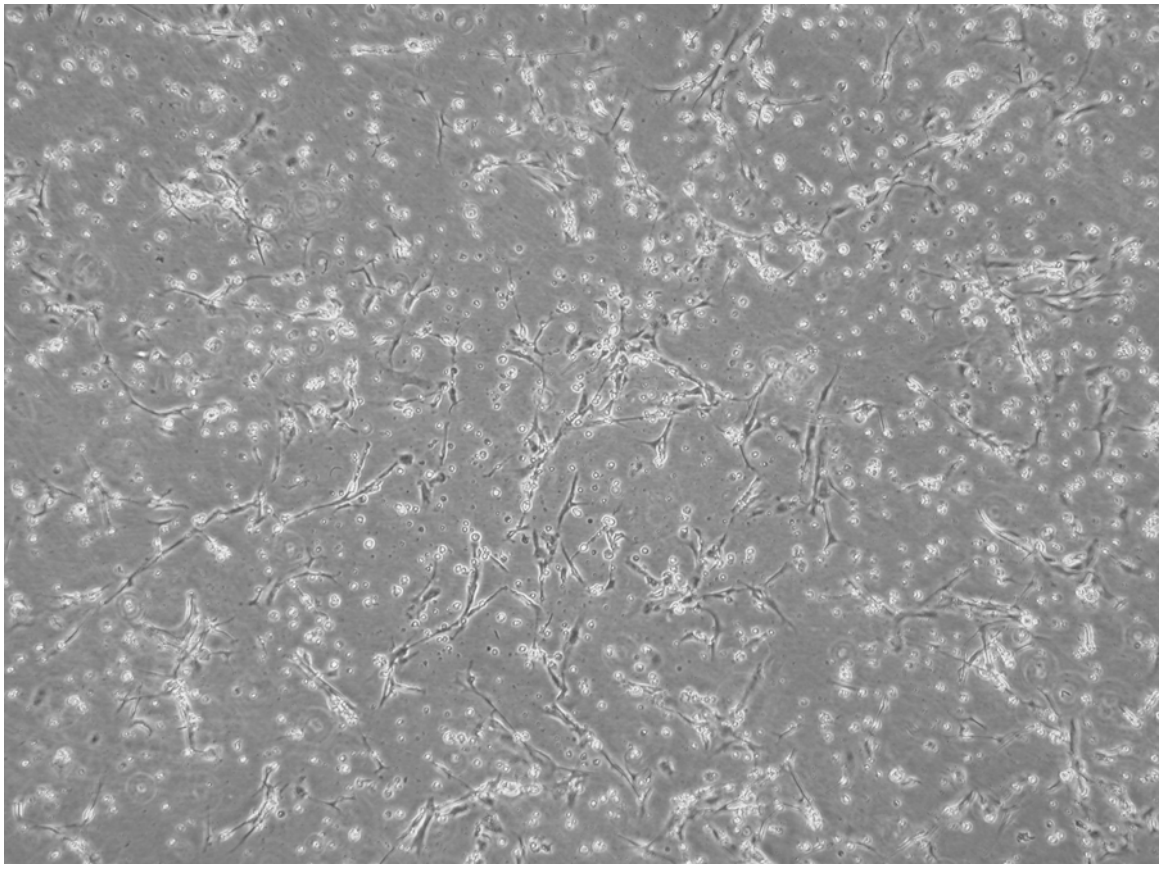


Figure 30b: Passage 29 LCB cells, 2 days old and 48 hours post infection with BNNV. Phase contrast, x40

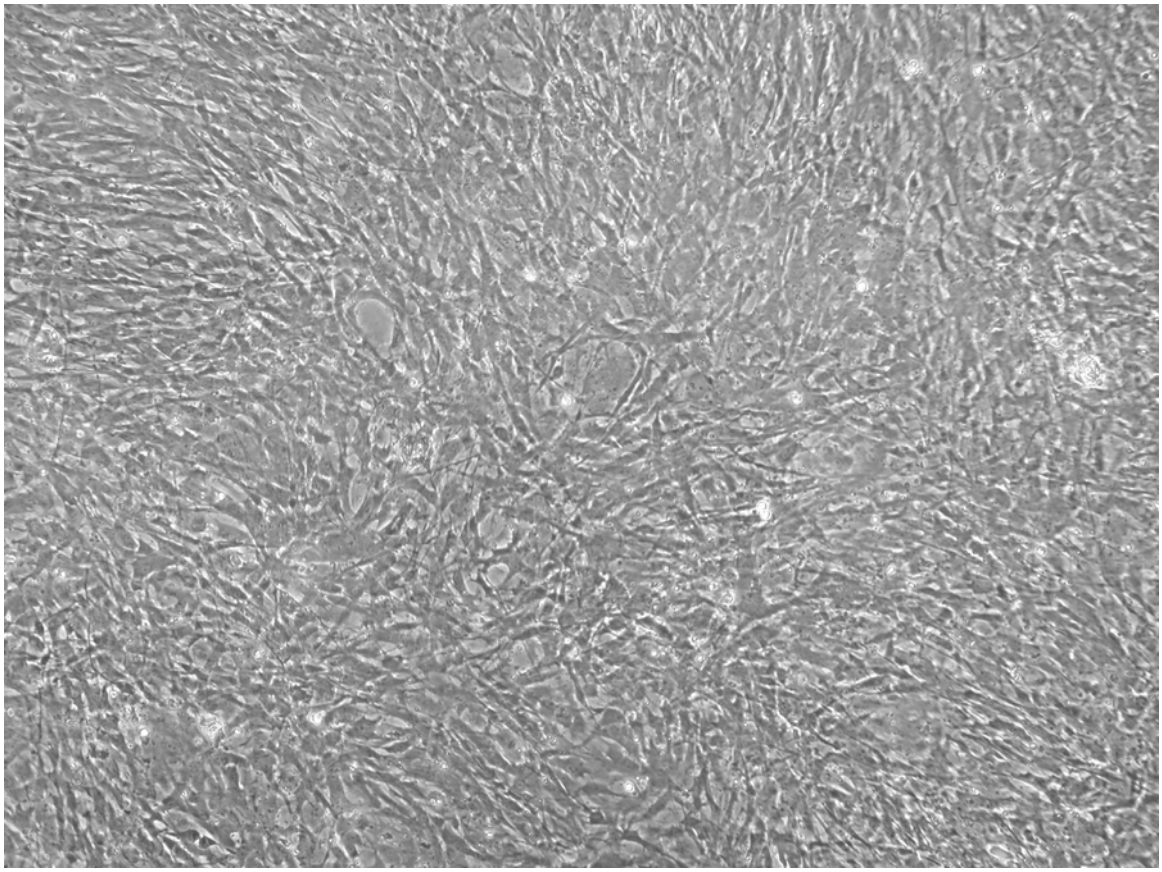


Figure 31a: Passage 31 LCB cells, 4 days old. Phase contrast, x40

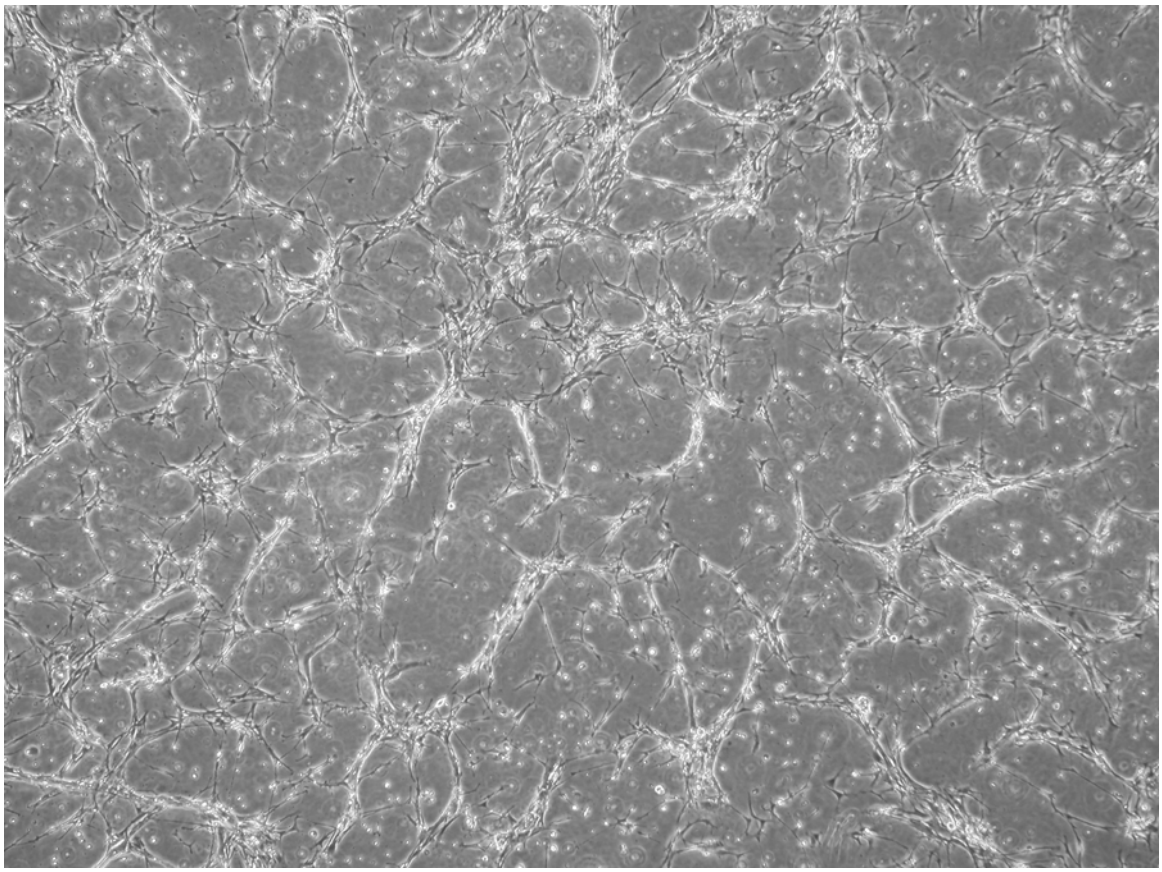


Figure 31b: Passage 31 LCB cells, 4 days old and 48 hours post infection with BNNV. Phase contrast, x40

6.2.4.3 Screening of clones

Screening of the eight clones derived from the LCB and LCB-O cell lines failed to identify a cell line that was susceptible to infection with nodavirus (Table 17). In the LCB-WT1, LCB-WT2 and LCB-WT3 cell lines CPE did develop after 4-5 days and was still present after seven days, but when new monolayers of cells were re-infected with the passage one material, no cellular changes were observed. This also occurred with the LCB-O-A and LCB-O-B cell lines, although the entire monolayer was destroyed within 48 hours after the first passage of infected material. Monolayer destruction was significantly reduced at passage two and took approximately four to six days to develop. As with all the other cell lines screened, no cellular changes were observed after three passages of a BNNV infected sample and no cloned cell line was produced that was susceptible to infection with nodavirus.

Table 17: Cloned brain cell lines derived from LCB and LCB-O cell lines and screened for susceptibility to nodavirus.

CELL LINE	PASSAGE	CPE	COMMENT
LCB-WT1	41	P1, P2	No cellular changes past passage 2
LCB-WT2	41	P1, P2	No cellular changes past passage 2
LCB-WT3	41	P1, P2	No cellular changes past passage 2
LCB-WT4	40	-	No cellular changes at all
LCB-WT5	42	P1	No cellular changes past passage 1
LCB-O-A	8	P1, P2	No cellular changes past passage 2
LCB-O-B	13	P1, P2	No cellular changes past passage 2
LCB-O-C	13	-	No cellular changes

6.2.4.4 Screening of the SSN-1 cell line

While the SSN-1 cell line was easily cultured, no CPE was observed after inoculation with samples from nodavirus infected barramundi larvae or nodavirus infected LCB cell culture supernatants. No cellular changes were observed and in all instances the monolayer continued to proliferate until it was overgrown and degenerated. Therefore, the SSN-1 cell lines used was not susceptible to the endemic barramundi or sleepy cod nodavirus isolates.

6.3 Conclusion

While primary brain cell lines and cloned brain cell lines were successfully produced, none of these cell lines were susceptible to infection with nodavirus. The original LCB cell line produced typical nodavirus CPE up to and including passage 35, but then transformed and was no longer susceptible to infection at passage 36 and above. Cloning of the LCB cell line and the newly initiated LCB-O cell lines resulted in the production of cell lines exhibiting the required growth characteristics but none of the cloned cell lines were susceptible to nodavirus. Cell lines were also successfully produced from tissue from specific regions of the brain, identified as most commonly infected with nodavirus by IHCT, but again none of these cell lines were susceptible to infection with nodavirus. The SSN-1 cell line was also not susceptible to infection with the nodavirus isolates used.

Why the original LCB cell line transformed, and why no susceptible cell lines were produced is unknown. The procedure used to initiate the cell lines was successful and was no different to the procedure used to develop the original susceptible LCB cell line so no modifications to

the initiation procedure were be considered. The samples used for screening the cell lines were known to be infected with viable nodavirus so failure to observe CPE was not due to lack of infectious virus in the inoculum. No cell lines were initiated from striped trumpeter due to logistical problems associated with live transport of the fish over the distances required and time constraints. Unfortunately, the objective to establish a cell line that could be used for the isolation, amplification and titration of endemic and exotic nodaviruses, from a range of fish species, was not achieved. A cell line susceptible to nodavirus infection is required and research to achieve this should be supported.

7. Immunodiagnostic test development

Objective 3: To produce immunodiagnostic tests, capable of localising endemic and exotic nodaviruses in fish tissues and cell cultures.

7.1 Methods

7.1.1 Inoculum preparation, vaccination procedure and blood collection

Recombinant coat protein (rCP), from a barramundi nodavirus isolate (BNNV rCP) and a sleepy cod nodavirus isolate (SCNNV rCP), was produced at the Queensland Agricultural Biotechnology Centre, Brisbane, from material provided by the Oonoonba Veterinary Laboratory. For the primary inoculation, each sheep was intramuscularly injected at two sites with 0.5ml of an emulsion containing equal volumes of rCP and Freund's Complete Adjuvant (FCA). For subsequent booster inoculations, each sheep was inoculated with 0.5ml of an emulsion containing equal volumes of rCP and Freund's Incomplete Adjuvant (FIA). The inoculation regime is described in Table 18.

Table 18: Polyclonal antibody production regime.

DAY	SHEEP	TREATMENT	COMMENT (Sample Identification)
0	8 10	SCNNV rCP with FCA BNNV rCP with FCA	Pre-inoculation samples taken Pre-inoculation samples taken
14	8 10	SCNNV rCP with FIA BNNV rCP with FIA	
28	8 10	SCNNV rCP with FIA BNNV rCP with FIA	
42	8 10	No inoculation No inoculation	Test bleed – 1 × clotted, 1 × lithium heparin (**2-46574-8)** Test bleed – 1 × clotted, 1 × lithium heparin (**2-46574-10)**
56	8 10	SCNNV rCP with FIA BNNV rCP with FIA	Test bleed – 1 × clotted, 1 × lithium heparin (**2-47496-8)** Test bleed – 1 × clotted, 1 × lithium heparin (**2-47496-10)**
70	8 10	No inoculation No inoculation	Trouble finding jugular – not bled Test bleed – 1 × clotted, 1 × lithium heparin (**2-48406-10)**
85	8 10	SCNNV rCP with FIA BNNV rCP with FIA	
102	8 10	SCNNV rCP with FIA BNNV rCP with FIA	
132	8 10	SCNNV rCP with FIA BNNV rCP with FIA	Trouble finding jugular – not bled ~450ml plasma - Blood-Pack® (**2-52296-10)**
156	8 10	SCNNV rCP with FIA SCNNV rCP with FIA	~450ml plasma - Blood-Pack® (**2-53906-8)** ~200ml plasma - Blood-Pack® (**2-53914-10)**
184	8 10	SCNNV rCP with FIA SCNNV rCP with FIA	~450ml plasma - Blood-Pack® (**2-55952-8)** ~450ml plasma - Blood-Pack® (**2-55960-10)**
212	8 10	No inoculation No inoculation	Trouble finding jugular – not bled ~450ml plasma - Blood-Pack® (**2-57990-10)**
362	8 10	No inoculation No inoculation	~450ml plasma - Blood-Pack® (**3-44656-8)** Trouble finding jugular – not bled

Prior to inoculation and during the development of the immune response after inoculation with the rCP, blood was collected into 10ml untreated and lithium heparin vacutainers from the jugular artery. After centrifugation at 900 × *g* for 10 minutes at 5°C, serum or plasma was collected and stored at -80°C until required. When blood was harvested during collection bleeds, jugular blood was collected into USP (CPDA-1) Blood-Pack® Units (Baxter, USA) containing citrate phosphate dextrose adenine solution as anticoagulant. The blood was clarified by centrifugation at 900 × *g* for 10 minutes at 5°C and plasma was stored at -80°C until required. The procedure for the inoculation and harvesting of blood had been approved by the Department of Primary Industries and Fisheries Townsville Local Animal Ethics Committee (No. TSV/26/00) with the volumes of inoculum and adjuvant (FCA and FIA) used determined from ANZCCART guidelines (Anon, 1998).

7.1.2 Dot-Blot analysis of sheep polyclonal antibodies

The development of polyclonal antibodies in the sheep after inoculation with the rCP was evaluated using Dot Bot apparatus (Bio-Rad, USA). The apparatus was designed to have sample flow through the blot substrate using a vacuum system. However, after blocking the reagent drain wells with Parafilm®, the apparatus was modified to produce a 96-well plate format with nitrocellulose as the substrate. This enable the test to be developed like an ELISA resolved issues associated with binding the antigen to the substrate and sample leakage through the apparatus.

The nitrocellulose used for all dot blots was Hybond-ECL (Amersham Pharmacia Biotech, Germany) with the Dot Blots designed to investigate the following:

- The titre of any antibodies produced.
- Any difference between serum and plasma as the format for the polyclonal antibodies.
- The development of the immune response and polyclonal antibody production.
- The sensitivity and specificity of the polyclonal antibodies.

7.1.2.1 Polyclonal Evaluation No. 1

To determine whether inoculation of the sheep with the rCP resulted in the production of polyclonal antibodies, whether serum or plasma could be used, and the broad range of dilutions to use for the primary (1°) antibody and secondary (2°) antibody conjugate, checkerboard titrations were undertaken as follows.

Nitrocellulose was equilibrated in Tris-buffered saline (TBS, 20mM Tris, 500mM NaCl) for at least 10 minutes. Parafilm® was placed over the vacuum template of the 96-well plate Dot Bot apparatus. Fifty microlitres of BNNV rCP, diluted to 1μg/ml (50ng/well) in Tris-buffered saline-Tween (TBST, 20mM TRIS, 500mM NaCl, 0.1% Tween–20), was added to each well using a multistepper. After incubation for 60 minutes at 100 rpm on an orbital shaker at room temperature, the plates were washed four times with TBST. Non-specific binding sites were blocked by addition of 200μl of 5% ECL Blocking agent (w/v in TBST) to each well, the plate was incubated as above then washed four times with TBST. Polyclonal antibody (Sheep α-BNNV rCP, 2-47496-10) dilutions of 1/50, 1/75, 1/125, 1/250, 1/500, 1/1000, 1/2000, 1/3000 and 1/4000 were prepared in 5% ECL Blocking agent (w/v in TBST). Dilutions were prepared for the serum and plasma samples obtained from the sheep. Negative sheep serum, diluted 1/50, was used as a negative control and one column had no polyclonal antibody added to act as a non-specific conjugate binding control. Fifty microlitres of the diluted Sheep α-NNV rCP samples were added to the appropriate wells of the Dot Blot apparatus (Figure

32) and the plate was incubated as above, then washed four times with TBST. Dilutions of conjugate (Rabbit α-Sheep IgG [H+L] HRP, Jackson ImmunoResearch, USA) of 1/500, 1/1000, 1/3000 and 1/5000 were prepared in 5% ECL Blocking agent (w/v in TBST) and 50µl of the diluted conjugate was added to the appropriate wells of the Dot Blot apparatus (Figure 32). The plate was incubated as above then washed four times with TBST. The nitrocellulose was removed from the Dot Blot apparatus and placed into a weigh boat for addition of the substrate. The HRP was developed using 3-Amino-9-ethylcarbozole in N,N-Dimethylformamide (AEC-DMF). AEC-DMF was prepared by adding 5ml of 0.4% AEC (w/v in DMF) to 100ml acetate buffer (14.8ml of 1.155% glacial acetic acid (v/v in deionised water), 35.3ml of 1.64% anhydrous sodium acetate (v/v in deionised water) and 50ml deionised water) followed by the addition of 100µl hydrogen peroxide immediately prior to use. The development was monitored and stopped by immersion of the nitrocellulose in deionised water.

	1	2	3	4	5	6	7	8	9	10	11	12	Conjugate Dilution	
A													1/500	
B													1/1000	1° Ab Serum
C													1/3000	
D													1/5000	
E													1/500	
F													1/1000	1° Ab Plasma
G													1/3000	
H													1/5000	
	1\50	1\75	1\125	1\250	1/500	1\1000	1/1500	1/2000	1/3000	1/4000	-ve sheep serum	-ve (no 1°Ab)	Antigen = BNNV rCP at 1µg/ml	

1° Antibody dilution (Sheep α-BNNV rCP, 2-47496-10)

Figure 32: Plate set-up for the checkerboard titration to determine broad primary (1°) antibody and conjugate (2°) dilutions and to compare serum and plasma as the primary antibody. Primary antibody used was 2-47496-10.

7.1.2.2 Polyclonal Evaluation No. 2

To determine the degree of cross-reactivity between SCNNV rCP and BNNV rCP, whether heat-inactivation of plasma improved the sensitivity of the polyclonal antibodies and narrow the range of dilutions to use for the primary (1°) antibody and secondary (2°) antibody conjugate optimisation, additional checkerboard titrations were conducted. The Dot Blot was conducted as described in Polyclonal Evaluation No. 1 with the plate set-up described in Figure 33. The nitrocellulose was coated with SCNNV rCP and BNNV rCP at a concentration of 10µg/ml (500ng/well) and duplicate aliquots of the same primary antibody, diluted to 1/250, 1/500, 1/1000, 1/1500 and 1/2500, were used. One aliquot was heat inactivated by incubation at 56°C for 30 minutes prior to use. Conjugate dilutions of 1/1000, 1/2000, 1/3000 and 1/4000 were used.

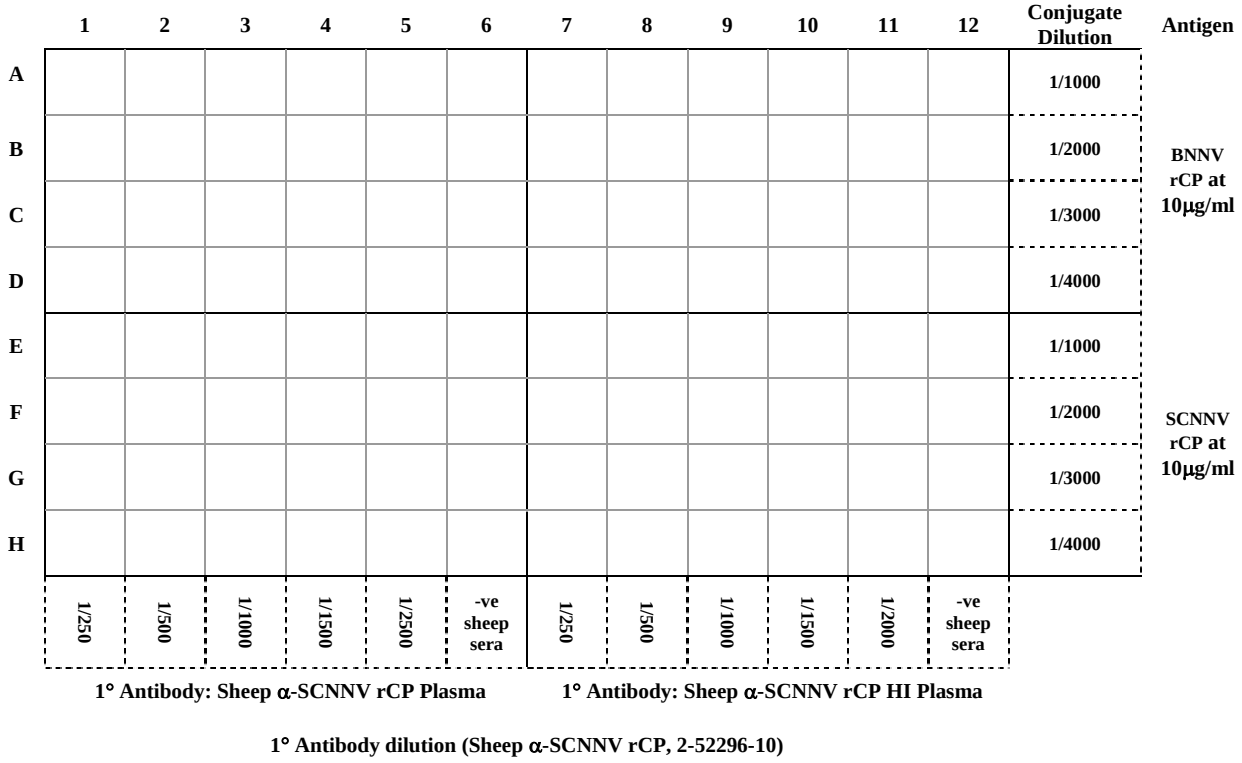


Figure 33: Plate set-up for the comparison between SCNNV rCP and BNNV rCP as antigen and between heat-inactivated and normal plasma as the primary antibody

7.1.2.3 Polyclonal Evaluation No. 3

As previous work had demonstrated that the sheep die produce antibodies after inoculation with NNV rCP, Dot Blots were used to analyse the development of the immune response, to develop a standard procedure for the production of Sheep α-NNV rCP polyclonal antibodies and identify collection bleeds which could be used for subsequent immunodiagnostics test development.

The Dot Blots were conducted as described in Polyclonal Evaluation No. 1. The blood samples used for the serial two-fold primary antibody dilutions and corresponding plate set-ups described in Figure 34 (Day 42 to Day 132 post inoculation) and Figure 35 (Day 132 to Day 362). The blood sample collection details are described in Table 18. The coating antigen used was SCNNV rCP at a concentration of 10µg/ml and the secondary antibody conjugate was used at a dilution 1/2000. With this series of plates, and all subsequent testing, the primary and secondary antibodies were diluted in 2.5% Skim milk powder (w/v in TBST, TBST-SMP) and all blocking was done with 5% TBST-SMP. While no comparative testing was conducted, using TBST-SMP as the primary and secondary antibody diluent and blocking buffer, led to the discovery that there was no significant difference when either TBST-SMP or 5% ECL Blocking agent were used. To reduce reagent costs, without compromising test integrity, TBST-SMP was used for all subsequent dilution and blocking steps for the Dot Blots.

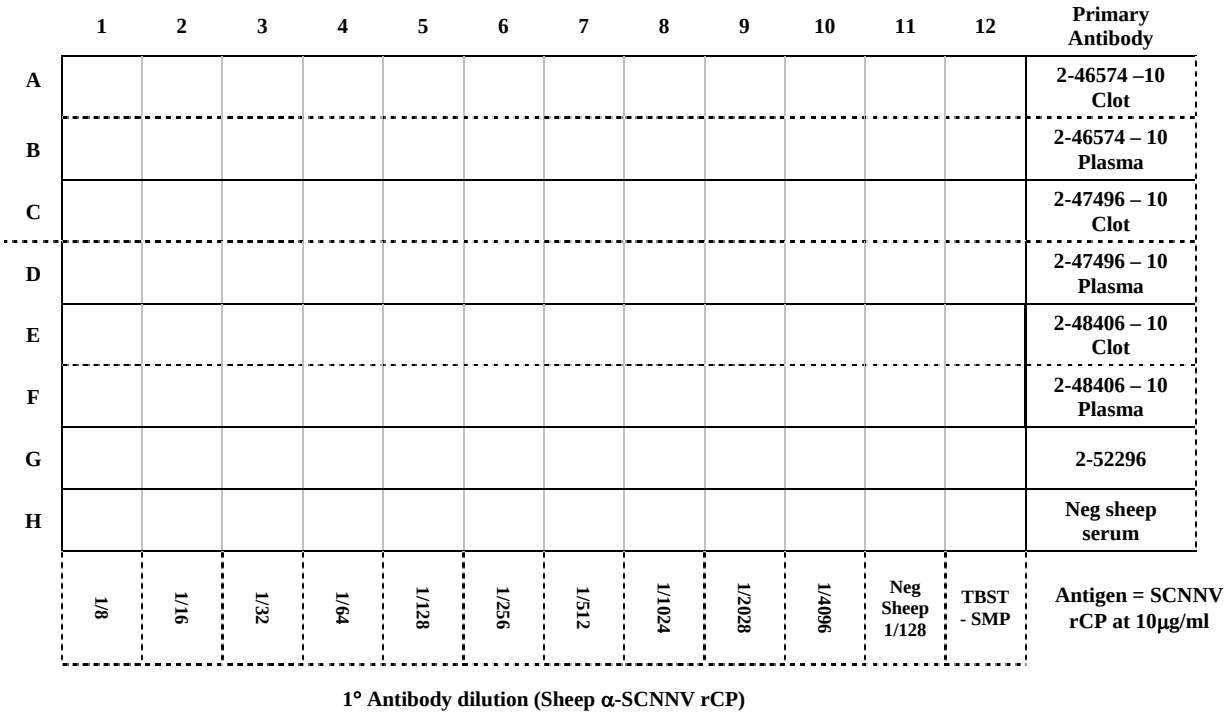


Figure 34: Plate set-up to evaluate the development of the sheep immune response to inoculation with recombinant coat protein from Day 42 to Day 132 post inoculation, and to assess any difference between antibodies collected as serum or plasma. Conjugate dilution was 1/2000.

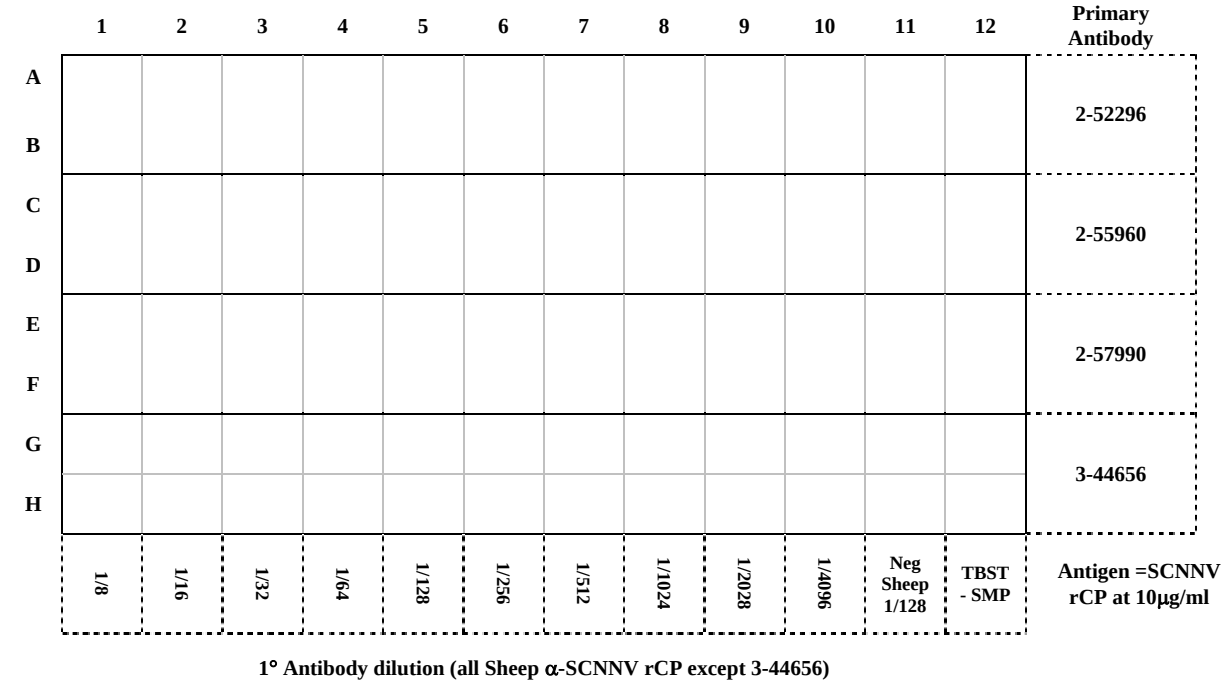


Figure 35: Plate set-up to evaluate the development of the sheep immune response to inoculation with recombinant coat protein from Day 132 to Day 362 post inoculation. Conjugate dilution was 1/2000.

7.1.3 SDS-PAGE of expressed coat protein Western Blot evaluation of the specificity and sensitivity of the polyclonal antibodies

To determine the purity of the recombinant coat protein preparation, samples were analysed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) based on the method of Laemmli (1970) using the Mini-PROTEAN® 3 Cell (Bio-Rad, USA). Ten percent separating gels, containing 4ml Acrylamide/bis (37.5:1, 2.6% C), 3ml 1.5M Tris (pH 8.8), 5ml deionised water, 100µl 10% SDS, 40µl 10% ammonium persulphate and 10µl N,N,N',N'-tetramethylethlyenediamine (TEMED), were overlaid with a 4% stacking gel, containing 0.75ml Acrylamide/bis (37.5:1, 2.6% C), 1.25ml 0.5M Tris (pH 6.8), 3ml deionised water, 10µl 10% SDS, 20µl 10% ammonium persulphate and 15µl TEMED. Gels were stored at 5ºC and used within two days of preparation.

To prepare the duplicate gels for SDS-PAGE and Western Blotting, eight five-fold serial dilutions of SCNNV rCP were prepared so the appropriate well of the gel received from 2.5µg to 0.00032µg of rCP in a 20µl well volume. Reducing sample buffer (RSB), containing 4ml deionised water, 1ml 0.5M Tris (pH 6.8), 0.8ml glycerol, 1.6ml 10% SDS, 0.4ml 2-β-mercaptoethanol and 0.2ml 0.05% bromophenol blue, was added to the sample at a ratio of 1:3 and the mixture was incubated at 97°C for 5 minutes then cooled on ice. The samples were centrifuged at 10,000 x *g* for 10 minutes at 5°C and 20µl of each clarified sample was added to the appropriate well of two polyacrylamide gels. The gels were electrophoresed at 200V until the dye front had reached the bottom of the gel. After removal from the apparatus, one gel was evaluated by Western Blot, as described below, and the proteins in the second gel were stained with Coomassie Brilliant Blue as follows. The gel was soaked in isopropanol:acetic acid:deionised water (25:10:65) for at least two hours to remove low molecular weight contaminants and SDS and improve the sensitivity of staining (Blank *et al*., 1982). Proteins were visualised by staining with Coomassie Brilliant Blue R-250 solution, containing 0.2% (w/v) Coomassie Brilliant Blue R-250, 20% (v/v) methanol and 20% (v/v) acetic acid for at least two hours. The gels were destained in 10% (v/v) ethanol and 10% (v/v) acetic acid and bands visualised over white light. Protein molecular weights were determined by comparing band migration with known protein standards.

The sensitivity and specificity of the polyclonal antibodies were evaluated by Western Blot as follows. The gel was equilibrated in transfer buffer (25mM Tris, 192mM glycine, 20% methanol, 80% deionised water) for 60 minutes and nitrocellulose, filter papers and fibre mats were equilibrated in transfer buffer for 15 minutes. The gel sandwich was prepared and placed in the Mini Trans-Blot® Electrophoretic Transfer Cell (Bio-Rad, USA) as per the manufacturer's instructions. After transfer at 100 volts for 90 minutes, the nitrocellulose was blocked with 5% TBST-SMP for 60 minutes at room temperature followed by four washes with TBST. The nitrocellulose was incubated with Sheep α-SCNNV rCP polyclonal antibodies, diluted 1/1000 in 50ml of 2.5% TBST-SMP, for 60 minutes at room temperature followed by four washes with TBST. The nitrocellulose was then incubated with Rabbit α-Sheep IgG [H+L] HRP (Jackson ImmunoResearch, USA) diluted 1/1000 in 50ml of 2.5% TBST-SMP, for 60 minutes at room temperature followed by four washes with TBST. The blot was developed using the ImmunoPure® Metal Enhanced DAB Substrate Kit (Pierce, USA) as per the manufacturer's instructions with development stopped by immersion of the nitrocellulose in deionised water.

7.1.4 Immunofluorescent Antibody Test (IFAT) development and optimisation

An indirect fluorescent antibody test (IFAT) format was used to evaluate the sheep polyclonal antibodies for the detection of antigen in histological sections. Clarified plasma was used as the primary antibody and a rabbit anti-sheep polyclonal antibody, conjugated to Cyanine 2 (Rabbit α-Sheep IgG [H+L] Cy2™ conjugate (Jackson ImmunoResearch, USA), was used as the indicator antibody. The Cy2™ fluorochrome has the same excitation and emission characteristics as the more common Fluorescein Isothyocyanate (FITC) but is more stable and less prone to photobleaching.

IFAT development was based on the method of Nguyen *et al*., (1997). In preparation for IFAT, 5µm tissue sections were applied to SuperFrost®Plus glass slides (Menzel-Glaser, Germany) and deparaffinised by incubation in xylene for 30 minutes followed by two further washes in fresh xylene. Tissue sections were rehydrated through a graded series of ethanol to TBS (100%, 96%, 75% 50%, TBS). The tissue sections were treated with prewarmed 0.1% trypsin (w/v in TBS) for 30 minutes at 37°C followed by three washes with TBS.
Non-specific binding sites were blocked by incubating the sections with 5% BSA (w/v in TBS) for 20 minutes followed by three washes with TBS.

7.1.4.1 IFAT Evaluation No 1

To determine whether the Sheep α-NNV rCP polyclonal antibodies would detect nodavirus in histological sections and the dilution range of primary antibody and secondary antibody conjugate to use, chequerboard titrations were conducted. Tissue sections used were replicated histological sections from known BNNV-infected and uninfected Golden perch (Table 19), produced after experimental infection trials which were a component of FRDC 1999/205 (Anderson and Moody, 2004).

Table 19: IFAT checkerboard titration set-up using Golden perch sections produced after infectivity trials described in Anderson and Moody (2004).

