# FINAL REPORT

# 2008/041 Aquatic Animal Health Subprogram: tools for investigation of the nodavirus carrier state in marine, euryhaline and freshwater fish and control of NNV through integrated management

**Richard Whittington**

**November 2012**

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# Aquatic Animal Health Subprogram: tools for investigation of the nodavirus carrier state in marine, euryhaline and freshwater fish and control of NNV through integrated management

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**2008/041 Aquatic Animal Health Subprogram: tools for investigation of the nodavirus carrier state in marine, euryhaline and freshwater fish and control of NNV through integrated management**

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**OBJECTIVES:**

1. To develop and validate a real-time PCR method for the detection and identification of Betanodaviruses

2. To develop and evaluate the applicability of serological tests for detection and identification of Betanodaviruses

3. To transfer developed technology to Australian diagnostic laboratories

4. To provide a basis for development of a national proficiency testing scheme for the detection and identification of betanodaviruses

5. To provide recommendations for improved biosecurity protocols in relation to nodavirus infection and fish translocation

**NON TECHNICAL SUMMARY**

**OUTCOMES ACHIEVED TO DATE**
This project will assist in ensuring the sustainability and profitability of the aquaculture industry and the health of natural resources by providing industry and governments with improved tools to make sound risk-based decisions for VNN management and control and for animal movements. The overall outcome of this project has been the successful development and transfer of technology to accurately identify NNV infection in barramundi and Australian bass, and to accurately identify the main means of infection of hatcheries, leading to reduced impact of disease in hatcheries, and lower risk of spread of disease to natural ecosystems, recreational and commercial fisheries. In addition, awareness of NNV has been enhanced in industry and also in government biosecurity agencies, and management approaches can now be aligned more strongly with scientific evidence. Thus Australia's capacity to prevent and control NNV is greatly improved.

This project was developed following national consultation and a scientific workshop convened by FRDC to ensure that research undertaken addressed the highest priorities of industry, governments and other stakeholders.

As a result of this project a new sensitive and specific test (qPCR) to detect NNV was successfully developed and transferred to five aquatic animal health diagnostic laboratories in Australia. The new test was established and run according to specifications and each laboratory has since participated in the Australian National Quality Assurance Program (ANQAP) for NNV with good results. The program is underpinned by an Australian and New Zealand Standard Diagnostic Test Procedure which was revised by researchers based on the findings of this project. The new qPCR test was shown to detect all of the known types of NNV and so can be used throughout Australia.

A disadvantage of qPCR test is that it requires samples of brain/eye from dead fish. However, another type of test was also developed which uses blood samples from live fish. This ELISA test measures the level of antibody against NNV. It was applied successfully in barramundi and Australian bass and individual fish were found that had both antibody in their blood and NNV present in their brain. Over time (months to years), the proportion of fish with antibodies in blood and virus in nervous tissue decreased, as did the levels of both antibodies and virus.

Using these new tests it was found that brood stock did not appear to be a source of infection for batches of larvae, even though some brood stock had been exposed to NNV. In fact there was no apparent relationship between infected batches of larvae and the test results of brood stock. Four batches of NNV-infected larval barramundi were found to have become infected during larval rearing or during grow out.

Based on these findings recommendations were developed to prevent outbreaks of NNV in hatcheries and to prevent spread of NNV through aquaculture. Emphasis should be placed on protecting larval fish from exposure to NNV rather than brood stock testing. The most likely source of infection is the water supply to the hatchery. Larval fish should be provided with UV-treated, filtered water to reduce the likelihood of exposure to NNV for as long as possible before transfer of fish to grow out. Hatcheries should adopt all-in-all-out batch culture and should develop individualised biosecurity protocols to reduce the risk of transmission of NNV associated with introduced brood stock, live feed, personnel movements, visitors and other factors.

Regardless of whether fish are required for grow out or for restocking, protocols to prevent translocation of NNV with movements of live cultured fish should recognise that subclinical infections may occur in young fish and that there is a high risk that the survivors of clinical NNV outbreaks will be sub-clinical carriers. Objective laboratory tests must be used to screen batches of fish for NNV infection. Histopathology should not be used for certification of freedom from infection.

Recommendations were made for further development to determine whether the ELISA can be used as a test for exposure to NNV infection at population level. This is important with respect to restocking waters for recreational fishing, as it is important to conduct surveys of wild fish to confirm that NNV is not already present. The biological significance of qPCR test positive fish that

contain very low levels of virus also requires further investigation to avoid unnecessary regulatory action such as destruction of batches of fish or quarantine. The effect of pooling tissues on test sensitivity in small fish also needs to be examined.

Other recommendations include developing better understanding of the distribution and abundance of wild fish with NNV infection, the behaviour of NNV in marine finfish, developing methods for disinfecting seawater to remove viable NNV, and determining the period of susceptibility of young fish to NNV.

In summary, this project achieved the successful development and transfer of new technology to accurately identify NNV infection in barramundi and Australian bass, and to accurately identify the main means of infection of fish in hatcheries. Thus Australia's capacity to prevent and control NNV is greatly improved.

**KEYWORDS: nervous necrosis virus, disease, barramundi, sea bass**

## ACKNOWLEDGEMENTS

Numerous individuals participated in this multidisciplinary research project, and they are listed in Appendix 2, Staff.

## BACKGROUND

FRDC has previously funded R&D on NNV infection in barramundi. The outcomes of these projects have informed disease control practices, and led to standard diagnostic tests being made available in aquatic animal health laboratories throughout Australia. In 2005-2006 there was a quantum leap in DNA-detection technology, with the advent of readily available real time quantitative PCR methods. Simultaneously there were outbreaks of NNV in barramundi in South Australia and Australian bass in New South Wales. The nodavirus isolates responsible for these two outbreaks were significantly different to the strains of nodavirus previously isolated in Australia and were not optimally targeted by the specific molecular detection test (nested RT-PCR) developed in the previous FRDC-funded projects. This has led to a major gap in molecular detection test capability for these strains. These events led to submission of research proposals in 2005 and 2006 to FRDC, which were not supported. The Aquatic Animal Health Subprogram noted that the applications had a state-based focus and suggested a national approach, consistent with the FRDC Aquatic Animal Health Subprogram's mandate. In addition, the Australian Chief Veterinary Officer wrote to FRDC requesting FRDC support to address research needs for NNV, noting that the disease was (then) internationally notifiable and to ensure a coordinated and collaborative approach so that research effort is efficient and not duplicated.

FRDC agreed to the Subprogram's recommendation for a strategic R&D plan and convened a workshop in Melbourne on 6-7 September 2007 to facilitate development of the plan. The workshop was attended by over 20 stakeholders representing FRDC, the Australian Barramundi Farmers Association, three commercial barramundi farmers, NSW Council of Freshwater Anglers, Commonwealth Department of Agriculture, Fisheries and Forestry, NSW Department of Primary Industries Biosecurity, Queensland Department of Primary Industries and Fisheries, South Australian Department of Primary Industries, NT Department of Primary Industry, Fisheries and Mines, Fisheries Western Australia, experts from aquatic animal health diagnostic laboratories, CSIRO and others. It was the largest consultative meeting ever convened in Australia to discuss NNV. The research application that led to this project was produced as a recommendation from the workshop and was closely aligned with the research and development plan that was produced by participants at the workshop.

It was determined at the workshop that the validated and routinely used tests for determining disease status, particularly of subclinically-infected fingerlings and brood stock, and for high-throughput sample testing requirements are inadequate. Real time quantitative PCR is a routinely used tool in most laboratories around Australia and has significant advantages over conventional gel-based PCR tests. These include a reduction in test turn-around times, increased sample throughput, reduced OH&S risks, reduced risk of contamination and false-positive results, and greater compatibility with robotic reagent preparation and delivery systems. Application of a standardised real time PCR test for the detection of betanodavirus is required to address this gap in capability. Development and application of serological tests, for the detection

of infection (antigen detection to indicate current infection) and exposure to the virus (antibody detection to indicate previous infection) will complement the real time PCR test and provide urgently required information regarding the merits of using antibody status as an indicator of infection in a wild or cultured fish population and whether antibody positive fish should or should not be used as brood stock.

## NEED

This project relates directly to the FRDC VNN Research and Development Plan, to facilitate industry profitability, sustainability, growth and development. There is an overarching need to measure and then reduce the risk to fisheries and aquaculture sectors (including natural resources) associated with transfer of nodaviruses. The aquaculture industry is proactive and responsible (eg triple bottom line reporting) and wishes to manage risks based on sound science. Industry acknowledges a residual risk that cannot be controlled: virus prevalence in the wild and natural fish movements. However, there is an immediate need for industry to conduct business in the face of unknowns with respect to true disease status. While there is a need to ensure that infected, but apparently healthy, stock are not moved to areas that are considered free of the disease/disease agent, it is of fundamental importance for the sustainability of the barramundi aquaculture industry and developing species ventures such as Australian bass that stock are translocated. Current tests for determining disease status are considered inadequate, therefore biosecurity protocols in the short-term are required to address the risk of introduction of disease with water, brood stock and fomites. As new information becomes available through R&D, these protocols will be revised and improved as needed to improve biosecurity. In the meantime, protocols are needed to manage risks with incomplete information and without overburdening industry with uneconomic or unwarranted requirements. There is need for mitigation of impacts on translocation: hatchery to nursery to grow-out; hatchery to wild (eg stocking for recreational fishing); both intra-state and interstate translocations, access to overseas markets; sourcing brood stock from the wild. Financial impacts, environmental impacts and mulitsector impacts at level of commercial, recreational and regulatory sectors all need to be addressed.

**OBJECTIVES**

1. To develop and validate a real-time PCR method for the detection and identification of Betanodaviruses

2. To develop and evaluate the applicability of serological tests for detection and identification of Betanodaviruses

3. To transfer developed technology to Australian diagnostic laboratories

4. To provide a basis for development of a national proficiency testing scheme for the detection and identification of betanodaviruses

5. To provide recommendations for improved biosecurity protocols in relation to nodavirus infection and fish translocation

## METHODS

There are five main objectives in this project, and each required the use of specific methodologies. To avoid confusion and because methodological development was required for a number of objectives, the results of many experiments were used to inform progressive development of the final methods, and so these are described in detail in the appropriate sections of the results. In summary, the methods that were used and/or developed under each objective included:

- To develop and validate a real-time PCR method for the detection and identification of Betanodaviruses. The methods used were reverse transcriptase real time polymerase chain reaction, virus isolation and analysis.

- To develop and evaluate the applicability of serological tests for detection and identification of Betanodaviruses. The methods used were indirect sandwich enzyme-linked immunosorbent assay (ELISA), serum neutralisation tests, competitive ELISA and analysis.

- To transfer developed technology to Australian diagnostic laboratories. The methods used were observational studies, production of plasmid and heat inactivated virus controls and analysis.

- To provide a basis for development of a national proficiency testing scheme for the detection and identification of betanodaviruses. The methods used were observational studies.

- To provide recommendations for improved biosecurity protocols in relation to nodavirus infection and fish translocation. The methods used were epidemiological studies, specifically cross sectional and prospective cohort studies, and analysis.

## RESULTS AND DISCUSSION

### Objective 1 and Objective 3

### To develop and validate a real-time PCR method for the detection and identification of Betanodaviruses

### To transfer developed technology to Australian diagnostic laboratories

Note: Objectives 1 and 3 are closely related and because test development and validation was carried out in conjunction with technology transfer the results are presented collectively. The following tasks were undertaken to achieve the objectives:

i. Distribution of laboratory protocols;
ii. Comparison of the new assay with the previous ANZSDP assay;
iii. Evaluation of alternative mastermix reagent kits;
iv. Detection of endemic and exotic betanodavirus genotypes;
v. Selection and storage of appropriate control reagents.

### i) Distribution of laboratory protocols

Standard operating procedures were prepared and distributed to each laboratory in year 1 of the project in addition to a flow chart for NNV detection. The SOPs are:

- NNV1 Sampling fish tissue for NNV 19-3-10
- NNV2 Tissue disruption for detection of NNV (microbead beating) 17-3-10
- NNV3 Tissue disruption for detection of NNV (pestle and tube) 19-3-10
- NNV4 Nucleic acid purification (spin column) 17-3-10
- NNV5 Nucleic acid purification (MagMax)19-3-10
- NNV6 Taqman RT-qPCR for detection of NNV 17-3-10
- NNV6 was replaced with an update on 13-6-12, with revised primer and probe concentrations and kit name. This is the final method adopted for this project

Methods for preparation of fish tissues, extraction of nucleic acid from tissue homogenates and qPCR were central to this project. The methods for preparation of fish tissues and extraction of nucleic acid from tissue homogenates were previously published (Hick et al., 2010). The qPCR method was named qR2T real-time reverse transcriptase-polymerase chain reaction (RT-qPCR) assay and was previously optimised and validated as a diagnostic assay for detection of betanodavirus from barramundi (*Lates calcarifer*) and Australian bass (*Macquaria novemaculeata*) fish tissue and striped snakehead (SSN-1) cell culture supernatant (Hick and Whittington, 2010). A plasmid control named p3.8qR2T was also developed for use in this assay.

When used together, tissue disruption by micro-bead beating and nucleic acid purification using a magnetic particle processor was predicted to result in a 15-fold increase in yield of virus from tissues, and increased the speed of sample preparation 10-fold compared to the use of manual tissue grinding and a silica-adsorption spin column methods. The mechanised and automated methods required less manual handling of samples, which may reduce the potential for false positive and false negative results arising through sample cross contamination, degradation and operator error, and high throughput applications are possible.

The University laboratory provided technical advice and support to the other laboratories during the initial phase. Variation in technical platforms at some laboratories led to a number of queries about assay ingredients. All laboratories that undertook the test obtained positive results for the plasmid control and the positive control fish and negative results for the negative control fish (Table 1). The plasmid standard was issued as an interim sample to enable labs to establish the assay, with a view to distribution of a permanent standard in the medium term. A surprising outcome was the durability of the spiked tissue homogenate positive control, which survived prolonged delay in transit and appeared to retain activity. The results provided a good indication that the assay had been transferred successfully.

Some variation in methodology between laboratories was needed to meet local requirements (Tables 2a, 2b). Nucleic acid purification was undertaken using relatively consistent approaches. The Kingfisher 96 used at EMAI is similar to that described in SOP NNV5. The QIAamp Viral RNA Mini Kit (QIAGEN) used at AAHL is similar to that described in SOP NNV4. AAHL used a protocol with a high primer concentration. Three mastermixes were used in addition to the one specified in SOP NNV6. At least 5 different thermocycler machine systems were used among laboratories. Each has a different means of determining threshold, and some machines offer several ways to set threshold, which impacts Ct values. Relatively consistent thermocycling parameters were used. AAHL used a longer denaturing step in the RT step (10 min instead of 5 min, specified for the kit), and a longer extension step during PCR (60 sec instead of 45 sec). Despite these variations, the limit of detection at each lab was similar, and the Cts reported for the positive control fish homogenate were very similar. This suggests that the qR2T assay is quite robust.

Protocols for RT-qPCR are idiosyncratic with respect to optima for a wide range of assay parameters, including type of enzyme (both reverse transcriptase and DNA polymerase), and buffering capacity and nature of ionic buffering environment, such that specific primer-probe combinations work optimally with one commercial kit and not another (reviewed by (Phillips, 2004). The importance of ring testing cannot be underestimated when protocols between laboratories cannot be completely standardised.