		1° Antibody dilution			
		1/10	1/50	1/100	1/200
Cy2™ conjugate dilution	1/50	GP +ve GP –ve	GP +ve GP –ve	GP +ve GP –ve	GP +ve GP –ve
	1/100	GP +ve GP –ve	GP +ve GP –ve	GP +ve GP –ve	GP +ve GP –ve
	1/200	GP +ve GP –ve	GP +ve GP –ve	GP +ve GP –ve	GP +ve GP –ve

Dilutions of Sheep α-SCNNV rCP polyclonal antibodies of 1/10, 1/50, 1/100 and 1/200 were prepared in 2.5% bovine serum albumin (BSA, w/v in TBS) and, after circling the tissue with a hydrophobic pen, 1ml was added to the appropriate slides and sections were incubated at 37°C for 60 minutes in a humid chamber. After washing the sections three times with TBS, 1ml of Rabbit α-Sheep IgG [H+L] Cy2™ conjugate (Jackson ImmunoResearch, USA), diluted 1/50, 1/100 and 1/200 in 2.5% BSA (w/v in TBS), was added to the appropriate slides and sections were incubated at 37°C for 60 minutes in a humid chamber. After washing the sections three times with TBS the sections were coverslipped using an aqueous mounting medium and were observed using a Leitz Orthopolan Fluorescent compound microscope equipped with a 50 Watt mercury burner and an Olympus IX70 Inverted fluorescent

microscope, equipped with a 100W Mercury burner. Images were captured using an Olympus C-4040 Zoom digital camera.

7.1.4.2 IFAT Evaluation No 2

Following the discovery that the initial antibody dilutions used in the IFAT were too low and to check repeatability of the IFAT with tissue sections from clinically infected barramundi, additional checkerboard titrations were undertaken. The tissue sections used were replicated sections from 42-day old barramundi with extensive BNNV lesions in the brain and retina and 42-day old barramundi with no lesions in the brain and retina. Dilutions of primary antibody of 1/500, 1/1000, 1/1500 and 1/2000 were prepared in 2.5% bovine serum albumin (BSA, w/v in TBS) and, after circling the tissue with a hydrophobic pen, 1ml was added to the appropriate slides and sections were incubated at 37°C for 60 minutes in a humid chamber. After washing the sections three times with TBS, 1ml of Rabbit α-Sheep IgG [H+L] Cy2™ conjugate (Jackson ImmunoResearch, USA), diluted 1/500, 1/1000, 1/1500 and 1/2000 in 2.5% BSA (w/v in TBS), was added to the appropriate slides and sections were incubated at 37°C for 60 minutes in a humid chamber. After washing the sections three times with TBS the sections were coverslipped and observed using the Olympus IX70 Inverted fluorescent microscope.

7.1.5 Immunofluorescent Antibody Test (IFAT) evaluation of exotic nodavirus isolates

To assess the cross-reactivity of the polyclonal antibodies with exotic nodavirus isolates, Dr Barry Munday (UTAS) facilitated the importation of formalin-fixed tissue, infected with nodavirus from several overseas countries (Table 20). The tissue sections were tested by IFAT as described above (section 7.1.4.2), with the Sheep α-SCNNV rCP polyclonal antibodies used at a dilution of 1/1000 and the Rabbit α-Sheep IgG [H+L] Cy2™ conjugate (Jackson ImmunoResearch, USA) used at a dilution of 1/1000.

Table 20: Sources of exotic nodavirus infected formalin-fixed material used to assess the cross-reactivity of the Sheep α-NNV polyclonal antibodies.

NAME	ORGANISATION	SPECIES	OVL ACCESSION
Dr Sindre Grotmol	Department of Zoology, University of Bergen. NORWAY	Atlantic halibut	3-40515
Dr Arik Diamant	Department of Pathobiology National Centre for Mariculture ISRAEL	*Lates calcarifer, Epinephelus tauvina*	2-57703
Dr Martine Vigneulle	AFSSA - Site de Brest Laboratoire d'études et de recherches en pathologie des poissons FRANCE	Sea bass	2-57034
Dr Yukio Maeno	Japan International Research Centre for Agricultural Sciences (JIRCAS) JAPAN	*Epinephelus coiodes* from the Philippines	2-57734
Dr Toshihiro Nakai	Graduate School of Biosphere Science Hiroshima University JAPAN	Japanese sea bass Japanese flounder	2-57726

7.1.6 Immunohistochemistry test (IHCT) development and optimisation

7.1.6.2 IHCT Evaluation No 1

Checkerboard titrations were conducted using tissue sections from submissions that were positive for nodavirus after analysis by histology and Nested RT-PCR. After de-paraffinisation as described above, endogenous peroxidase was blocked by immersing the slides in 3% H_2O_2 (v/v in methanol) for 20 minutes at room temperature, followed by three washes with TBS. Non-specific binding sites were blocked by incubating the sections with 5% BSA (w/v in TBS) for 20 minutes followed by three washes with TBS. Dilutions of primary antibody of 1/500, 1/1000, 1/1500 and 1/2000 were prepared in 2.5% BSA (w/v in TBS) and after circling the tissue with a hydrophobic pen, 1ml was added to the appropriate slides and sections were incubated at 37°C for 60 minutes in a humid chamber. After washing the sections three times with TBS, 1ml of Rabbit α-Sheep IgG [H+L] HRP conjugate (Jackson ImmunoResearch, USA), diluted 1/500, 1/1000, 1/1500 and 1/2000 in 2.5% BSA (w/v in TBS), was added to the appropriate slides and sections were incubated at 37°C for 60 minutes in a humid chamber. After washing three times with TBS, the HRP was visualised by addition of 1ml of 3-Amino-9-ethylcarbozole in N,N-Dimethylformamide (AEC-DMF). AEC-DMF was prepared by adding 5ml of 0.4% AEC (w/v in DMF) to 100ml acetate buffer (14.8ml of 1.155% glacial acetic acid (v/v in deionised water), 35.3ml of 1.64% anhydrous sodium acetate (v/v in deionised water) and 50ml deionised water) followed by the addition of 100µl hydrogen peroxide immediately prior to use. One millilitre was added to each section and the development of a positive section was monitored. Colour development was stopped (usually after 10-15 minutes) by immersing the slides in deionised water. The tissue sections were counterstained with Mayer's haematoxylin for 60 seconds, rinsed in tap water for 60 seconds, blued in lithium carbonate for 60 seconds and rinsed in tap water for 60 seconds (Sheehan and Hrapchak, 1980). Sections were coverslipped using aqueous mounting medium and observed using the Olympus BX-51 compound microscope. Images were captured using an Olympus C-4040 Zoom digital camera.

7.1.6.3 IHCT Evaluation No 2

As the production of the AEC-DMF substrate was hazardous to the operator and labour intensive, a commercially available HRP substrate, the ImmunoPure® Metal Enhanced DAB Substrate Kit (Pierce, USA) was evaluated. The testing was conducted using tissue samples from submissions that were positive for nodavirus after testing by histology and Nested RT-PCR. After de-paraffinisation as described above, endogenous peroxidase was blocked by immersing the slides in 3% H_2O_2 (v/v in methanol) for 20 minutes at room temperature, followed by three washes with TBS. Non-specific binding sites were blocked by incubating the sections with 5% BSA (w/v in TBS) for 20 minutes followed by three washes with TBS. One millilitre of Sheep α-SCNNV rCP polyclonal antibodies, diluted 1/1000 in 2.5% BSA (w/v in TBS), was added to the sections, which were incubated at 37°C for 60 minutes in a humid chamber. After three washes in TBS, 1ml of Rabbit α-Sheep IgG [H+L] HRP conjugate (Jackson ImmunoResearch, USA), diluted 1/1000 in 2.5% BSA (w/v in TBS), was added and the sections were incubated at 37°C for 60 minutes in a humid chamber. Substrate preparation and HRP development was performed according to the manufacturer's instructions as follows. The DAB Metal Concentrate (10x) was mixed well and diluted 1/10 in the Stable Peroxide Buffer to produce working solution. One millilitre was added to each section and after incubation for 10 minutes substrate development was stopped by immersing the slides in deionised water. Sections were counterstained, coverslipped and observed as above.

7.1.7 Immunodiagnostic test evaluation

7.1.7.1 IHCT testing of known BNNV-positive and negative sections

Known nodavirus negative and positive tissue sections were tested by IHCT to check that the test would consistently produce positive results for positive sections and negative results for negative sections, to determine the degree of any non-specific background staining and to generally monitor the repeatability of the test. The IHCT procedure used was as described in Section 7.1.6.3. The tissue sections included twenty positive and negative sections from barramundi larvae and fry, four positive and negative sections from silver perch fry and nine positive and negative sections from sleepy cod. The sections were tested

7.1.7.2 Comparison of Nested RT-PCR histology, IHCT and IFAT for the detection of nodavirus in fish infected with BNNV and sampled daily.

To compare the capability of Nested RT-PCR, histology, IFAT and IHCT at detecting nodavirus in barramundi during an infection with BNNV, material was collected from a population of two month old barramundi that had been bath infected with BNNV (Anderson and Moody, 2004). Briefly, each day post infection for 28 days, five fish were euthanased, pooled, homogenised and tested using the Nested RT-PCR. An additional five fish were also euthanased, fixed in Bouin's fixative and tissue sections were prepared for histology, IFAT and IHCT work. Three serial sections were cut for each fish, one stained with H&E, one tested using IFAT (Section 7.1.5) and the third tested using IHCT (Section 7.1.6.3). A total of 29 fresh and 145 fixed fish samples were collected and tested. Aside from the euthanased fish, no other mortalities occurred during the trial.

7.1.7.3 Comparison of histology and IHCT for the detection of nodavirus in fish surviving experimental challenge with BNNV.

To compare the capability of histology and IHCT at detecting nodavirus in fish surviving an infection with BNNV, serial tissue sections were prepared from fish surviving a 28-day experimental infection trial with BNNV (Anderson and Moody, 2004) (Table 21). From four species, tissue sections were prepared from 249 fish. Two serial sections were cut for each fish, one stained with H&E and the other tested using IHCT (Section 7.1.6.3)

Table 21: List of infectivity trial species and sample numbers for fish surviving experimental infection with BNNV and tested by histology and IHCT.

SPECIES	AGE	TREATMENT	NUMBER OF FISH
Barramundi	20 days	Bath survivors	17
Barramundi	7 weeks	Inject survivors	19
Barramundi	7 weeks	Inject survivors	16
Barramundi	12 weeks	Inject survivors	35
Barcoo Grunter	6 weeks	Inject survivors	35
Barcoo Grunter	12 weeks	Inject survivors	35
Golden perch	12 weeks	Inject survivors	35
Silver perch	6 weeks	Inject survivors	13
Silver perch	12 weeks	Inject survivors	44
		TOTAL	**249**

7.2 Results and Discussion

7.2.1 Inoculum preparation, vaccination procedure and sample collection

The procedure used for inoculation of the sheep with FCA and FIA produced no detectable discomfort, lesions or stress to the sheep. Collection and processing unclotted blood to produce plasma, was significantly easier than harvesting and processing clotted blood to produce serum and resulted in a greater volume of antibody.

7.2.2 Dot-Blot analysis of polyclonal antibodies

7.2.2.1 Polyclonal Evaluation No. 1

Positive reactions appeared as dark spots on the nitrocellulose. While a strong signal was produced with primary antibody dilutions up to 1/4000 (Figure 36, Column 10) and a conjugate dilution up to 1/1000 (Figure 36, Row B), there was also significant non-specific binding when negative sheep serum was used at a dilution of 1/50 (Figure 36, Column 11). Given the much higher end point of the primary antibody, the negative sheep serum should be diluted further and still be an appropriate negative control. There was no significant difference when serum or plasma was used as the primary antibody. Collection of plasma resulted in a greater volume of product so subsequent harvest bleeds were collected to yield plasma, not serum. The clarified Sheep α-SCNNV rCP and Sheep α-BNNV rCP antibodies were stored as 0.9ml and 4.5ml aliquots at -80°C.

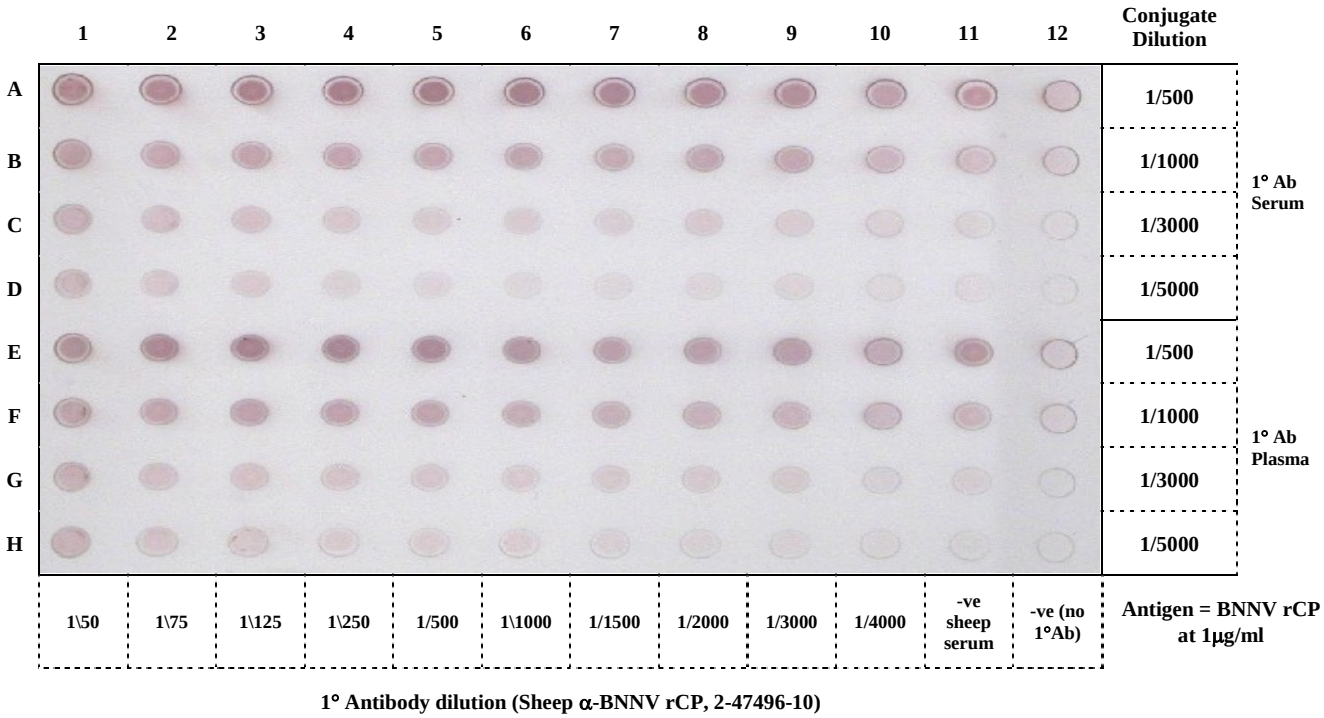


Figure 36: Checkerboard titration of primary (1°) antibody and conjugate (2°) dilutions and comparison of serum and plasma as the primary antibody. Primary antibody was diluted across the plate with secondary antibody dilutions running down the plate. The degree of reactivity was determined by the intensity of coloured product in the well.

The concentration of antigen (1µg/ml) used to coat the nitrocellulose was too dilute and increasing the concentration in subsequent blots will be investigated. The concentration used

was based on concentrations reported in the literature for ELISA tests that ranged from 100ng/well to 500ng/well (Mushiake *et al*., 1992; Watanabe *et al*., 2000). However, incubation times ranged from two hours to overnight. Increasing the concentration of antigen from 50ng/well to 500ng/well should create better contrast between positive, negative and non-specific results, while still maintaining antigen levels similar to those reported in the literature for other nodavirus antibodies. The Bio-Rad 96-well Dot Blot apparatus worked extremely well when the vacuum gasket was covered with Parafilm®. This enabled reagents to be added with multichannel and multistepper pipettors and the blot to be treated in the same way as an ELISA plate, which resulted in a very efficient and reproducible Dot Blot procedure. Care needed to be taken with the final wash, to remove unbound conjugate, to ensure excessive background did not occur when the substrate was added.

7.2.2.2 Polyclonal Evaluation No. 2

There was no significant difference in intensity when either SCNNV rCP or BNNV rCP was used as the coating antigen and either heat-inactivated or normal Sheep α-SCNNV rCP plasma was used as the primary antibody (Figure 37). Increasing the concentration of antigen applied to the nitrocellulose to 10μg/ml did not result in an increase in non-specific binding when negative sheep serum was used at a dilution of 1/128 (Figure 37, Column 6 and 12). The primary antibody could be used at dilution of at least 1/2500 and the conjugate could be used at a dilution up to 1/3000. However, conjugate used at 1/2000 would produce a strong signal, does not require addition of very small volumes, was considered to be more efficient and there would still be minimal cross-reactivity with negative serum.

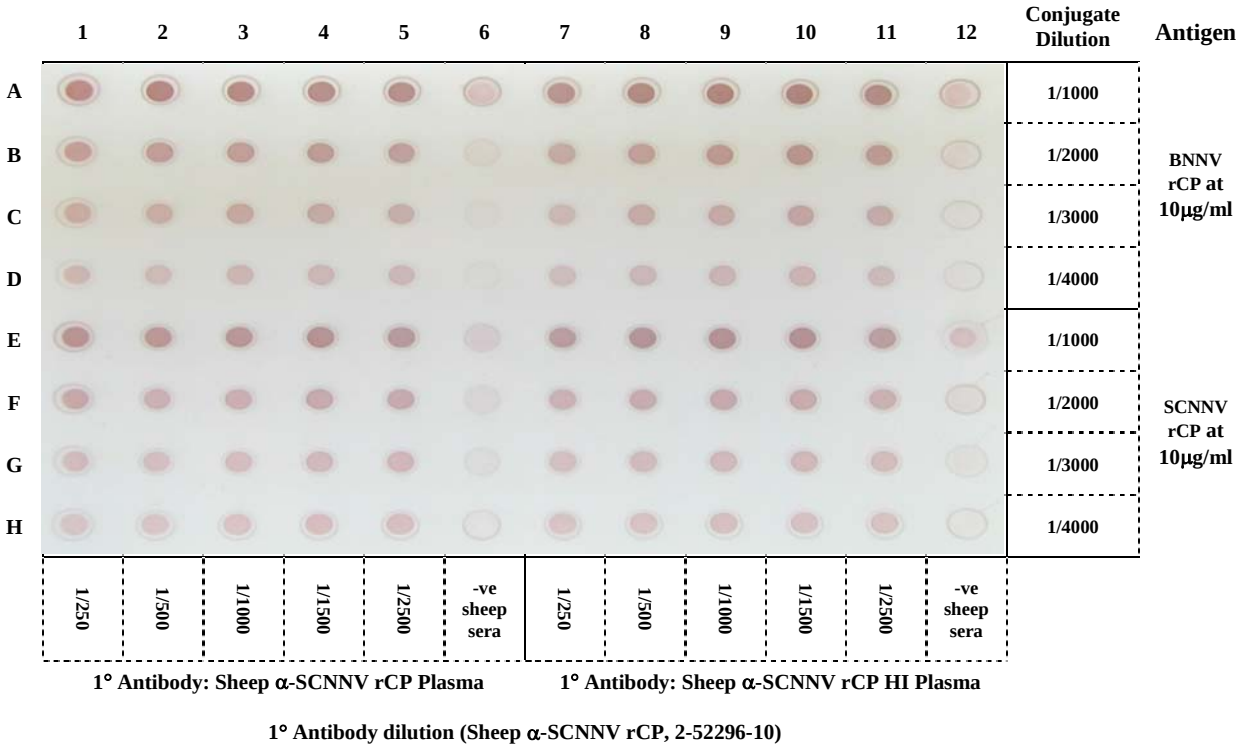


Figure 37: Evaluation of cross-reactivity between the polyclonal antibodies, raised against the Sleepy cod nodavirus isolate (sheep α-SCNNV), with BNNV recombinant coat protein (rCP) and SCNNV rCP as the antigen. This blot also evaluated any difference between primary antibody, in the form of plasma, that had been heat-inactivated (HI) and not heat inactivated and narrowed the dilution range of the antibodies. The degree of reactivity was determined by the intensity of coloured product in the well.

7.2.2.3 Polyclonal Evaluation No. 3

Evaluation of the development of the sheep immune response to inoculation with recombinant coat protein.

A significant antibody response was first detected in sample 2-48406-10, collected 70 days after the first inoculations and 14 days after the third booster inoculation (Figure 38, Row E and F). Antibody levels were detectable to a dilution of at least 1/4096. There was no reaction when negative sheep serum was tested (Figure 38, Row H). The immune response was higher in sample 2-52296, collected after 132 days after the first inoculations and 30 days after the fifth booster inoculation (Figure 38, Row G).

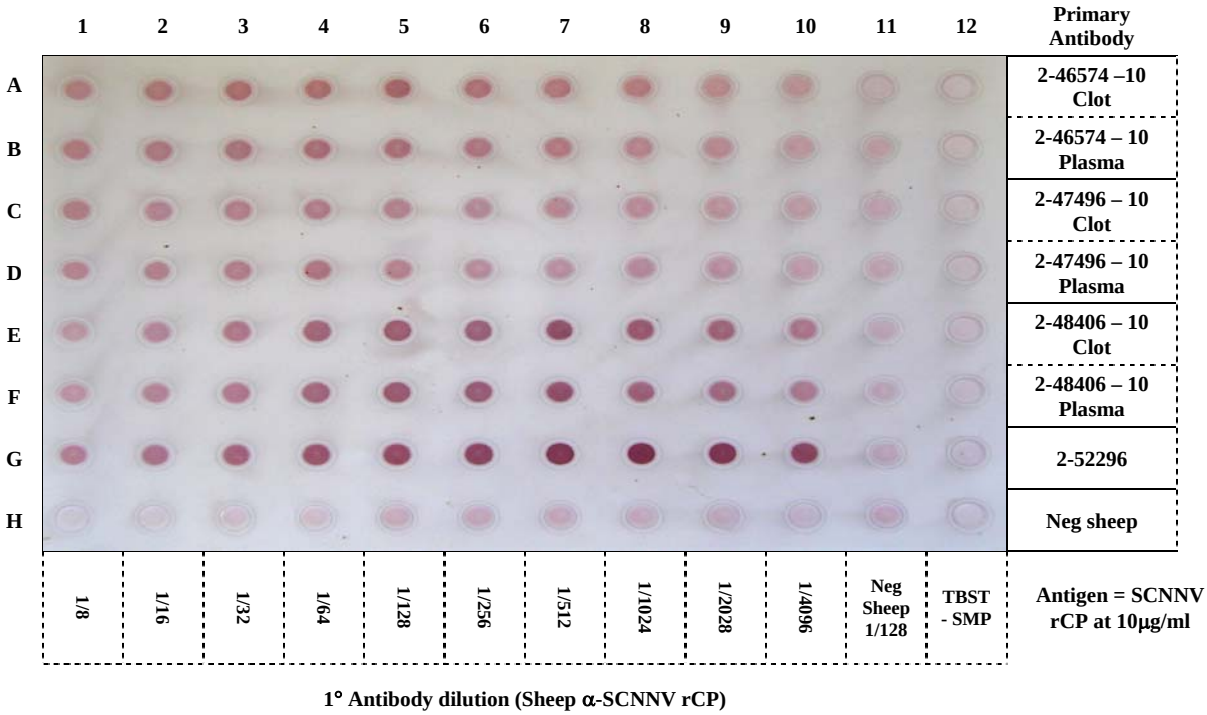


Figure 38: Development of the sheep immune response after inoculation with recombinant coat protein, from Day 42 to Day 132 post inoculation, and assessment of any difference between antibody collected as serum or plasma. Conjugate dilution was 1/2000. The degree of reactivity was determined by the intensity of coloured product in the well.

Subsequent bleeds, obtained from 24 to 30 days post booster inoculation, consistently produced antibodies that were detectable to a dilution of at least 1/4096 (Figure 38 and Figure 39). There was a significant reduction in antibody titre after 178 days if no booster inoculations were undertaken (Figure 39). While this sheep had been inoculated with BNNV rCP and the other samples were from Sheep 8, inoculated with SCNNV rCP, pervious work had determined there was no significant difference in cross-reactivity, regardless of the inoculum or antibodies used. Therefore, it has been assumed that this would be similar to reduced antibody response in Sheep 10 at that time. After the 362-day bleed no further production bleeds were undertaken as a stockpile of 1.5 litres of antibody detectable to a dilution of 1/4096 had been produced, aliquoted and stored at -80°C. If required, the procedure described here could be used to immunise naïve sheep to produce more polyclonal antibodies.

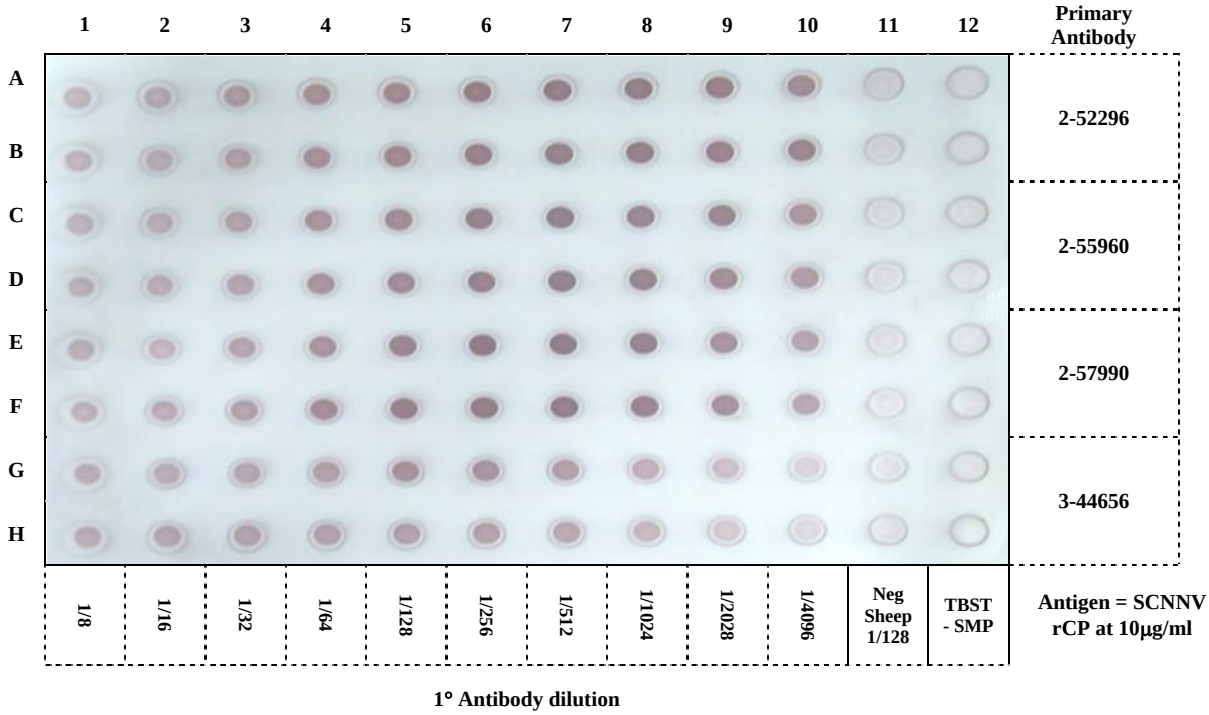


Table 39: Development of the sheep immune response after inoculation with recombinant coat protein, from Day 132 to Day 362 post inoculation. Conjugate dilution was 1/2000. The degree of reactivity was determined by the intensity of coloured product in the well.

7.2.3 SDS-PAGE of expressed coat protein Western Blot evaluation of the specificity and sensitivity of the polyclonal antibodies

Polyacrylamide gel analysis of the SCNNV rCP demonstrated that the recombinant coat protein was the major protein present in the sample (Figure 40, Lane 2). While other proteins were present these disappeared as the samples was diluted. When 0.5µg was loaded and electrophoresed through the gel, the recombinant coat protein was the only protein observed (Figure 40, Lane 3).

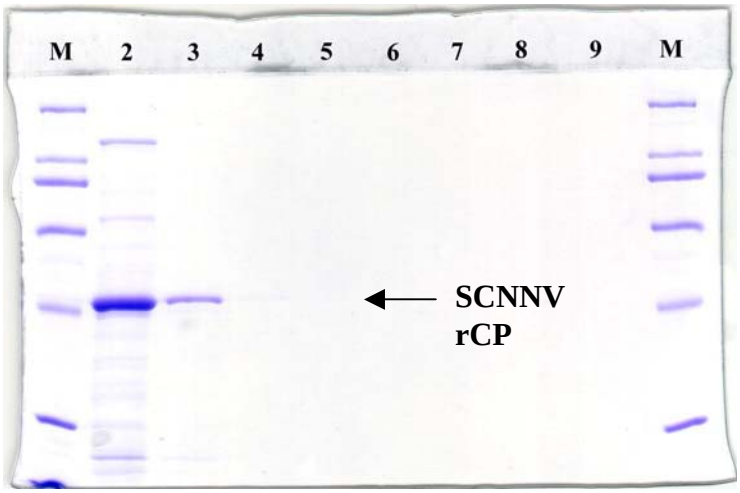


Figure 40: Coomassie Blue R-250 stained 10% SDS-PAGE gel of serial, 5-fold dilutions of SCNNV rCP.
M = BioRad broad range markers
Lane 2 = 2.5µg
Lane 3 = 0.5µg
Lane 4 = 0.1µg
Lane 5 = 0.02µg
Lane 6 = 0.004µg
Lane 7 = 0.0008µg
Lane 8 = 0.00016µg
Lane 9 = 0.000032µg

Western blot of the duplicate 10% SDS-PAGE gel, using the Sheep α-SCNNV rCP polyclonal antibodies, demonstrated that, as expected, antibodies were produced against all the proteins in the samples (Figure 41, Lane 2). However, as the amount of the recombinant coat protein preparation added to the well decreased the only reactive band was that of the

recombinant coat protein (Figure 41, Lane 5 and 6). Therefore, while the polyclonal antibodies did cross-react with other proteins in the inoculum the major target protein of the antibodies was the SCNNV rCP.

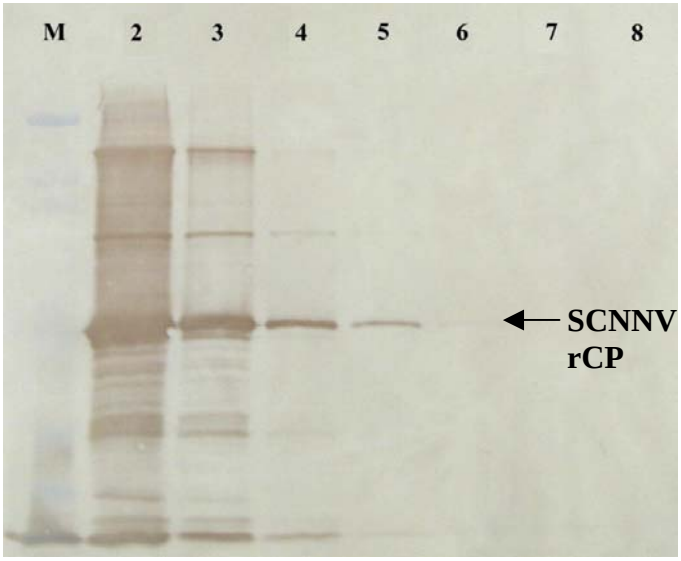


Figure 41: Western Blot of the 10% SDS-PAGE gel of serial, 5-fold dilutions of SCNNV rCP (Figure 40).
M = BioRad broad range markers
Lane 2 = 2.5µg
Lane 3 = 0.5µg
Lane 4 = 0.1µg
Lane 5 = 0.02µg
Lane 6 = 0.004µg
Lane 7 = 0.0008µg
Lane 8 = 0.00016µg
Lane 9 = 0.000032µg

7.2.4 Immunofluorescent Antibody Test (IFAT) development and optimisation

7.2.4.1 IFAT Evaluation No 1

With sections examined using a Leitz Orthoplan compound fluorescent microscope, equipped with a 50W Mercury burner, specific fluorescence was observed in all BNNV-positive sections and no specific fluorescence was observed in BNNV-negative sections (Figure 42). However, the fluorescence was difficult to see unless the room lights were turned off and it was impossible to determine which organ the fluorescent signal was present in. As an alternative, the Olympus IX70 inverted fluorescent microscope, equipped with a 100W Mercury burner was used to observe the sections. The IX70 inverted microscope produced excellent fluorescence in a bright room, the low power objectives allowed rapid screening of sections and the analyst could determine which section of the brain the antibodies had bound to. The specific fluorescence originated from the lesions in the brain and retina (Figure 43 and Figure 44).

When tissue sections from clinically BNNV-infected 42-day old barramundi were tested there was specific fluorescence originating from the extensive viral induced vacuolation in the brain (Figure 44a and 44b) and retina (Figure 44c). No non-specific fluorescence was seen in any tissue of the uninfected 42-day old barramundi (Figure 44d). There was a higher degree of background fluorescence, or brightness in all sections due to the increased power of the light source, especially at lower magnifications. As the IFAT had been successfully used to detect positive material from fish that had survived experimental infection with BNNV (and were possible carriers) (Figure 43) and from clinically infected fish (Figure 44) higher dilutions of primary and secondary antibodies could be used to reduce non-specific fluorescence and increase the sensitivity of the test.