Table 1. Results obtained at six participating laboratories for positive and negative control fish homogenates and an interim plasmid standard in the qR2T real time PCR assay when first established

| Test location | Plasmid control | | | | Positive control fish homogenate | Negative control fish homogenate |
|---|---|---|---|---|---|---|
| | Copy number in first standard used in 10 fold dilution series | Nominal limit of detection* | Ct for $10^7$ copies | Efficiency of reaction if provided | | |
| University of Sydney | 1 x $10^7$ | $10^3$ | ~12 | 91 | Positive (Ct ~14) | Negative (no Ct) |
| EMAI | 1 x $10^7$ | $10^3$ | ~13 | | Positive (Ct~14) | Negative (no Ct) |
| AAHL | 1x $10^6$ | >$10^2$ | ~17 | 88-96 | Na | na |
| Oonoonba Vet Lab | 1 x $10^7$ | not determined** | ~22 | | Positive (Ct ~ 13) | Negative |
| Berrimah Vet Lab | 1 x $10^7$ | $10^3$ | ~24 | 80 | Positive (Ct ~14) | Negative (no Ct) |
| Fisheries WA | 1 x $10^7$ | $10^{4**}$ | ~23 | 82 | Positive (Ct ~ 18) | Negative (no Ct) |

* lowest copy number detected in at least one replicate with Ct<~40
** plasmid degradation likely to have occurred due to transport delay
na, not available

Table 2a. Application of standard operating procedures at each laboratory at project commencement.

| Step | University protocol | Procedure used or variation noted | | | | |
|---|---|---|---|---|---|---|
| | | EMAI | AAHL | Oonoonba Vet Lab | Berrimah Vet Lab | Fisheries WA* |
| Fish homogenisation | NNV2 or NNV3 | Sample supplied as homogenate. | Sample supplied as homogenate. Otherwise will use mortar and pestle or MagNA Lyser bead beater | Sample supplied as homogenate. | Sample supplied as homogenate.<br>Otherwise NNV2 will be used | Sample supplied as homogenate.<br>Otherwise NNV3 protocol will be used |
| Nucleic acid purification | NNV4 or NNV5 | EMAI SOP<br>Kingfisher 96 | QIAamp Viral RNA Mini Kit (QIAGEN) | NNV4 | NNV5 | NNV4 |
| PCR overall protocol | NNV6 | NNV6 | | NNV 6 | Previous SOP | NNV6 |
| | Primers: 250nM<br>Probe: 200nM<br>Template: 5μL | Primers: 250nM<br>Probe: 200nM<br>Template: 5μL | Primers: 0.9μM<br>Probe: 0.25μM<br>Template: 2μL | Template: 5μL | Primers: 250 nM, Probe: 250 nM<br>Template: 5μL | Template: TBAμL |
| PCR mastermix | QuantiTect Virus + ROX Vial Kit (Qiagen) | QuantiTect Virus + ROX Vial Kit (Qiagen) | TaqMan One-Step RT-PCR Master Mix (Applied Biosystems)<br>AgPath-ID One-Step RT-PCR Kit (Ambion) | Quanti Tect Virus did not use the ROX (added water instead)<br>Ag-Path-ID One-Step RT-PCR Kit (Ambion) | Superscript III Platinum One-step Quantitative PCR System (Invitrogen) | Quantitect Virus + Rox Vial Kit (Qiagen) |
| PCR thermocycler | Stratagene MX3000 | ABI 7900 | ABI 7500 Fast Instrument | Corbett Rotor-Gene 6000 | Corbett Research Rotor-Gene, | Corbett Rotor-Gene Q (Qiagen) |
| | Automatic | Manual threshold set at 0.5 | | No ROX. | No ROX | |

| Step | University protocol | Procedure used or variation noted | | | | |
|---|---|---|---|---|---|---|
| | | EMAI | AAHL | Oonoonba Vet Lab | Berrimah Vet Lab | Fisheries WA* |
| | threshold<br><br>with ROX | | | | | |
| PCR cycling steps | 50°C 10 min,<br><br>95°C 5 min.<br><br>45 cycles:<br><br>95°C 15 sec,<br><br>60°C 45 sec. | 50°C 20 min,<br><br>95°C 5 min.<br><br>45 cycles:<br><br>95°C 15 sec,<br><br>60°C 45 sec. | 50°C 20 min,<br><br>95°C 10 min.<br><br>45 cycles:<br><br>95°C 15 sec,<br><br>60°C 60 sec | 50°C 20 min,<br><br>95°C 5 min.<br><br>45 cycles:<br><br>95°C 15 sec,<br><br>60°C 45 sec. | 50°C 20 min,<br><br>95°C 5 min.<br><br>45 cycles:<br><br>95°C 15 sec,<br><br>60°C 45 sec | 50°C 20 min,<br><br>95°C 5 min.<br><br>45 cycles:<br><br>95°C 15 sec,<br><br>60°C 45 sec |

Table 2b. Application of standard operating procedures at each laboratory at project end.

| *Step* | *Comment or action* | *University protocol* | *Procedure used or variation noted* | | | | |
|---|---|---|---|---|---|---|---|
| | | | *EMAI* | *AAHL* | *Oonoonba Vet Lab* | *Berrimah Vet Lab* | *Fisheries WA** |
| *Nucleic acid purification* | *required for HI Nodavirus batch # V104* | *NNV4 or NNV5* | *EMAI SOP Kingfisher 96* | *QIAamp Viral RNA Mini Kit (QIAGEN)* | *NNV4* | *NNV5* | *NNV4* |
| *PCR overall protocol* | | *NNV6 Primers: 250nM Probe: 200nM Template: 5μL* | *NNV6 Primers: 250nM Probe: 200nM Template: 5μL* | *Primers: 0.9μM Probe: 0.25μM Template: 2μL* | *NNV 6 Template: 5μL* | *Previous SOP Primers: 250 nM, Probe: 250 nM Template: 5μL* | *NNV6 Template: TBAμL* |
| *PCR mastermix* | | *AgPath-ID One-Step RT-PCR Kit (Applied Biosystems)*** | *AgPath-ID One-Step RT-PCR Kit (Applied Biosystems)* | *TaqMan One-Step RT-PCR Master Mix (Applied Biosystems) AgPath-ID One-Step RT-PCR Kit (Ambion)*** | *Quanti Tect Virus did not use the ROX (added water instead) Ag-Path-ID One-Step RT-PCR Kit (Ambion)*** | *Superscript III Platinum One-step Quantitative PCR System (Invitrogen)* | *Quantitect Virus + Rox Vial Kit (Qiagen)* |
| *PCR thermocycler* | | *Stratagene MX3000 Automatic threshold with ROX* | *ABI 7900 Automatic baseline; manual threshold set at 0.5* | *ABI 7500 Fast Instrument* | *Corbett Rotor-Gene 6000 No ROX.* | *Corbett Research Rotor-Gene, No ROX* | *Corbett Rotor-Gene Q (Qiagen)* |
| *PCR cycling steps* | | *45°C 10 m 95°C 10 m 45 cycles: 95°C 15 sec 60°C 45 sec* | *50°C 20m 95°C 10 m 45 cycles: 95°C 15s 60°C 45s* | *50°C 20 m 95°C 10 m 45 cycles : 95°C 15 s 60°C 60 s* | *50°C20m 95°C 5m 45 cycles: 95°C 15s 60°C 45 s* | *50°C 20m 95°C 5m 45 cycles: 95°C 15s 60°C 45s* | *50°C 20m 95°C 5m 45 cycles: 95°C 15s 60°C 45s* |

* the numbers such as NNV6 refer to standard operating procedures that were circulated to each lab previously

** manufactured and distributed by Applied Biosystems; formerly Invitrogen/Ambion

### ii) Comparison of the new assay with the previous ANZSDP assay

Although the new method was known to have high sensitivity it was necessary to compare it with other methods in an independent laboratory. Consequently the new real time assay was compared at AAHL to the Australian and New Zealand Standard Diagnostic Test, which is a reverse transcriptase nested PCR (RT-nPCR), which yields results at two steps (firstly RT-PCR and then nPCR). The qR2T assay appears to be $10^2$ to $10^3$ times more sensitive than the ANZSDP method (Table 3).

Table 3. Comparative testing of NNV (isolate BNNV-QLD) 10-fold dilutions by the NNV qR2T RT-qPCR and ANZSDP NNV RT-nPCR. The qR2T assay was run with the TaqMan One-Step RT-PCR Master Mix Reagents (Applied Biosystems) and the AgPath-ID One-Step RT-PCR Kit (Ambion) mastermixes. Samples were tested in single tubes.

| Dilution | qR2T assay | | ANZSDP | |
|---|---|---|---|---|
| | TaqMan $C_T$ | AgPath $C_T$ | RT-PCR | nPCR |
| $10^{-1}$ | 20.33 | 17.63 | + | + |
| $10^{-2}$ | 24.01 | 20.73 | + | + |
| $10^{-3}$ | 27.14 | 23.85 | - | + |
| $10^{-4}$ | 30.67 | 27.46 | - | + |
| $10^{-5}$ | 35.87 | 30.56 | - | $\pm$ |
| $10^{-6}$ | 38.95 | 34.92 | - | - |
| $10^{-7}$ | Undet | 38.96 | - | - |
| $10^{-8}$ | Undet | Undet | - | - |
| Slope | -3.78 | -3.42 | | |
| Efficiency | 83.96 | 96.23 | | |

Undet, not detected

### iii) Evaluation of alternative mastermix reagent kits

Two PCR mastermixes were used in a number of experiments at AAHL. These were the TaqMan One-Step RT-PCR Master Mix Reagents Kit (Applied Biosystems) and AgPath-ID One-Step RT-PCR Kit (Ambion). Similar results were obtained with either mastermix (Tables 4 and 7).

In a further experiment, serial 10-fold dilutions were prepared from qR2T plasmid provided at a concentration of $2 \times 10^7$ copies/µL. Plasmid dilutions were tested with the qR2T assay and results are presented in Table 5. The correspondence between results from the two kits was very high.

Table 4. Comparison of PCR master mixes. Samples were tested in triplicate on the same plate.

| | qR2T Plasmid | | | |
|---|---|---|---|---|
| | TaqMan | | AgPath | |
| Copies | $C_T$ | SD | $C_T$ | SD |
| $10^6$ | 17.37 | 0.09 | 17.31 | 0.27 |
| $10^5$ | 20.83 | 0.21 | 20.25 | 0.32 |
| $10^4$ | 24.20 | 0.07 | 23.62 | 0.34 |
| $10^3$ | 27.83 | 0.24 | 27.24 | 0.40 |
| $10^2$ | 31.12 | 0.40 | 30.45 | 0.60 |
| $10^1$ | 36.34* | 0.75 | 34.54* | 0.02 |
| $10^0$ | Undet | N/A | Undet | N/A |
| | | | | |
| Slope | -3.648 | | -3.417 | |
| Efficiency | 87.975 | | 96.172 | |

*2 replicates producing acceptable $C_T$ values, one replicate produced an undetermined result
Undet, not detected

Mirroring experience at the University of Sydney during routine use, the Berrimah Veterinary Laboratory reported in August 2010 unsatisfactory results obtained from a series of ANQAP NNV samples when QuantiTect Virus + ROX Vial Kit (Qiagen) mastermix was used, but satisfactory results using Superscript III Platinum One-Step Quantitative RT-PCR system (Invitrogen) mastermix. The results were puzzling because the assay had been developed using the QuantiTect Virus + ROX Vial Kit (Qiagen) mastermix and this prompted an investigation.

Four alternative RT-PCR kits were sourced for comparison with the Qiagen kit which was used to develop and optimise this assay (Table 5).

Table 5. Mastermix kits that were compared for inclusion in the qR2T qPCR assay for NNV

| Supplier | Manufacturer | Product | Cat # |
|---|---|---|---|
| Qiagen | Qiagen | QuantiTect Virus + ROX Vial Kit | 211031 |
| Invitrogen | Invitrogen[1] | SuperScript III One-Step RT-PCR System with Platinum Taq DNA Polymerase | 12574-018 |
| Invitrogen | Invitrogen[2] | SuperScript III One-Step Quantitative RT-PCR System | 11732-020 |
| Bioline | Bioline | BioScript One-Step RT-PCR Kit | BIO-65030 |
| Invitrogen | Applied Biosystems | AgPath-ID One-Step RT-PCR Kit | 4387424M |

The PCR master mixes were prepared in a clean area designated for this purpose. The mastermixes were aliquoted into PCR tubes then nuclease-free water and negative control RNA were added in triplicate to NTC (no template control) and negative control tubes for each test mix. Plasmid standard dilutions

were prepared and added in triplicate to each test mix starting with the highest dilution. Finally positive RNA control was added to the appropriate tubes. Three RT-PCR kits were compared directly in a single run; two runs were required to compare all four products. The qR2T assay PCR cycling conditions were used throughout this evaluation to enable reactions using different mastermixes to be compared in the same run. Cycling parameters varied slightly from those recommended by the individual manufacturers, but this was not expected to be a significant factor as product information indicated that a range of parameters were acceptable for each kit, with optimisation for each target recommended.

The results indicated that use of the QIAGEN, Invitrogen[2] or Bioline products for the qR2T assay led to 1 log lower analytical sensitivity compared to the Invitrogen[1] and Applied Biosystems products (Table 6).

Table 6. A comparison of the analytical sensitivity of the qR2T qPCR assay for NNV including different commercial mastermixes, using a plasmid dilution series. Thresholds were adjusted manually to obtain optimal reaction efficiency. Data are the mean responses of two replicates.

| | Qiagen | Invitrogen[1] | Bioline | Qiagen | Invitrogen[2] | Applied Biosystems |
|---|---|---|---|---|---|---|
| Threshold | 0.0954 | 0.157 | 0.106 | 0.06 | 0.1 | 0.14 |
| Efficiency (%) | 95 | 95 | 98 | 95 | 83.7 | 95 |
| Plasmid Copy no | | $Ct_{Av}$ | | | $Ct_{Av}$ | |
| $1 \times 10^7$ | 15.65 | 17.11 | 16.26 | 13.99 | 16.78 | 15.83 |
| $1 \times 10^6$ | 18.97 | 20.48 | 19.51 | 17.64 | 20.78 | 19.26 |
| $1 \times 10^5$ | 21.95 | 23.51 | 22.7 | 20.79 | 24.23 | 22.73 |
| $1 \times 10^4$ | 25.49 | 27.05 | 25.96 | 24.21 | 27.93 | 26.08 |
| $1 \times 10^3$ | 29.63 | 30.49 | 29.89 | 28.73 | 32.15 | 29.91 |
| $1 \times 10^2$ | No Ct | 33.77 | No Ct | 43.46 | 40.93 | 32.64 |
| $1 \times 10^1$ | No Ct | 39.11 | No Ct | No Ct | No Ct | 39.54 |
| $1 \times 10^0$ | No Ct | No Ct | No Ct | No Ct | No Ct | No Ct |
| Pos RNA control | 20.02 | 23.11 | 18.96 | 15.21 | 14.37 | 13.91 |
| Neg RNA control | No Ct | 35.8 | No Ct | No Ct | 36.86 | No Ct |
| NTC | No Ct | No Ct | No Ct | No Ct | No Ct | No Ct |

Based on these results it was assumed that there had been a “non-notified” change in QIAGEN kit specifications, or deterioration of some component in the current lot of this kit. As a newly ordered QIAGEN kit behaved the same way, the former possibility seems more likely. It was therefore recommended that Invitrogen[1] and Applied Biosystems products be used in place of the QIAGEN product in the qR2T qPCR assay for NNV. Data presented above from AAHL suggest that the TaqMan One-Step RT-PCR Master Mix Reagents Kit (Applied Biosystems) may also be used successfully.