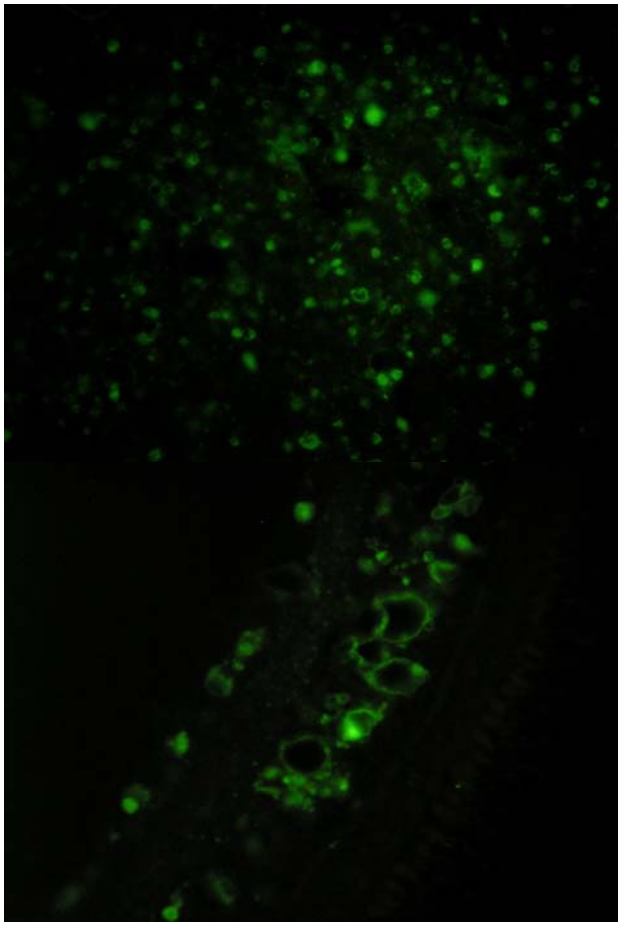


Figure 42a: IFAT result for a brain section from a 6-week old Golden perch experimentally infected with BNNV. x100
1°Ab; 1/50
2°Ab; 1/100

Section observed with the Leitz Orthoplan compound fluorescent microscope, with a 50W Mercury burner

Figure 42b: IFAT result for a brain section from a 6-week old Golden perch experimentally infected with BNNV. x200
1°Ab; 1/50
2°Ab; 1/100

Section observed with the Leitz Orthoplan compound fluorescent microscope, with a 50W Mercury burner

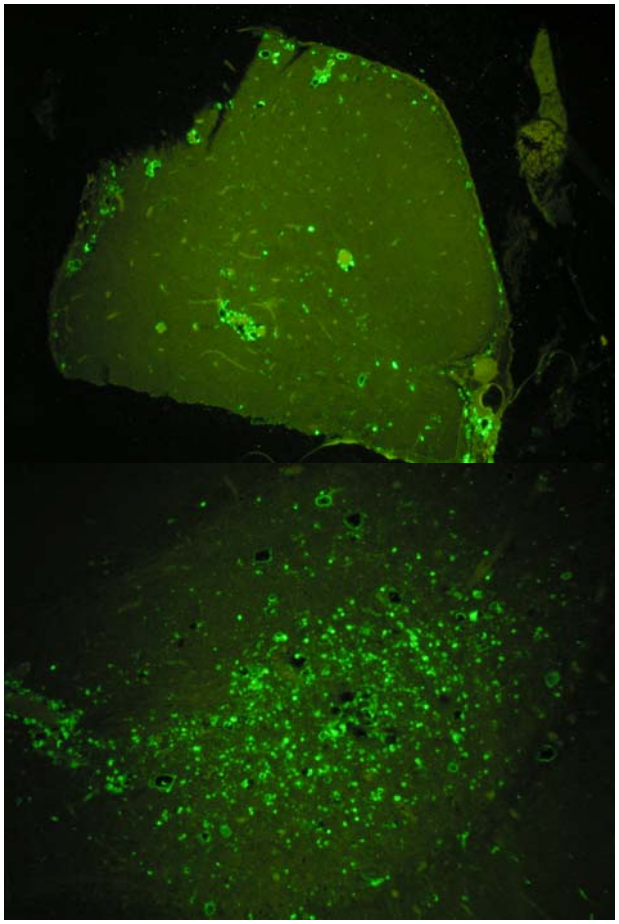


Figure 43a: IFAT result for a brain section from a 6-week old Golden perch experimentally infected with BNNV. x40
1°Ab; 1/50
2°Ab; 1/100

Section observed with the Olympus IX70 Inverted fluorescent microscope, with a 100W Mercury burner

Figure 43b: IFAT result for a brain section from a 6-week old Golden perch experimentally infected with BNNV. x100
1°Ab; 1/50
2°Ab; 1/100

Section observed with the Olympus IX70 Inverted fluorescent microscope, with a 100W Mercury burner

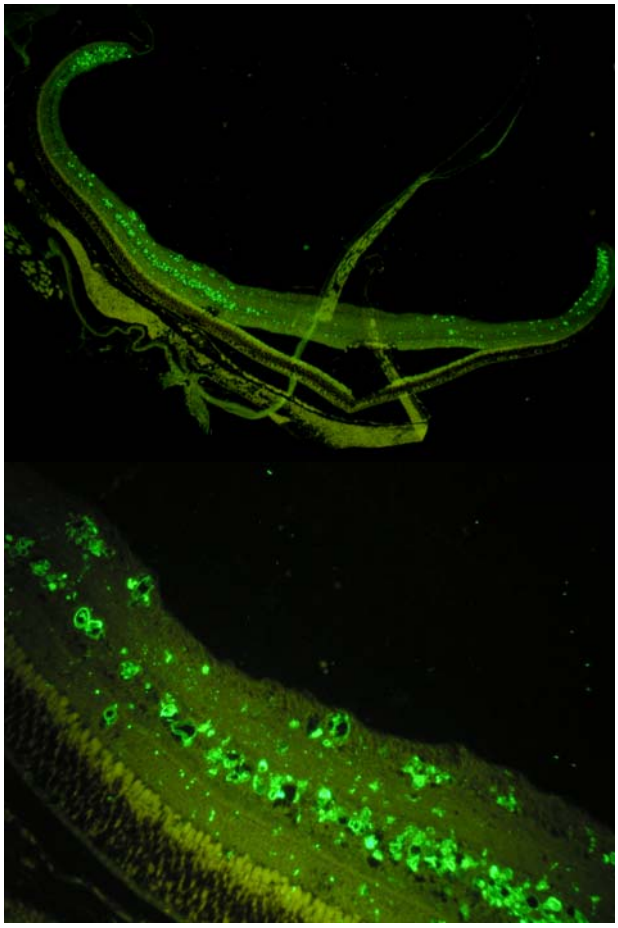


Figure 43c: IFAT result for a retina section from a 6-week old Golden perch experimentally infected with BNNV. x40
1°Ab; 1/50
2°Ab; 1/100

Section observed with the Olympus IX70 Inverted fluorescent microscope, equipped with a 100W Mercury burner

Figure 43d: IFAT result for a retina section from a 6-week old Golden perch experimentally-infected with BNNV. x100
Primary antibody dilution; 1/50
Conjugate dilution; 1/100

Section observed with the Olympus IX70 Inverted fluorescent microscope, equipped with a 100W Mercury burner

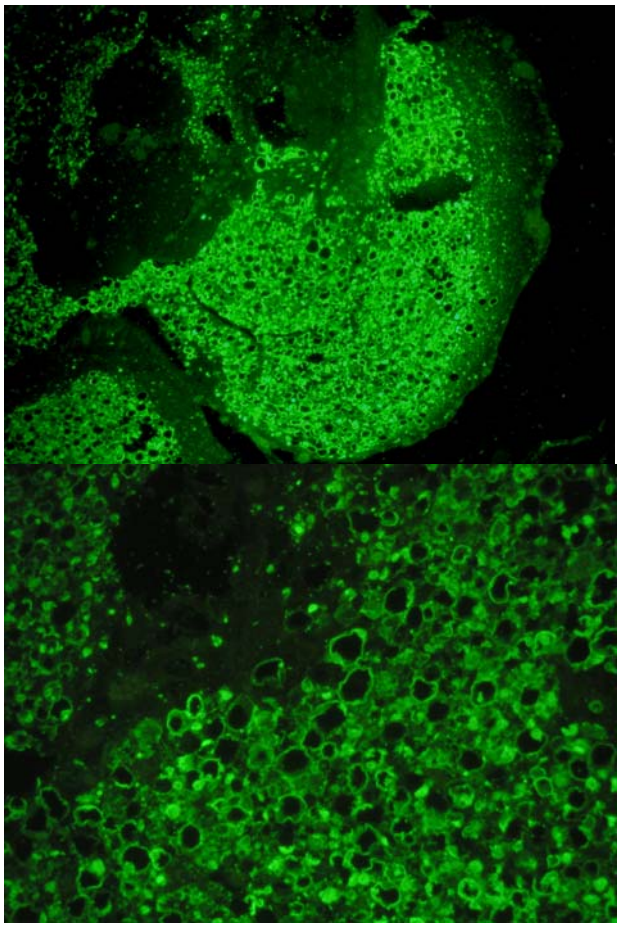


Figure 44a: IFAT result for a brain section from a 42-day old barramundi infected with BNNV. x40
Primary antibody dilution; 1/50
Conjugate dilution; 1/100

Section observed with the Olympus IX70 Inverted fluorescent microscope, equipped with a 100W Mercury burner

Figure 44b: IFAT result for a brain section from a 42-day old barramundi infected with BNNV. x150
Primary antibody dilution; 1/50
Conjugate dilution; 1/100

Section observed with the Olympus IX70 Inverted fluorescent microscope, equipped with a 100W Mercury burner

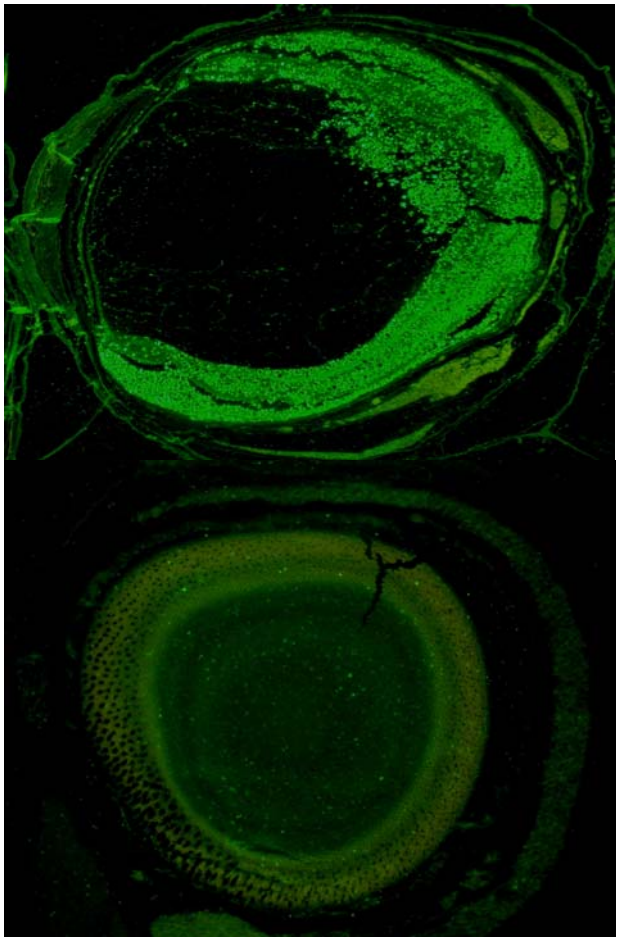


Figure 44c: IFAT result for a retina section from a 42-day old barramundi infected with BNNV. x40
Primary antibody dilution; 1/50
Conjugate dilution; 1/100

Section observed with the Olympus IX70 Inverted fluorescent microscope, equipped with a 100W Mercury burner

Figure 44d: IFAT result for a retina section from an uninfected 42-day old barramundi. x40
Primary antibody dilution; 1/50
Conjugate dilution; 1/100

Section observed with the Olympus IX70 Inverted fluorescent microscope, equipped with a 100W Mercury burner

7.2.4.2 IFAT Evaluation No 2

Strong, specific fluorescence was observed in all sections when the Sheep α-SCNNV rCP antibodies were used at dilutions of 1/500, 1/1000, 1/1500 or 1/2000 and the Rabbit α-Sheep IgG [H+L] Cy2™ conjugate used at dilutions of 1/500, 1/1000, 1/1500 or 1/2000. No non-specific background staining was observed with any concentration of either the primary or secondary antibodies and there was no non-specific fluorescence observed in negative control sections. The dilutions it was decided to use in subsequent the IFAT work was 1/1000 for Sheep α-SCNNV rCP and α-BNNV rCP antibodies and 1/1000 for the Cy2™ conjugate as these dilutions would detect antigen in sub-clinically and clinically-infected fish (Figure 45).

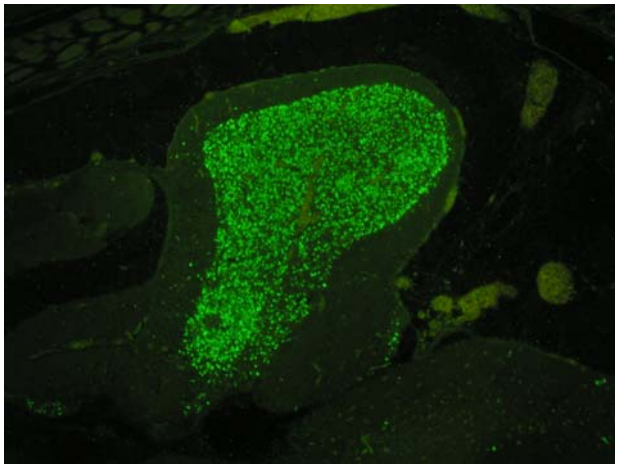


Figure 45a: IFAT result for a brain section from a 2-month-old barramundi, experimentally infected with BNNV. x40
Primary antibody dilution; 1/1000
Conjugate dilution; 1/1000

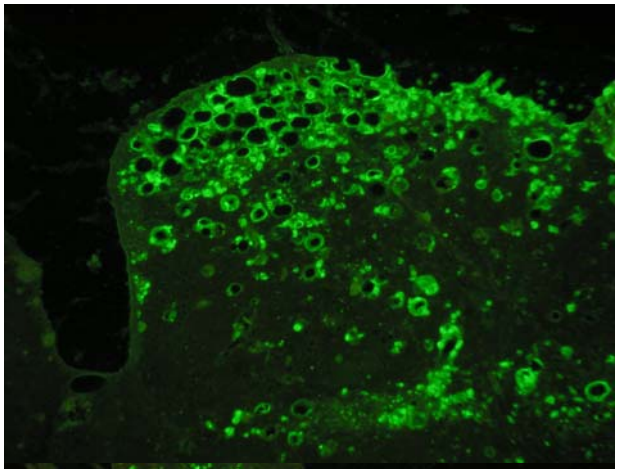


Figure 45b: IFAT result for a brain section from a 2-month-old barramundi, experimentally-infected with BNNV. x100
Primary antibody dilution; 1/1000
Conjugate dilution; 1/1000

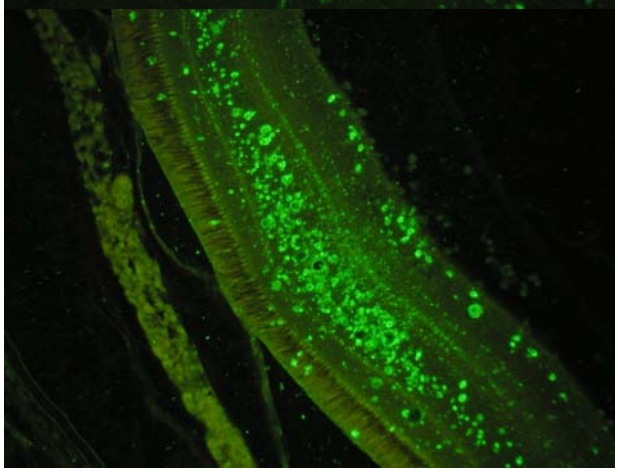


Figure 45c: IFAT result for a retina section from a 2-month-old barramundi, experimentally-infected with BNNV. x100
Primary antibody dilution; 1/1000
Conjugate dilution; 1/1000

7.2.5 Immunofluorescent Antibody Test (IFAT) evaluation of exotic nodavirus isolates

All tissue sections tested produced strong, specific fluorescence in the brain and eye (Figures 46, 47, 48, 49 and 50). As with all other previous samples tested, no specific fluorescence was detected in any tissue other than the brain and eye. While this was the first time it had been demonstrated that antibodies raised against Australian nodavirus isolates would detect exotic nodavirus isolates, antibodies raised against exotic nodavirus isolates have detected Australian nodavirus isolates (Munday *et al*., 1994; Mori *et al*., 2003). As the nodavirus coat protein is conserved among different isolates, as indicated by the ability of the primers developed by Nishizawa *et al*., (1994) and Thiery *et al*., (1999) to detect the vast majority of nodavirus isolates tested (OIE, 2003) it was not surprising that the Sheep α-BNNV rCP and α-SCNNV rCP polyclonal antibodies detected the exotic. Interestingly, specific fluorescence was not always associated with lesions (Figure 47a), raising the possibility that the presence of specific VNN lesions does not always indicate the presence of viral antigen (in this case the coat protein). This raises the possibility that tissue sections, designated as nodavirus positive by histology, due to the presence of specific lesions in the brain and eye, may in fact be negative, or un-infectious, and may be considered false positive results.

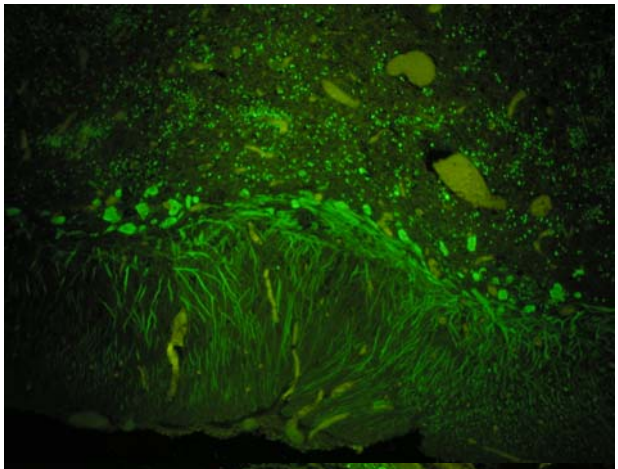


Figure 46a: IFAT result for a brain section from a sea bass infected with nodavirus from France. x100
Primary antibody dilution; 1/1000
Conjugate dilution; 1/1000

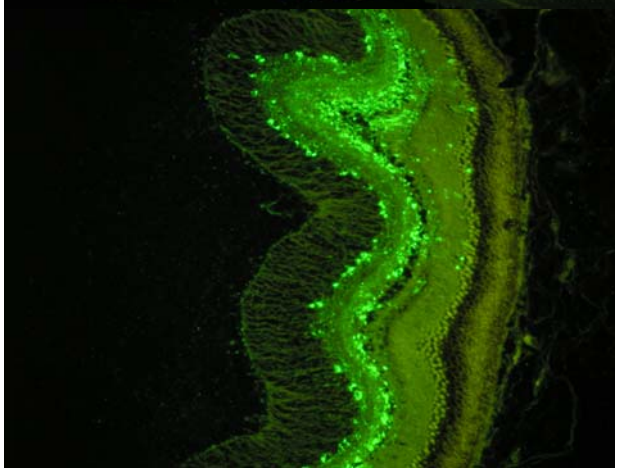


Figure 46b: IFAT result for a brain section from a sea bass infected with nodavirus from France. x100
Primary antibody dilution; 1/1000
Conjugate dilution; 1/1000

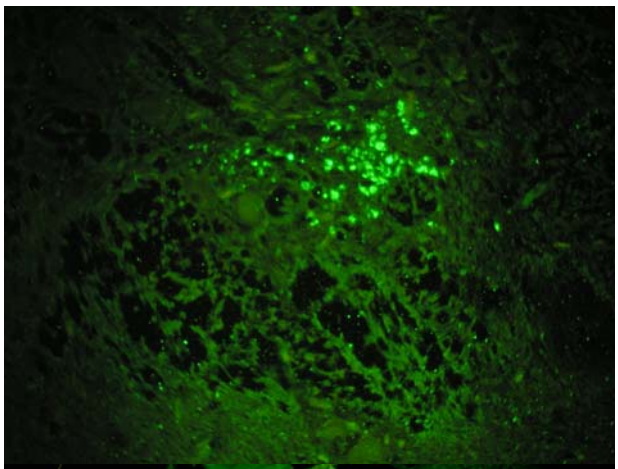


Figure 47a: IFAT result for a brain section from *Lates calcarifer* infected with nodavirus in Israel. x100
Primary antibody dilution; 1/1000
Conjugate dilution; 1/1000

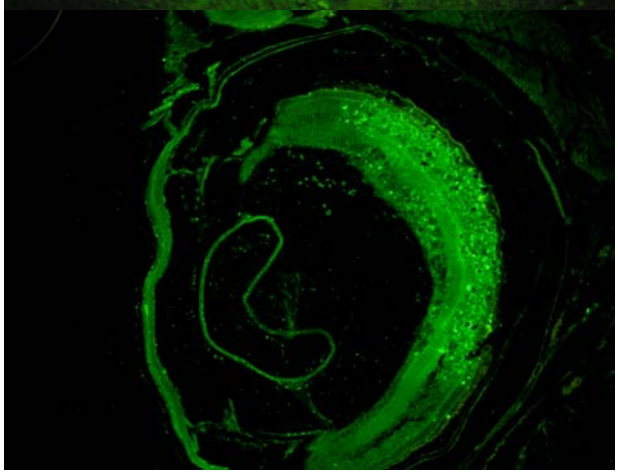


Figure 47b: IFAT result for an eye section from *Lates calcarifer* infected with nodavirus in Israel. x100
Primary antibody dilution; 1/1000
Conjugate dilution; 1/1000

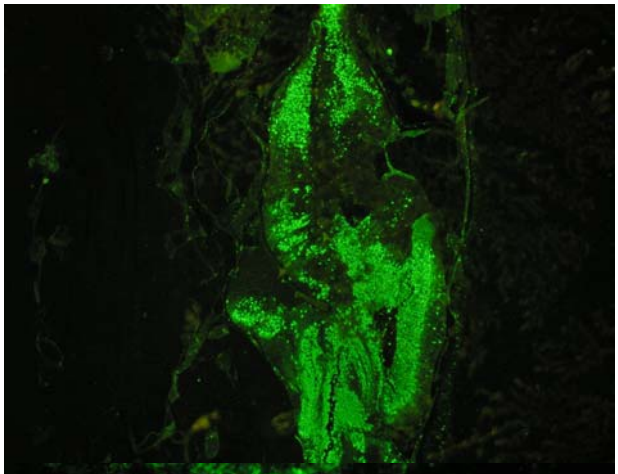


Figure 48a: IFAT result for a brain section from a Japanese sea bass infected with nodavirus in Japan. x100
Primary antibody dilution; 1/1000
Conjugate dilution; 1/1000

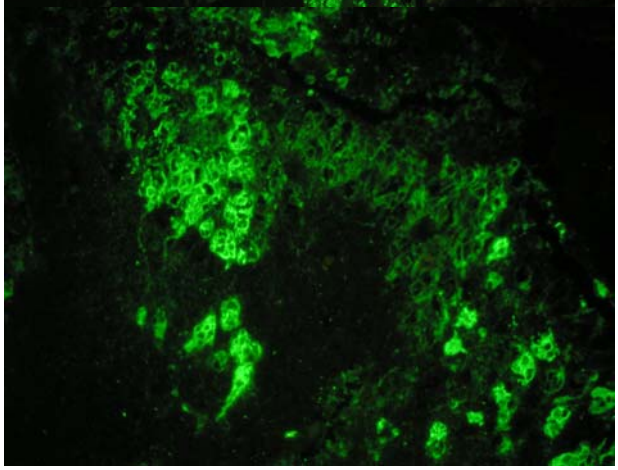


Figure 48b: IFAT result for a brain section from a Japanese sea bass infected with nodavirus in Japan. x200
Primary antibody dilution; 1/1000
Conjugate dilution; 1/1000

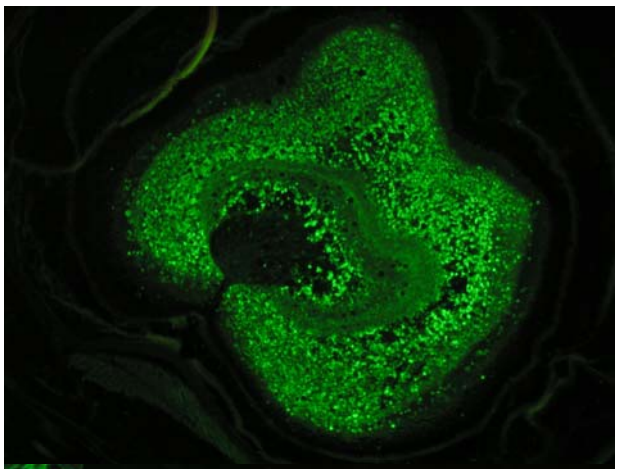


Figure 49a: IFAT result for a brain section from an Atlantic halibut infected with nodavirus in Norway. x100
Primary antibody dilution; 1/1000
Conjugate dilution; 1/1000

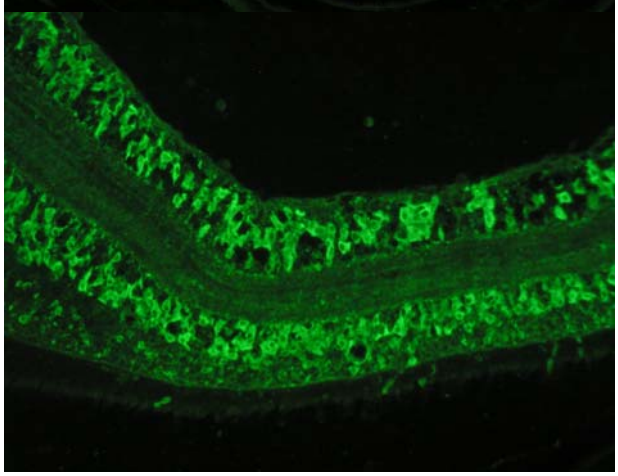


Figure 49b: IFAT result for a retina section from an Atlantic halibut infected with nodavirus in Norway. x200
Primary antibody dilution; 1/1000
Conjugate dilution; 1/1000

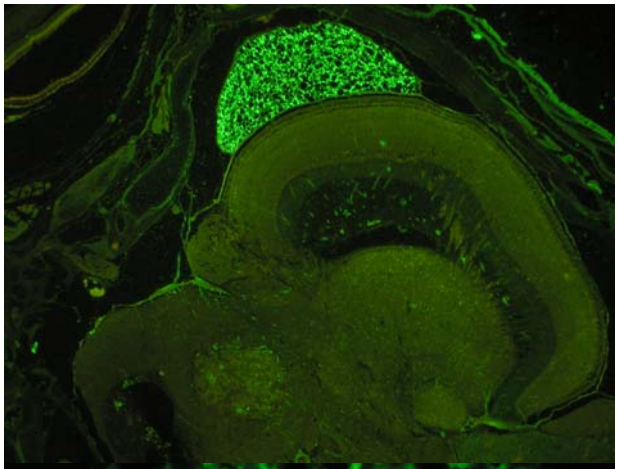


Figure 50a: IFAT result for a brain section from an *Epinephelus coiodes* infected with nodavirus in the Philippines. x40
Primary antibody dilution; 1/1000
Conjugate dilution; 1/1000

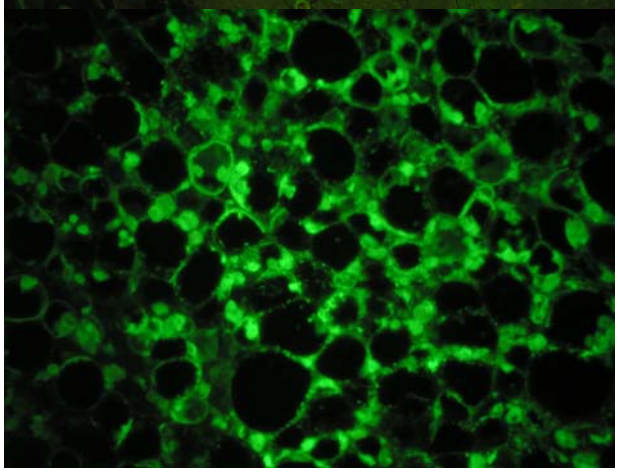


Figure 50b: IFAT result for a brain section from an *Epinephelus coiodes* infected with nodavirus in the Philippines. x300
Primary antibody dilution; 1/1000
Conjugate dilution; 1/1000

7.2.6 Immunohistochemistry test (IHCT) development and optimisation

7.2.6.1 IHCT Evaluation No 1

Strong, specific positive reactions, characterised by reddish-brown staining were observed in BNNV-positive tissue sections when both primary and secondary antibodies were used at dilutions of 1/500, 1/1000 or 1/1500. As with the IFAT results, the positive staining was restricted to the nervous tissue of the brain and eye (Figure 51). No non-specific staining was observed in negative sections. Dilutions of 1/1000, for the primary and secondary antibodies, will be used for future testing. Aside from producing acceptable, repeatable results, these dilutions are the same as for the IFAT, which will maintain consistency between the two tests.

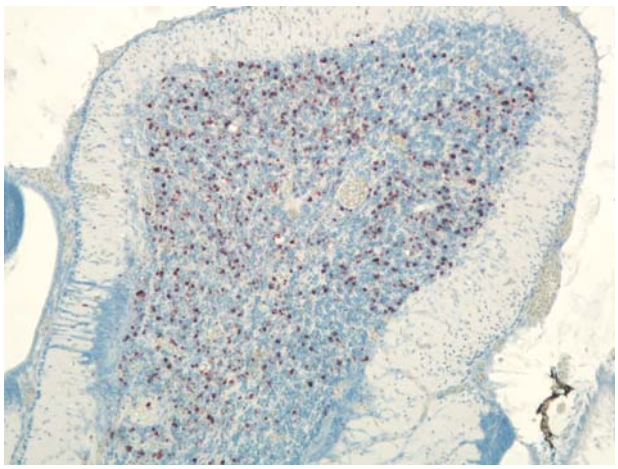


Figure 51a: IHCT result for a brain section from a 2-month-old barramundi, 12 days post infection with BNNV. x200
Primary antibody dilution; 1/1000
Conjugate dilution; 1/1000

Substrate – AEC-DMF

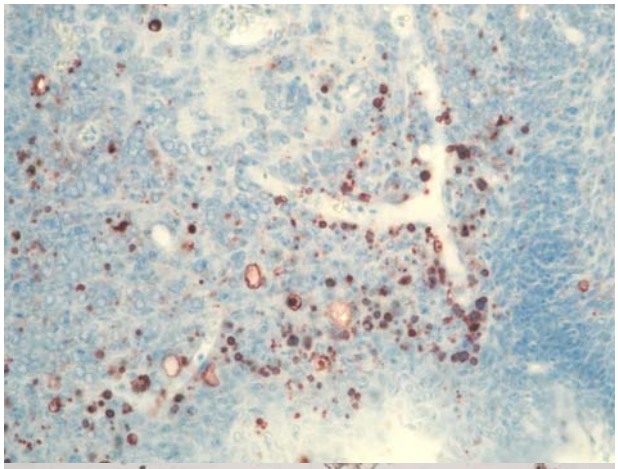


Figure 51b: IHCT result for a brain section from a 2-month-old barramundi, 14 days post infection with BNNV. x400
Primary antibody dilution; 1/1000
Conjugate dilution; 1/1000

Substrate – AEC-DMF

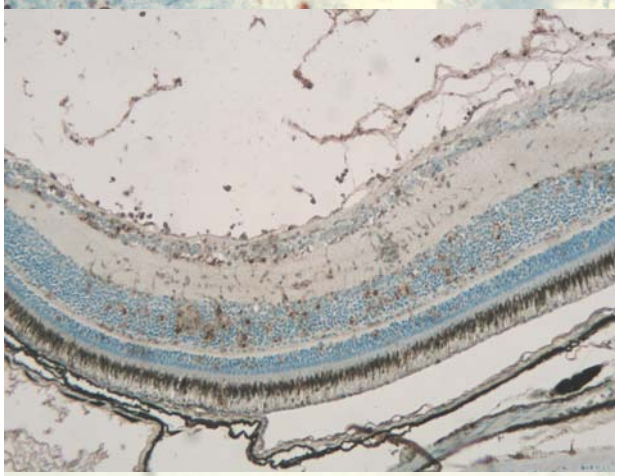


Figure 51c: IHCT result for a retina section from a 2-month-old barramundi, 14 days post infection with BNNV. x200
Primary antibody dilution; 1/1000
Conjugate dilution; 1/1000

Substrate – AEC-DMF

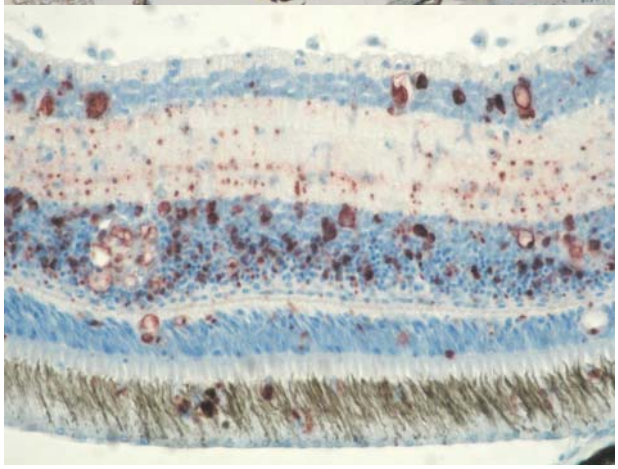


Figure 51d: IHCT result for a retina section from a 2-month-old barramundi, 14 days post infection with BNNV. x400
Primary antibody dilution; 1/1000
Conjugate dilution; 1/1000

Substrate – AEC-DMF

7.2.6.2 IHCT Evaluation No 2

The ImmunoPure® Metal Enhanced DAB Substrate Kit (Pierce, USA) produced strong, dark positive reactions with minimal non-specific background staining (Figure 52). The DAB solution was easy to prepare and staining for a set period of 10 minutes produced consistent results, eliminating the requirement of monitoring the development with a positive tissue section. The positive staining was more obvious, intense and detailed compared to development using AEC-DMF. Therefore, the ImmunoPure® Metal Enhanced DAB Substrate Kit (Pierce, USA) will be used in all future IHCT work.

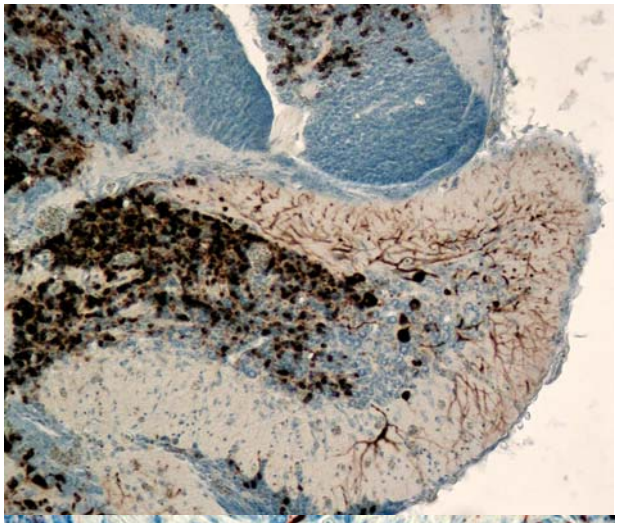


Figure 52a: IHCT result for a brain section from a 42-day old barramundi infected with BNNV. x200
Primary antibody dilution; 1/1000
Conjugate dilution; 1/1000

Substrate – ImmunoPure® Metal Enhanced DAB Substrate Kit

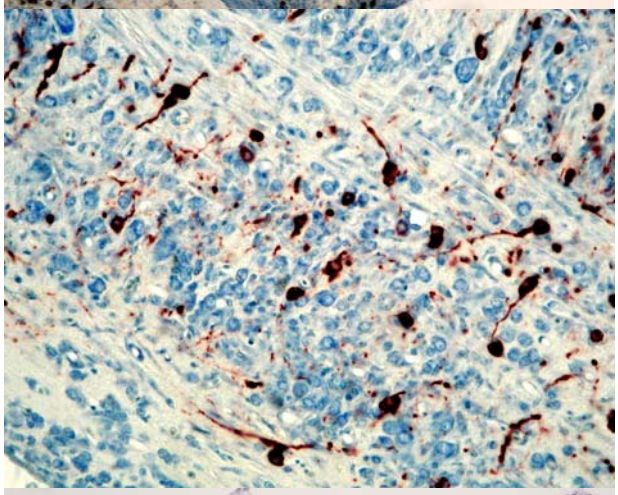


Figure 5 2b: IFAT result for a brain section from a 42-day old barramundi infected with BNNV. x400
Primary antibody dilution; 1/1000
Conjugate dilution; 1/1000

Substrate – ImmunoPure® Metal Enhanced DAB Substrate Kit

Figure 52c: IFAT result for a retina section from a 42-day old barramundi infected with BNNV. x200
Primary antibody dilution; 1/1000
Conjugate dilution; 1/1000

Substrate – ImmunoPure® Metal Enhanced DAB Substrate Kit

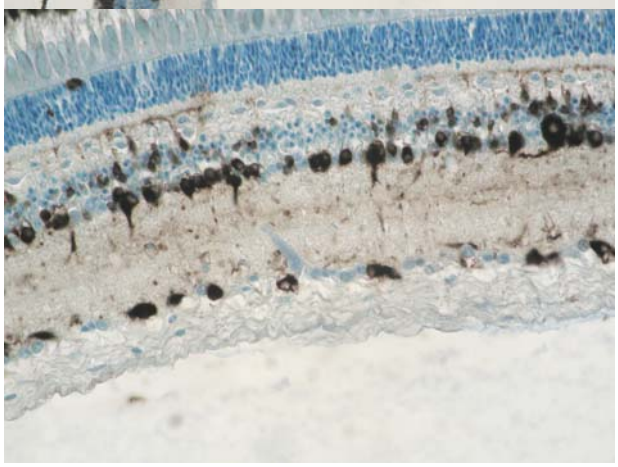


Figure 52d: IFAT result for a retina section from an uninfected 42-day old barramundi. x400
Primary antibody dilution; 1/1000
Conjugate dilution; 1/1000

Substrate – ImmunoPure® Metal Enhanced DAB Substrate Kit

Initially, the IFAT was superior to the IHCT when a simple positive/negative result was all that was required due to the increased intensity of the positive staining. However, the use of the ImmunoPure® Metal Enhanced DAB Substrate Kit (Pierce, USA) for the IHCT, instead of the AEC-DMF substrate, increased the sensitivity to levels comparable to the IFAT, based on the results from the tissue sections tested so far. Counterstaining dramatically improved the quality of the positive results and the IHCT was adopted as the test of choice for nodavirus detection in formalin fixed tissue sections. However, the IFAT is still an appropriate tool for detection of nodavirus in tissue sections and adoption of both tests would ensure capability is maintained if one test failed, for reasons other than primary antibody failure.

7.2.7 Immunodiagnostic test evaluation

7.2.7.1 IHCT of known positive and negative sections

Positive, intense and extensive reactions were observed in all BNNV-positive tissue sections. The specific staining was associated with the extensive lesions caused by the nodavirus (Figure 53). No non-specific staining was seen in any of the BNNV-negative sections. As with previous work, all positive staining was restricted to the nervous tissue of the brain and retina.

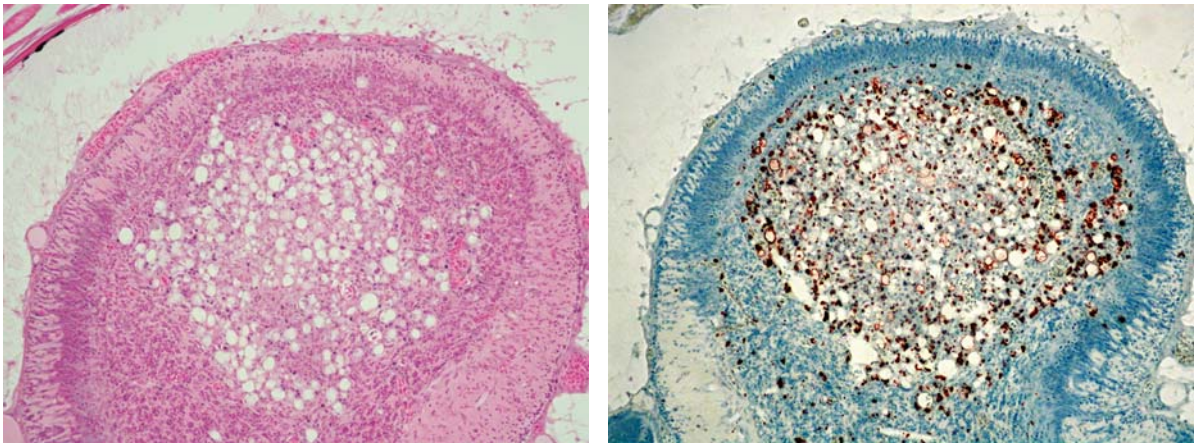


Figure 53a: Histology (H&E, left) and IHCT (right) results from a brain section from a barramundi fry, clinically infected with BNNV. x200. Primary antibody dilution; 1/1000, Conjugate dilution; 1/1000.

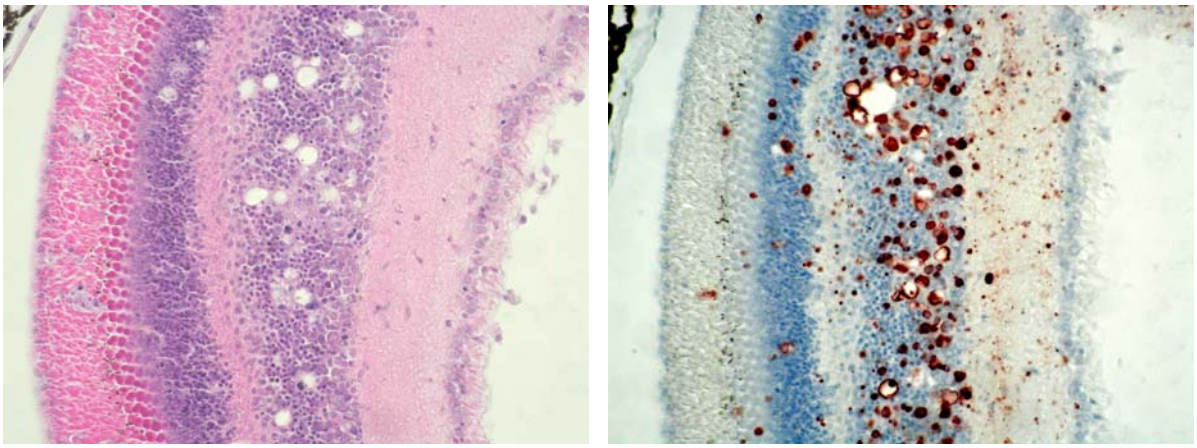


Figure 53b: Histology (H&E, left) and IHCT (right) results from a retina section from a barramundi fry, clinically infected with BNNV. x400. Primary antibody dilution; 1/1000, Conjugate dilution; 1/1000.