**iv) Detection of endemic and exotic betanodavirus genotypes**

AAHL undertook validation of the assay using a range of exotic strains of NNV. These were grown in cell culture to produce material for analysis. The first trial of the NNV qR2T RT-nPCR assay involved endemic (BNNV-QLD and ABNNV) and exotic (BFNNV, RGNNV and SJNNV) isolates which had been amplified for use as positive control material. Template was extracted from 140µL cell culture supernatant and diluted 10-fold in sterile distilled water. The NNV qR2T RT-qPCR detected all enzootic and exotic nodavirus isolates tested (Table 7). The Cts suggested similar analytical sensitivity for each genotype, assuming a similar titre of virus in parent cell cultures. Previously Hick and Whittington (2010) found lower sensitivity for BFNNV compared to other genotypes, but that work was performed on archival nucleic acid samples sent from Japan. The present work at AAHL suggests potential application for the assay in detection of genotypes in addition to RGNNV.

Table 7. Detection of endemic and exotic NNV isolates using the qR2T assay. Samples were tested with the TaqMan One-Step RT-PCR Master Mix Reagents (Applied Biosystems) and the AgPath-ID One-Step RT-PCR Kit (Ambion) mastermixes on the same plate. Samples were tested in single wells.

| | BFNNV | | RGNNV | | SJNNV | | ABNNV | | BNNV-QLD | |
|---|---|---|---|---|---|---|---|---|---|---|
| | TaqMan | AgPath | TaqMan | AgPath | TaqMan | TaqMan | AgPath | TaqMan | AgPath | TaqMan |
| Dilution | $C_T$ | $C_T$ | $C_T$ | $C_T$ | $C_T$ | $C_T$ | $C_T$ | $C_T$ | $C_T$ | $C_T$ |
| Neat | 14.17 | 15.70 | 11.53 | N/A | 11.86 | 11.52 | 12.70 | 13.05 | 8.58 | 7.71 |
| $10^{-1}$ | 17.40 | 18.53 | 15.04 | 14.31 | 15.20 | 14.65 | 16.28 | 17.00 | 12.63 | 12.08 |
| $10^{-2}$ | 21.06 | 21.42 | 18.70 | 17.63 | 18.62 | 18.45 | 19.94 | 20.00 | 15.88 | 15.24 |
| $10^{-3}$ | 24.99 | 24.88 | 22.19 | 21.23 | 22.28 | - | 23.49 | 23.14 | 19.44 | 18.36 |
| $10^{-4}$ | 28.81 | 28.38 | 25.76 | 24.50 | 25.93 | 24.91 | 26.71 | 26.19 | 22.90 | 21.63 |
| $10^{-5}$ | 32.31 | 32.44 | 29.09 | 27.57 | 29.04 | 28.40 | 30.23 | 29.88 | 26.39 | 24.62 |
| $10^{-6}$ | 36.89 | 37.82 | 33.56 | 31.67 | 33.67 | 32.87 | 35.04 | 35.27 | 29.57 | 28.07 |
| $10^{-7}$ | Undet | Undet | Undet | 37.29 | Undet | 38.65 | Undet | Undet | 34.04 | 31.76 |
| $10^{-8}$ | Undet | Undet | Undet | Undet | Undet | Undet | Undet | Undet | Undet | Undet |
| Slope | -3.86 | -3.80 | -3.64 | -3.69 | -3.64 | -3.84 | -3.65 | -3.54 | -3.52 | -3.25 |
| Efficiency | 81.64 | 83.32 | 88.30 | 86.60 | 88.37 | 82.26 | 87.79 | 91.50 | 92.37 | 103.16 |

**v) Selection and storage of appropriate control reagents**

There are two main applications of qPCR – diagnosis in which a positive or negative result is sufficient, and quantification. Different controls may be used for different applications. Robust controls are required for use in each laboratory, and ideally, the same controls would be used at each laboratory to enable standardisation of the assay. However prior to this project there were no data on the types of controls that could be used for NNV assays. Therefore preliminary NNV control materials were shipped on dry ice by courier or by hand delivery to each participating laboratory in Year 1 to enable the assay to be established. These materials comprised: negative control barramundi clarified tissue

homogentate batch # V046; positive control barramundi clarified tissue homogentate spiked with nodavirus infected SSN-1 tissue culture supernatant (LcNNV 09/07 Genbank:GQ402010, GQ402011) batch # V047; and linearised plasmid qR2T batch # V050 adjusted to give 2 x $10^7$ copies /ul batch # V051.

An extensive investigation into the stability of the plasmid under a range of laboratory handling and storage conditions was then undertaken. In addition the suitability of live and heat inactivated virus and RNA controls was tested and an interlaboratory comparison was undertaken. The aim of this work was to define a suitable control/controls for inclusion in real time quantitative PCR (qCR) assays for NNV.

*Stability of plasmid p3.8 qR2T DNA in RLT buffer*

RNeasy Kits are designed to isolate total RNA from small quantities of starting material utilising a lysis and homogenisation buffer which contains guanidinium thiocyanate, such as buffer RLT. Homogenised lysates in RLT can be stored at –80°C for several months. Here we tested the hypothesis that buffer RLT could be used to stabilise plasmid stocks for long term storage at -$80^0$C.

Linearised plasmid p3.8qR2T at 5 x $10^8$ copies/ul was prepared in buffer RLT (Qiagen RNeasy kit lysis buffer). Copy number of the linearised plasmid was estimated using the formula:

$$\frac{\text{conc. plasmid DNA} \times (6.022 \times 10^{14})}{\text{total plasmid bp} \times 330}$$

$$= \frac{25 \times (6.022 \times 10^{14})}{4946 \times 330}$$

= 9.22 x $10^9$ copies /ul lot #V056

Plasmid V056 was diluted to 5 x $10^8$ copies/ul in buffer RLT and 15 x 100ul aliquots were prepared. Three aliquots were placed at each of 5 temperatures: $37^0$C, $22^0$C, $4^0$C, -$20^0$C, -$80^0$C for 7 days. A ten fold serial dilution was prepared from each of two randomly selected aliquots and the endpoint was determined in the qR2T qPCR assay using QIAGEN mastermix but excluding the reverse transcription step ($50^0$C for 20min) in the thermocycling parameters. The Ct threshold was set at the value derived from the Day 0 standard curve (0.1431).

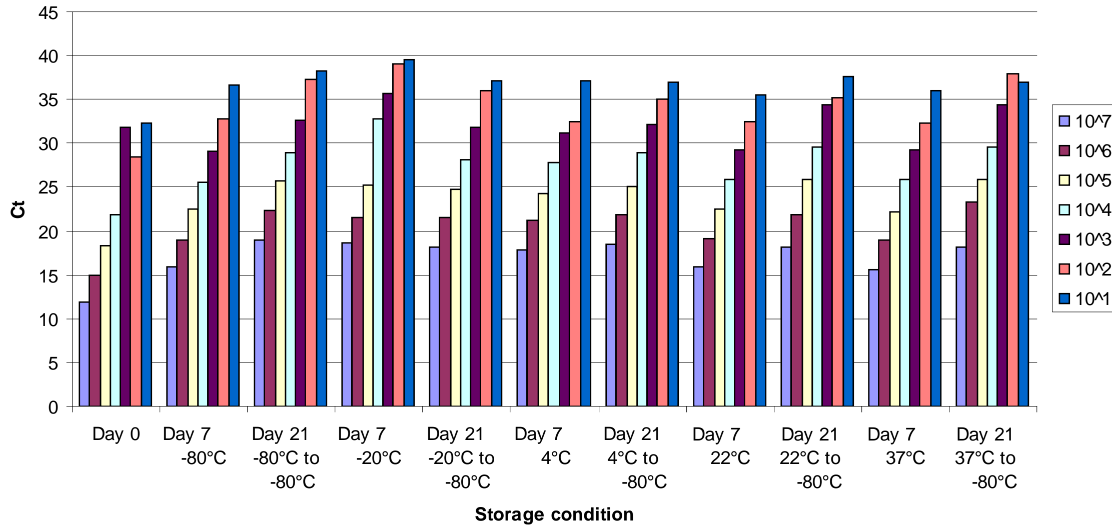
To simulate transport conditions during shipment of plasmid between laboratories, and common storage conditions upon arrival at the laboratory, all 3 aliquots from each temperature were then stored at -$80^0$C and re-tested after a further 14 days to evaluate the effect of a freeze-thaw step. The same two aliquots from each temperature regime were tested with and without the reverse transcription step.

The results indicated that there was a loss of approximately one log of copy number (i.e. 90%) between day 0 and day 7 regardless of storage temperature (Table 8, Figure 1). Efficiency was poor with storage at 4°C and -20°C suggesting changes to the plasmid which interfere with its even suspension/distribution in the buffer.There was a further but lower loss between day 7 and day 21 for storage at -80°C, 22°C and 37°C but apparently not at 4°C or -20°C. Overall there was little difference between storage temperatures. The data for day 21 represent storage at the nominated temperature for 7 days, followed by 14 days at -80°C. Therefore there were two freeze-thaw cycles for temperatures -80°C and -20°C, and one freeze-thaw cycle for 4°C, 22°C and 37°C. It is uncertain whether the duration of storage or the freeze-thaw event is deleterious.

Table 8. Endpoint of detection of plasmid DNA following storage of plasmid for 7 days and a further 14 days. The endpoint was defined as the highest dilution in which both duplicates of both replicate dilution series gave a Ct. A plasmid standard curve ($1x10^7$ – $1x10^0$ copies) was generated on Day 0 (qR2T assay, QuantiTect Virus + ROX Vial Kit) before incubating 2 x 100ul aliquots of plasmid at each test temperature. Independent 10-fold dilutions were then prepared from each plasmid aliquot and standard curves were generated. Day 0, plasmid control before incubation at test temperatures; day 7, plasmid control after 7days at test temperatures; Day 21, plasmid control after 7 days at test temperature followed by 14 days at -$80^0$C

| Temperature of storage °C | Day 0 | Day 7 | Efficiency | Day 21 | Efficiency |
|---|---|---|---|---|---|
| | | | | No RT, with RT | No RT, with RT |
| Control | $1 \times 10^0$ | | | | |
| 37 | | $1 \times 10^1$ | 95.8 | $1 \times 10^3$, $1 \times 10^4$ | 87.0, 102.5 |
| 22 | | $1 \times 10^1$ | 100.3 | $1 \times 10^3$, $1 \times 10^3$ | 94.8, 84.8 |
| 4 | | $1 \times 10^1$ | 86.8 | $1 \times 10^2$, $1 \times 10^3$ | 101.1, 93.3 |
| -20 | | $1 \times 10^2$ | 77.6 | $1 \times 10^2$, $1 \times 10^3$ | 97.3, 81.7 |
| -80 | | $1 \times 10^1$ | 96.3 | $1 \times 10^2$, $1 \times 10^3$ | 95.2, 99.2 |

Figure 1. Titration of plasmid stored for 7 days in buffer RLT at a range of temperatures before transfer to -80C and storage for a further 14 days and testing in qPCR.



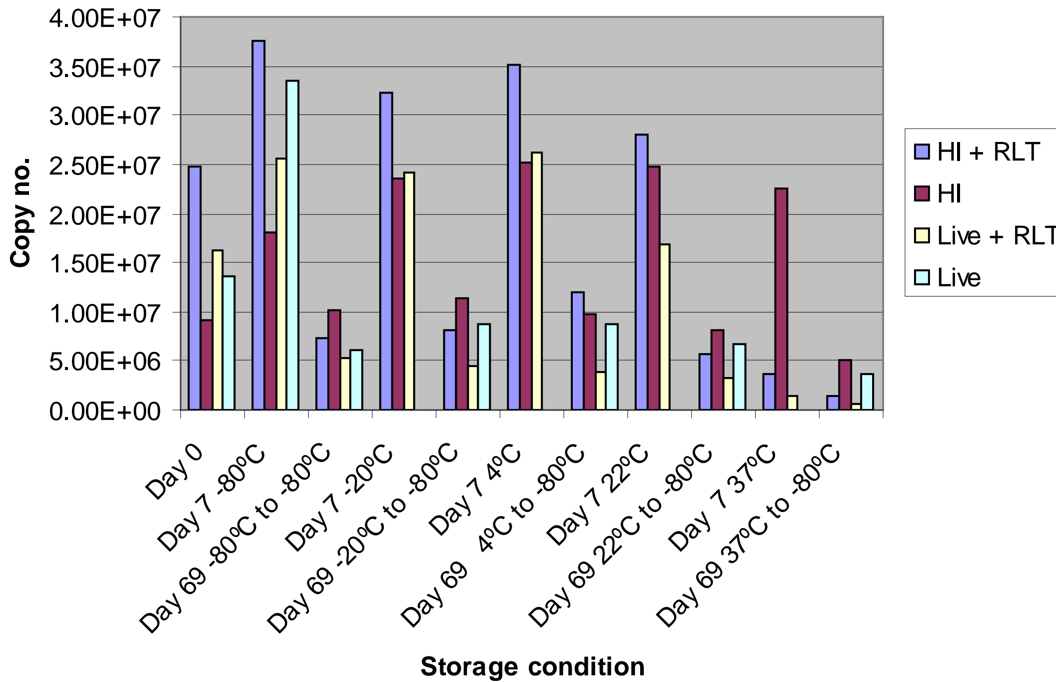

*Stability of heat inactivated and live virus tissue culture supernatant as standards*
Tissue homogenate spiked with LcNNV lot #V027 was used to inoculate SSN-1 cells. Positive tissue culture supernatant (TCSN) was harvested and pooled and an end point titration was performed to determine the mean titre – it was $10^{7.7}$ $TCID_{50}$/ml. Heat Inactivation was for 1hour at $60^{0}$C. For RLT treatments 200ul virus was combined with 1200ul buffer RLT. For the other treatments 200ul virus was combined with 1200ul culture medium.

RNA extractions were performed for qPCR analysis (AgPath-ID One-Step RT-PCR, Applied Biosystems). For each treatment group 15 x 75ul aliquots were prepared. Three aliquots of each were incubated at $37^{0}$C, $22^{0}$C, $4^{0}$C, $-20^{0}$C and $-80^{0}$C respectively for 7 days. Each treatment group was then transferred to $-80^{0}$C and retested after approximately 2 months.

After incubation at each temperature RNA extractions were performed and qPCR analysis was completed using the AgPath-ID One-Step RT-PCR kit (Applied Biosystems) quantified with a plasmid standard curve. For Day 0 samples, RNA extractions were performed on selected samples, with two independent 10-fold dilutions (neat – $10^{-7}$) prepared from each extraction for qPCR analysis. For samples collected after 7 days at each temperature and then after 2 months at -80C, RNA extractions were performed on 3 aliquots of each treatment group.

The results are shown in Figure 2.

Figure 2. Detection of NNV (copy number) in samples that were stored at a range of temperatures for 7 days prior to storage at -80C for a further two months.




There was little difference in detection of NNV in heat inactivated or live NNV TCSN stored for 7 days at -80, -20, 4, or 22$^0$C. However, storage at 37C was detrimental, with >0.5 log loss. Note that a freeze-thaw was required for -80 and -20 storage condition. The conditions 4, 22 and 37$^0$C can be used to predict the effect of inter-laboratory transport on plasmid activity.