7.2.7.2 Comparison of histology, IHCT and IFAT for the detection of nodavirus in fish infected with BNNV and sampled daily.

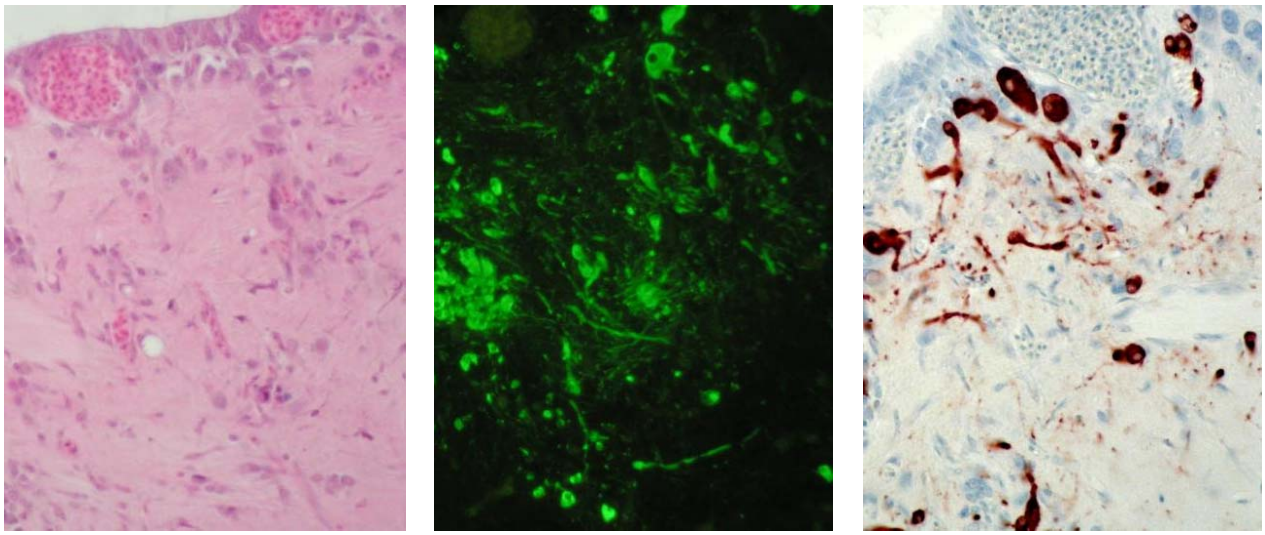
Results of the comparative testing of the 280 samples collected during the experimental bath infection of the 2-month-old barramundi are presented in Table 21. All fish were positive by Nested RT-PCR from one day post infection. The IHCT consistently detected nodavirus in fish from four days post infection (although positive staining was only seen in the spinal cord in one of the Day 4 tissue sections). Samples were consistently RT-PCR positive from 5 days post infection and positive IFAT and histology results were only consistently observed from eight days post infection. Generally, as the trial progressed a much higher proportion of tissue samples were nodavirus positive when the immunodiagnostic tests were used, compared to the proportion of tissue samples diagnosed positive by the observation of histological lesions. A similar finding was reported after experimental infections of sea bass (*Dicentrarchus labrax*) larvae where positive IHCT staining was observed in nervous tissue four days post infection, in the absence of histological lesions (Breuil *et al*., 2001).

Table 21: Comparison of Nested RT-PCR, histology (H&E), IFAT and IHCT for the detection of nodavirus in two month old barramundi, collected daily after experimental bath infection with BNNV. Each test was conducted on serial tissue sections from the same fish (B = Brain, E = Eye).

DAY SAMPLED	RT-PCR	nRT-PCR	HISTOLOGY (H&E)		IFAT		IHCT	
			B	E	B	E	B	E
Pre-trial	-	-	0/5	0/5	0/5	0/5	0/5	0/5
1	-	+ ve	0/4	0/4	0/5	0/5	0/4	0/5
2	-	+ ve	0/5	0/4	0/4	0/5	0/4	0/5
3	-	+ ve	0/3	0/2	0/3	0/5	0/3	0/5
4	-	+ ve	0/5	0/3	0/4	0/5	2/4*	0/3
5	+ ve	+ ve	0/4	0/3	0/4	0/4	2/4	0/4
6	+ ve	+ ve	N/A	0/2	0/4	0/2	2/4	0/4
7	+ ve	+ ve	0/5	0/5	0/5	0/5	2/3	1/4
8	+ ve	+ ve	2/5	0/2	4/5	0/2	3/4	1/2
9	+ ve	+ ve	1/5	0/3	4/5	2/3	4/5	3/3
10	+ ve	+ ve	2/5	0/3	4/5	2/3	5/5	2/2
11	+ ve	+ ve	0/4	N/A	2/4	N/A	2/4	N/A
12	+ ve	+ ve	1/4	1/2	2/4	1/2	2/3	2/3
13	+ ve	+ ve	5/5	4/5	5/5	5/5	5/5	4/4
14	+ ve	+ ve	3/5	2/2	5/5	2/2	4/4	2/2
15	+ ve	+ ve	1/3	4/5	2/4	4/5	1/1	4/5
16	+ ve	+ ve	3/4	2/2	1/1	4/4	4/4	2/2
17	+ ve	+ ve	2/2	4/5	2/2	5/5	2/2	5/5
18	+ ve	+ ve	2/4	3/4	5/5	3/3	4/4	2/3
19	+ ve	+ ve	3/4	3/4	4/4	3/3	4/4	3/3
20	+ ve	+ ve	2/2	1/1	2/2	1/1	2/4	1/1
21	+ ve	+ ve	4/4	1/2	4/4	2/2	4/4	1/1
22	+ ve	+ ve	5/5	3/3	5/5	3/3	5/5	3/3
23	+ ve	+ ve	3/5	1/2	3/3	3/3	3/3	1/1
24	+ ve	+ ve	2/4	0/3	3/4	2/3	3/4	3/3
25	+ ve	+ ve	3/5	2/4	3/5	3/3	4/4	4/4
26	+ ve	+ ve	2/5	0/1	5/5	1/1	5/5	2/2
27	+ ve	+ ve	1/5	0/2	3/4	2/3	5/5	2/2
28	+ ve	+ ve	1/5	0/2	4/5	1/2	1/4	1/2

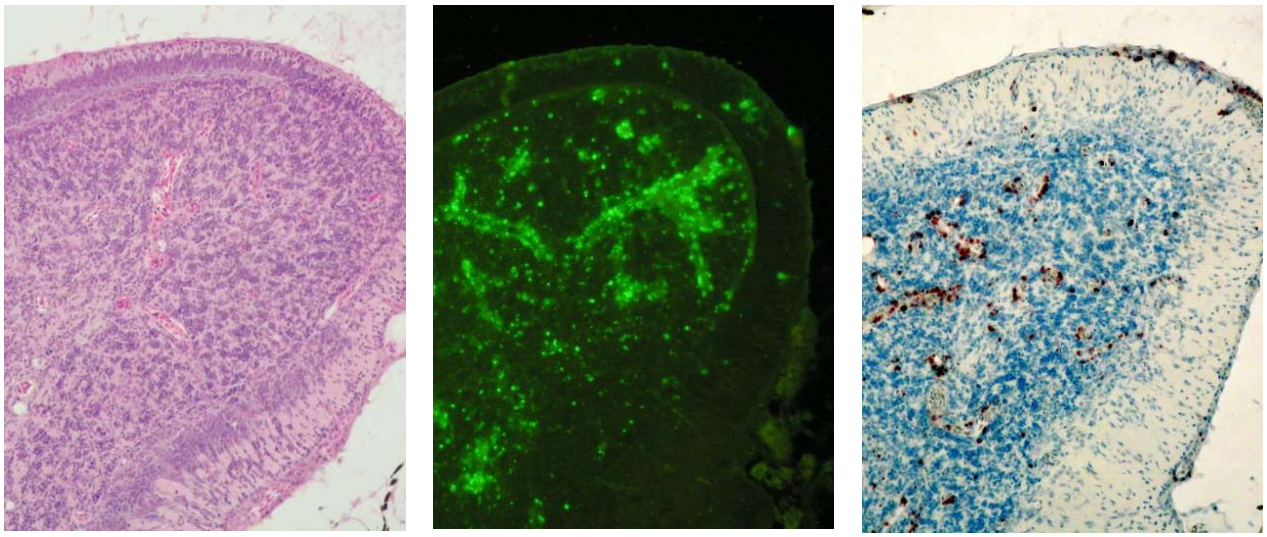
N/A = No tissue present in the section *One section contained spinal cord.

The increased sensitivity of the immunodiagnostics was apparent when tissue sections were tested from sub-clinically infected fish. A higher proportion of these fish were positive by IHCT compared to detection of lesions using histology (Table 21). A significant proportion of the tissue sections, especially those from fish euthanased during the early stages of the infection, were obviously IHCT and IFAT positive, but histological lesions were not apparent (Figure 54). Therefore, the immunodiagnostic tests, especially the IHCT, were more sensitive than histology for the detection of nodavirus in the sub-clinically infected fish produced during the trial.



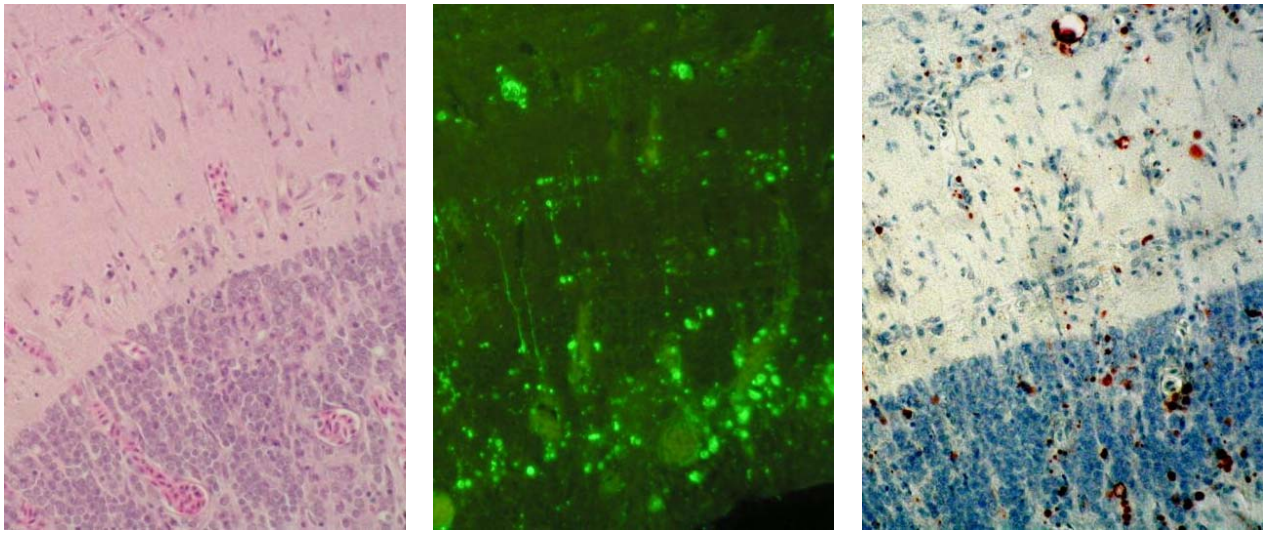
Histology (H&E) **IFAT** **IHCT**

Figure 54a: Histology (H&E), IFAT and IHCT on serial sections from the same barramundi brain, 9 days post infection with BNNV. x400.



Histology (H&E) **IFAT** **IHCT**

Figure 54b: Histology (H&E), IFAT and IHCT on serial sections from the same barramundi brain, 21 days post infection with BNNV. x200



Histology (H&E) **IFAT** **IHCT**

Figure 54c: Histology (H&E), IFAT and IHCT on serial sections from the same barramundi brain, 26 days post infection with BNNV. x400

7.2.7.3 Comparison of histology and IHCT for the detection of nodavirus in fish surviving experimental challenge with BNNV.

The results of comparative testing of the fish surviving experimental infection with BNNV are in Table 22 with descriptions of the experimental infection trials described in Anderson and Moody (2004). From these samples, from four different species, the IHCT detected nodavirus in 194/249 (77.9%) of the surviving fish. When examined histologically, BNNV lesions were present in 124/249 (49.8%) of these samples. A Chi Squared test on these combined results indicated that this difference was significant ($p<0.001$) (A. Reid, pers. com.).

Table 22: Comparative histology (H&E), IFAT and IHCT analysis of fish surviving experimental infection with BNNV (B = Brain, E = Eye, B+E = Brain and Eye combined).

SPECIES	AGE	TREATMENT	HISTOLOGY	IHCT		
			B + E	B + E	B	E
Barramundi	20 days	Bath survivors	16/17 94.1%	17/17 100%	61/66 92.4%	49/52 94.2%
Barramundi	7 weeks	Inject survivors	9/19 50.0%	16/20 80.0%	26/30 86.7%	19/20 95.0%
Barramundi	7 weeks	Inject survivors	11/16 68.75%	12/13 92.3%	23/31 74.2%	12/14 85.7%
Barramundi	12 weeks	Inject survivors	5/35 14.2%	16/36 44.4%	14/29 48.3%	8/36 22.2%
Barcoo Grunter	6 weeks	Inject survivors	18/35 51.4%	31/35 88.6%	18/35 54.4%	28/34 82.4%
Barcoo Grunter	12 weeks	Inject survivors	13/35 37.4%	29/35 82.9%	12/35 34.3%	28/35 80.0%
Golden perch	12 weeks	Inject survivors	19/35 54.3%	30/35 85.7%	16/22 72.7%	29/35 82.9%
Silver perch	6 weeks	Inject survivors	5/13 38.5%	6/13 46.2%	4/13 30.8%	6/13 46.2%
Silver perch	12 weeks	Inject survivors	29/44 65.9%	37/44 84.1%	24/36 66.7%	34/44 77.3%
		TOTAL	**124/249 49.8%**	**194/249 77.9%**	**198/266 74.4%**	**211/283 74.5%**

Examples of these comparative test results, for individual fish from the same population, surviving experimental infection with BNNV are presented in Appendix 3. While RT-PCR results were variable, all fish were positive by Nested RT-PCR, when tested in pools of four with the number of samples positive by IHCT higher than those positive by histology. Therefore, the IHCT was significantly more sensitive than histology for the diagnosis of nodavirus, especially when sub-clinically infected fish were tested (Table 22, Appendix 3). Negative control sections were always negative and no non-specific staining was observed in any tissue sections. From the combined results in Table 22, the proportion of positive samples was almost identical when either brain or eye tissue was tested by IHCT. However, it is recommended that both eye and brain tissue is tested and both tissues are usually present in the same tissue sections if the fish tissues are prepared by longitudinal sectioning. Overall, specific staining was consistently observed in the nervous tissue of the spinal cord, brain and retina after testing using the IHCT. The regions of the brain most commonly infected are presented in Figure 55. The three most commonly infected regions were the cerebellum, optic tectum and valvula cerebelli. The IHCT was sensitive and specific enough to stain the bodies of the neuronal cells (Figure 56) and in many instances only reacted in the cytoplasm and not the nucleus of the cells (Figure 56 E and F) confirming the specific nature of the Sheep α-NNV polyclonal antibodies and the cytoplasmic nature of the virus.

In general, more intense and obvious IHCT staining was observed in clinically-infected fish, with staining closely associated with the lesions caused by the virus. As the lesions in the fish became less obvious, so too did the distribution and abundance of positive IHCT staining. However, all fish that had lesions observed by histology were consistently positive after testing by IHCT. When the infectivity status of samples was unknown, due to the absence of lesions after examination of the histological sections, positive IHCT staining was usually less obvious and in many cases, especially with older, surviving fish, the staining was restricted to a very small number (>10) of cells. However, due to the sensitive and specific nature of the IHCT, and the complete lack of non-specific staining in negative tissue sections, the positive IHCT staining for these types of samples was sufficient to unequivocally diagnose nodavirus as present in the tissue sections.

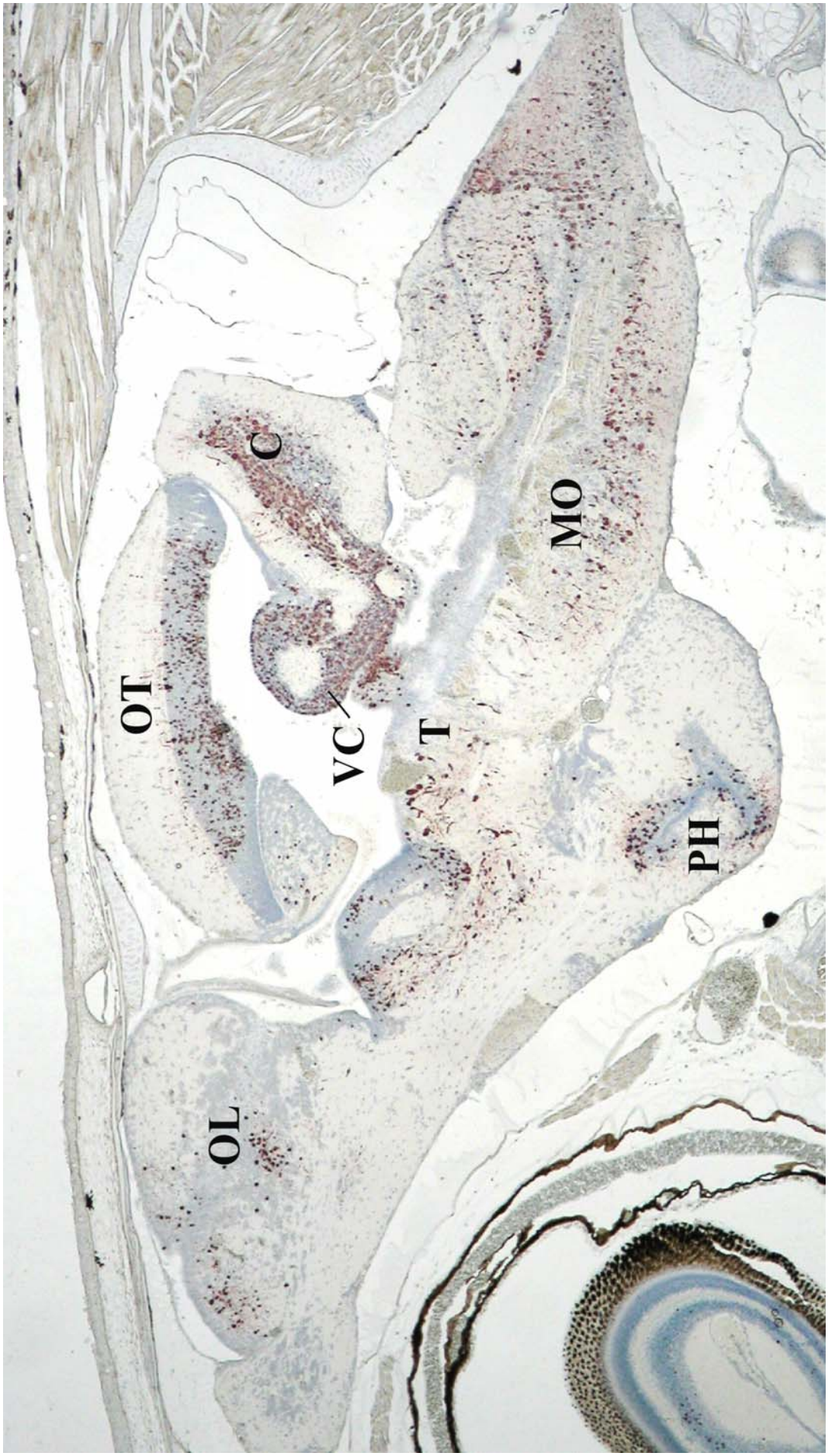


Figure 55: IHCT of a BNNV-infected barramundi brain, showing the major regions of the brain where staining is consistently seen as dense, black staining in neuronal cells. x100. E = eye, OL = olfactory lobe, PH = periventricular hypothalamic region, T = tegmentum VC = valvula cerebelli, OT = optic tectum, C = cerebellum, MO = medulla oblongata. (brain regions from Groman, 1982)

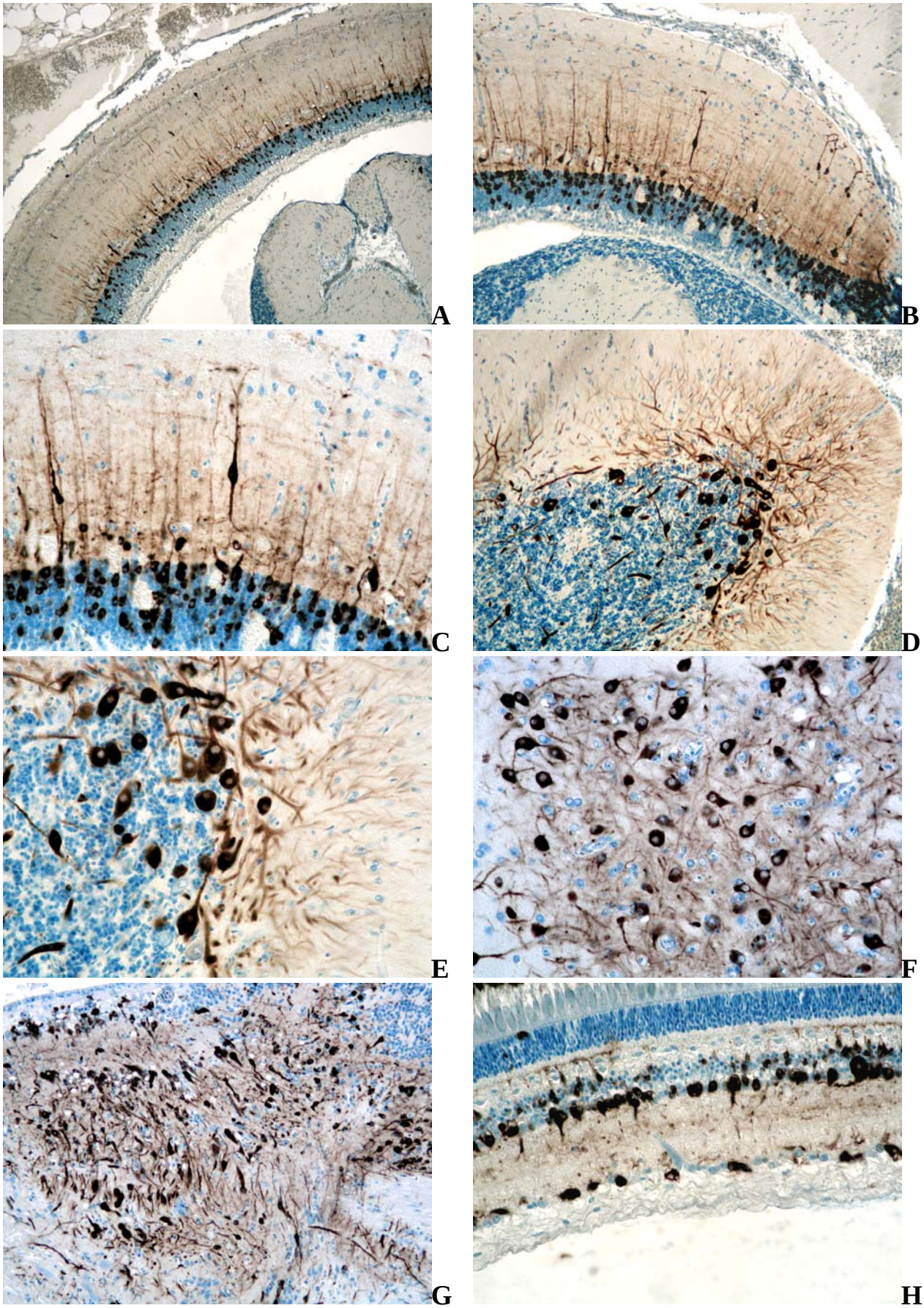


Figure 56: IHCT results for 12-week-old Barcoo grunter surviving experimental infection with BNNV. A; optic tectum (x100), B; optic tectum (x400), C; optic tectum (x400), D; cerebellum (x200), E; cerebellum (x400), F; optic lobe (x400), G; tegmentum (x200), H; Retina (x200)

As mentioned previously, all antibody reactions were restricted to the nervous tissue of the brain, spinal cord and retina. The vast majority of the tissue sections tested only contained brain and eye as these organs were easier to prepare for testing and as a result the spinal cord was only infrequently observed after testing. However, one interesting point to note was the appearance of what appeared to be specific staining in the atrium of the heart (Figure 57a) and intestine of one adult barramundi tested (Figure 57b). As these tissues were not routinely tested the significance of this staining is unknown. However, as the staining appeared to be specific, and positive IHCT results have been observed in the intestine from Atlantic halibut (*Hippoglossus hippoglossus*) following experimental infection (Grotmol *et al*., 1999), the results have been included here. Ongoing testing with the IHCT should clarify whether this staining is specific and what it actually means.

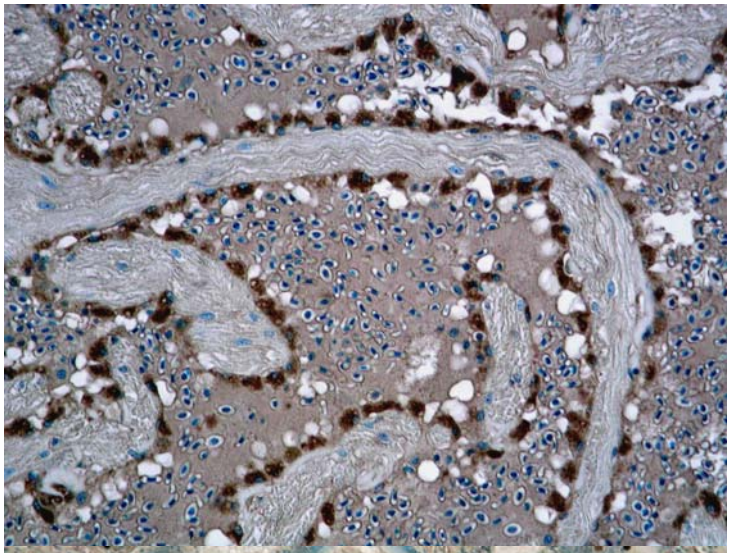


Figure 57a: IHCT result for the atrium of the heart of an adult barramundi. x400

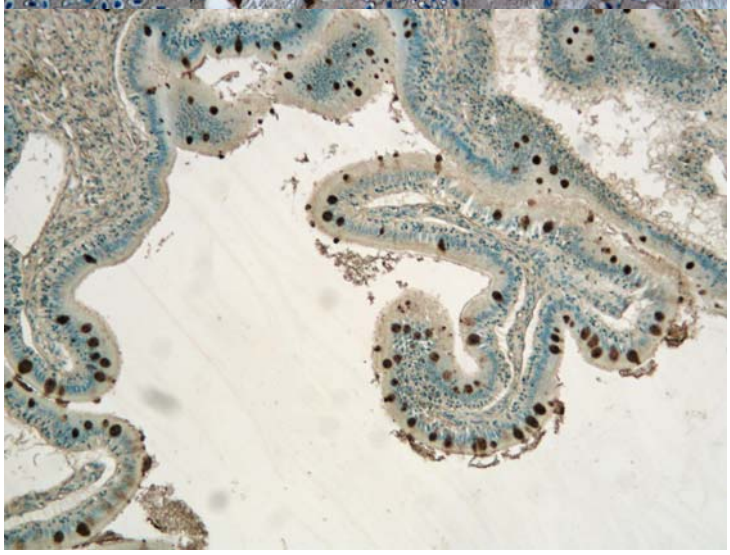


Figure 57b: IHCT result for the intestine of the same adult barramundi as in Figure 57a. x400

7.3 Conclusions

The objective to produce immunodiagnostic tests, capable of localising endemic and exotic nodaviruses in fish tissues and cell cultures, has been achieved. The project produced sensitive, specific and high titre polyclonal antibodies directed against the recombinant coat protein of two Australian nodavirus isolates, one from barramundi (BNNV) and the other from sleepy cod (SCNNV). The IHCT and IFAT procedures produced clear, specific staining in tissue sections of nodavirus infected fish from five overseas countries and from tissue sections from a range of clinically and sub-clinically infected fish species from Queensland.

There was a 100% correlation between the presence of typical nodavirus lesions and positive staining using the immunodiagnostics. No staining was ever observed in negative tissue sections. The IHCT was slightly more sensitive than the IFAT when tissue sections from sub-clinically infected material were tested. Both immunodiagnostic tests were significantly more sensitive than histology for the diagnosis of nodavirus in tissue sections from these sub-clinically infected fish. While only a small number of positively stained cells were observed in some tissue sections from fish surviving infection with BNNV, the location and appearance of the staining in these cells was sufficient to confidently identify the tissue as positive for nodavirus. This was especially important when tissue sections were tested after histological lesions were not observed. Therefore, the immunodiagnostics are useful for confirming nodavirus as the agent responsible for lesions in larvae and fry and juvenile fish and essential for identification of nodavirus in tissue sections where lesions are unapparent or inconclusive.

The sensitivity and specificity of the polyclonal antibodies compare very favourably with polyclonal antibodies produced and used in IHCT and IFAT procedures in use overseas (Breuil *et al*., 2001; Munday *et al*., 2002; OIE, 2003, Tanaka *et al*., 2004). While these procedures used serum instead of plasma, the project determined that plasma was equivalent to serum and had the advantage of being easier to harvest and process. The published procedures also used antisera produced in rabbits, while the antibodies described in this report were produced in sheep. There were two advantages to using sheep for the production of the polyclonal antibodies instead of rabbits. One was the relative ease of maintaining the experimental animals. The sheep used were part of the Oonoonba Veterinary Laboratory flock, which is used for the production of biologicals for routine serology testing. These sheep required less maintenance, handling and labour than rabbits. The second advantage is the significantly larger volume of plasma that can be harvested from sheep, compared to rabbits, significantly reducing the amount of validation required. There is currently over one litre of validated, aliquoted Sheep α-NNV rCP polyclonal antibodies available for laboratories requiring nodavirus immunodiagnostic capability. These polyclonal antibodies could be used to develop an IFAT for confirmation of nodavirus as the virus causing the CPE, when a nodavirus susceptible cell line becomes readily available.

Development of two immunodiagnostic procedures, the IFAT and IHCT, gives choice to laboratories that require this capability. The IFAT procedure developed during this project used a Cy2™ conjugate (Jackson ImmunoResearch, USA) instead of the more common FITC conjugate. This is a newer conjugate, which is more stable and exhibits brighter fluorescence than FITC (but has the same excitation and emission characteristics), is widely available and at a comparative cost to FITC. Laboratories using FITC would either need to change conjugates or re-optimised dilutions of primary and secondary antibodies before implementing the IFAT. Similarly, the IHCT procedure uses a commercially available substrate (ImmunoPure® Metal Enhanced DAB Substrate Kit, Pierce, USA) that is more sensitive, easier to prepare and poses less workplace health and safety risk to the analyst performing the test than the AEC-DMF substrate. Other commercial substrate kits were not evaluated, however most should be appropriate for use in the IHCT after appropriate validation.

The IFAT and IHCT procedures developed, optimised and validated in this report will be incorporated into the Australian and New Zealand Standard Diagnostic Procedure for the detection of nodavirus.

8. Technology and test transfer

Objective 4: "To distribute the above technology and procedures to laboratories as soon as optimisation and validation are complete".

The NNV Nested RT-PCR, IFAT and IHCT procedures have been distributed to the Co-Investigators. The synthetic RNA and polyclonal antibodies have also been distributed.

Comparison of the procedure developed during this project, and a commercially available NNV Nested RT-PCR kit, has been undertaken at Berrimah Veterinary Laboratory in Darwin. Preliminary results have indicated that the procedure developed at the OVL was at least 1000-fold more sensitive than the commercial kit (Dr Colin Shelly, personal communication).

The results of the project were formally presented and/or discussed with Co-Investigators at the:

- Aquatic Animal Health National Technical Working Group (NAAH-TWG) Forum in Perth (2-6 June, 2003)
- Australian Prawn and Barramundi Farmers Conference in Darwin (24-26 July, 2003)
- FRDC Aquatic Animal Health Subprogram Scientific Conference in Geelong (8–10 October, 2003)
- SCAHLS Molecular Diagnosis and PCR related Technologies for Pathogens in Veterinary Diagnostic Laboratories in Geelong (25-27 November, 2003)

The procedures developed are in the draft Australian and New Zealand Standard Diagnostic Procedure for the detection of nodavirus (Chapter 9).

9. Draft ANZSDP for detection of nodavirus

Objective 5: "To produce an Australian and New Zealand Standard Diagnostic Procedure for the detection of nodavirus".

Detection of aquatic nodaviruses

NJ Moody
Oonoonba Veterinary Laboratory
Department of Primary Industries and Fisheries
PO Box 1085
Townsville QLD 4810
nick.moody@dpi.qld.gov.au

PF Horwood
Oonoonba Veterinary Laboratory
Department of Primary Industries and Fisheries
PO Box 1085
Townsville QLD 4810
paul.horwood@dpi.qld.gov.au

SUMMARY

Aquatic nodaviruses are non-enveloped, icosahedral viruses of the Betanodavirus genus of the Family Nodaviridae and contain two segments of positive sense, single stranded RNA. The disease, termed Viral Nervous Necrosis (VNN) or Viral Encephalopathy and Retinopathy (VER), is a serious disease predominantly observed in larvae, fry and juveniles of marine finfish, although infections in older fish and fish cultured in freshwater have been reported. Nodavirus infections have been reported from over 37 fish species, in temperate and tropical environments, from all continents except Africa.

Infection with nodavirus can produce acute disease, resulting in 100% mortality in larvae and fry. Disease can still occur in juvenile fish although mortalities are reduced. Surviving fish and non-susceptible species may act as carriers. Diagnosis of VNN is made by observation of typical nodavirus vacuolation in the nervous tissue, or detection of antigen by IHCT, IFAT or Nested RT-PCR.

***Identification of the agent:** Diagnosis of nodavirus infection can be made by observation of clinical signs and histology when fish are clinically infected. A definitive diagnosis of nodavirus infection and identification of the agent in subclinical infections requires the detection of virus, viral antigen or viral RNA in affected tissues.*

***Status of Australia and New Zealand:** Nodaviruses have been detected in Queensland, the Northern Territory, Tasmania, Western Australia and South Australia. However, management of the disease has since limited the occurrence and frequency of reports from a number of areas. Nodavirus infections have not been reported in New Zealand.*

Introduction

The disease caused by nodavirus infections is termed Viral Nervous Necrosis (VNN) or Viral Encephalopathy and Retinopathy (VER). VNN is the more common name used in the recent literature. Nodavirus infections have been described from over 37 fish species, and have an almost worldwide distribution. [1,2,3]

Within Australia, nodavirus infections have been reported from Queensland, the Northern Territory, Tasmania, Western Australia and South Australia and while the majority of outbreaks have been reported from aquaculture establishments, a number of isolates have been obtained from wild-caught fish.[3] The most recent review of nodaviruses was by Munday *et al*.[2]

Aetiology

Nodaviruses are members of the Betanodavirus genus of the Family Nodaviridae and are non-enveloped, icosahedral viruses with a diameter of approximately 25 to 30nm. Virions contain two segments (RNA1 and RNA2) of positive sense single stranded RNA (ssRNA). The RNA2 segment contains the sequence for the viral coat protein[4], which is highly conserved among isolates and is used in the production of antibodies and is the target for the detection of viral RNA by molecular detection methods.

Comparison of partial coat protein gene sequences from three Australian nodavirus isolates demonstrated the high sequence homology between Australian isolates, although the two marine isolates, from barramundi (BNNV) and barramundi cod (BCNNV), were most closely related to the exotic grouper nodavirus isolates than they were to the endemic freshwater isolate from sleepy cod (SCNNV).[3]

Epidemiology

VNN is primarily an acute infectious disease of cultured larvae and fry from marine environments. Generally, mortalities of up to 100% are common in infected fry although susceptibility tends to decrease as the age of the fish increases. [1,2,5,6] However, significant mortalities have been reported from harvest size fish of some species.[7,8]

Fish surviving infection may become carriers and other non-susceptible species may act as carriers. Nodaviruses have been detected in juvenile fish surviving experimental and natural infection [3,5,6,9], from adult fish from susceptible species. [3,8,9] and in asymptomatic fish from a number of species where disease has not been reported or transmitted.[12,13,14]

The most common mode of transmission is thought to be vertically from broodstock to progeny though infected eggs and sperm or horizontally by co-habitation. [1,2] Anecdotal evidence exists to suggest influent water may also be a source of infection.

While data is limited, nodaviruses appear to be relatively stable to a range of environmental conditions. Titres remained the same after storage in tissue culture medium for 6 months at 15°C and no noticeable decrease in titre was observed after storage at pH 3 to 7 after 6 weeks. [15]

Clinical signs and gross pathology

Most obvious clinical signs are observed in larvae and fry and are due to the damage to the nervous tissue of the spinal cord, brain and retina caused by the virus. Typically, affected fish display abnormal swimming behaviour, including spiral swimming and rapid uncoordinated darting movement, with mass mortalities occurring over a short period of time. [2,5] Colour changes, cessation of feeding and increased

susceptibility to cannibalism may also be observed.

Gross pathological changes are not common. However, overinflation of the swimbladder in NNV-infected sevenband grouper [8] and red drum.[16] has been reported

Histopathology

VNN is characterised by vacuolation and necrosis of the nervous tissue of the spinal cord, brain and retina. The severity of the vacuolation can range from one or two affected cells to necrosis of entire regions of the brain. Detailed descriptions of the histopathology caused by nodavirus are described in Munday *et al*.[2]

Control

Exclusion of the virus from aquaculture premises, good facility hygiene and reduced stocking densities has decreased the incidence of VNN outbreaks. Screening by RT-PCR and use of only NNV-negative broodstock has reduced the occurrence of disease in larvae.[17,18,19] Testing with the RT-PCR did not detect all infected broodstock.[20] Screening broodstock with the Nested RT-PCR will further reduce this although the virus may be present at levels below the detection limit of the test.[3] Disinfection of eggs by ozone is also used to reduce the incidence of infection.[21,22]

Diagnosis

General comments

This standard diagnostic procedure describes the procedures currently in use at the Department Of Primary Industries and Fisheries' Oonoonba Veterinary Laboratory (OVL), which were developed through the Fisheries Research and Development Corporation Project "Development of diagnostic tests for the detection of nodavirus" (FRDC 2001/626). The Project Final Report[3] describes the optimisation and validation of these procedures, which are based on the recommendations in the OIE Manual of Diagnostic Tests for Aquatic Animals for VER[1].

The reagents described in this procedure are those used at the Oonoonba Veterinary Laboratory. Alternate reagents are obviously available, however it is strongly recommended that comparative testing be undertaken to assess any changes in test performance.

Transport and storage of samples

Samples for Nested RT-PCR testing should be kept cool at all times and transported to the laboratory within 24 hours, on ice. If samples cannot be transported within 24 hours they should be frozen at -80°C and transported on dry ice. Samples can be stored at -80°C for two years before loss of integrity is observed.

Samples for histology, immunodiagnostics or TEM testing should be placed in fixative immediately after euthanasia of the fish and processed using standard histology procedures.

Tests available

Clinical signs in larvae and fry are an indication of nodavirus infection, however definitive diagnosis requires observation of vacuoles in tissue sections or detection of the viral antigen or viral RNA in the tissues.

Diagnosis can be made in fixed material by the observation of vacuoles in the nervous tissue of the spinal cord, brain and retina, combined with observation of clinical signs, or by detection of the viral coat protein in the tissue sections using the immunohistochemistry test (IHCT; Appendix 1) or the indirect fluorescent

antibody test (IFAT; Appendix 2). The IHCT is significantly more sensitive at detecting nodavirus in sub-clinically infected fish or fish with equivocal lesions than histology or IFAT.[3]

Detection of the virus in unfixed samples, which include fish tissue and broodstock eggs, sperm and blood, is accomplished using the Nested RT-PCR test (Appendix 3). While the RT-PCR will detect nodavirus in clinically infected fry and larvae exhibiting significant mortality, the Nested RT-PCR is required for testing all other samples[3]. It is strongly recommended that all samples be tested using the complete Nested RT-PCR regardless of any observed clinical signs.

Acknowledgements

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Appendices

Appendix 1
Immunohistochemistry Test (IHCT) procedure

Equipment

Humid chamber
37°C incubator
Staining jars
Pipettors and tips
Compound microscope

Reagents

Deparaffinised, rehydrated tissue sections mounted on positively charged glass histology slides
Tris buffered saline (TBS; 20mM Tris, 500mM NaCl)
0.1% trypsin in TBS
3% H_2O_2 in methanol
5% bovine serum albumin (BSA) in TBS
2.5% BSA in TBS
Primary antibody: Sheep α-NNV rCP polyclonal antibody (Oonoonba Veterinary Laboratory)

Secondary antibody: Rabbit α-Sheep IgG [H+L] HRP conjugate (Jackson ImmunoResearch, USA)
ImmunoPure® Metal Enhanced DAB Substrate Kit (Pierce, USA)
Deionised water
Mayer's haematoxylin
Lithium carbonate
Aqueous mounting medium

Procedure

a) Circle the tissue sections with a hydrophobic marker. Sections should not be allowed to dry at dry at any stage
b) Add 1ml 0.1% trypsin (w/v in TBS) for 30 minutes at 37°C followed by three washes with TBS
c) Block endogenous peroxidase by immersing the slides in 3% H_2O_2 (v/v in methanol) for 20 minutes at room temperature then wash three times with TBS.
d) Block non-specific binding sites by incubating the sections with 5% BSA (w/v in TBS) for 20 minutes then wash three times with TBS.
e) Add 1ml Sheep α-NNV rCP polyclonal antibody, diluted 1/1000 in 2.5% BSA (w/v in TBS), and incubate at 37°C for 60 minutes in a humid chamber.
f) Wash three times with in TBS.
g) Add 1ml of Rabbit α-Sheep IgG [H+L] HRP conjugate, diluted 1/1000 in 2.5% BSA (w/v in TBS), and incubate at 37°C for 60 minutes in a humid chamber.
h) Wash three times with in TBS.
i) Prepare the substrate as follows: The DAB Metal Concentrate (10x) is mixed well and diluted 1/10 in the Stable Peroxide Buffer. Add 1ml to each section and incubate for 10 minutes. Stop substrate development by immersing the slides in deionised water.
j) Counterstain 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.
k) Coverslip sections using an aqueous mounting medium and observed microscopically.

Interpretation

Positive reaction: dense, black staining of cells in the nervous tissue of the spinal cord, brain and retina (Figure 1).

Negative reaction: No dark staining in any nervous tissue.

Background staining: No significant background staining is usually observed.

Appendix 2
Indirect Fluorescent Antibody Test (IFAT) procedure

Equipment

Humid chamber
37°C incubator
Staining jars
Pipettors and tips
Fluorescent microscope

Reagents

Deparaffinised, rehydrated tissue sections mounted on positively charged glass histology slides
Tris buffered saline (TBS; 20mM Tris, 500mM NaCl)
0.1% trypsin in TBS
5% bovine serum albumin (BSA) in TBS
2.5% BSA in TBS
Primary antibody: Sheep α-NNV rCP polyclonal antibody (Oonoonba Veterinary Laboratory)
Secondary antibody: Rabbit α-Sheep IgG [H+L] Cy2™ conjugate (Jackson ImmunoResearch, USA)
Aqueous mounting medium

Procedure

a) Circle the tissue sections with a hydrophobic marker. Sections should not be allowed to dry at dry at any stage
b) Block non-specific binding sites by incubating the sections with 5% BSA (w/v in TBS) for 20 minutes then wash three times with TBS.
c) Add 1ml Sheep α-NNV rCP polyclonal antibody, diluted 1/1000 in 2.5% BSA (w/v in TBS), and incubate at 37°C for 60 minutes in a humid chamber.
d) Wash three times with TBS.
e) Add 1ml of Rabbit α-Sheep IgG [H+L] Cy2™ conjugate, diluted 1/1000 in 2.5% BSA (w/v in TBS), and incubate at 37°C for 60 minutes in a humid chamber.
f) Wash the sections three times with TBS.
g) Coverslip sections using an aqueous mounting medium and observe microscopically using a fluorescent microscope.

Interpretation

Positive reaction: bright green fluorescence of cells in the nervous tissue of the spinal cord, brain and retina (Figure 2).

Negative reaction: No specific green fluorescence in nervous tissue

Background staining: No significant background staining is usually observed.

Appendix 3
Nested RT-PCR procedure

The NNV Nested RT-PCR test is based on the R3-F2 primers and RT-PCR cycling conditions described in Nishizawa, et al.[23] to produce a 426bp amplicon and the nested primers NR'3-NF'2 and Nested PCR cycling conditions described in Thiery, et al.[24] to produce a 294bp amplicon. This procedure uses the modifications described in Moody *et al*.[3] One modification exists for testing of blood where BSA is added at 1µg/µl to the primary PCR.

Table 1: Primers use din the NNV Nested RT-PCR test (5' to 3')

R3	CGAGTCAACACGGGTGAAGA
F2	CGTGTCAGTCATGTGTCGCT
NR'3	GGATTTGACGGGGCTGCTCA
NF'2	GTTCCCTGTACAACGATTCC

Good aseptic technique and Quality Control procedures are required for Nested RT-PCR testing to avoid contamination and production false positive results.

Equipment

Filter tips (1-1000µl range)
Pipettes
Vortex mixer
200µl PCR tubes
500µl PCR tubes
1.5 or 2ml microcentrifuge tubes
Thermal Cycler (Eppendorf MasterCycler)
Microcentrifuge
0°C Thermoblock
UV transilluminator
Gel documentation system

Reagents

Viral Transport Medium (VTM; Medium 199 supplemented with 1000IU/ml benzylpenicillin, 1mg/ml streptomycin sulphate and 2µg/ml amphotericin B)
High Pure Viral RNA Extraction Kit (ROCHE, USA)
StrataScript™ RT (Stratagene)
StrataScript™ buffer (Stratagene, USA), 25mM Magnesium chloride (Promega)
Primers; R3, F2, NR'3, NF'2 (20µM stock)
dNTP's (Promega)
Recombinant RNasin Ribonuclease Inhibitor (Promega)
RNase-free deionised water

HotStarTaq™ Master Mix Kit (QIAGEN)
10mg/ml Bovine Serum Albumin (for testing blood)
DNA ladder and loading dye
Agarose
Ethidium bromide

Procedure

Sample preparation

a) Fish or organ samples are homogenised in approximately two volumes of Viral Transport Medium (VTM; Medium 199 supplemented with 1000IU/ml benzylpenicillin, 1mg/ml streptomycin sulphate and 2µg/ml amphotericin B). For small sample volumes (i.e. < 2ml fluid), tissue is homogenised in a stomacher bag using a 2lb hammer. The homogenate is transferred to 1.5ml O-ring, screw-cap microcentrifuge tubes and stored at -80°C until required. For larger sample volumes, tissue is homogenised using a rotor-stator (Heidolph, Germany) fitted with an 18G attachment. The homogenates are clarified by centrifugation at 900 × g for 20 minutes at 5°C and the supernatant stored in 0.9ml aliquots in O-ring screw-cap microcentrifuge tubes at -80°C until required. Immediately prior to use, the sample is thawed, centrifuged at 10,000 × g for 10 minutes and the supernatant used for the analysis.
b) Samples obtained from broodstock (eggs and sperm) are homogenized by drawing the sample repeatedly drawn back and forth through an 18 Gauge needle, until the viscosity is reduced and an even homogenate produced.
c) Blood samples are lysed by addition of an equal volume of sterile deionised water and incubation at 4°C for 60 minutes. Cellular debris is removed by centrifugation at 10,000 × g for 10 minutes and the supernatant used for the analysis.

Nested RT-PCR procedure

a) RNA is extracted with the High Pure Viral Nucleic Acid Kit according to the manufacturer's instructions. The optional homogenisation buffer and sample incubation step described in the kit is required.
b) Aliquots of sample RNA are heated to 90°C for 5 minutes and cDNA is transcribed in a 20µl reaction mix, containing 9.5µl RNA sample, 1x StrataScript™ buffer, 5U RNasin, 0.5mM dNTPs, 1.0µM R3 primer, 20U StrataScript™ RT and DEPC-treated deionised water, at 42°C for 60 minutes then 90°C for 5 minutes.
c) PCR amplification is carried out in a 50µl reaction mix, containing 5µl of the RT reaction, 0.2µM of each primer (R3 and F2), 2.5mM $MgCl_2$, 25µl of HotStarTaq™ Master Mix and deionised water (Note §), in a Mastercycler thermal cycler programmed with the following cycles; 95°C for 15 minutes, 30 cycles of 95°C for 40 seconds, 50°C for 40 seconds, 72°C for 40 seconds and finally 72°C for 10 minutes.
   Note §: add BSA at 1µg/µl if blood is being tested
d) Nested PCR amplification is carried out in a 50µl reaction mix, containing 1µl of the PCR reaction, 1.0µM NR'3 and NF'2 primers, 25µl of HotStarTaq™ Master Mix and deionised water, in a Mastercycler thermal cycler programmed with the following cycles; 95°C for 15 minutes, 25

cycles of 94°C for 40 seconds, 50°C for 40 seconds, 72°C for 40 seconds and finally 72°C for 10 minutes.

e) Reaction products are analysed after electrophoresis through a 2% agarose gel containing 10mg/ml ethidium bromide. Amplicons are visualised using a UV transilluminator

Interpretation

Positive reaction: For the RT-PCR a 426bp amplicon is produced and for the Nested PCR a 294bp amplicon is produced (Figure 3).

Negative reaction: No amplicons are produced. Some non-specific banding is observed after RT-PCR testing of broodstock spawning fluids and blood by this is not seen after Nested PCR testing and does not affect the test.

Figure Legends

Figure 1: Positive IHCT result. BNNV-infected barramundi brain

Figure 2: Positive IFAT result. BNNV-infected barramundi brain

Figure 3: Positive Nested RT-PCR result. Upper gel: RT-PCR – positive amplicon of 426bp. Lower gel: Nested PCR – positive amplicon of 294bp.

Figure 1

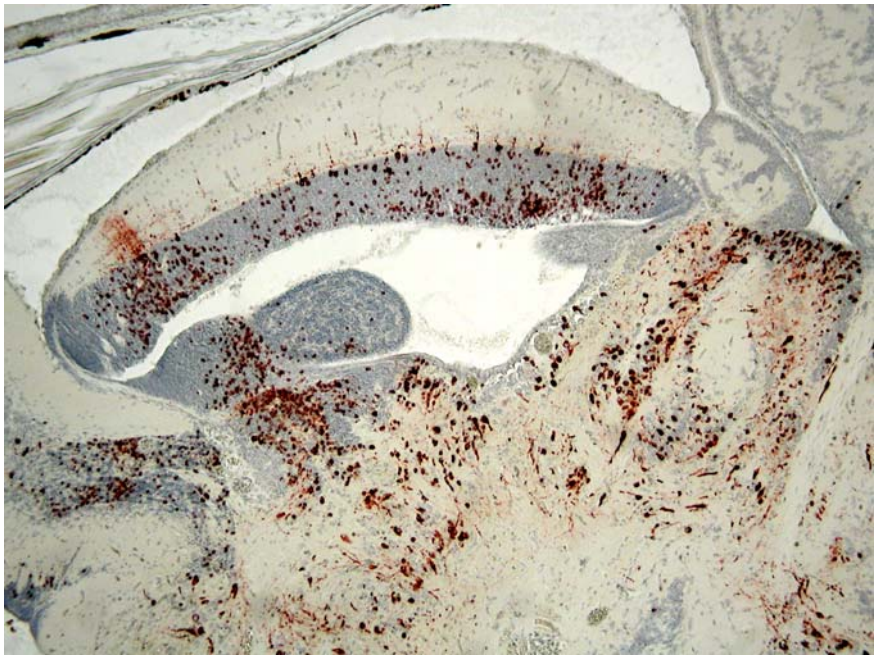


Figure 2

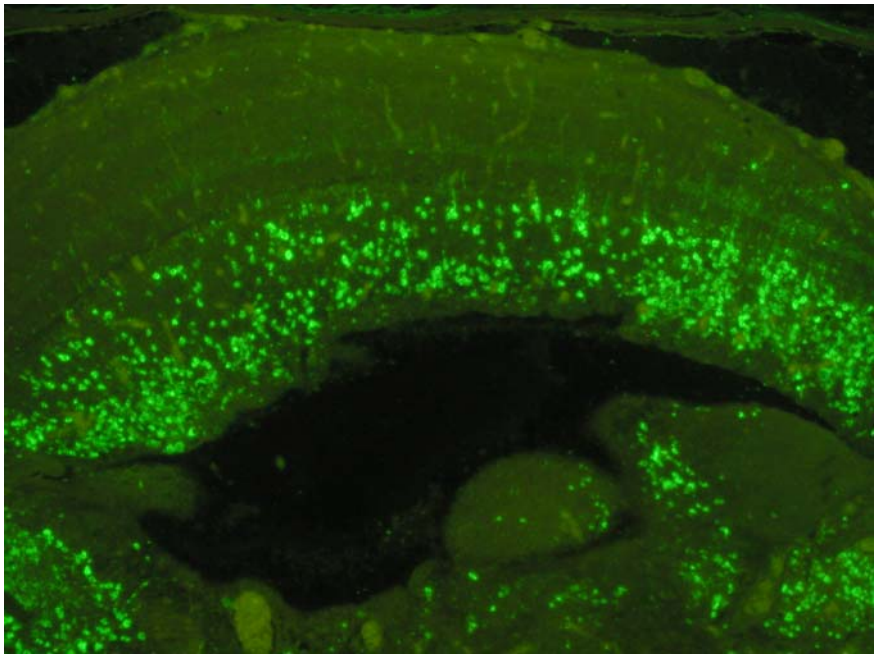
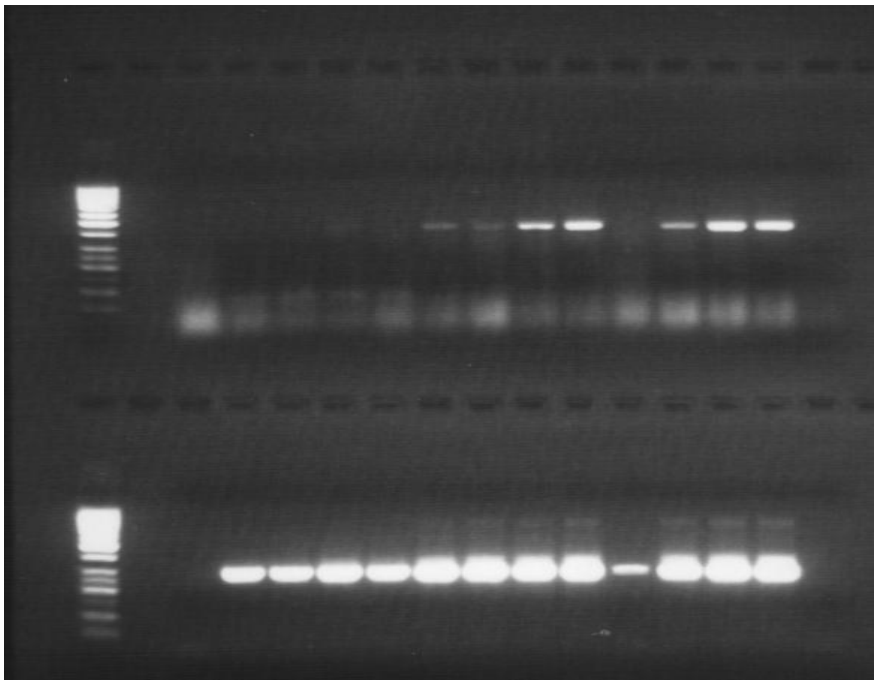


Figure 3



10. Benefits and adoption

The major benefits of the project are the nodavirus diagnostic procedures (Nested RT-PCR, IFAT and IHCT) and sequencing RT-PCR procedure that were developed. These procedures have been distributed to the Co-Investigators. Adoption of the procedures will significantly improve the sensitivity, specificity, quality and range of diagnostic tests available for the detection of nodavirus throughout Australia. The robust and standardised procedures can be used to detect and identify nodavirus in a variety of fresh or fixed material, from a range of temperate and tropical fish species and are applicable to both cultured and wild fish. This report details the development of the reagents, optimisation of the test components, validation of the final standardised procedures and recommendations for the application of the tests. As many diagnostic laboratories have, or are implementing, quality assurance programs, and test procedures must be fully validated before they can be implemented, the work described in this report has significantly reduced the resources required by individual laboratories to optimise and validate the tests before implementation. The majority of the reagents required are commercially available, although comparative testing is strongly recommended if alternate reagents are used, to check that the test is still performing as expected. Other reagents produced for the project at Oonoonba Veterinary Laboratory (OVL) include batches of sheep α-NNV polyclonal antibodies, synthetic RNA for use as a positive control in the Nested RT-PCR and infected and uninfected fixed material for use in the IFAT and IHCT. These reagents have been tested and validated and are available from OVL.

The Nested RT-PCR procedure is currently being used in an FRDC funded project to produce nodavirus free fry and has been incorporated into the suite of tests available at the AFDL-AAHL and OVL. The Nested RT-PCR testing was conducted on striped trumpeter broodstock from Tasmania. While further work is required regarding sample preparation procedures for broodstock blood and spawning material to optimise virus release, the detection test required is available to do this. Sequencing demonstrated the strong homology of the Australian nodavirus isolates to the exotic grouper isolates and that there are at least two distinct nodavirus strains in Australia (barramundi and sleepy cod isolates). While no striped trumpeter nodavirus sequence data could be obtained, due to the low levels of virus in the samples, the sequencing RT-PCR procedure will allow sequencing and comparison of this isolate when suitable material becomes available. Similarly, nodavirus isolates detected from new species can be amplified, sequenced and compared with the coat protein sequences generated for three endemic isolates.

While brain cell lines were successfully produced, none were susceptible to infection with nodavirus. This is disappointing, however the procedure used to initiate, clone and screen the cells was successful and could be used in future work looking at developing tropical finfish cell lines. The inability to produce a nodavirus susceptible cell line also highlights the difficulty in establishing cell lines to detect a specific virus and the requirement to exploit and preserve susceptible cell lines as soon as they are developed.

The immunodiagnostic procedures (IHCT and IFAT) can be used to confirm the identity of lesions in clinically infected fish. However, the significant benefit of these immunodiagnostic procedures is their ability to detect nodavirus in sub-clinically infected material. Clinical signs and presence of lesions in the spinal cord, brain and eye of histological sections indicate nodavirus is the causative agent. However, the intensity of lesions is dependent on the severity of the infection and the age and species of the infected fish. During the project the benefit of the IHCT became apparent during testing of samples from fish surviving experimental infection with BNNV. A significantly higher number of positive tissue sections

were identified using the IHCT compared to histology. All these samples were positive after Nested RT-PCR testing. The IHCT was also used during the project to definitively identify nodavirus in tissue sections from diagnostic submissions where histological analysis was inconclusive. Therefore, the major benefit of the immunodiagnostics tests is the ability to unequivocally identify nodavirus in tissue sections where histological lesions are unapparent or equivocal.

The procedures developed during the project have formed the basis for the Australian and New Zealand Standard Diagnostic Procedure (ANZSDP) for the detection of nodavirus. The implementation of the procedures, through the adoption of the ANZSDP will continue to expand the knowledge base regarding nodavirus infections and increase the confidence of industry and fisheries managers in the quality of information supplied.

11. Further development

Establishment of a cell line, capable of the isolation and amplification of nodaviruses, is essential. Cell lines are the only alternative to live animal bioassays for the identification of infectious virus in a sample and are considered the "gold standard" test for virus detection. The ability to isolate and grow nodavirus in cell culture would significantly improve our understanding of these viruses, especially with regard to management, control and prevention. It is unknown why none of the cell lines produce during this project were susceptible to infection with nodavirus, but the fact that the original LCB cell line developed was susceptible to nodavirus, indicated it is possible to produce one. The ability to identify and quantify infectious virus in samples would enable determination of the sensitivity and specificity of the procedures described in this report. This is especially critical for sub-clinically infected material. As there was other procedure available to measure the sensitivity and specificity of the Nested RT-PCR and immunodiagnostic tests, only general comparative assessments of sensitivity could be made.

Improvements to the detecting of nodavirus infections in broodstock samples are required. It is thought that the virus was present in very low levels in these samples, and at the limits of detection of the Nested RT-PCR test. Sub-optimal preparation of the samples was a contributing to this issue. While the test itself needs no refinement, optimising of sample preparation would eliminate this as a factor. Ongoing testing of broodstock and their progeny is currently a component of another FRDC project (2002/043). Detection of antibodies in broodstock may also improve the management of nodavirus in aquaculture facilities. Antigen (RT-PCR) and antibody (ELISA) detection tests have been used overseas to reduce the incidence of infection in the larvae by eliminating either antibody or antigen positive broodstock from the spawning population (Watanabe *et al*., 1998; Breuil *et al*., 2000). Implementation of an antibody detection test would further improve the ability to screen broodstock and reduce the impact of nodavirus in aquaculture hatcheries. Preliminary agar gel immunodiffusion (AGID) experiments, using the recombinant coat protein and sheep α-NNV polyclonal antibodies as the antigen and antibody, are very encouraging and further work will be undertaken to determine whether an NNV AGID will be appropriate for the detection of antibodies in broodstock fish.

12. Planned outcomes

The Nested RT-PCR, IHCT and IFAT procedures significantly contribute to the major planned outcome from the project, which was the availability of a suite of tests (Nested RT-PCR, cell line and immunodiagnostics) for the detection and/or identification of nodaviruses by laboratories in Australia.

The Nested RT-PCR is being used screen broodstock prior to introduction into aquaculture facilities and prior to spawning. This test is also being used by an FRDC funded project with the aims of producing nodavirus free fry and determining the natural distribution of nodavirus in the environment. Both the Nested RT-PCR and immunodiagnostics can detect nodavirus in clinical and sub-clinical infections in fry and larvae, and offer a significant improvement in sensitivity compared to identification of specific lesions by histology. Both the Nested RT-PCR and IHCT have subsequently been used to identify nodavirus as the cause of disease in new species being evaluated for aquaculture. Testing of the broodstock has since been implemented to determine the proportion of infected broodstock. The Nested RT-PCR and immunodiagnostics will enable more sensitive screening of larvae prior to release, significantly reducing the risk of introduction of nodavirus, with sub-clinically infected fish, into the environment.

Incorporation of the tests into the range used at the AAHL Fish Diseases Laboratory will enable rapid identification of any exotic nodavirus incursions into Australia and incorporation into state laboratories will enable rapid identification of any endemic nodavirus outbreaks.

13. Conclusion

The Nested RT-PCR and immunodiagnostic procedures developed during the project will detect endemic and exotic nodavirus isolates in fresh and fixed material. The procedures have been optimised and validated, distributed to Co-Investigators laboratories and incorporated into a draft Australian and New Zealand Standard Diagnostic Procedure (ANZSDP) for the detection of nodavirus. The development, optimisation and validation work conducted during the project was more extensive than any similar research reported in the literature, however, the procedures developed and conclusions drawn compare very favourably with the results that are available. Only one of the project objectives; "to produce a cell line that can be used for the isolation, amplification and titration of endemic and exotic nodaviruses", was not achieved.

The Nested RT-PCR detected endemic nodavirus infections in material obtained from Tasmania, the Northern Territory and Queensland and two exotic nodavirus isolates tested at AFDL-AAHL. The results obtained from tissue culture supernatant, whole larvae and brain tissue demonstrated the sensitive, specific, repeatable and robust nature of the procedure for these sample types. No negative results were obtained from samples that were known to be nodavirus positive (false-negative) and no positive results were obtained from samples that were known to be nodavirus negative (false-positives). For broodstock testing, blood appeared to be more appropriate than spawning material although sample preparation of broodstock ovarian tissue and sperm requires further investigation before a standard sample preparation procedure can be developed. A number of different methods were trialed, with the most successful adopted, however improvements need to made to ensure a higher degree of repeatability for sample preparation. Confounding this is the theory that levels of virus in broodstock material were approaching the limits of detection of the Nested RT-PCR test. This was indicated by the results of Nested RT-PCR testing of broodstock clotted blood, whole blood and spawning material, where no three samples from the one fish all gave positive results. Consecutive testing of the same samples was also inconclusive. Addressing the issue of broodstock spawning sample preparation and subsequent testing of broodstock is required. While complete confidence could be given to positive results, it is unknown what proportion of negative results were false negative results.

Most RNA extraction procedures, reverse transcriptases and DNA polymerases could be used in the Nested RT-PCR, although sensitivity was variable. Some reagents produced false-negative results so when alternatives to the reagents described in the final Nested RT-PCR procedure are used, comparative testing is strongly recommended to ensure the reagents are appropriate. Synthetic coat protein RNA was produced for use as positive control in the Nested RT-PCR. The synthetic RNA will eliminate the reliance on nodavirus outbreaks to obtain clinical material, eliminate the need to conduct *in vivo* amplification work to generate clinically infected material and reduce of the requirement for interstate transport of infectious material. The sequencing RT-PCR demonstrated that while the three Australian nodavirus isolates were highly homologous (97.7%-99.8% similarity) the barramundi and barramundi cod isolates were most closely related to the overseas grouper isolates while the sleepy cod was more closely related to the other, non-grouper isolates.

Over 50 primary brain cell lines and 39 cloned brain cell lines were successfully produced. The primary cell lines were developed from both whole brain tissue and from tissue from specific regions of the brain, identified as most commonly infected with nodavirus by IHCT. Only 15 of the primary cell lines and 8 of the cloned cell lines were considered viable candidates for screening, based primarily on growth rate comparisons with the original

nodavirus-susceptible LCB cell line. None of the screened cell lines were susceptible to infection with nodavirus. The SSN-1 cell line was also not susceptible to infection with the nodavirus isolates used. The original LCB cell line was thawed from storage in liquid nitrogen and produced typical nodavirus CPE up to and including passage 35, but transformed somehow and was no longer susceptible to infection at passage 36 and above. Cloning of the LCB cell line was successfully undertaken, but failed to result in a cell line susceptible to nodavirus. The samples used for the screening were known to be infectious so failure to observe any CPE was not due to lack of infectious virus in the inoculum. No cell lines were initiated from striped trumpeter due to logistical problems associated with live transport of the fish over the distances required and time constraints. A cell line susceptible to nodavirus infection is required and research to achieve this should be supported.

The project developed procedures for the production of sensitive, specific and high titre polyclonal antibodies directed against the recombinant coat protein of two Australian nodavirus isolates, in sheep. Over one litre of validated Sheep α-NNV rCP polyclonal antibodies was produced and is available for laboratories requiring nodavirus immunodiagnostic capability. These antibodies, when used at a dilution of 1/1000 in IFAT and IHCT procedures, successfully detected nodavirus infections in formalin fixed tissue sections. No negative results were obtained from samples that were known to be positive (false-negative) and no positive results were obtained from samples that were known to be negative (false-positives). The IHCT was used during the project to confirm the identity of nodavirus in diagnostic submissions where histology was inconclusive and nodavirus had not been reported from that species before. The IFAT and IHCT procedures successfully detected nodavirus infections in formalin fixed tissue from five overseas countries. Although two procedures were developed, the IHCT was used for most of the comparative testing as this procedure did not require specialised equipment (fluorescent microscope) and was most likely to be adopted laboratories as the immunodiagnostics test of choice. Comparative testing of serial tissue sections (n=249), of fish surviving experimental infection with BNNV, by IHCT and histology demonstrated the significant improvement in the sensitivity of nodavirus detection by the IHCT. Only 49.8% of these tissue sections were positive by histology whereas 77.9% were positive by IHCT. Therefore, the immunodiagnostics tests have significantly increased the sensitivity of nodavirus diagnosis in fixed material, especially with regard to fish that are not clinically affected or where lesions were equivocal.

The Nested RT-PCR, IFAT and IHCT test procedures and polyclonal antibodies and synthetic RNA have been distributed to Co-Investigators All the procedures will be made freely available though this report and the Australian and New Zealand Standard Diagnostic Procedure (ANZSDP) for the detection of nodavirus.

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Appendix 1: BNNV, BCNNV and SCNNV partial coat protein sequences

Appendix 1.1: BNNV nodavirus isolate sequences obtained from the four plasmids. A, B, C and D; plasmids sequenced, F; sequence generated using the forward primer, R; sequence generated using the reverse primer.

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                *        20         *        40         *        60         *        80
3AF1 : ----------------------------CGAACCTCTAGTTGTCGAG-ATCTCCCAGGCCGTCCTCCCAGCCGGGACAGGAACAGAC :  58
3AF2 : ----------------TTTGGGGGGGACTNNNNTTNAGGNTGTCGAG-ATCTCCCAGGCCGTCCTCCCAGCCGGGACAGGAACAGAC :  70
3AR1 : GGATTTGGACGTGCGACCAATGACGTCCATCTCTCAGGTATGTCGAGAATCTCCCAGGCCGTCCTCCCAGCCGGGACAGGAACAGAC :  87
3AR2 : GGATTTGGACGTGCGACCAATGACGTCCATCTCTCAGGTATGTCGAGAATCTCCCAGGCCGTCCTCCCAGCCGGGACAGGAACAGAC :  87
3BF1 : -------------------------AACTCNGNCTCTAGTTGTCGAG-ATCTCCCAGGCCGTCCTCCCAGCCGGGACAGGAACAGAC :  61
3BF2 : ------------------------ACTCNANCTTCTAGTTGTCGAG-ATCTCCCAGGCCGTCCTCCCAGCCGGGACAGGAACAGAC :  61
3BR1 : GGATTTGGACGTGCGACCAATGACGTCCATCTCTCAGGTATGTCGAGAATCTCCCAGGCCGTCCTCCCAGCCGGGACAGGAACAGAC :  87
3BR2 : GGATTTGGACGTGCGACCAATGACGTCCATCTCTCAGGTATGTCGAGAATCTCCCAGGCCGTCCTCCCAGCCGGGACAGGAACAGAC :  87
3CF1 : --------------------CGCATTANNGNNNANTAGNTGTCGAG-ATCTCCCAGGCCGTCCTCCCAGCCGGGACAGGAACAGAC :  65
3CF2 : -------------------CTGCCGTTTAANGNANTTAGTTGTCGAG-ATCTCCCAGGCCGTCCTCCCAGCCGGGACAGGAACAGAC :  67
3CR1 : GGATTTGGACGTGCGACCAATGACGTCCATCTCTCAGGTATGTCGAGAATCTCCCAGGCCGTCCTCCCAGCCGGGACAGGAACAGAC :  87
3CR2 : GGATTTGGACGTGCGACCAATGACGTCCATCTCTCAGGTATGTCGAGAATCTCCCAGGCCGTCCTCCCAGCCGGGACAGGAACAGAC :  87
3DF1 : ----------------CTAGAAAAGTTANNNANNNTTAGNTGTCGAG-ATCTCCCAGGCCGTCCTCCCAGCCGGGACAGGAACAGAC :  70
3DF2 : ------------------CGAGACGGTTANNNNNNTTAGNTGTCGAG-ATCTCCCAGGCCGTCCTCCCAGCCGGGACAGGAACAGAC :  68
3DR1 : GGATTTGGACGTGCGACCAATGACGTCCATCTCTCAGGTATGTCGAGAATCTCCCAGGCCGTCCTCCCAGCCGGGACAGGAACAGAC :  87
3DR2 : GGATTTGGACGTGCGACCAATGACGTCCATCTCTCAGGTATGTCGAGAATCTCCCAGGCCGTCCTCCCAGCCGGGACAGGAACAGAC :  87
                                            TGTCGAG ATCTCCCAGGCCGTCCTCCCAGCCGGGACAGGAACAGAC

          *       100         *       120         *       140         *       160         *
3AF1 : GGATACGTTGTTGTTGATGCAACCATCGTCCCCGACCTCCTGCCACGACTGGGACACGCTGCTAGAATCTTCCAGCGATACGCTGTT : 145
3AF2 : GGATACGTTGTTGTTGATGCAACCATCGTCCCCGACCTCCTGCCACGACTGGGACACGCTGCTAGAATCTTCCAGCGATACGCTGTT : 157
3AR1 : GGATACGTTGTTGTTGATGCAACCATCGTCCCCGACCTCCTGCCACGACTGGGACACGCTGCTAGAATCTTCCAGCGATACGCTGTT : 174
3AR2 : GGATACGTTGTTGTTGATGCAACCATCGTCCCCGACCTCCTGCCACGACTGGGACACGCTGCTAGAATCTTCCAGCGATACGCTGTT : 174
3BF1 : GGATACGTTGTTGTTGATGCAACCATCGTCCCCGACCTCCTGCCACGACTGGGACACGCTGCTAGAATCTTCCAGCGATACGCTGTT : 148
3BF2 : GGATACGTTGTTGTTGATGCAACCATCGTCCCCGACCTCCTGCCACGACTGGGACACGCTGCTAGAATCTTCCAGCGATACGCTGTT : 148
3BR1 : GGATACGTTGTTGTTGATGCAACCATCGTCCCCGACCTCCTGCCACGACTGGGACACGCTGCTAGAATCTTCCAGCGATACGCTGTT : 174
3BR2 : GGATACGTTGTTGTTGATGCAACCATCGTCCCCGACCTCCTGCCACGACTGGGACACGCTGCTAGAATCTTCCAGCGATACGCTGTT : 174
3CF1 : GGATACGTTGTTGTTGATGCAACCATCGTCCCCGACCTCCTGCCACGACTGGGACACGCTGCTAGAATCTTCCAGCGATACGCTGTT : 152
3CF2 : GGATACGTTGTTGTTGATGCAACCATCGTCCCCGACCTCCTGCCACGACTGGGACACGCTGCTAGAATCTTCCAGCGATACGCTGTT : 154
3CR1 : GGATACGTTGTTGTTGATGCAACCATCGTCCCCGACCTCCTGCCACGACTGGGACACGCTGCTAGAATCTTCCAGCGATACGCTGTT : 174
3CR2 : GGATACGTTGTTGTTGATGCAACCATCGTCCCCGACCTCCTGCCACGACTGGGACACGCTGCTAGAATCTTCCAGCGATACGCTGTT : 174
3DF1 : GGATACGTTGTTGTTGATGCAACCATCGTCCCCGACCTCCTGCCACGACTGGGACACGCTGCTAGAATCTTCCAGCGATACGCTGTT : 157
3DF2 : GGATACGTTGTTGTTGATGCAACCATCGTCCCCGACCTCCTGCCACGACTGGGACACGCTGCTAGAATCTTCCAGCGATACGCTGTT : 155
3DR1 : GGATACGTTGTTGTTGATGCAACCATCGTCCCCGACCTCCTGCCACGACTGGGACACGCTGCTAGAATCTTCCAGCGATACGCTGTT : 174
3DR2 : GGATACGTTGTTGTTGATGCAACCATCGTCCCCGACCTCCTGCCACGACTGGGACACGCTGCTAGAATCTTCCAGCGATACGCTGTT : 174
       GGATACGTTGTTGTTGATGCAACCATCGTCCCCGACCTCCTGCCACGACTGGGACACGCTGCTAGAATCTTCCAGCGATACGCTGTT