Compared to Day 7, there was a loss of NNV between Day 7 and Day 69 associated with either storage of samples at -80C or freeze-thaw. The magnitude of the loss was about 1 log (i.e. 90%) and was similar for each storage temperature in the range -80 to 22$^0$C; the loss appeared to be slightly greater at 37$^0$C. With the exception of storage at 37$^0$C, buffer RLT appeared to better maintain heat inactivated NNV at each temperature compared to samples which did not contain this buffer. Heat inactivated NNV appeared to be slightly more stable than live NNV at each storage temperature.

*Inter-laboratory testing of plasmid, RNA and virus control material*

Three control reagents were sent to each laboratory on dry ice using World Couriers.

1. Plasmid standard qR2T batch # V105. Linearised plasmid qR2T batch # V105 adjusted to give 2 x $10^8$ copies /ul.

2. Positive RNA control reagent batch # V064. RNA extracted from 200ul of reagent # V047(Clarified tissue homogenate (barramundi) spiked with Nodavirus infected SSN-1 tissue culture supernatant (LcNNV 09/07 Genbank:GQ402010, GQ402011)) using Roche HighPure Viral RNA Extraction Kit .

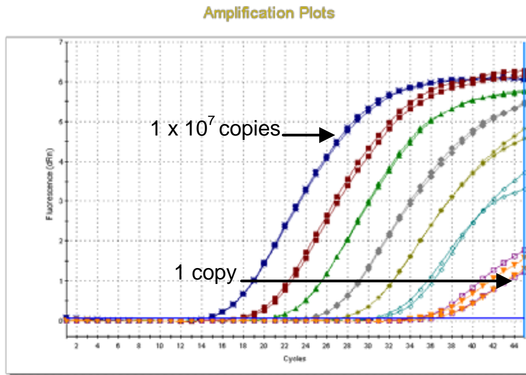
3. Heat inactivated tissue culture supernatant batch # V104. Heat inactivated Nodavirus infected SSN-1 tissue culture supernatant (LcNNV 09/07 Genbank:GQ402010, GQ402011) from which RNA is to be extracted and tested.

Each laboratory was requested to confirm the methodology in use and to prepare a 10-fold serial dilution of each, out to 8 steps, and run the real time PCR using the protocols indicated below. Tabulated results including Ct for each dilution and the limit of detection were to be collated together with comments on the utility of the reagents as controls and indication of preference from each user group.

Three laboratories responded with data (EMAI, BVL and USyd). The methods used these laboratories are shown in Table 2b, together with the most recently available data for the other laboratories with capacity for this test.

As result of inter-laboratory comparisons it was shown that the analytical sensitivity of the qR2T real time PCR assay at the University of Sydney had fallen by 2 to 3 logs. A complex experiment was conducted to identify the components of the qR2T assay that was out of specification. Individual components of the University of Sydney assay were swapped out and replaced with a reagent prepared by and supplied from EMAI. The results indicated that University of Sydney primers (20 uM stock) were faulty. Several batches of primers were used in 2011 therefore it was unlikely to be a batch-related issue. Storage at 20 uM appears to be detrimental. Storing the qR2T assay primers as concentrated 100uM stocks and adding directly to PCR master mix was shown to maintain reaction efficiency and analytical sensitivity within the limits established during assay development and validation. The SOP for the qR2T assay was amended on 13/3/12. A typical plasmid standard curve is shown in Figure 3.

Figure 3. Representative plasmid standard curve




The three potential control reagents for the qR2T assay were re-evaluated using primers from 100uM stocks. The results are shown in Table 9. The assay had similar analytical sensitivity at each of 3 laboratories and efficiency was satisfactory in each case.

Table 9. Comparison of control reagents for NNV real time PCR at three laboratories. Results are shown for primer stocks at 100 uM and 20 uM for the USyd laboratory.

| *Plasmid V105* | Threshold | Efficiency | Copy number | | | | | | | | | limit of detection |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | $1X10^8$ | $1X10^7$ | $1X10^6$ | $1X10^5$ | $1X10^4$ | $1X10^3$ | $1X10^2$ | $1X10^1$ | $1X10^0$ | |
| Usyd (100uM) | 0.088 | 103.3 | nd | 13.58 | 16.49 | 19.54 | 23.05 | 25.98 | 29.34 | 33.25 | 35.87 | $1X10^0$ copies |
| Usyd (20 uM) | 0.1376 | 75.9 | nd | 16.36 | 20.52 | 23.86 | 27.33 | 30.67 | 35.04 | 44.65 | No Ct | $1X10^1$ copies |
| Berrimah Vet Lab | 0.0116 | 92.1 | 12.78 | 16.77 | 20.6 | 24.43 | 28.13 | 31.09 | 34.08 | 37.7 | nd | $1X10^1$ copies (limit tested) |
| EMAI | no data | 104 | nd | 12.14 | 15.12 | 19.26 | 22.53 | 25.78 | 28.98 | 31.33 | 32.02 | |
| *Control RNA V064* | Threshold | Efficiency | dilution | | | | | | | | | |
| | | | neat | $10^{-1}$ | $10^{-2}$ | $10^{-3}$ | $10^{-4}$ | $10^{-5}$ | $10^{-6}$ | $10^{-7}$ | $10^{-8}$ | |
| Usyd (100uM) | 0.088 | 103.3 | 13.99 | 16.78 | 20.26 | 23.95 | 27.37 | 31.23 | 38.5 | 43.49 | nd | $4.075 X 10^{-3}$ copies (limit tested) |
| Usyd (20 uM) | 0.1376 | 75.9 | nd | 19.55 | 23.76 | 27.18 | 31.98 | 37.5 | No Ct | No Ct | No Ct | $2.814 X 10^1$ copies |
| Berrimah Vet Lab | 0.0116 | 92.1 | 13.61 | 18.37 | 22.54 | 26.55 | 30.29 | 33.17 | 38.5 | 40.41 | 38.66 | not reported |
| EMAI | no data | 97? | nd | nd | 21.65 | 25.65 | 29.29 | 32.84 | 35.02 | 35.7 | No Ct | not reported |
| *HI tcsn V104* | Threshold | Efficiency | dilution | | | | | | | | | |
| | | | neat | $10^{-1}$ | $10^{-2}$ | $10^{-3}$ | $10^{-4}$ | $10^{-5}$ | $10^{-6}$ | $10^{-7}$ | $10^{-8}$ | |
| Usyd (100uM) | 0.088 | 103.3 | 10.17 | 12.88 | 16.04 | 19.47 | 22.68 | 25.8 | 29.55 | 34.03 | nd | 3.96 copies (limit tested) |
| Usyd (20 uM) | 0.1376 | 75.9 | nd | 15.55 | 19.05 | 22.75 | 26.37 | 30.42 | 35.71 | No Ct | No Ct | $1.168 X 10^2$ copies |
| Berrimah Vet Lab | 0.0116 | 92.1 | 7.08 | 10.3 | 16.25 | 21.03 | 23.54 | 26.85 | 32.2 | 33.92 | 35.16 | not reported |
| EMAI | no data | 87? | nd | nd | 17.8 | 21.51 | 25.23 | 28.3 | 32.35 | 36.58 | No Ct | not reported |

*Conclusions*

1. Plasmid containing NNV sequence is a suitable control for enumeration in qPCR while heat inactivated NNV-infected TCSN in RLT buffer is a suitable positive control.

2. The plasmid in RLT buffer can be stored at -20$^0$C or -80$^0$C in a concentrated solution in small aliquots which are used only once. It is likely that plasmid can be shipped at 4$^0$C or 22$^0$C and then frozen at -80$^0$C without substantial loss of activity.