         180         *       200         *       220         *       240         *       260
3AF1 : GAAACACTGGAGTTTGAAATTCAGCCAATGTGCCCCGCAAACACGGGCGGTGGTTACGTTGCTGGCTTCCTGCCTGATCCAACTGAC : 232
3AF2 : GAAACACTGGAGTTTGAAATTCAGCCAATGTGCCCCGCAAACACGGGCGGTGGTTACGTTGCTGGCTTCCTGCCTGATCCAACTGAC : 244
3AR1 : GAAACACTGGAGTTTGAAATTCAGCCAATGTGCCCCGCAAACACGGGCGGTGGTTACGTTGCTGGCTTCCTGCCTGATCCAACTGAC : 261
3AR2 : GAAACACTGGAGTTTGAAATTCAGCCAATGTGCCCCGCAAACACGGGCGGTGGTTACGTTGCTGGCTTCCTGCCTGATCCAACTGAC : 261
3BF1 : GAAACACTGGAGTTTGAAATTCAGCCAATGTGCCCCGCAAACACGGGCGGTGGTTACGTTGCTGGCTTCCTGCCTGATCCAACTGAC : 235
3BF2 : GAAACACTGGAGTTTGAAATTCAGCCAATGTGCCCCGCAAACACGGGCGGTGGTTACGTTGCTGGCTTCCTGCCTGATCCAACTGAC : 235
3BR1 : GAAACACTGGAGTTTGAAATTCAGCCAATGTGCCCCGCAAACACGGGCGGTGGTTACGTTGCTGGCTTCCTGCCTGATCCAACTGAC : 261
3BR2 : GAAACACTGGAGTTTGAAATTCAGCCAATGTGCCCCGCAAACACGGGCGGTGGTTACGTTGCTGGCTTCCTGCCTGATCCAACTGAC : 261
3CF1 : GAAACACTGGAGTTTGAAATTCAGCCAATGTGCCCCGCAAACACGGGCGGTGGTTACGTTGCTGGCTTCCTGCCTGATCCAACTGAC : 239
3CF2 : GAAACACTGGAGTTTGAAATTCAGCCAATGTGCCCCGCAAACACGGGCGGTGGTTACGTTGCTGGCTTCCTGCCTGATCCAACTGAC : 241
3CR1 : GAAACACTGGAGTTTGAAATTCAGCCAATGTGCCCCGCAAACACGGGCGGTGGTTACGTTGCTGGCTTCCTGCCTGATCCAACTGAC : 261
3CR2 : GAAACACTGGAGTTTGAAATTCAGCCAATGTGCCCCGCAAACACGGGCGGTGGTTACGTTGCTGGCTTCCTGCCTGATCCAACTGAC : 261
3DF1 : GAAACACTGGAGTTTGAAATTCAGCCAATGTGCCCCGCAAACACGGGCGGTGGTTACGTTGCTGGCTTCCTGCCTGATCCAACTGAC : 244
3DF2 : GAAACACTGGAGTTTGAAATTCAGCCAATGTGCCCCGCAAACACGGGCGGTGGTTACGTTGCTGGCTTCCTGCCTGATCCAACTGAC : 242
3DR1 : GAAACACTGGAGTTTGAAATTCAGCCAATGTGCCCCGCAAACACGGGCGGTGGTTACGTTGCTGGCTTCCTGCCTGATCCAACTGAC : 261
3DR2 : GAAACACTGGAGTTTGAAATTCAGCCAATGTGCCCCGCAAACACGGGCGGTGGTTACGTTGCTGGCTTCCTGCCTGATCCAACTGAC : 261
       GAAACACTGGAGTTTGAAATTCAGCCAATGTGCCCCGCAAACACGGGCGGTGGTTACGTTGCTGGCTTCCTGCCTGATCCAACTGAC

          *       280         *       300         *       320         *       340
3AF1 : AACGATCACACCTTCGACGCGCTTCAAGCAACTCGTGGTGCAGTCGTTGCCAAATGGTGGGAAAGCAGAACAGTCCGACCTCAGTAC : 319
3AF2 : AACGATCACACCTTCGACGCGCTTCAAGCAACTCGTGGTGCAGTCGTTGCCAAATGGTGGGAAAGCAGAACAGTCCGACCTCAGTAC : 331
3AR1 : AACGATCACACCTTCGACGCGCTTCAAGCAACTCGTGGTGCAGTCGTTGCCAAATGGTGGGAAAGCAGAACAGTCCGACCTCAGTAC : 348
3AR2 : AACGATCACACCTTCGACGCGCTTCAAGCAACTCGTGGTGCAGTCGTTGCCAAATGGTGGGAAAGCAGAACAGTCCGACCTCAGTAC : 348
3BF1 : AACGATCACACCTTCGACGCGCTTCAAGCAACTCGTGGTGCAGTCGTTGCCAAATGGTGGGAAAGCAGAACAGTCCGACCTCAGTAC : 322
3BF2 : AACGATCACACCTTCGACGCGCTTCAAGCAACTCGTGGTGCAGTCGTTGCCAAATGGTGGGAAAGCAGAACAGTCCGACCTCAGTAC : 322
3BR1 : AACGATCACACCTTCGACGCGCTTCAAGCAACTCGTGGTGCAGTCGTTGCCAAATGGTGGGAAAGCAGAACAGTCCGACCTCAGTAC : 348
3BR2 : AACGATCACACCTTCGACGCGCTTCAAGCAACTCGTGGTGCAGTCGTTGCCAAATGGTGGGAAAGCAGAACAGTCCGACCTCAGTAC : 348
3CF1 : AACGATCACACCTTCGACGCGCTTCAAGCAACTCGTGGTGCAGTCGTTGCCAAATGGTGGGAAAGCAGAACAGTCCGACCTCAGTAC : 326
3CF2 : AACGATCACACCTTCGACGCGCTTCAAGCAACTCGTGGTGCAGTCGTTGCCAAATGGTGGGAAAGCAGAACAGTCCGACCTCAGTAC : 328
3CR1 : AACGATCACACCTTCGACGCGCTTCAAGCAACTCGTGGTGCAGTCGTTGCCAAATGGTGGGAAAGCAGAACAGTCCGACCTCAGTAC : 348
3CR2 : AACGATCACACCTTCGACGCGCTTCAAGCAACTCGTGGTGCAGTCGTTGCCAAATGGTGGGAAAGCAGAACAGTCCGACCTCAGTAC : 348
3DF1 : AACGATCACACCTTCGACGCGCTTCAAGCAACTCGTGGTGCAGTCGTTGCCAAATGGTGGGAAAGCAGAACAGTCCGACCTCAGTAC : 331
3DF2 : AACGATCACACCTTCGACGCGCTTCAAGCAACTCGTGGTGCAGTCGTTGCCAAATGGTGGGAAAGCAGAACAGTCCGACCTCAGTAC : 329
3DR1 : AACGATCACACCTTCGACGCGCTTCAAGCAACTCGTGGTGCAGTCGTTGCCAAATGGTGGGAAAGCAGAACAGTCCGACCTCAGTAC : 348
3DR2 : AACGATCACACCTTCGACGCGCTTCAAGCAACTCGTGGTGCAGTCGTTGCCAAATGGTGGGAAAGCAGAACAGTCCGACCTCAGTAC : 348
       AACGATCACACCTTCGACGCGCTTCAAGCAACTCGTGGTGCAGTCGTTGCCAAATGGTGGGAAAGCAGAACAGTCCGACCTCAGTAC
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Appendix 1.1 (cont'd): BNNV nodavirus isolate sequences obtained from the four plasmids. A, B, C and D; plasmids sequenced, F; sequence generated using the forward primer, R; sequence generated using the reverse primer.

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        *       360         *       380         *       400         *       420         *
3AF1 : ACCCGCACGCTCCTCTGGACCTCGTCGGGAAAGGAGCAGCGTCTCACGTCACCTGGTCGGCTGATACTCCTGTGTGTCGGCAACAAC : 406
3AF2 : ACCCGCACGCTCCTCTGGACCTCGTCGGGAAAGGAGCAGCGTCTCACGTCACCTGGTCGGCTGATACTCCTGTGTGTCGGCAACAAC : 418
3AR1 : ACCCGCACGCTCCTCTGGACCTCGTCGGGAAAGGAGCAGCGTCTCACGTCACCTGGTCGGCTGATACTCCTGTGTGTCGGCAACAAC : 435
3AR2 : ACCCGCACGCTCCTCTGGACCTCGTCGGGAAAGGAGCAGCGTCTCACGTCACCTGGTCGGCTGATACTCCTGTGTGTCGGCAACAAC : 435
3BF1 : ACCCGCACGCTCCTCTGGACCTCGTCGGGAAAGGAGCAGCGTCTCACGTCACCTGGTCGGCTGATACTCCTGTGTGTCGGCAACAAC : 409
3BF2 : ACCCGCACGCTCCTCTGGACCTCGTCGGGAAAGGAGCAGCGTCTCACGTCACCTGGTCGGCTGATACTCCTGTGTGTCGGCAACAAC : 409
3BR1 : ACCCGCACGCTCCTCTGGACCTCGTCGGGAAAGGAGCAGCGTCTCACGTCACCTGGTCGGCTGATACTCCTGTGTGTCGGCAACAAC : 435
3BR2 : ACCCGCACGCTCCTCTGGACCTCGTCGGGAAAGGAGCAGCGTCTCACGTCACCTGGTCGGCTGATACTCCTGTGTGTCGGCAACAAC : 435
3CF1 : ACCCGCACGCTCCTCTGGACCTCGTCGGGAAAGGAGCAGCGTCTCACGTCACCTGGTCGGCTGATACTCCTGTGTGTCGGCAACAAC : 413
3CF2 : ACCCGCACGCTCCTCTGGACCTCGTCGGGAAAGGAGCAGCGTCTCACGTCACCTGGTCGGCTGATACTCCTGTGTGTCGGCAACAAC : 415
3CR1 : ACCCGCACGCTCCTCTGGACCTCGTCGGGAAAGGAGCAGCGTCTCACGTCACCTGGTCGGCTGATACTCCTGTGTGTCGGCAACAAC : 435
3CR2 : ACCCGCACGCTCCTCTGGACCTCGTCGGGAAAGGAGCAGCGTCTCACGTCACCTGGTCGGCTGATACTCCTGTGTGTCGGCAACAAC : 435
3DF1 : ACCCGCACGCTCCTCTGGACCTCGTCGGGAAAGGAGCAGCGTCTTACGTCACCTGGTCGGCTGATACTCCTGTGTGTCGGCAACAAC : 418
3DF2 : ACCCGCACGCTCCTCTGGACCTCGTCGGGAAAGGAGCAGCGTCTTACGTCACCTGGTCGGCTGATACTCCTGTGTGTCGGCAACAAC : 416
3DR1 : ACCCGCACGCTCCTCTGGACCTCGTCGGGAAAGGAGCAGCGTCTTACGTCACCTGGTCGGCTGATACTCCTGTGTGTCGGCAACAAC : 435
3DR2 : ACCCGCACGCTCCTCTGGACCTCGTCGGGAAAGGAGCAGCGTCTTACGTCACCTGGTCGGCTGATACTCCTGTGTGTCGGCAACAAC : 435
       ACCCGCACGCTCCTCTGGACCTCGTCGGGAAAGGAGCAGCGTCT ACGTCACCTGGTCGGCTGATACTCCTGTGTGTCGGCAACAAC

         440         *       460         *       480         *       500         *       520
3AF1 : ACTGATGTGGTCAACGTGTCAGTGCTGTGTCGCTGGAGTGTTCGACTGAGCGTTCCATCTCTTGAGACACCTGAAGAGACCACCGCT : 493
3AF2 : ACTGATGTGGTCAACGTGTCAGTGCTGTGTCGCTGGAGTGTTCGACTGAGCGTTCCATCTCTTGAGACACCTGAAGAGACCACCGCT : 505
3AR1 : ACTGATGTGGTCAACGTGTCAGTGCTGTGTCGCTGGAGTGTTCGACTGAGCGTTCCATCTCTTGAGACACCTGAAGAGACCACCGCT : 522
3AR2 : ACTGATGTGGTCAACGTGTCAGTGCTGTGTCGCTGGAGTGTTCGACTGAGCGTTCCATCTCTTGAGACACCTGAAGAGACCACCGCT : 522
3BF1 : ACTGATGTGGTCAACGTGTCAGTGCTGTGTCGCTGGAGTGTTCGACTGAGCGTTCCATCTCTTGAGACACCTGAAGAGACCACCGCT : 496
3BF2 : ACTGATGTGGTCAACGTGTCAGTGCTGTGTCGCTGGAGTGTTCGACTGAGCGTTCCATCTCTTGAGACACCTGAAGAGACCACCGCT : 496
3BR1 : ACTGATGTGGTCAACGTGTCAGTGCTGTGTCGCTGGAGTGTTCGACTGAGCGTTCCATCTCTTGAGACACCTGAAGAGACCACCGCT : 522
3BR2 : ACTGATGTGGTCAACGTGTCAGTGCTGTGTCGCTGGAGTGTTCGACTGAGCGTTCCATCTCTTGAGACACCTGAAGAGACCACCGCT : 522
3CF1 : ACTGATGTGGTCAACGTGTCAGTGCTGTGTCGCTGGAGTGTTCGACTGAGCGTTCCATCTCTTGAGACACCTGAAGAGACCACCGCT : 500
3CF2 : ACTGATGTGGTCAACGTGTCAGTGCTGTGTCGCTGGAGTGTTCGACTGAGCGTTCCATCTCTTGAGACACCTGAAGAGACCACCGCT : 502
3CR1 : ACTGATGTGGTCAACGTGTCAGTGCTGTGTCGCTGGAGTGTTCGACTGAGCGTTCCATCTCTTGAGACACCTGAAGAGACCACCGCT : 522
3CR2 : ACTGATGTGGTCAACGTGTCAGTGCTGTGTCGCTGGAGTGTTCGACTGAGCGTTCCATCTCTTGAGACACCTGAAGAGACCACCGCT : 522
3DF1 : ACTGATGTGGTCAACGTGTCAGTGCTGTGTCGCTGGAGTGTTCGACTGAGCGTTCCATCTCTTGAGACACCTGAAGAGACCACCGCT : 505
3DF2 : ACTGATGTGGTCAACGTGTCAGTGCTGTGTCGCTGGAGTGTTCGACTGAGCGTTCCATCTCTTGAGACACCTGAAGAGACCACCGCT : 503
3DR1 : ACTGATGTGGTCAACGTGTCAGTGCTGTGTCGCTGGAGTGTTCGACTGAGCGTTCCATCTCTTGAGACACCTGAAGAGACCACCGCT : 522
3DR2 : ACTGATGTGGTCAACGTGTCAGTGCTGTGTCGCTGGAGTGTTCGACTGAGCGTTCCATCTCTTGAGACACCTGAAGAGACCACCGCT : 522
       ACTGATGTGGTCAACGTGTCAGTGCTGTGTCGCTGGAGTGTTCGACTGAGCGTTCCATCTCTTGAGACACCTGAAGAGACCACCGCT

             *       540         *       560         *       580         *       600
3AF1 : CCCATCATGACACAAGGTTCCCTGTACAACGATTCCCTTTCCACAAATGACTTCAAGTCCATCCTCCTAGGATCCACACCACTGGAC : 580
3AF2 : CCCATCATGACACAAGGTTCCCTGTACAACGATTCCCTTTCCACAAATGACTTCAAGTCCATCCTCCTAGGATCCACACCACTGGAC : 592
3AR1 : CCCATCATGACACAAGGTTCCCTGTACAACGATTCCCTTTCCACAAATGACTTCAAGTCCATCCTCCTAGGATCCACACCACTGGAC : 609
3AR2 : CCCATCATGACACAAGGTTCCCTGTACAACGATTCCCTTTCCACAAATGACTTCAAGTCCATCCTCCTAGGATCCACACCACTGGAC : 609
3BF1 : CCCATCATGACACAAGGTTCCCTGTACAACGATTCCCTTTCCACAAATGACTTCAAGTCCATCCTCCTAGGATCCACACCACTGGAC : 583
3BF2 : CCCATCATGACACAAGGTTCCCTGTACAACGATTCCCTTTCCACAAATGACTTCAAGTCCATCCTCCTAGGATCCACACCACTGGAC : 583
3BR1 : CCCATCATGACACAAGGTTCCCTGTACAACGATTCCCTTTCCACAAATGACTTCAAGTCCATCCTCCTAGGATCCACACCACTGGAC : 609
3BR2 : CCCATCATGACACAAGGTTCCCTGTACAACGATTCCCTTTCCACAAATGACTTCAAGTCCATCCTCCTAGGATCCACACCACTGGAC : 609
3CF1 : CCCATCATGACACAAGGTTCCCTGTACAACGATTCCCTTTCCACAAATGACTTCAAGTCCATCCTCCTAGGATCCACACCACTGGAC : 587
3CF2 : CCCATCATGACACAAGGTTCCCTGTACAACGATTCCCTTTCCACAAATGACTTCAAGTCCATCCTCCTAGGATCCACACCACTGGAC : 589
3CR1 : CCCATCATGACACAAGGTTCCCTGTACAACGATTCCCTTTCCACAAATGACTTCAAGTCCATCCTCCTAGGATCCACACCACTGGAC : 609
3CR2 : CCCATCATGACACAAGGTTCCCTGTACAACGATTCCCTTTCCACAAATGACTTCAAGTCCATCCTCCTAGGATCCACACCACTGGAC : 609
3DF1 : CCCATCATGACACAAGGTTCCCTGTACAACGATTCCCTTTCCACAAATGACTTCAAGTCCATCCTCCTAGGATCCACACCACTGGAC : 592
3DF2 : CCCATCATGACACAAGGTTCCCTGTACAACGATTCCCTTTCCACAAATGACTTCAAGTCCATCCTCCTAGGATCCACACCACTGGAC : 590
3DR1 : CCCATCATGACACAAGGTTCCCTGTACAACGATTCCCTTTCCACAAATGACTTCAAGTCCATCCTCCTAGGATCCACACCACTGGAC : 609
3DR2 : CCCATCATGACACAAGGTTCCCTGTACAACGATTCCCTTTCCACAAATGACTTCAAGTCCATCCTCCTAGGATCCACACCACTGGAC : 609
       CCCATCATGACACAAGGTTCCCTGTACAACGATTCCCTTTCCACAAATGACTTCAAGTCCATCCTCCTAGGATCCACACCACTGGAC

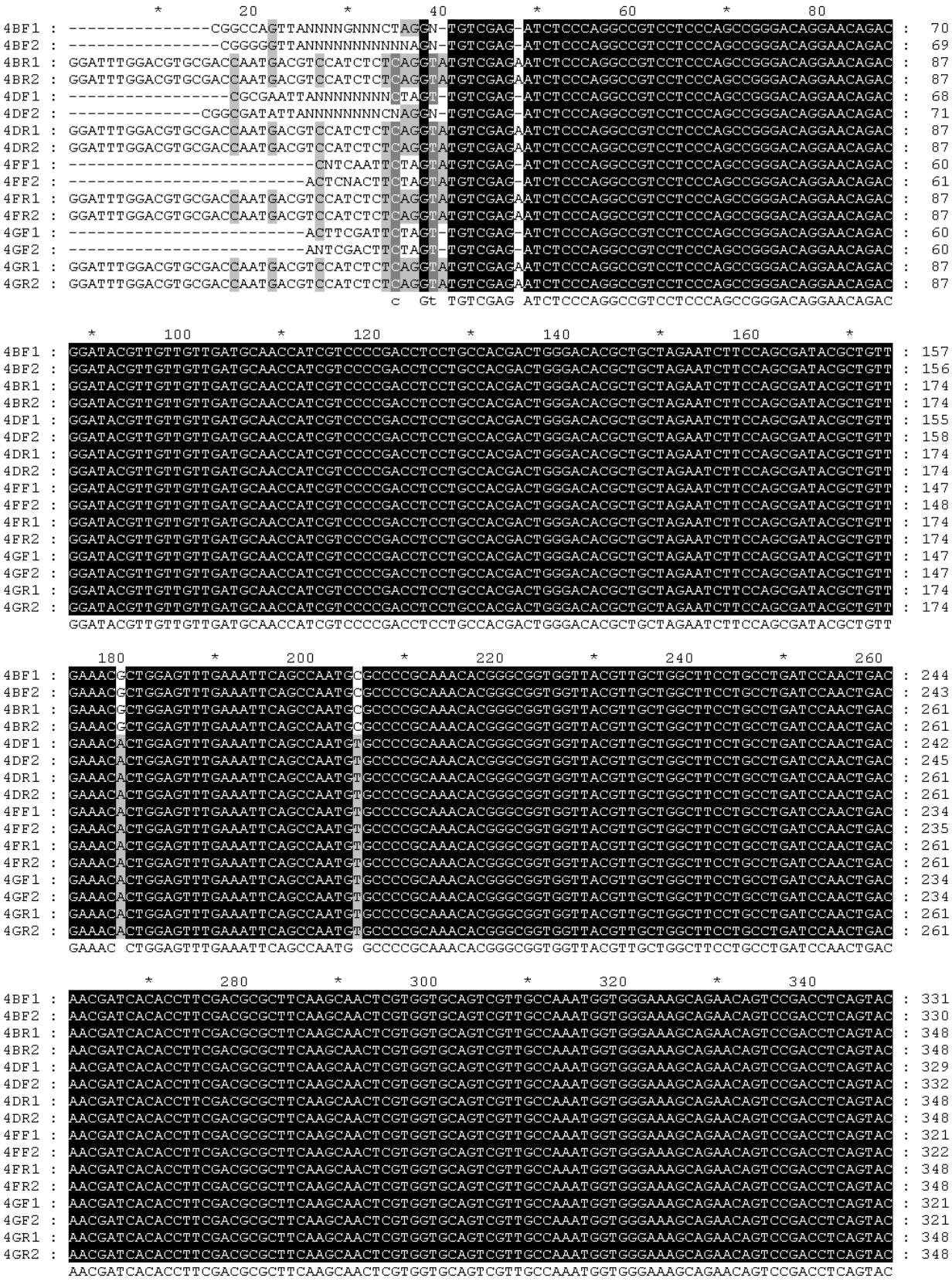
       *       620         *       640         *       660         *       680         *
3AF1 : ATTGCTCCTGATGGAGCAGTCTTCCAGCTGGACCGTCCGCTGTCCATTGACTACAGCCTTGGAACTGGAGATGTTGACCGTGCTGTT : 667
3AF2 : ATTGCTCCTGATGGAGCAGTCTTCCAGCTGGACCGTCCGCTGTCCATTGACTACAGCCTTGGAACTGGAGATGTTGACCGTGCTGTT : 679
3AR1 : ATTGCTCCTGATGGAGCAGTCTTCCAGCTGGACCGTCCGCTGTCCATTGACTACAGCCTTGGAACTGGAGATGTTGACCGTGCTGTT : 696
3AR2 : ATTGCTCCTGATGGAGCAGTCTTCCAGCTGGACCGTCCGCTGTCCATTGACTACAGCCTTGGAACTGGAGATGTTGACCGTGCTGTT : 696
3BF1 : ATTGCTCCTGATGGAGCAGTCTTCCAGCTGGACCGTCCGCTGTCCATTGACTACAGCCTTGGAACTGGAGATGTTGACCGTGCTGTT : 670
3BF2 : ATTGCTCCTGATGGAGCAGTCTTCCAGCTGGACCGTCCGCTGTCCATTGACTACAGCCTTGGAACTGGAGATGTTGACCGTGCTGTT : 670
3BR1 : ATTGCTCCTGATGGAGCAGTCTTCCAGCTGGACCGTCCGCTGTCCATTGACTACAGCCTTGGAACTGGAGATGTTGACCGTGCTGTT : 696
3BR2 : ATTGCTCCTGATGGAGCAGTCTTCCAGCTGGACCGTCCGCTGTCCATTGACTACAGCCTTGGAACTGGAGATGTTGACCGTGCTGTT : 696
3CF1 : ATTGCTCCTGATGGAGCAGTCTTCCAGCTGGACCGTCCGCTGTCCATTGACTACAGCCTTGGAACTGGAGATGTTGACCGTGCTGTT : 674
3CF2 : ATTGCTCCTGATGGAGCAGTCTTCCAGCTGGACCGTCCGCTGTCCATTGACTACAGCCTTGGAACTGGAGATGTTGACCGTGCTGTT : 676
3CR1 : ATTGCTCCTGATGGAGCAGTCTTCCAGCTGGACCGTCCGCTGTCCATTGACTACAGCCTTGGAACTGGAGATGTTGACCGTGCTGTT : 696
3CR2 : ATTGCTCCTGATGGAGCAGTCTTCCAGCTGGACCGTCCGCTGTCCATTGACTACAGCCTTGGAACTGGAGATGTTGACCGTGCTGTT : 696
3DF1 : ATTGCCCCTGATGGAGCAGTCTTCCAGCTGGACCGTCCGCTGTCCATTGACTACAGCCTTGGAACTGGAGATGTTGACCGTGCTGTT : 679
3DF2 : ATTGCCCCTGATGGAGCAGTCTTCCAGCTGGACCGTCCGCTGTCCATTGACTACAGCCTTGGAACTGGAGATGTTGACCGTGCTGTT : 677
3DR1 : ATTGCCCCTGATGGAGCAGTCTTCCAGCTGGACCGTCCGCTGTCCATTGACTACAGCCTTGGAACTGGAGATGTTGACCGTGCTGTT : 696
3DR2 : ATTGCCCCTGATGGAGCAGTCTTCCAGCTGGACCGTCCGCTGTCCATTGACTACAGCCTTGGAACTGGAGATGTTGACCGTGCTGTT : 696
       ATTGC CCTGATGGAGCAGTCTTCCAGCTGGACCGTCCGCTGTCCATTGACTACAGCCTTGGAACTGGAGATGTTGACCGTGCTGTT
```

Appendix 1.1 (cont'd): BNNV nodavirus isolate sequences obtained from the four plasmids. A, B, C and D; plasmids sequenced, F; sequence generated using the forward primer, R; sequence generated using the reverse primer.

```
        700         *       720         *       740         *       760         *       780
3AF1 : TACTGGCACCTCAAGAAGTTTGCTGGAAATGCTGGCACACCTGCAGGCTGGTTTCGCTGGGGCATCTGGGACAACTTCAACAAGACG : 754
3AF2 : TACTGGCACCTCAAGAAGTTTGCTGGAAATGCTGGCACACCTGCAGGCTGGTTTCGCTGGGGCATCTGGGACAACTTCAACAAGACG : 766
3AR1 : TACTGGCACCTCAAGAAGTTTGCTGGAAATGCTGGCACACCTGCAGGCTGGTTTCGCTGGGGCATCTGGGACAACT-CAACAAGACG : 782
3AR2 : TACTGGCACCTCAAGAAGTTTGCTGGAAATGCTGGCACACCTGCAGGCTGGTTTCGCTGGGGCATCTGGGACAACT-CAACAAGACG : 782
3BF1 : TACTGGCACCTCAAGAAGTTTGCTGGAAATGCTGGCACACCTGCAGGCTGGTTTCGCTGGGGCATCTGGGACAACTTCAACAAGACG : 757
3BF2 : TACTGGCACCTCAAGAAGTTTGCTGGAAATGCTGGCACACCTGCAGGCTGGTTTCGCTGGGGCATCTGGGACAACTTCAACAAGACG : 757
3BR1 : TACTGGCACCTCAAGAAGTTTGCTGGAAATGCTGGCACACCTGCAGGCTGGTTTCGCTGGGGCATCTGGGACAACT-CAACAAGACG : 782
3BR2 : TACTGGCACCTCAAGAAGTTTGCTGGAAATGCTGGCACACCTGCAGGCTGGTTTCGCTGGGGCATCTGGGACAACT-CAACAAGACG : 782
3CF1 : TACTGGCACCTCAAGAAGTTTGCTGGAAATGCTGGCACACCTGCAGGCTGGTTTCGCTGGGGCATCTGGGACAACTTCAACAAGACG : 761
3CF2 : TACTGGCACCTCAAGAAGTTTGCTGGAAATGCTGGCACACCTGCAGGCTGGTTTCGCTGGGGCATCTGGGACAACTTCAACAAGACG : 763
3CR1 : TACTGGCACCTCAAGAAGTTTGCTGGAAATGCTGGCACACCTGCAGGCTGGTTTCGCTGGGGCATCTGGGACAACTTCAACAAGACG : 783
3CR2 : TACTGGCACCTCAAGAAGTTTGCTGGAAATGCTGGCACACCTGCAGGCTGGTTTCGCTGGGGCATCTGGGACAACT-CAACAAGACG : 782
3DF1 : TACTGGCACCTCAAGAAGTTTGCTGGAAATGCTGGCACACCTGCAGGCTGGTTTCGCTGGGGCATCTGGGACAACTTCAACAAGACG : 766
3DF2 : TACTGGCACCTCAAGAAGTTTGCTGGAAATGCTGGCACACCTGCAGGCTGGTTTCGCTGGGGCATCTGGGACAACTTCAACAAGACG : 764
3DR1 : TACTGGCACCTCAAGAAGTTTGCTGGAAATGCTGGCACACCTGCAGGCTGGTTTCGCTGGGGCATCTGGGACAACTTCAACAAGACG : 783
3DR2 : TACTGGCACCTCAAGAAGTTTGCTGGAAATGCTGGCACACCTGCAGGCTGGTTTCGCTGGGGCATCTGGGACAACTTCAACAAGACG : 783
       TACTGGCACCTCAAGAAGTTTGCTGGAAATGCTGGCACACCTGCAGGCTGGTTTCGCTGGGGCATCTGGGACAACT CAACAAGACG

            *       800         *       820         *
3AF1 : TTCACAGATGGCGTTGCCTACTACTCTGATGAGCAGCCCCGTCAAATCC : 803
3AF2 : TTCACAGATGGCGTTGCCTACTACTCTGATGAGCAGCCCCGTCAAATCC : 815
3AR1 : T-CACAGATGCGTGCACGAAGTCCG----------------------- : 806
3AR2 : T-CACAGATGGCGTGCNAGNNGG------------------------- : 804
3BF1 : TTCACAGATGGCGTTGCCTACTACTCTGATGAGCAGCCCCGTCAAATCC : 806
3BF2 : TTCACAGATGGCGTTGCCTACTACTCTGATGAGCAGCCCCGTCAAATCC : 806
3BR1 : T-CACAGATGGCGNGCAATCGG---------------------------- : 803
3BR2 : T-CACAGATGGCGTGCGANNC---------------------------- : 802
3CF1 : TTCACAGATGGCGTTGCCTACTACTCTGATGAGCAGCCCCGTCAAATCC : 810
3CF2 : TTCACAGATGGCGTTGCCTACTACTCTGATGAGCAGCCCCGTCAAATCC : 812
3CR1 : T-CACAGATGGCNGCNCNTATCTAATCGAG------------------- : 812
3CR2 : T-CACAGATGGCNGCNCNTAAGGCTCG---------------------- : 808
3DF1 : TTCACAGATGGCGTTGCCTACTACTCTGATGAGCAGCCCCGTCAAATCC : 815
3DF2 : TTCACAGATGGCGTTGCCTACTACTCTGATGAGCAGCCCCGTCAAATCC : 813
3DR1 : T-CACAGATGGCNGCNNNNATTCAAGGTG-------------------- : 811
3DR2 : T-CACAGATGGCNGCNTNNTAATGGCCTC-------------------- : 811
       T CACAGATGgc
```

Appendix 1.2: BCNNV nodavirus isolate sequences obtained from the four plasmids. A, B, C and D; plasmids sequenced, F; sequence generated using the forward primer, R; sequence generated using the reverse primer.



Appendix 1.2 (cont'd): BCNNV nodavirus isolate sequences obtained from the four plasmids. A, B, C and D; plasmids sequenced, F; sequence generated using the forward primer, R; sequence generated using the reverse primer.

```
          *       360         *       380         *       400         *       420         *
4BF1 : ACCCGCACGCTCCTCTGGACCTCGTCGGGAAAGGAGCAGCGTCTCACGTCACCTGGTCGGCTGATACTCCTGTGTGTCGGCAACAAC : 418
4BF2 : ACCCGCACGCTCCTCTGGACCTCGTCGGGAAAGGAGCAGCGTCTCACGTCACCTGGTCGGCTGATACTCCTGTGTGTCGGCAACAAC : 417
4BR1 : ACCCGCACGCTCCTCTGGACCTCGTCGGGAAAGGAGCAGCGTCTCACGTCACCTGGTCGGCTGATACTCCTGTGTGTCGGCAACAAC : 435
4BR2 : ACCCGCACGCTCCTCTGGACCTCGTCGGGAAAGGAGCAGCGTCTCACGTCACCTGGTCGGCTGATACTCCTGTGTGTCGGCAACAAC : 435
4DF1 : ACCCGCACGCTCCTCTGGACCTCGTCGGGAAAGGAGCAGCGTCTCACGTCACCTGGTCGGCTGATACTCCTGTGTGTCGGCAACAAC : 416
4DF2 : ACCCGCACGCTCCTCTGGACCTCGTCGGGAAAGGAGCAGCGTCTCACGTCACCTGGTCGGCTGATACTCCTGTGTGTCGGCAACAAC : 419
4DR1 : ACCCGCACGCTCCTCTGGACCTCGTCGGGAAAGGAGCAGCGTCTCACGTCACCTGGTCGGCTGATACTCCTGTGTGTCGGCAACAAC : 435
4DR2 : ACCCGCACGCTCCTCTGGACCTCGTCGGGAAAGGAGCAGCGTCTCACGTCACCTGGTCGGCTGATACTCCTGTGTGTCGGCAACAAC : 435
4FF1 : ACCCGCACGCTTCTCTGGACCTCGTCGGGAAAGGAGCAGCGTCTCACGTCACCTGGTCGGCTGATACTCCTGTGTGTCGGCAACAAC : 408
4FF2 : ACCCGCACGCTTCTCTGGACCTCGTCGGGAAAGGAGCAGCGTCTCACGTCACCTGGTCGGCTGATACTCCTGTGTGTCGGCAACAAC : 409
4FR1 : ACCCGCACGCTTCTCTGGACCTCGTCGGGAAAGGAGCAGCGTCTCACGTCACCTGGTCGGCTGATACTCCTGTGTGTCGGCAACAAC : 435
4FR2 : ACCCGCACGCTTCTCTGGACCTCGTCGGGAAAGGAGCAGCGTCTCACGTCACCTGGTCGGCTGATACTCCTGTGTGTCGGCAACAAC : 435
4GF1 : ACCCGCACGCTCCTCTGGACCTCGTCGGGAAAGGAGCAGCGTCTCACGTCACCTGGTCGGCTGATACTCCTGTGTGTCGGCAACAAC : 408
4GF2 : ACCCGCACGCTCCTCTGGACCTCGTCGGGAAAGGAGCAGCGTCTCACGTCACCTGGTCGGCTGATACTCCTGTGTGTCGGCAACAAC : 408
4GR1 : ACCCGCACGCTCCTCTGGACCTCGTCGGGAAAGGAGCAGCGTCTCACGTCACCTGGTCGGCTGATACTCCTGTGTGTCGGCAACAAC : 435
4GR2 : ACCCGCACGCTCCTCTGGACCTCGTCGGGAAAGGAGCAGCGTCTCACGTCACCTGGTCGGCTGATACTCCTGTGTGTCGGCAACAAC : 435
       ACCCGCACGCT CTCTGGACCTCGTCGGGAAAGGAGCAGCGTCTCACGTCACCTGGTCGGCTGATACTCCTGTGTGTCGGCAACAAC

          440         *       460         *       480         *       500         *       520
4BF1 : ACTGATGTGGTCAACGTGTCAGTACTGTGTCGCTGGAGTGTTCGACTGAGCGTTCCATCTCTTGAGACACCTGAAGAGACCACCGCT : 505
4BF2 : ACTGATGTGGTCAACGTGTCAGTACTGTGTCGCTGGAGTGTTCGACTGAGCGTTCCATCTCTTGAGACACCTGAAGAGACCACCGCT : 504
4BR1 : ACTGATGTGGTCAACGTGTCAGTACTGTGTCGCTGGAGTGTTCGACTGAGCGTTCCATCTCTTGAGACACCTGAAGAGACCACCGCT : 522
4BR2 : ACTGATGTGGTCAACGTGTCAGTACTGTGTCGCTGGAGTGTTCGACTGAGCGTTCCATCTCTTGAGACACCTGAAGAGACCACCGCT : 522
4DF1 : ACTGATGTGGTCAACGTGTCAGTACTGTGTCGCTGGAGTGTTCGACTGAGCGTTCCATCTCTTGAGACACCTGAAGAGACCACCGCT : 503
4DF2 : ACTGATGTGGTCAACGTGTCAGTACTGTGTCGCTGGAGTGTTCGACTGAGCGTTCCATCTCTTGAGACACCTGAAGAGACCACCGCT : 506
4DR1 : ACTGATGTGGTCAACGTGTCAGTACTGTGTCGCTGGAGTGTTCGACTGAGCGTTCCATCTCTTGAGACACCTGAAGAGACCACCGCT : 522
4DR2 : ACTGATGTGGTCAACGTGTCAGTACTGTGTCGCTGGAGTGTTCGACTGAGCGTTCCATCTCTTGAGACACCTGAAGAGACCACCGCT : 522
4FF1 : ACTGATGTGGTCAACGTGTCAGTACTGTGTCGCTGGAGTGTTCGACTGAGCGTTCCATCTCTTGAGACACCTGAAGAGACCACCGCT : 495
4FF2 : ACTGATGTGGTCAACGTGTCAGTACTGTGTCGCTGGAGTGTTCGACTGAGCGTTCCATCTCTTGAGACACCTGAAGAGACCACCGCT : 496
4FR1 : ACTGATGTGGTCAACGTGTCAGTACTGTGTCGCTGGAGTGTTCGACTGAGCGTTCCATCTCTTGAGACACCTGAAGAGACCACCGCT : 522
4FR2 : ACTGATGTGGTCAACGTGTCAGTACTGTGTCGCTGGAGTGTTCGACTGAGCGTTCCATCTCTTGAGACACCTGAAGAGACCACCGCT : 522
4GF1 : ACTGATGTGGTCAACGTGTCAGTACTGTGTCGCTGGAGTGTTCGACTGAGCGTTCCATCTCTTGAGACACCTGAAGAGACCACCGCT : 495
4GF2 : ACTGATGTGGTCAACGTGTCAGTACTGTGTCGCTGGAGTGTTCGACTGAGCGTTCCATCTCTTGAGACACCTGAAGAGACCACCGCT : 495
4GR1 : ACTGATGTGGTCAACGTGTCAGTACTGTGTCGCTGGAGTGTTCGACTGAGCGTTCCATCTCTTGAGACACCTGAAGAGACCACCGCT : 522
4GR2 : ACTGATGTGGTCAACGTGTCAGTACTGTGTCGCTGGAGTGTTCGACTGAGCGTTCCATCTCTTGAGACACCTGAAGAGACCACCGCT : 522
       ACTGATGTGGTCAACGTGTCAGTACTGTGTCGCTGGAGTGTTCGACTGAGCGTTCCATCTCTTGAGACACCTGAAGAGACCACCGCT

              *       540         *       560         *       580         *       600
4BF1 : CCCATCATGACACAAGGTTCCCTGTACAACGATTCCCTTTCCACAAATGACTTCAAGTCCATCCTCCTAGGATCCACACCACTGGAC : 592
4BF2 : CCCATCATGACACAAGGTTCCCTGTACAACGATTCCCTTTCCACAAATGACTTCAAGTCCATCCTCCTAGGATCCACACCACTGGAC : 591
4BR1 : CCCATCATGACACAAGGTTCCCTGTACAACGATTCCCTTTCCACAAATGACTTCAAGTCCATCCTCCTAGGATCCACACCACTGGAC : 609
4BR2 : CCCATCATGACACAAGGTTCCCTGTACAACGATTCCCTTTCCACAAATGACTTCAAGTCCATCCTCCTAGGATCCACACCACTGGAC : 609
4DF1 : CCCATCATGACACAAGGTTCCCTGTACAACGATTCCCTTTCCACAAATGACTTCAAGTCCATCCTCCTAGGATCCACACCACTGGAC : 590
4DF2 : CCCATCATGACACAAGGTTCCCTGTACAACGATTCCCTTTCCACAAATGACTTCAAGTCCATCCTCCTAGGATCCACACCACTGGAC : 593
4DR1 : CCCATCATGACACAAGGTTCCCTGTACAACGATTCCCTTTCCACAAATGACTTCAAGTCCATCCTCCTAGGATCCACACCACTGGAC : 609
4DR2 : CCCATCATGACACAAGGTTCCCTGTACAACGATTCCCTTTCCACAAATGACTTCAAGTCCATCCTCCTAGGATCCACACCACTGGAC : 609
4FF1 : CCCATCATGACACAAGGTTCCCTGTACAACGATTCCCTTTCCACAAATGACTTCAAGTCCATCCTCCTAGGATCCACACCACTGGAC : 582
4FF2 : CCCATCATGACACAAGGTTCCCTGTACAACGATTCCCTTTCCACAAATGACTTCAAGTCCATCCTCCTAGGATCCACACCACTGGAC : 583
4FR1 : CCCATCATGACACAAGGTTCCCTGTACAACGATTCCCTTTCCACAAATGACTTCAAGTCCATCCTCCTAGGATCCACACCACTGGAC : 609
4FR2 : CCCATCATGACACAAGGTTCCCTGTACAACGATTCCCTTTCCACAAATGACTTCAAGTCCATCCTCCTAGGATCCACACCACTGGAC : 609
4GF1 : CCCATCATGACACAAGGTTCCCTGTACAACGATTCCCTTTCCACAAATGACTTCAAGTCCATCCTCCTAGGATCCACACCACTGGAC : 582
4GF2 : CCCATCATGACACAAGGTTCCCTGTACAACGATTCCCTTTCCACAAATGACTTCAAGTCCATCCTCCTAGGATCCACACCACTGGAC : 582
4GR1 : CCCATCATGACACAAGGTTCCCTGTACAACGATTCCCTTTCCACAAATGACTTCAAGTCCATCCTCCTAGGATCCACACCACTGGAC : 609
4GR2 : CCCATCATGACACAAGGTTCCCTGTACAACGATTCCCTTTCCACAAATGACTTCAAGTCCATCCTCCTAGGATCCACACCACTGGAC : 609
       CCCATCATGACACAAGGTTCCCTGTACAACGATTCCCTTTCCACAAATGACTTCAAGTCCATCCTCCTAGGATCCACACCACTGGAC

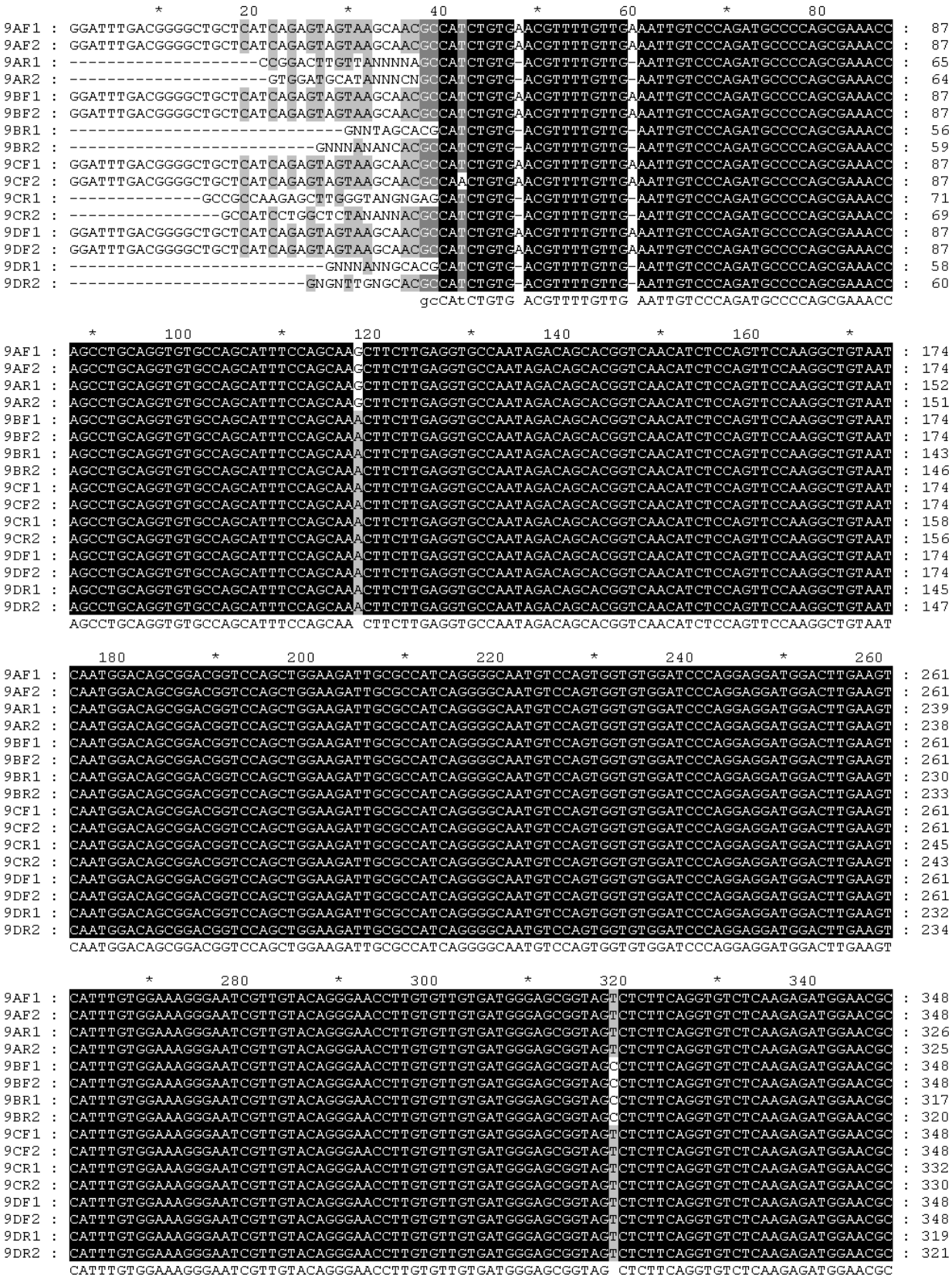
          *       620         *       640         *       660         *       680         *
4BF1 : ATTGCCCCTGATGGAGCAGTCTTCCAGCTGGACCGTCCGCTGTCCATTGACTACAGCCTTGGAACTGGAGATGTTGACCGTGCTGTT : 679
4BF2 : ATTGCCCCTGATGGAGCAGTCTTCCAGCTGGACCGTCCGCTGTCCATTGACTACAGCCTTGGAACTGGAGATGTTGACCGTGCTGTT : 678
4BR1 : ATTGCCCCTGATGGAGCAGTCTTCCAGCTGGACCGTCCGCTGTCCATTGACTACAGCCTTGGAACTGGAGATGTTGACCGTGCTGTT : 696
4BR2 : ATTGCCCCTGATGGAGCAGTCTTCCAGCTGGACCGTCCGCTGTCCATTGACTACAGCCTTGGAACTGGAGATGTTGACCGTGCTGTT : 696
4DF1 : ATTGCCCCTGATGGAGCAGTCTTCCAGCTGGACCGTCCGCTGTCCATTGACTACAGCCTTGGAACTGGAGATGTTGACCGTGCTGTT : 677
4DF2 : ATTGCCCCTGATGGAGCAGTCTTCCAGCTGGACCGTCCGCTGTCCATTGACTACAGCCTTGGAACTGGAGATGTTGACCGTGCTGTT : 680
4DR1 : ATTGCCCCTGATGGAGCAGTCTTCCAGCTGGACCGTCCGCTGTCCATTGACTACAGCCTTGGAACTGGAGATGTTGACCGTGCTGTT : 696
4DR2 : ATTGCCCCTGATGGAGCAGTCTTCCAGCTGGACCGTCCGCTGTCCATTGACTACAGCCTTGGAACTGGAGATGTTGACCGTGCTGTT : 696
4FF1 : ATTGCCCCTGATGGAGCAGTCTTCCAGCTGGACCGTCCGCTGTCCATTGACTACAGCCTTGGAACTGGAGATGTTGACCGTGCTGTT : 669
4FF2 : ATTGCCCCTGATGGAGCAGTCTTCCAGCTGGACCGTCCGCTGTCCATTGACTACAGCCTTGGAACTGGAGATGTTGACCGTGCTGTT : 670
4FR1 : ATTGCCCCTGATGGAGCAGTCTTCCAGCTGGACCGTCCGCTGTCCATTGACTACAGCCTTGGAACTGGAGATGTTGACCGTGCTGTT : 696
4FR2 : ATTGCCCCTGATGGAGCAGTCTTCCAGCTGGACCGTCCGCTGTCCATTGACTACAGCCTTGGAACTGGAGATGTTGACCGTGCTGTT : 696
4GF1 : ATTGCCCCTGATGGAGCAGTCTTCCAGCTGGACCGTCCGCTGTCCATTGACTACAGCCTTGGAACTGGAGATGTTGACCGTGCTGTT : 669
4GF2 : ATTGCCCCTGATGGAGCAGTCTTCCAGCTGGACCGTCCGCTGTCCATTGACTACAGCCTTGGAACTGGAGATGTTGACCGTGCTGTT : 669
4GR1 : ATTGCCCCTGATGGAGCAGTCTTCCAGCTGGACCGTCCGCTGTCCATTGACTACAGCCTTGGAACTGGAGATGTTGACCGTGCTGTT : 696
4GR2 : ATTGCCCCTGATGGAGCAGTCTTCCAGCTGGACCGTCCGCTGTCCATTGACTACAGCCTTGGAACTGGAGATGTTGACCGTGCTGTT : 696
       ATTGCCCCTGATGGAGCAGTCTTCCAGCTGGACCGTCCGCTGTCCATTGACTACAGCCTTGGAACTGGAGATGTTGACCGTGCTGTT
```

Appendix 1.2 (cont'd): BCNNV nodavirus isolate sequences obtained from the four plasmids. A, B, C and D; plasmids sequenced, F; sequence generated using the forward primer, R; sequence generated using the reverse primer.

```
         700         *       720         *       740         *       760         *       780
4BF1 : TACTGGCACCTCAAGAAGTTTGCTGGAAATGCTGGCACACCTGCAGGCTGGTTTCGCTGGGGCATCTGGGACAACTTCAACAAGACG : 766
4BF2 : TACTGGCACCTCAAGAAGTTTGCTGGAAATGCTGGCACACCTGCAGGCTGGTTTCGCTGGGGCATCTGGGACAACTTCAACAAGACG : 765
4BR1 : TACTGGCACCTCAAGAAGTTTGCTGGAAATGCTGGCACACCTGCAGGCTGGTTTCGCTGGGGCATCTGGGACAACTTCAACAAGACG : 783
4BR2 : TACTGGCACCTCAAGAAGTTTGCTGGAAATGCTGGCACACCTGCAGGCTGGTTTCGCTGGGGCATCTGGGACAACT-CAACAAGACG : 782
4DF1 : TACTGGCACCTCAAGAAGTTTGCTGGAAATGCTGGCACACCTGCAGGCTGGTTTCGCTGGGGCATCTGGGACAACTTCAACAAGACG : 764
4DF2 : TACTGGCACCTCAAGAAGTTTGCTGGAAATGCTGGCACACCTGCAGGCTGGTTTCGCTGGGGCATCTGGGACAACTTCAACAAGACG : 767
4DR1 : TACTGGCACCTCAAGAAGTTTGCTGGAAATGCTGGCACACCTGCAGGCTGGTTTCGCTGGGGCATCTGGGACAACT-CAACAAGACG : 782
4DR2 : TACTGGCACCTCAAGAAGTTTGCTGGAAATGCTGGCACACCTGCAGGCTGGTTTCGCTGGGGCATCTGGGACAACTTCAACAAGACG : 783
4FF1 : TACTGGCACCTCAAGAAGTTTGCTGGAAATGCTGGCACACCTGCAGGCTGGTTTCGCTGGGGCATCTGGGACAACTTCAACAAGACG : 756
4FF2 : TACTGGCACCTCAAGAAGTTTGCTGGAAATGCTGGCACACCTGCAGGCTGGTTTCGCTGGGGCATCTGGGACAACTTCAACAAGACG : 757
4FR1 : TACTGGCACCTCAAGAAGTTTGCTGGAAATGCTGGCACACCTGCAGGCTGGTTTCGCTGGGGCATCTGGGACAACT-CAACAAGACG : 782
4FR2 : TACTGGCACCTCAAGAAGTTTGCTGGAAATGCTGGCACACCTGCAGGCTGGTTTCGCTGGGGCATCTGGGACAACT-CAACAAGACG : 782
4GF1 : TACTGGCACCTCAAGAAGTTTGCTGGAAATGCTGGCACACCTGCAGGCTGGTTTCGCTGGGGCATCTGGGACAACTTCAACAAGACG : 756
4GF2 : TACTGGCACCTCAAGAAGTTTGCTGGAAATGCTGGCACACCTGCAGGCTGGTTTCGCTGGGGCATCTGGGACAACTTCAACAAGACG : 756
4GR1 : TACTGGCACCTCAAGAAGTTTGCTGGAAATGCTGGCACACCTGCAGGCTGGTTTCGCTGGGGCATCTGGGACAACT-CAACAAGACG : 782
4GR2 : TACTGGCACCTCAAGAAGTTTGCTGGAAATGCTGGCACACCTGCAGGCTGGTTTCGCTGGGGCATCTGGGACAACT-CAACAAGACG : 782
       TACTGGCACCTCAAGAAGTTTGCTGGAAATGCTGGCACACCTGCAGGCTGGTTTCGCTGGGGCATCTGGGACAACT CAACAAGACG

             *       800         *       820         *
4BF1 : TTCACAGATGGCGTTGCCTACTACTCTGATGAGCAGCCCCGTCAAATCC : 815
4BF2 : TTCACAGATGGCGTTGCCTACTACTCTGATGAGCAGCCCCGTCAAATCC : 814
4BR1 : T-CACAGATGCNNCNGNNNNAACTAGCCTATGGCA-------------- : 817
4BR2 : T-CACAGATGGCGNCNNTNNNTATAGCCGGCATA--------------- : 815
4DF1 : TTCACAGATGGCGTTGCCTACTACTCTGATGAGCAGCCCCGTCAAATCC : 813
4DF2 : TTCACAGATGGCGTTGCCTACTACTCTGATGAGCAGCCCCGTCAAATCC : 816
4DR1 : T-CACAGATGCNCNTNNTTCTCCGAG----------------------- : 807
4DR2 : T-CACAGATGGCGNCNNNNATAACACGCC-------------------- : 811
4FF1 : TTCACAGATGGCGTTGCCTACTACTCTGATGAGCAGCCCCGTCAAATCC : 805
4FF2 : TTCACAGATGGCGTTGCCTACTACTCTGATGAGCAGCCCCGTCAAATCC : 806
4FR1 : T-CACAGATGGCGTGCGAANCNC-------------------------- : 804
4FR2 : T-CACAGATGCGTGCTNATTNC-------------------------- : 803
4GF1 : TTCACAGATGGCGTTGCCTACTACTCTGATGAGCAGCCCCGTCAAATCC : 805
4GF2 : TTCACAGATGGCGTTGCCTACTACTCTGATGAGCAGCCCCGTCAAATCC : 805
4GR1 : T-CACAGATGAGTGCGATTCGG-------------------------- : 803
4GR2 : T-CACAGATGGCGTGCGAANGG-------------------------- : 803
       T CACAGATG
```

Appendix 1.3: SCNNV nodavirus isolate sequences obtained from the four plasmids. A, B, C and D; plasmids sequenced, F; sequence generated using the forward primer, R; sequence generated using the reverse primer.



Appendix 1.3 (cont'd): SCNNV nodavirus isolate sequences obtained from the four plasmids. A, B, C and D; plasmids sequenced, F; sequence generated using the forward primer, R; sequence generated using the reverse primer.

```
          *       360         *       380         *       400         *       420         *
9AF1 : TCAGTCGAACACTCCAGCGACACAGCACCGAAACGTTGACCACATCAGTGTTGTTGCCGGCACACAGGAGTATCAGCCGACCAGGTG : 435
9AF2 : TCAGTCGAACACTCCAGCGACACAGCACCGAAACGTTGACCACATCAGTGTTGTTGCCGGCACACAGGAGTATCAGCCGACCAGGTG : 435
9AR1 : TCAGTCGAACACTCCAGCGACACAGCACCGAAACGTTGACCACATCAGTGTTGTTGCCGGCACACAGGAGTATCAGCCGACCAGGTG : 413
9AR2 : TCAGTCGAACACTCCAGCGACACAGCACCGAAACGTTGACCACATCAGTGTTGTTGCCGGCACACAGGAGTATCAGCCGACCAGGTG : 412
9BF1 : TCAGTCGAACACTCCAGCGACACAGCACCGAAACGTTGACCACATCAGTGTTGTTGCCGACACACAGGAGTATCAGCCGACCAGGTG : 435
9BF2 : TCAGTCGAACACTCCAGCGACACAGCACCGAAACGTTGACCACATCAGTGTTGTTGCCGACACACAGGAGTATCAGCCGACCAGGTG : 435
9BR1 : TCAGTCGAACACTCCAGCGACACAGCACCGAAACGTTGACCACATCAGTGTTGTTGCCGACACACAGGAGTATCAGCCGACCAGGTG : 404
9BR2 : TCAGTCGAACACTCCAGCGACACAGCACCGAAACGTTGACCACATCAGTGTTGTTGCCGACACACAGGAGTATCAGCCGACCAGGTG : 407
9CF1 : TCAGTCGAACACTCCAGCGACACAGCACCGAAACGTTGACCACATCAGTGTTGTTGCCGACACACAGGAGTATCAGCCGACCAGGTG : 435
9CF2 : TCAGTCGAACACTCCAGCGACACAGCACCGAAACGTTGACCACATCAGTGTTGTTGCCGACACACAGGAGTATCAGCCGACCAGGTG : 435
9CR1 : TCAGTCGAACACTCCAGCGACACAGCACCGAAACGTTGACCACATCAGTGTTGTTGCCGACACACAGGAGTATCAGCCGACCAGGTG : 419
9CR2 : TCAGTCGAACACTCCAGCGACACAGCACCGAAACGTTGACCACATCAGTGTTGTTGCCGACACACAGGAGTATCAGCCGACCAGGTG : 417
9DF1 : TCAGTCGAACACTCCAGCGACACAGCACCGAAACGTTGACCACATCAGTGTTGTTGCCGACACACAGGAGTATCAGCCGACCAGGTG : 435
9DF2 : TCAGTCGAACACTCCAGCGACACAGCACCGAAACGTTGACCACATCAGTGTTGTTGCCGACACACAGGAGTATCAGCCGACCAGGTG : 435
9DR1 : TCAGTCGAACACTCCAGCGACACAGCACCGAAACGTTGACCACATCAGTGTTGTTGCCGACACACAGGAGTATCAGCCGACCAGGTG : 406
9DR2 : TCAGTCGAACACTCCAGCGACACAGCACCGAAACGTTGACCACATCAGTGTTGTTGCCGACACACAGGAGTATCAGCCGACCAGGTG : 408
       TCAGTCGAACACTCCAGCGACACAGCACCGAAACGTTGACCACATCAGTGTTGTTGCCG CACACAGGAGTATCAGCCGACCAGGTG

          440         *       460         *       480         *       500         *       520
9AF1 : ACGTGAGACGCTGCTCCTTTCCCGACGAGGTCCAGAGGAGCGTGCGGGTGTACTGCCGTCGGACTGTTCTGCTTTCCCACCATTTGG : 522
9AF2 : ACGTGAGACGCTGCTCCTTTCCCGACGAGGTCCAGAGGAGCGTGCGGGTGTACTGCCGTCGGACTGTTCTGCTTTCCCACCATTTGG : 522
9AR1 : ACGTGAGACGCTGCTCCTTTCCCGACGAGGTCCAGAGGAGCGTGCGGGTGTACTGCCGTCGGACTGTTCTGCTTTCCCACCATTTGG : 500
9AR2 : ACGTGAGACGCTGCTCCTTTCCCGACGAGGTCCAGAGGAGCGTGCGGGTGTACTGCCGTCGGACTGTTCTGCTTTCCCACCATTTGG : 499
9BF1 : ACGTGAGACGCTGCTCCTTTCCCGACGAGGTCCAGAGGAGCGTGCGGGTGTACTGTAGTCGGACTGTTCTGCTTTCCCACCATTTGG : 522
9BF2 : ACGTGAGACGCTGCTCCTTTCCCGACGAGGTCCAGAGGAGCGTGCGGGTGTACTGTAGTCGGACTGTTCTGCTTTCCCACCATTTGG : 522
9BR1 : ACGTGAGACGCTGCTCCTTTCCCGACGAGGTCCAGAGGAGCGTGCGGGTGTACTGTAGTCGGACTGTTCTGCTTTCCCACCATTTGG : 491
9BR2 : ACGTGAGACGCTGCTCCTTTCCCGACGAGGTCCAGAGGAGCGTGCGGGTGTACTGTAGTCGGACTGTTCTGCTTTCCCACCATTTGG : 494
9CF1 : ACGTGAGACGCTGCTCCCTTCCCGACGAGGTCCAGAGGAGCGTGCGGGTGTACTGTGGTCGGACTGTTCTGCTTTCCCACCATTTGG : 522
9CF2 : ACGTGAGACGCTGCTCCCTTCCCGACGAGGTCCAGAGGAGCGTGCGGGTGTACTGTGGTCGGACTGTTCTGCTTTCCCACCATTTGG : 522
9CR1 : ACGTGAGACGCTGCTCCCTTCCCGACGAGGTCCAGAGGAGCGTGCGGGTGTACTGTGGTCGGACTGTTCTGCTTTCCCACCATTTGG : 506
9CR2 : ACGTGAGACGCTGCTCCCTTCCCGACGAGGTCCAGAGGAGCGTGCGGGTGTACTGTGGTCGGACTGTTCTGCTTTCCCACCATTTGG : 504
9DF1 : ACGTGAGACGCTGCTCCTTTCCCGACGAGGTCCAGAGGAGCGTGCGGGTGTACTGTGGTCGGACTGTTCTGCTTTCCCACCATTTGG : 522
9DF2 : ACGTGAGACGCTGCTCCTTTCCCGACGAGGTCCAGAGGAGCGTGCGGGTGTACTGTGGTCGGACTGTTCTGCTTTCCCACCATTTGG : 522
9DR1 : ACGTGAGACGCTGCTCCTTTCCCGACGAGGTCCAGAGGAGCGTGCGGGTGTACTGTGGTCGGACTGTTCTGCTTTCCCACCATTTGG : 493
9DR2 : ACGTGAGACGCTGCTCCTTTCCCGACGAGGTCCAGAGGAGCGTGCGGGTGTACTGTGGTCGGACTGTTCTGCTTTCCCACCATTTGG : 495
       ACGTGAGACGCTGCTCC TTCCCGACGAGGTCCAGAGGAGCGTGCGGGTGTACTG  GTCGGACTGTTCTGCTTTCCCACCATTTGG

              *       540         *       560         *       580         *       600
9AF1 : CAACGACTGCACCACGAGTTGCTTGAAGCGCGTCGAAGGTGTGATCGTTGTCAGTTGGATCAGGCAGGAAGCCAGCAACGTAACCAC : 609
9AF2 : CAACGACTGCACCACGAGTTGCTTGAAGCGCGTCGAAGGTGTGATCGTTGTCAGTTGGATCAGGCAGGAAGCCAGCAACGTAACCAC : 609
9AR1 : CAACGACTGCACCACGAGTTGCTTGAAGCGCGTCGAAGGTGTGATCGTTGTCAGTTGGATCAGGCAGGAAGCCAGCAACGTAACCAC : 587
9AR2 : CAACGACTGCACCACGAGTTGCTTGAAGCGCGTCGAAGGTGTGATCGTTGTCAGTTGGATCAGGCAGGAAGCCAGCAACGTAACCAC : 586
9BF1 : CAACGACTGCACCACGAGTTGCTTGAAGCGCGTCGAAGGTGTGATCGTTGTCAGTTGGATCAGGCAGGAAGCCAGCAACGTAACCAC : 609
9BF2 : CAACGACTGCACCACGAGTTGCTTGAAGCGCGTCGAAGGTGTGATCGTTGTCAGTTGGATCAGGCAGGAAGCCAGCAACGTAACCAC : 609
9BR1 : CAACGACTGCACCACGAGTTGCTTGAAGCGCGTCGAAGGTGTGATCGTTGTCAGTTGGATCAGGCAGGAAGCCAGCAACGTAACCAC : 578
9BR2 : CAACGACTGCACCACGAGTTGCTTGAAGCGCGTCGAAGGTGTGATCGTTGTCAGTTGGATCAGGCAGGAAGCCAGCAACGTAACCAC : 581
9CF1 : CAACGACTGCACCACGAGTTGCTTGAAGCGCGTCGAAGGTGTGATCGTTGTCAGTTGGATCAGGCAGGAAGCCAGCAACGTAACCAC : 609
9CF2 : CAACGACTGCACCACGAGTTGCTTGAAGCGCGTCGAAGGTGTGATCGTTGTCAGTTGGATCAGGCAGGAAGCCAGCAACGTAACCAC : 609
9CR1 : CAACGACTGCACCACGAGTTGCTTGAAGCGCGTCGAAGGTGTGATCGTTGTCAGTTGGATCAGGCAGGAAGCCAGCAACGTAACCAC : 593
9CR2 : CAACGACTGCACCACGAGTTGCTTGAAGCGCGTCGAAGGTGTGATCGTTGTCAGTTGGATCAGGCAGGAAGCCAGCAACGTAACCAC : 591
9DF1 : CAACGACTGCACCACGAGTTGCTTGAAGCGCGTCGAAGGTGTGATCGTTGTCAGTTGGATCAGGCAGGAAGCCAGCAACGTAACCAC : 609
9DF2 : CAACGACTGCACCACGAGTTGCTTGAAGCGCGTCGAAGGTGTGATCGTTGTCAGTTGGATCAGGCAGGAAGCCAGCAACGTAACCAC : 609
9DR1 : CAACGACTGCACCACGAGTTGCTTGAAGCGCGTCGAAGGTGTGATCGTTGTCAGTTGGATCAGGCAGGAAGCCAGCAACGTAACCAC : 580
9DR2 : CAACGACTGCACCACGAGTTGCTTGAAGCGCGTCGAAGGTGTGATCGTTGTCAGTTGGATCAGGCAGGAAGCCAGCAACGTAACCAC : 582
       CAACGACTGCACCACGAGTTGCTTGAAGCGCGTCGAAGGTGTGATCGTTGTCAGTTGGATCAGGCAGGAAGCCAGCAACGTAACCAC

          *       620         *       640         *       660         *       680         *
9AF1 : CGCCCGTGTTTGCGGGGCACATTGGCTGAATTTCAAACTCCAGTGTTTCAACAGCGTATCGCTGGAAGATTCTAGCAGCGTGTCCCA : 696
9AF2 : CGCCCGTGTTTGCGGGGCACATTGGCTGAATTTCAAACTCCAGTGTTTCAACAGCGTATCGCTGGAAGATTCTAGCAGCGTGTCCCA : 696
9AR1 : CGCCCGTGTTTGCGGGGCACATTGGCTGAATTTCAAACTCCAGTGTTTCAACAGCGTATCGCTGGAAGATTCTAGCAGCGTGTCCCA : 674
9AR2 : CGCCCGTGTTTGCGGGGCACATTGGCTGAATTTCAAACTCCAGTGTTTCAACAGCGTATCGCTGGAAGATTCTAGCAGCGTGTCCCA : 673
9BF1 : CGCCCGTGTTTGCGGGGCACATTGGCTGAATTTCAAACTCCAGTGTTTCAACAGCGTATCGCTGGAAGATTCTAGCAGCGTGTCCCA : 696
9BF2 : CGCCCGTGTTTGCGGGGCACATTGGCTGAATTTCAAACTCCAGTGTTTCAACAGCGTATCGCTGGAAGATTCTAGCAGCGTGTCCCA : 696
9BR1 : CGCCCGTGTTTGCGGGGCACATTGGCTGAATTTCAAACTCCAGTGTTTCAACAGCGTATCGCTGGAAGATTCTAGCAGCGTGTCCCA : 665
9BR2 : CGCCCGTGTTTGCGGGGCACATTGGCTGAATTTCAAACTCCAGTGTTTCAACAGCGTATCGCTGGAAGATTCTAGCAGCGTGTCCCA : 668
9CF1 : CGCCCGTGTTTGCGGGGCACATTGGCTGAATTTCAAACTCCAGTGTTTCAACAGCGTATCGCTGGAAGATTCTAGCAGCGTGTCCCA : 696
9CF2 : CGCCCGTGTTTGCGGGGCACATTGGCTGAATTTCAAACTCCAGTGTTTCAACAGCGTATCGCTGGAAGATTCTAGCAGCGTGTCCCA : 696
9CR1 : CGCCCGTGTTTGCGGGGCACATTGGCTGAATTTCAAACTCCAGTGTTTCAACAGCGTATCGCTGGAAGATTCTAGCAGCGTGTCCCA : 680
9CR2 : CGCCCGTGTTTGCGGGGCACATTGGCTGAATTTCAAACTCCAGTGTTTCAACAGCGTATCGCTGGAAGATTCTAGCAGCGTGTCCCA : 678
9DF1 : CGCCCGTGTTTGCGGGGCACATTGGCTGAATTTCAAACTCCAGTGTTTCAACAGCGTATCGCTGGAAGATTCTAGCAGCGTGTCCCA : 696
9DF2 : CGCCCGTGTTTGCGGGGCACATTGGCTGAATTTCAAACTCCAGTGTTTCAACAGCGTATCGCTGGAAGATTCTAGCAGCGTGTCCCA : 696
9DR1 : CGCCCGTGTTTGCGGGGCACATTGGCTGAATTTCAAACTCCAGTGTTTCAACAGCGTATCGCTGGAAGATTCTAGCAGCGTGTCCCA : 667
9DR2 : CGCCCGTGTTTGCGGGGCACATTGGCTGAATTTCAAACTCCAGTGTTTCAACAGCGTATCGCTGGAAGATTCTAGCAGCGTGTCCCA : 669
       CGCCCGTGTTTGCGGGGCACATTGGCTGAATTTCAAACTCCAGTGTTTCAACAGCGTATCGCTGGAAGATTCTAGCAGCGTGTCCCA
```

Appendix 1.3 (cont'd): SCNNV nodavirus isolate sequences obtained from the four plasmids. A, B, C and D; plasmids sequenced, F; sequence generated using the forward primer, R; sequence generated using the reverse primer.

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        700         *       720         *       740         *       760         *       780
9AF1 : GTCGCGGCAGGAGGTCGGGAACGATGGTTGCGTCAACAACAACGTATCCGTCTGTTCCTGTCCCGGCTGGGAGGACGGCCTGGGAGA : 783
9AF2 : GTCGCGGCAGGAGGTCGGGAACGATGGTTGCGTCAACAACAACGTATCCGTCTGTTCCTGTCCCGGCTGGGAGGACGGCCTGGGAGA : 783
9AR1 : GTCGCGGCAGGAGGTCGGGAACGATGGTTGCGTCAACAACAACGTATCCGTCTGTTCCTGTCCCGGCTGGGAGGACGGCCTGGGAGA : 761
9AR2 : GTCGCGGCAGGAGGTCGGGAACGATGGTTGCGTCAACAACAACGTATCCGTCTGTTCCTGTCCCGGCTGGGAGGACGGCCTGGGAGA : 760
9BF1 : GTCGCGGCAGGAGGTCGGGAACGATGGTTGCGTCAACAACAACGTATCCGTCTGTTCCTGTCCCGGCTGGGAGGACGGCCTGGGAGA : 783
9BF2 : GTCGCGGCAGGAGGTCGGGAACGATGGTTGCGTCAACAACAACGTATCCGTCTGTTCCTGTCCCGGCTGGGAGGACGGCCTGGGAGA : 783
9BR1 : GTCGCGGCAGGAGGTCGGGAACGATGGTTGCGTCAACAACAACGTATCCGTCTGTTCCTGTCCCGGCTGGGAGGACGGCCTGGGAGA : 752
9BR2 : GTCGCGGCAGGAGGTCGGGAACGATGGTTGCGTCAACAACAACGTATCCGTCTGTTCCTGTCCCGGCTGGGAGGACGGCCTGGGAGA : 755
9CF1 : GTCGCGGCAGGAGGTCGGGAACGATGGTTGCGTCAACAACAACGTATCCGTCTGTTCCTGTCCCGGCTGGGAGGACGGCCTGGGAGA : 783
9CF2 : GTCGCGGCAGGAGGTCGGGAACGATGGTTGCGTCAACAACAACGTATCCGTCTGTTCCTGTCCCGGCTGGGAGGACGGCCTGGGAGA : 783
9CR1 : GTCGCGGCAGGAGGTCGGGAACGATGGTTGCGTCAACAACAACGTATCCGTCTGTTCCTGTCCCGGCTGGGAGGACGGCCTGGGAGA : 767
9CR2 : GTCGCGGCAGGAGGTCGGGAACGATGGTTGCGTCAACAACAACGTATCCGTCTGTTCCTGTCCCGGCTGGGAGGACGGCCTGGGAGA : 765
9DF1 : GTCGCGGCAGGAGGTCGGGAACGATGGTTGCGTCAACAACAACGTATCCGTCTGTTCCTGTCCCGGCTGGGAGGACGGCCTGGGAGA : 783
9DF2 : GTCGCGGCAGGAGGTCGGGAACGATGGTTGCGTCAACAACAACGTATCCGTCTGTTCCTGTCCCGGCTGGGAGGACGGCCTGGGAGA : 783
9DR1 : GTCGCGGCAGGAGGTCGGGAACGATGGTTGCGTCAACAACAACGTATCCGTCTGTTCCTGTCCCGGCTGGGAGGACGGCCTGGGAGA : 754
9DR2 : GTCGCGGCAGGAGGTCGGGAACGATGGTTGCGTCAACAACAACGTATCCGTCTGTTCCTGTCCCGGCTGGGAGGACGGCCTGGGAGA : 756
       GTCGCGGCAGGAGGTCGGGAACGATGGTTGCGTCAACAACAACGTATCCGTCTGTTCCTGTCCCGGCTGGGAGGACGGCCTGGGAGA

            *       800         *       820         *
9AF1 : T-CTCGACANCTAGNNNNNNNTAATTCGCCCG---------------- : 814
9AF2 : T-CTCNACANCTNGNNNNNNTNNTAAAACGGCCG--------------- : 816
9AR1 : TTCTCGACATACCTGAGAGATGGACGTCATTGGTCGCACGTCCAAATCC : 810
9AR2 : TTCTCGACATACCTGAGAGATGGACGTCATTGGTCGCACGTCCAAATCC : 809
9BF1 : T-CTCGACATACTAGAGAGNCGAGT------------------------ : 807
9BF2 : T-CTCGACATACTAGAATCGAG--------------------------- : 804
9BR1 : TTCTCGACATACCTGAGAGATGGACGTCATTGGTCGCACGTCCAAATCC : 801
9BR2 : TTCTCGACATACCTGAGAGATGGACGTCATTGGTCGCACGTCCAAATCC : 804
9CF1 : T-CTCGACANCTNANNNNCNNNTAACGGCCANT---------------- : 815
9CF2 : TTCNTCGGACA------------------------------------- : 794
9CR1 : TTCTCGACATACCTGAGAGATGGACGTCATTGGTCGCACGTCCAAATCC : 816
9CR2 : TTCTCGACATACCTGAGAGATGGACGTCATTGGTCGCACGTCCAAATCC : 814
9DF1 : T-CTCGACATACTAGAAGNCGAGT------------------------ : 806
9DF2 : T-CTCGACATACTAGAANTNGGAGT----------------------- : 807
9DR1 : TTCTCGACATACCTGAGAGATGGACGTCATTGGTCGCACGTCCAAATCC : 803
9DR2 : TTCTCGACATACCTGAGAGATGGACGTCATTGGTCGCACGTCCAAATCC : 805
       T CtcgacA a
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Appendix 2: Comparison of endemic and exotic nodavirus coat protein sequences

Appendix 2.1: Multiple nucleotide sequence alignment of the consensus partial coat protein sequence of the three Australian nodavirus isolates with the exotic nodavirus isolates.

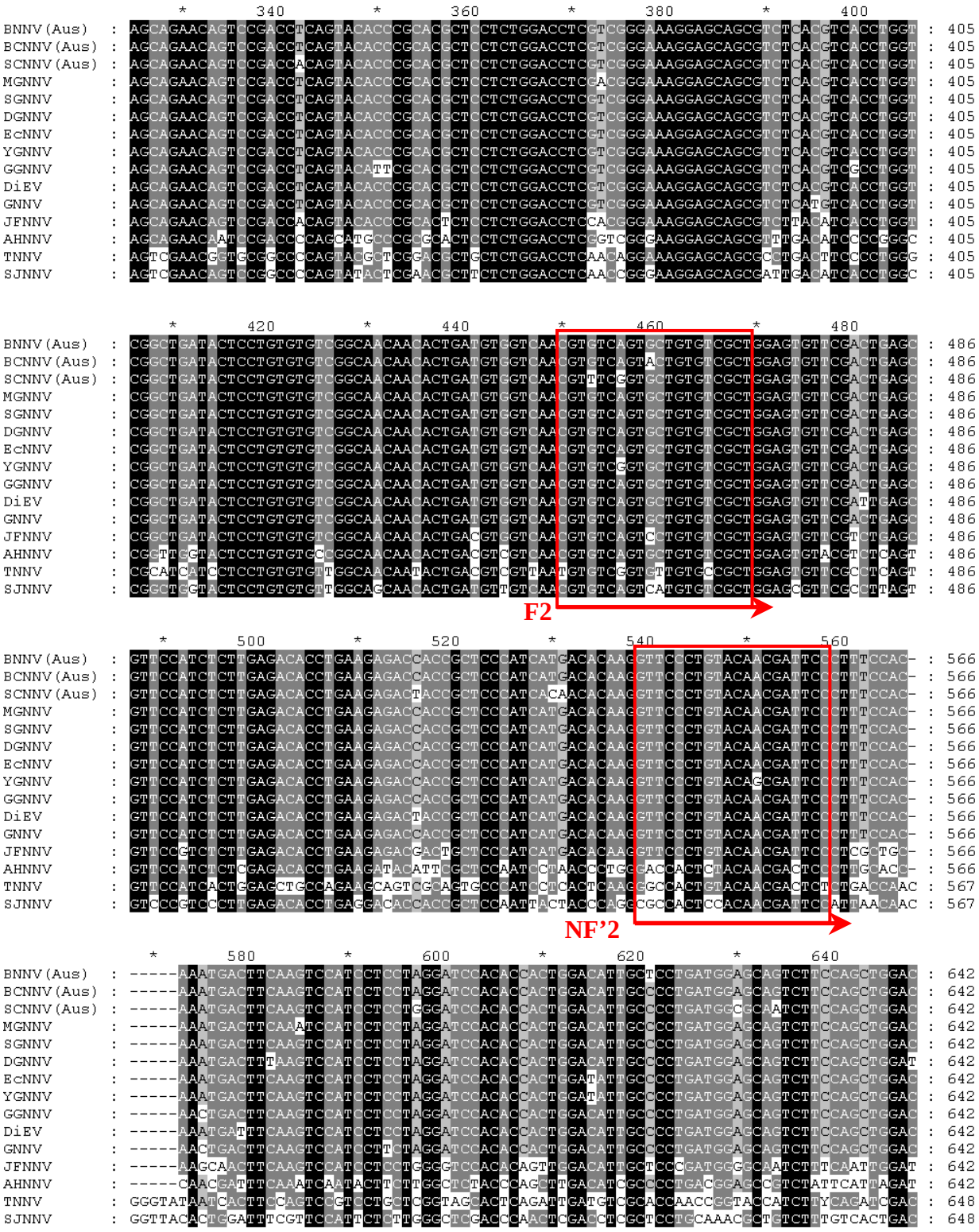
```
                    *         20         *         40         *         60         *         80
BNNV(Aus)   : GGATTTGGACGTGCGACCAATGACGTCCATCTCTCAGGTATGTCGAGAATCTCCCAGGCCGTCCTCCCAGCCGGGACAGGA : 81
BCNNV(Aus)  : GGATTTGGACGTGCGACCAATGACGTCCATCTCTCAGGTATGTCGAGAATCTCCCAGGCCGTCCTCCCAGCCGGGACAGGA : 81
SCNNV(Aus)  : GGATTTGGACGTGCGACCAATGACGTCCATCTCTCAGGTATGTCGAGAATCTCCCAGGCCGTCCTCCCAGCCGGGACAGGA : 81
MGNNV       : GGATTTGGACGTGGGACCAATGACGTCCATCTCTCAGGTATGTCGAGAATCTCCCAGGCCGTCCTCCCAGCCGGGACAGGA : 81
SGNNV       : GGATTTGGACGTGGGACCAATGACGTCCATCTCTCAGGTATGTCGAGAATCTCCCAGGCCGTCCTCCCAGCCGGGACAGGA : 81
DGNNV       : GGATTTGGACGTGGGACCAATGACGTCCATCTCTCAGGTATGTCGAGAATCTCCCAGGCCGTCCTCCCAGCCGGGACAGGA : 81
EcNNV       : GGATTTGGACGTGGGACCAATGACGTCCATCTCTCAGGTATGTCGAGAATCTCCCAGGCCGTCCTCCCAGCCGGGACAGGA : 81
YGNNV       : GGATTTGGACGTGCGACCAATGACGTCCATCTCTCAGGTATGTCGAGAATCTCCCAGGCCGTCCTCCCAGCCGGGACAGGA : 81
GGNNV       : GGATTCGGACGTGGGACCAATGACGTCCATCTCTCAGGTATGTCGAGAATCTCCCAGGCCGTCCTCCCAGCCGGGACAGGA : 81
DiEV        : GGATTTGGACGTGGGACCAATGACGTCCATCTCTCAGGTATGTCGAGAATCTCCCAGGCCGTCCTCCCAGCCGGGACAGGA : 81
GNNV        : GGATTCGGACGTGGGACCAATGAGTCTATCTCTCAGGTATGTCGAGAATCTCCCAGGCCGTCCTCCCAGCCGGGACAGGA : 81
JFNNV       : GGATTTGGACGTGGGACCAATGATGTCCATCTCTCAGGTATATCGAGAATCTCCCAGGCCGTTCTCCCAGCCGGGACAGGA : 81
AHNNV       : GGATTTGGACGTGGGACCAATGACGTCCATCTCACGGGTATGTCGAGAATCGCCCAAGCGGTTATCCCAGCTGGCACCGGC : 81
TNNV        : GGATTTGGACGTCCGACCAATGATGTCCACTCTCTCGGGTATGTCGAGAATTGCCCAGGCGGTCATCCCAGCMGGGACCGGA : 81
SJNNV       : GGATTTGGACGTGCGACCAATGATGTCCATATCTCGGGAATGTCACGCATCGCTCAAGCAGTTGTTCCAGCCGGGACAGGA : 81

                    *         100        *         120        *         140        *         160
BNNV(Aus)   : ACAGACGGATACGTTGTTGTTGATGCAACCATCGTCCCCGACCTCCTGCCACGACTGGGACACGCTGCTAGAATCTTCCAG : 162
BCNNV(Aus)  : ACAGACGGATACGTTGTTGTTGATGCAACCATCGTCCCCGACCTCCTGCCACGACTGGGACACGCTGCTAGAATCTTCCAG : 162
SCNNV(Aus)  : ACAGACGGATACGTTGTTGTTGACGCAACCATCGTTCCCGACCTCCTGCCGCGACTGGGACACGCTGCTAGAATCTTCCAG : 162
MGNNV       : ACAGACGGATACGTTGTTGTTGATGCAACCATCGTCCCCGACCTCCTGCCACGACTGGGACACGCTGCTAGAATCTTCCAG : 162
SGNNV       : ACAGACGGATACGTTGTTGTTGACGCAACCATCGTCCCCGACCTCCTGCCACGACTGGGACACGCTGCTAGAATCTTCCAG : 162
DGNNV       : ACAGACGGATACGTTGTTGTTGACGCAACCATCGTCCCCGACCTCCTGCCACGACTGGGACACGCTGCTAGAATCTTCCAG : 162
EcNNV       : ACTGACGGATACGTTGTCGTTGACGCAACCATCGTCCCCGACCTCCTGCCACGACTGGGACACGCTGCTAGAATCTTCCAG : 162
YGNNV       : ACTGACGGATACGTTGTTGTTGACGCAACCATCGTCCCCGACCTCCTGCCACGACTGGGACACGCTGCTAGAATCTTCCAG : 162
GGNNV       : ACAGACGGATACGTTGTTGTTGACGCAACCATCGTCCCCGACCTCCTGCCACGACTGGGACACGCTGCTAGAATCTTCCAG : 162
DiEV        : ACAGACGGATACGTTGTTGTTGACGCAACCATCGTCCCCGACCTCCTGCCACGACTGGGACACGCTGCTAGAATCTTCCAG : 162
GNNV        : ACAGACGGATACGTTGTTGTTGACGCAACCATCGTCCCCGACCTCCTGCCACGACTGGGACACGCTGCTAGAATCTTCCAG : 162
JFNNV       : ACTGACGGATACCTTGTTGTTGACGCAACTATCGTCCCCGAACTCCTGCCACGACTGGGACACGCTGCTAGAATCTTCCAG : 162
AHNNV       : ACGGACGGATACATCGTGGTTGACCAAACCATCGTCCCCGAGCTCTTGCCAAGACTGGGATTTGCTGCTAGAATCTTCCAG : 162
TNNV        : ACTGACGGACATGTCGTCCTTGACCAGATCATTGTCCCTGAGCTCCTGCCACGACTGGGACATGCTAGCCGAATCTTCCAG : 162
SJNNV       : ACAGATGGAAAGATTGTCGTCGATTCCACAATCGTTCCAGAACTCCTGCCACGGCTTGGACACGCTGCTCGAATCTTCCAG : 162

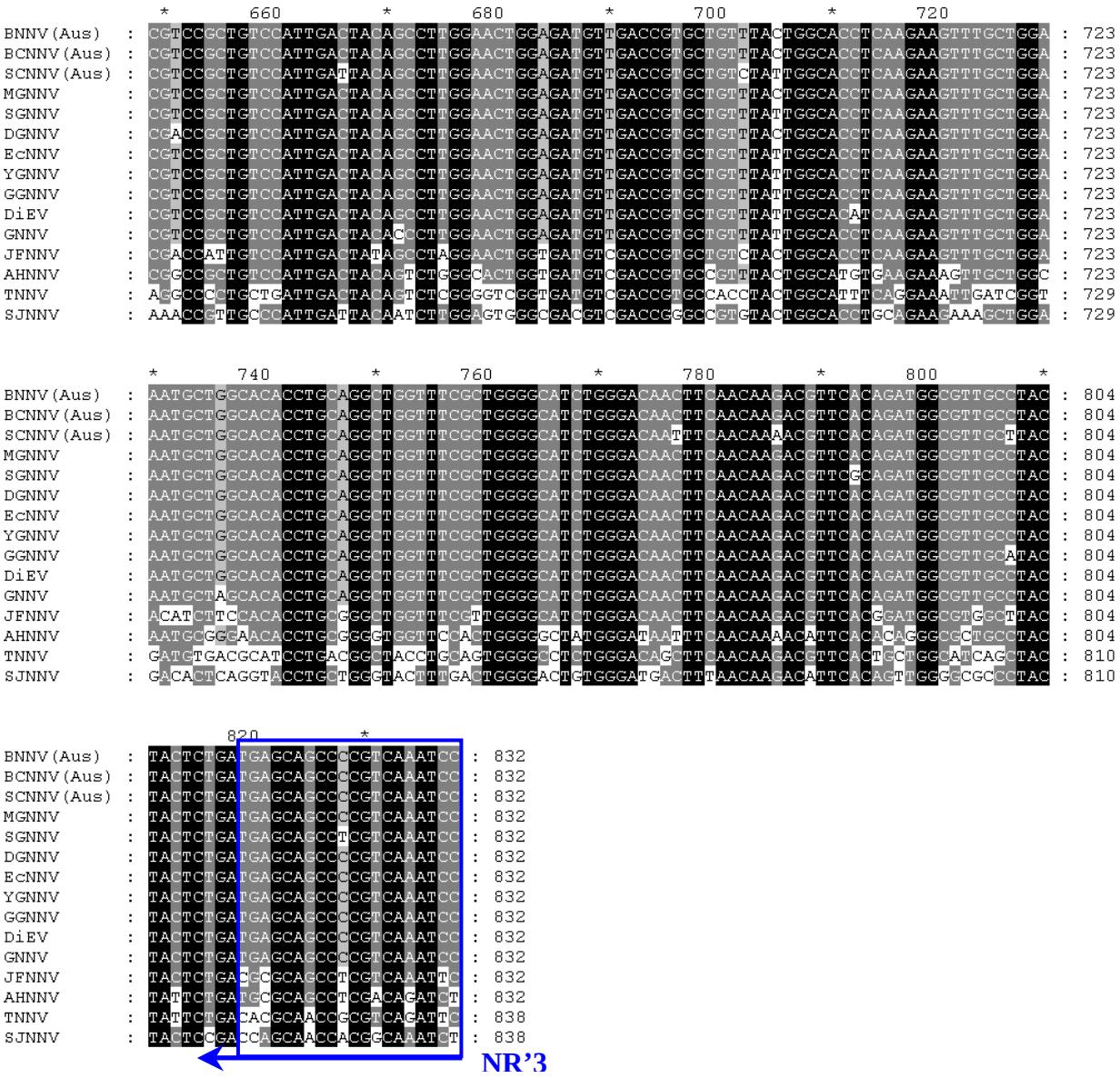
                    *         180        *         200        *         220        *         240
BNNV(Aus)   : CGATACGCTGTTGAAACACTGGAGTTTGAAATTCAGCCAATGTGCCCCGCAAACACGGGCGGTGGTTACGTTGCTGGCTTC : 243
BCNNV(Aus)  : CGATACGCTGTTGAAACACTGGAGTTTGAAATTCAGCCAATGTGCCCCGCAAACACGGGCGGTGGTTACGTTGCTGGCTTC : 243
SCNNV(Aus)  : CGATACGCTGTTGAAACACTGGAGTTTGAAATTCAGCCAATGTGCCCCGCAAACACGGGCGGTGGTTACGTTGCTGGCTTC : 243
MGNNV       : CGATACGCTGTTGAAGCACTGGAGTTTGAAATTCAGCCAATGTGCCCCGCAAACACGGGCGGTGGTTACGTTGCTGGCTTC : 243
SGNNV       : CGATACGCTGTTGAAACACTGGAGTTTGAAATTCAGCCAATGTGCCCCGCAAACACGGGCGGTGGTTACGTTGCTGGCTTC : 243
DGNNV       : CGATACGCTGTTGAAACACTGGAGTTTGAAATTCAGCCAATGTGCCCCGCAAACACGGGCGGTGGTTACGTTGCTGGCTTC : 243
EcNNV       : CGATACGCTGTTGAAACACTGGAGTTTGAAATTCAGCCAATGTGCCCCGCAAACACGGGCGGTGGTTACGTTGCTGGCTTC : 243
YGNNV       : CGATACGCTGTTGAAACACTGGAGTTTGAAATTCAGCCAATGTGCCCCGCAAACACGGGCGGTGGTTACGTTGCTGGCTTC : 243
GGNNV       : CGATACGCTGTTGAAACACTGGAGTTTGAAATTCAGCCAATGTGCCCCGCAAACACGGGCGGTGGTTACGTTGCTGGCTTC : 243
DiEV        : CGATACGCTGTTGAAACACTGGAGTTTGAAATTCAGCCAATGTGCCCCGCAAACACGGGCGGTGGTTACGTTGCTGGCTTC : 243
GNNV        : CGATACGCTGTTGAAACACTGGAGTTTGAAATTCAGCCAATGTGCCCCGCAAACACGGGCGGTGGTTACGTTGCTGGCTTC : 243
JFNNV       : CGATACGCTGTTGAAACACTGGAGTTCGAAATTCAGCCAATGTGCCCCGCAAACACGGGCGGTGGTTACGTTGCTGGCTTC : 243
AHNNV       : CGATACGCTGTTGAGACACTGGAGTTCGAAATTCAGCCAATGTGCCCCGCAAACACGGGCGGTGGTTACGTGGCTGGCTTC : 243
TNNV        : CGATACATTGTTGACACACTGGAGTTCGAAATTCAGCCAATGTGCCCCGCAAACACGGGCGGTGGTTACGTTGCTGGCTTC : 243
SJNNV       : CGATACGCTGTTGAAACACTGGAGTTCGAAATTCAGCCAATGTGCCCCGCAAACACGGGCGGTGGTTACGTTGCTGGCTTC : 243

                    *         260        *         280        *         300        *         320
BNNV(Aus)   : CTGCCTGATCCAACTGACAACGATCACACCTTCGACGCGCTTCAAGCAACTCGTGGTGCAGTCGTTGCCAAATGGTGGGAA : 324
BCNNV(Aus)  : CTGCCTGATCCAACTGACAACGATCACACCTTCGACGCGCTTCAAGCAACTCGTGGTGCAGTCGTTGCCAAATGGTGGGAA : 324
SCNNV(Aus)  : CTGCCTGATCCAACTGACAACGATCACACCTTCGACGCGCTTCAAGCAACTCGTGGTGCAGTCGTTGCCAAATGGTGGGAA : 324
MGNNV       : CTGCCTGATCCAACTGACAACGATCACACCTTCGACGCGCTTCAAGCAACTCGTGGTGCAGTCGTTGCCAAATGGTGGGAA : 324
SGNNV       : CTGCCTGATCCAACTGACAACGATCACACCTTCGACGCGCTTCAAGCAACTCGTGGTGCAGTCGTTGCCAAATGGTGGGAA : 324
DGNNV       : CTGCCTGATCCAACTGACAACGACCACACCTTCGACGCGCTTCAAGCAACTCGTGGTGCAGTCGTTGCCAAATGGTGGGAA : 324
EcNNV       : CTGCCTGATCCAACTGACAACGACCACACCTTCGACGCGCTTCAAGCAACTCGTGGTGCAGTCGTTGCCAAATGGTGGGAA : 324
YGNNV       : CTGCCTGATCCAACTGACAACGACCACACCTTCGACGCGCTTCAAGCAACTCGTGGTGCAGTCGTTGCCAAATGGTGGGAA : 324
GGNNV       : CTGCCTGATCCAACTGACAACGATCACACCTTCGACGCGCTTCAAGCAACTCGTGGTGCAGTCGTTGCCAAATGGTGGGAA : 324
DiEV        : CTGCCTGATCCAACTGACAACGATCACACCTTCGACGCGCTTCAAGCAACTCGTGGTGCAGTCGTTGCCAAATGGTGGGAA : 324
GNNV        : CTGCCTGATCCAACTGACAACGATCACACCTTCGACGCGCTTCAAGCAACTCGTGGTGCAGTCGTTGCCAAATGGTGGGAA : 324
JFNNV       : CTGCCTGATCCAACTGACAACGACCACACCTTCGACGCGCTTCAAGCAACTCGTGGTGCGGTCGTGGCCAAATGGTGGGAA : 324
AHNNV       : CTGCCTGATCCAACTGACAGCGACCACACCTTCGACGCGAATTCAAGCGCACTCGCGGTGCGGTCGTTGCCAAATGGTGGGAA : 324
TNNV        : CTGCCTGACCCAACTGACAGCGACCACACCTTCGACGCGCTTCAGGCGCACTCGTGGTGCGGTCGTTGCCAAATGGTGGGAA : 324
SJNNV       : CTGCCTGATCCAACTGACAACGACCACACCTTCGATGCGCTCCAAGCAACTCGTGGTGCAGTCGTCGCCAAATGGTGGGAA : 324
```

Appendix 2.1 (cont'd): Multiple nucleotide sequence alignment of the consensus partial coat protein sequence of the three Australian nodavirus isolates with the exotic nodavirus isolates. F2 and NF'2 indicate the forward primer binding sites for the RT-PCR and Nested PCR.



Appendix 2.1 (cont'd): Multiple nucleotide sequence alignment of the consensus partial coat protein sequence of the three Australian nodavirus isolates with the exotic nodavirus isolates. NR'3 indicates the reverse primer binding site for the Nested PCR.



Appendix 2.2: Deduced amino acid sequence alignment of the partial coat protein sequence of the three Australian nodavirus isolates with the exotic nodavirus isolates.

```
                    *         20         *         40         *         60         *         80
BNNV(Aus)   : GFGRATNDVHLSGMSRISQAVLPAGTGTDGYVVVDATIVPDLLPRLGHAARIFQRYAVETLEFEIQPMCPANTGGGYVAGF :  81
BCNNV(Aus)  : GFGRATNDVHLSGMSRISQAVLPAGTGTDGYVVVDATIVPDLLPRLGHAARIFQRYAVETLEFEIQPMCPANTGGGYVAGF :  81
SCNNV(Aus)  : GFGRATNDVHLSGMSRISQAVLPAGTGTDGYVVVDATIVPDLLPRLGHAARIFQRYAVETLEFEIQPMCPANTGGGYVAGF :  81
MGNNV       : GFGRGTNDVHLSGMSRISQAVLPAGTGTDGYVVVDATIVPDLLPRLGHAARIFQRYAVEALEFEIQPMCPANTGGGYVAGF :  81
SGNNV       : GFGRGTNDVHLSGMSRISQAVLPAGTGTDGYVVVDATIVPDLLPRLGHAARIFQRYAVETLEFEIQPMCPANTGGGYVAGF :  81
DGNNV       : GFGRGTNDVHLSGMSRISQAVLPAGTGTDGYVVVDATIVPDLLPRLGHAARIFQRYAVETLEFEIQPMCPANTGGGYVAGF :  81
EcNNV       : GFGRGTNDVHLSGMSRISQAVLPAGTGTDGYVVVDATIVPDLLPRLGHAARIFQRYAVETLEFEIQPMCPANTGGGYVAGF :  81
YGNNV       : GFGRATNDVHLSGMSRISQAVLPAGTGTDGYVVVDATIVPDLLPRLGHAARIFQRYAVETLEFEIQPMCPANTGGGYVAGF :  81
GGNNV       : GFGRGTNDVHLSGMSRISQAVLPAGTGTDGYVVVDATIVPDLLPRLGHAARIFQRYAVETLEFEIQPMCPANTGGGYVAGF :  81
DiEV        : GFGRGTNDVHLSGMSRISQAVLPAGTGTDGYVVVDATIVPDLLPRLGHAARIFQRYAVETLEFEIQPMCPANTGGGYVAGF :  81
GNNV        : GFGRGTNEVYLSGMSRISQAVLPAGTGTDGYVVVDATIVPDLLPRLGHAARIFQRYAVETLEFEIQPMCPANTGGGYVAGF :  81
JFNNV       : GFGRGTNDVHLSGISRISQAVLPAGTGTDGYLVVDATIVPELLPRLGHAARIFQRYAVETLEFEIQPMCPANTGGGYVAGF :  81
AHNNV       : GFGRGTNDVHLTGMSRIAQAVIPAGTGTDGYIVVDETIVPELLPRLGPAARIFQRYAVETLEFEIQPMCPANTGGGYVAGF :  81
TNNV        : GFGRPTNDVHLSGMSRIAQAVIP?GTGTDGHVVLDEIIVPELLPRLGHASRIFQRYIVDTLEFEIQPMCPANTGGGYVAGF :  80
SJNNV       : GFGRATNDVHISGMSRIAQAVVPAGTGTDGKIVVDSTIVPELLPRLGHAARIFQRYAVETLEFEIQPMCPANTGGGYVAGF :  81

                        *       100         *       120         *       140         *       160
BNNV(Aus)   : LPDPTDNDHTFDALQATRGAVVAKWWESRTVRPQYTRTLLWTSSGKEQRLTSPGRLILLCVGNNTDVVNVSVLCRWSVRLS : 162
BCNNV(Aus)  : LPDPTDNDHTFDALQATRGAVVAKWWESRTVRPQYTRTLLWTSSGKEQRLTSPGRLILLCVGNNTDVVNVSVLCRWSVRLS : 162
SCNNV(Aus)  : LPDPTDNDHTFDALQATRGAVVAKWWESRTVRPQYTRTLLWTSSGKEQRLTSPGRLILLCVGNNTDVVNVSVLCRWSVRLS : 162
MGNNV       : LPDPTDNDHTFDALQATRGAVVAKWWESRTVRPQYTRTLLWTSTGKEQRLTSPGRLILLCVGNNTDVVNVSVLCRWSVRLS : 162
SGNNV       : LPDPTDNDHTFDALQATRGAVVAKWWESRTVRPQYTRTLLWTSSGKEQRLTSPGRLILLCVGNNTDVVNVSVLCRWSVRLS : 162
DGNNV       : LPDPTDNDHTFDALQATRGAVVAKWWESRTVRPQYTRTLLWTSSGKEQRLTSPGRLILLCVGNNTDVVNVSVLCRWSVRLS : 162
EcNNV       : LPDPTDNDHTFDALQATRGAVVAKWWESRTVRPQYTRTLLWTSSGKEQRLTSPGRLILLCVGNNTDVVNVSVLCRWSVRLS : 162
YGNNV       : LPDPTDNDHTFDALQATRGAVVAKWWESRTVRPQYTRTLLWTSSGKEQRLTSPGRLILLCVGNNTDVVNVSVLCRWSVRLS : 162
GGNNV       : LPDPTDNDHTFDALQATRGAVVAKWWESRTVRPQYIRTLLWTSSGKEQRLTSPGRLILLCVGNNTDVVNVSVLCRWSVRLS : 162
DiEV        : LPDPTDNDHTFDALQATRGAVVAKWWESRTVRPQYTRTLLWTSSGKEQRLTSPGRLILLCVGNNTDVVNVSVLCRWSVRLS : 162
GNNV        : LPDPTDNDHTFDALQATRGAVVAKWWESRTVRPQYTRTLLWTSSGKEQRLMSPGRLILLCVGNNTDVVNVSVLCRWSVRLS : 162
JFNNV       : LPDPTDNDHTFDALQATRGAVVAKWWESRTVRPQYTRTLLWTSTGKEQRLTSPGRLILLCVGNNTDVVNVSVLCRWSVRLS : 162
AHNNV       : LPDPTDSDHTFDAIQATRGAVVAKWWESRTIRPQHARALLWTSVGKEQRLTSPGRLVLLCAGNNTDVVNVSVLCRWSVRLS : 162
TNNV        : LPDPTDSDHTFDALQATRGAVVAKWWESRTVRPQYARTLLWTSTGKEQRLTSPGRIILLCVGNNTDVVNVSVLCRWSVRLS : 161
SJNNV       : LPDPTDNDHTFDALQATRGAVVAKWWESRTVRPQYTRTLLWTSTGKEQRLTSPGRLVLLCVGSNTDVVNVSVMCRWSVRLS : 162

                    *       180         *       200         *       220         *       240
BNNV(Aus)   : VPSLETPEETTAPIMTQGSLYNDSLS?-?NDFKSILLGSTPLDIAPDGAVFQLDRPLSIDYSLGTGDVDRAVYWHLKKFAG : 240
BCNNV(Aus)  : VPSLETPEETTAPIMTQGSLYNDSLS?-?NDFKSILLGSTPLDIAPDGAVFQLDRPLSIDYSLGTGDVDRAVYWHLKKFAG : 240
SCNNV(Aus)  : VPSLETPEETTAPITTQGSLYNDSLS?-?NDFKSILLGSTPLDIAPDGAIFQLDRPLSIDYSLGTGDVDRAVYWHLKKFAG : 240
MGNNV       : VPSLETPEETTAPIMTQGSLYNDSLS?-?NDFKSILLGSTPLDIAPDGAVFQLDRPLSIDYSLGTGDVDRAVYWHLKKFAG : 240
SGNNV       : VPSLETPEETTAPIMTQGSLYNDSLS?-?NDFKSILLGSTPLDIAPDGAVFQLDRPLSIDYSLGTGDVDRAVYWHLKKFAG : 240
DGNNV       : VPSLETPEETTAPIMTQGSLYNDSLS?-?NDFKSILLGSTPLDIAPDGAVFQLDRPLSIDYSLGTGDVDRAVYWHLKKFAG : 240
EcNNV       : VPSLETPEETTAPIMTQGSLYNDSLS?-?NDFKSILLGSTPLDIAPDGAVFQLDRPLSIDYSLGTGDVDRAVYWHLKKFAG : 240
YGNNV       : VPSLETPEETTAPIMTQGSLYSDSLS?-?NDFKSILLGSTPLDIAPDGAVFQLDRPLSIDYSLGTGDVDRAVYWHLKKFAG : 240
GGNNV       : VPSLETPEETTAPIMTQGSLYNDSLS?-?TDFKSILLGSTPLDIAPDGAVFQLDRPLSIDYSLGTGDVDRAVYWHLKKFAG : 240
DiEV        : VPSLETPEETTAPIMTQGSLYNDSLS?-?NDFKSILLGSTPLDIAPDGAVFQLDRPLSIDYSLGTGDVDRAVYWHIKKFAG : 240
GNNV        : VPSLETPEETTAPIMTQGSLYNDSLS?-?TDFKSILLGSTPLDIAPDGAVFQLDRPLSIDYTLGTGDVDRAVYWHLKKFAG : 240
JFNNV       : VPSLETPEETTAPIMTQGSLYNDSLA?-?SNFKSILLGSTQLDIAPDGAIFQLDRPLSIDYSLGTGDVDRAVYWHLKKFAG : 240
AHNNV       : VPSLETPEDTFAPILTLGPLYNDSLA?-?NDFKSILLGSTQLDIAPDGAVYSLDRPLSIDYSLGTGDVDRAVYWHVKKVAG : 240
TNNV        : VPSLELPEAVAVPILTQGPLYNDSLTNGYNHFQSVLLGSTQIDVAPTETIIQIDRPLLIDYSLGVGDVDRATYWHFRKLIG : 241
SJNNV       : VPSLETPEDTTAPITTQAPLHNDSINNGYTGFFSILLGSTQLDLAPANAVFVTDKPLPIDYNLGVGDVDRAVYWHLQKKAG : 243

                    *       260         *
BNNV(Aus)   : NAGTPAGWFRWGIWDNFNKTFTDGVAYYSDEQPRQI : 276
BCNNV(Aus)  : NAGTPAGWFRWGIWDNFNKTFTDGVAYYSDEQPRQI : 276
SCNNV(Aus)  : NAGTPAGWFRWGIWDNFNKTFTDGVAYYSDEQPRQI : 276
MGNNV       : NAGTPAGWFRWGIWDNFNKTFTDGVAYYSDEQPRQI : 276
SGNNV       : NAGTPAGWFRWGIWDNFNKTFADGVAYYSDEQPRQI : 276
DGNNV       : NAGTPAGWFRWGIWDNFNKTFTDGVAYYSDEQPRQI : 276
EcNNV       : NAGTPAGWFRWGIWDNFNKTFTDGVAYYSDEQPRQI : 276
YGNNV       : NAGTPAGWFRWGIWDNFNKTFTDGVAYYSDEQPRQI : 276
GGNNV       : NAGTPAGWFRWGIWDNFNKTFTDGVAYYSDEQPRQI : 276
DiEV        : NAGTPAGWFRWGIWDNFNKTFTDGVAYYSDEQPRQI : 276
GNNV        : NASTPAGWFRWGIWDNFNKTFTDGVAYYSDEQPRQI : 276
JFNNV       : TSSTPAGWFRWGIWDNFNKTFTDGVAYYSDAQPRQI : 276
AHNNV       : NAGTPAGWERWGLWDNFNKTFTQGAAYYSDAQPRQI : 276
TNNV        : DVTHPDGYLQWGLWDSFNKTFTAGISYYSDTQPRQI : 277
SJNNV       : DTQVPAGYFDWGLWDDFNKTFTVGAPYYSDQQPRQI : 279
```

Appendix 3: Examples of Nested RT-PCR, Histology and IHCT results for fish surviving experimental infection with barramundi nodavirus (BNNV)

Appendix 3.1: Comparative Nested RT-PCR, Histology (H&E) and IHCT test results for individual barcoo grunter, surviving injection infection with BNNV at the end of the 28-day trial. Fish were 6 weeks old at the commencement of the infectivity trial. (For the IHCT, E = Eye, B = Brain, N/A = not in section, + = lightly infected, +++ = heavily infected).

SAMPLE	RT-PCR	nRT-PCR	HISTOLOGY	IHCT	
				E	B
1	-	+	-	++	+
2			-	+	+
3			+ (E)	+++	-
4			+ (E)	++	-
5	+	+	+ (E) ?	++	-
6			+ (E)	+++	+
7			-	-	-
8			+ (E)	++	++
9	+	+	-	+	+
10			+ (E)	+++	++
11			-	-	+
12			-	+++	+
13	-	+	+ (E)	++	++
14			+ (E)	++	+
15			+(E)	++	+++
16			-	+	-
17	-	+	+ (E,B)	+	-
18			-	+++	++
19			-	-	+
20			+ (E)	N/A	-
21	+	+	+ (E,B)	++	+++
22			-	-	+
23			-	+	+
24			-	+	-
25	+	+	+ (E)	+++	-
26			+ (E)	++	-
27			-	-	-
28			-	+	-
29	-	+	+ (E) ?	++	-
30			+ (E) ?	+++	-
31			-	-	-
32			+ (E,B)	++	+
33	+	+	+ (E)	+	+
34			-	+	-
35			-	+	-
TOTAL POSITIVE	**5/9 55.5%**	**9/9 100%**	**18/35 51.4%**	**28/34 82.4%**	**18/35 54.4%**

Appendix 3.2: Comparative Nested RT-PCR, Histology (H&E) and IHCT test results for individual silver perch, surviving injection infection with BNNV at the end of the 28-day trial. Fish were 12 weeks old at the commencement of the infectivity trial. (E = Eye, B = Brain, N/A = not in section, + = lightly infected, +++++ = heavily infected).

SAMPLE	RT-PCR	nRT-PCR	HISTOLOGY	IHCT	
				E	B
1	Not recorded	+	+ (E)	++	+
2			+ (E)	+++++	N/A
3			-	++	-
4			-	-	+
5	Not recorded	+	-	+	+
6			-	+	+
7			-	+	-
8			+ (E,B) ?	+	-
9	Not recorded	+	-	++	++
10			-	-	++
11			+ (E)	+++	+
12			+ (E)	++	+
13	Not recorded	+	+ (E)	+	+
14			+ (E,B)	+++	+
15			+ (E)	+	+
16			+ (E,B)	+++	+
17	Not recorded	+	+ (E) ?	++	N/A
18			+ (E) ?	+	N/A
19			+ (E,B)	++	+
20			-	+	++
21	Not recorded	+	+ (E)	+	N/A
22			+ (E,B)	++	++
23			+ (E)	+	+
24			-	-	-
25	Not recorded	+	+ (E) ?	+	-
26			+ (E,B)	++	+
27			+ (E,B)	+++	+
28			-	-	-
29	Not recorded	+	+ (E)	+++	N/A
30			+ (E)	+++	N/A
31			+ (E)	-	++
32			-	-	-
33	Not recorded	+	+ (E) ?	++	-
34			-	-	-
35			-	-	-
36			+ (E)	+++	+
37	Not recorded	+	+ (E)	+	+
38			+ (E)	+	N/A
39			+ (B) ?	-	-
40			-	-	-
41	Not recorded	+	+ (E)	+++	+
42			+ (E)	+++	++
43			+ (E)	+	N/A
44			-	+	+
TOTAL POSITIVE		**11/11 100%**	**29/44 65.9%**	**34/44 77.3%**	**24/36 66.7%**

Appendix 3.3: Comparative Nested RT-PCR, Histology (H&E) and IHCT test results for individual barramundi, surviving injection infection with BNNV at the end of the 28-day trial. Fish were 12 weeks old at the commencement of the infectivity trial. (E = Eye, B = Brain, N/A = not in section, + = lightly infected, +++ = heavily infected).

SAMPLE	RT-PCR	nRT-PCR	HISTOLOGY	IHCT	
				E	B
1	Not recorded	+	+ (E)		
2			+ (B)	-	+
3			-	-	+
4			+ (E)	-	-
5	Not recorded	+	-	+	++
6			-	+	+
7			-	-	-
8			-	-	N/A
9	Not recorded	+	-	-	-
10			-	-	-
11			-	++	+
12			-	++	++
13	Not recorded	+	-	-	N/A
14			-	+	+
15			-	-	N/A
16			-	-	-
17	Not recorded	+	-	-	-
18			-	-	+++
19			-	-	++
20			+ (E) ?	+++	N/A
21	Not recorded	+	-	-	+
22			-	-	-
23			-	-	+
24			-	-	-
25	Not recorded	+	-	+	++
26			-	-	+
27			-	-	-
28			-	-	-
29	Not recorded	+	-	-	-
30			-	-	-
31			-	-	-
32			-	-	N/A
33	Not recorded	+	-	-	-
34			-	-	N/A
35			+ (E)	+++	N/A
36			-	-	+
TOTAL POSITIVE	**N/A**	**9/9 100%**	**5/36 14.2%**	**8/36 22.2%**	**14/29 48.3%**

Appendix 3.4: Comparative Nested RT-PCR, Histology (H&E) and IHCT test results for individual golden perch, surviving injection infection with BNNV at the end of the 28-day trial. Fish were 12 weeks old at the commencement of the infectivity trial. (E = Eye, B = Brain, N/A = not in section, + = lightly infected, +++++ = heavily infected).

SAMPLE	RT-PCR	nRT-PCR	HISTOLOGY	IHCT	
				E	B
1	+	+	+ (B) ?	+++	+++
2			-	-	+
3			-	-	-
4	+	+	+ (B,E)	++++	+++
5			+ (E) ?	+++	++
6			+ (E) ?	+++	+
7			+ (B,E)	+++	N/A
8	-	+	-	+++	+
9			+ (E)	++++	+
10			+ (E)	++++	++++
11			-	+	++
12	-	+	+ (E) ?	+++	++++
13			-	-	-
14			+ (E,B)	+++++	N/A
15			+ (E) ?	+++	++
16	-	+	+ (E)	+++	N/A
17			-	+++	-
18			+ (B,E)	+++	N/A
19			-	+	-
20	-	+	+ (E,B)	++++	+++
21			-	++	-
22			+ (E)	++	+++
23			+ (E)	+++	N/A
24	-	+	-	-	-
25			-	-	N/A
26			-	-	N/A
27			-	+	N/A
28	-	+	-	+	N/A
29			+ (B,E)	++	++
30			-	++	N/A
31			-	++	N/A
32	-	+	+ (B,E)	+++	++
33			-	+	N/A
34			+ (E,B)	++++	++++
35			+ (E,B)	+++	N/A
TOTAL POSITIVE	**2/9 22.2%**	**9/9 100%**	**19/35 54.3%**	**29/35 82.9%**	**16/22 72.7%**

Appendix 4: Intellectual property

There is no specific intellectual property arising from this project. All information generated is on the public domain.

Issues associated with selling the polyclonals antibodies will be discussed and approved by all stakeholders before any action is taken.

Appendix 5: Staff list

Oonoonba Veterinary Laboratory, Biosecurity, Department of Primary Industries and Fisheries;

NJ Moody, Virologist
PF Horwood, Research Technician
S McHardy, Research Technician

Australian Fish Diseases Laboratory, Australian Animal Health Laboratory, CSIRO;

M Crane, Project Leader and Officer-in-Charge, AFDL
K McKoll, Veterinary Specialist, AFDL

Tasmanian Aquaculture and Fisheries Institute (TAFI), University of Tasmania:

S Battaglene, Aquaculture Section Leader, Marine Research Laboratories
D Morehead, Research Fellow (Aquaculture), Marine Research Laboratories
J Carson, Principal Microbiologist, Fish Health Unit

Northern Territory Department of Business, Industry and Resource Development

J Humphrey, Manager, Aquatic Animal Health, Berrimah Veterinary Laboratories
C Shelley, Manager, Darwin Aquaculture Centre

IDEXX Laboratories

B Munday, Fish Health Division

Notes