3. Heat inactivated NNV TCSN in RLT buffer should be stored at -20$^0$C or -80$^0$C in a concentrated solution in small aliquots which are used only once. It is likely that heat inactivated TCSN can be shipped at 4$^0$C or 22$^0$C and then frozen at -80$^0$C without substantial loss of activity compared to continuous cold storage.

4. Although the test procedures appear quite robust, ring testing (for example through ANQAP) should be undertaken to ensure that variations in protocol which exist between laboratories do not result in inconsistent diagnostic outcomes.

## Objective 2

## To develop and evaluate the applicability of serological tests for detection and identification of Betanodaviruses

The following tasks were undertaken in order to complete this objective:

i) Purification of antigens of betanodavirus: recombinant coat protein (rCP) and native virus

ii) Production of antisera against the rCP and native virus antigens of betanodavirus in mammals and fish

iii) Development of ELISA for detection of anti-NNV antibodies in fish

iv) Evaluation of alternative test formats: competitive ELISA and serum neutralisation test

v) Validation of the ELISA using serum samples from exposed/infected and control fish

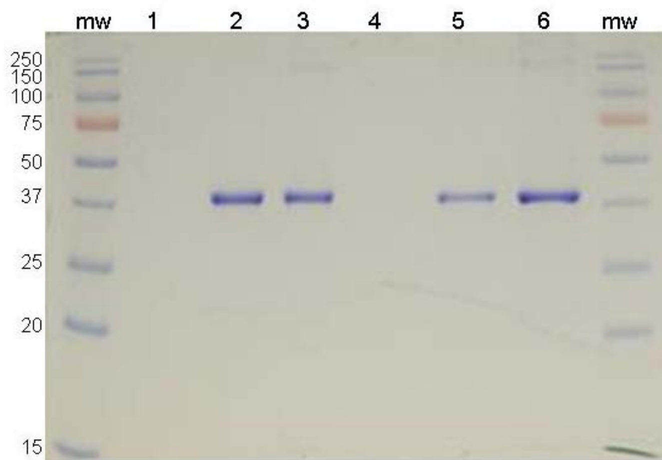
### i) Purification of antigens of betanodavirus: recombinant coat protein (rCP) and native virus

Prior work conducted at the University of Sydney by Kylie Gore during an ARC-funded project indicated that purification of NNV was not straightforward, despite several reports to the contrary in the literature. Standard methods resulted in high yields of impure virus which was deemed to be unsuitable for production of antisera. Specifically, density gradient purification of NNV did not remove all traces of cell culture proteins. An extremely clean virus preparation is desirable for use as antigen when generating mammalian antibodies which are to be used as reagents in immunological assays. Antibodies to SSN-1 (fish) cells in which NNV is usually cultivated, might cross-react with proteins in fish samples, for example in immunohistochemistry. ELISA formats which use antigen generated in SSN-1 cells would be susceptible to non-specific reactivity if antibodies to the cells were present together with the anti-NNV antibodies. Reports of immunological studies using antibodies produced against NNV include only limited data indicating the effectiveness of the purification procedures (Breuil and Romestand, 1999; Kuo et al., 2011; Nguyen et al., 1996). For this reason two approaches were used in this project: production of antisera against a recombinant coat protein of NNV, as the coat protein is the dominant antigen of the virus, and; novel strategies to obtain purified native virus. The information in this section of the report is important because it describes the antigens which were used to immunise sheep, rabbits and fish for ELISA development, and may be informative in the future in relation to assay specificity.

*Purification of rCP antigen*

In a previous FRDC project at QDPI the coat protein of NNV was cloned, expressed and used to immunize in sheep. This reagent is now used in immunohistochemistry tests for NNV in Australia (Moody et al., 2004a). However, this batch of rCP is no longer available. For this reason a new source of rCP was required. rCP samples #R752 and #753 which were prepared by Paul Hick and Satoko Kawaji at the University of Sydney in 2009 were gifted to the project, thawed on ice from -80°C and dialysed at a volume ratio of 1:1000 for 40 hours across a tube membrane with 12,900 Da cut-off (Sigma) against two changes of PBS with 0.01% w/v sodium azide at 4°C. This resulted in a fine precipitate in both samples; the buffer was changed and dialysis continued for 24 hours after which the buffer was changed to PBS without sodium azide and dialysis continued for 12 hours. The floccular precipitate was suspended in the buffer and well-mixed samples were stored in 1.5 ml tubes at -80°C and labeled: R752 one step 3.9.10 and R753 refolding 3.9.10. The Protein Assay (BioRad) was used to determine the protein content with BSA as the standard according to the manufacturer's instructions; protein concentrations were estimated to be 0.7 and 0.4 mg/ml respectively prior to dialysis. Samples were submitted to EMAI for negative contrast electron microscopy; no viral-like particles were observed. SDS-PAGE analysis confirmed bands of approximately 40 kDa with minimal amounts of contaminating protein (Figure 4); there was little or no soluble protein as the supernatant of the suspension was devoid of visible bands.

Figure 4. SDS-PAGE analysis of NNV recombinant coat protein preparations after dialysis. Coomassie brilliant blue stain. MW, molecular weight markers; lane 1, R752 supernatant; lane 2 R752 pellet; lane 3 R752 whole sample; lane 4, R753 supernatant; lane 5, R753 pellet; lane 6, R753 whole sample



*Purification of native virus antigen*

Two purified NNV preparations were used as antigen for inoculation of sheep and rabbits (sample 1: 1201/08-1 and sample 2: 1201/08-2). Both preparations were prepared by repeating the following steps with NNV isolate D236 (EMAI) (isolated in 2006 from 150 day old clinically normal Australian bass, Port Stephens) cultured in SSN-1 cells in 2 different cell culture factories. Sheep serum with antibodies specific for SSN-1 cell proteins was used to remove contaminating cell culture proteins by immuno-precipitation. Preparations of purified NNV treated in this way were more pure than gradient purified preparations based on the evidence from analyses using electron microscopy and SDS PAGE (Gore et al unpublished).

1. Propagation of NNV in SSN-1 cells in a 10 deck cell culture factory.
2. Removal of SSN-1 cell debris. A clarified cell culture supernatant containing NNV was prepared by pooling sonicated SSN-1cells and culture medium, performing low speed centrifugation and discarding the pellet.
3. Concentration of NNV by centrifugation at 10 000 *x g* for 17 h. The pellet was resuspended in 10 ml of cell culture medium.
4. The concentrated NNV preparation was sonicated and then passed through a 0.45 µm filter membrane.
5. Sucrose cushion centrifugation. NNV was further purified and concentrated by ultracentrifugation at 100 000 *x g* for 2.5 h in tubes containing 10 ml of 15% sucrose with a 0.5 ml 60% sucrose cushion. The 0.5 ml fraction of 60% sucrose was retained.
6. Linear sucrose gradient centrifugation. The fraction from step 5 was applied to a linear sucrose gradient and centrifuged at 100 000 *x g* for 16 h. A 0.5 ml fraction collected from Band 3 was retained.
7. Immuno-precipitation. The sample collected from step 6 was mixed with 1 ml of sheep anti-SSN-1 serum and 8.5 ml of TEN buffer and incubated with shaking at 37$^0$C for 1 hour.
8. Affinity chromatography. Sheep IgG and precipitated SSN-1 cell antigens were removed using a protein-G in a sepharose column.
9. Sucrose cushion centrifugation. The flow-through from step 8 was applied to 6 tubes containing 10 ml of 15% sucrose with a 0.5 ml 60% sucrose cushion. Concentrated NNV was collected in a 0.6 ml fraction from the sucrose cushion.
10. Discontinuous sucrose gradient centrifugation. The sample from step 9 was applied to two discontinuous sucrose gradients and centrifuged at 100 000 *x g* for 16 h. Purified NNV was collected in a 0.5 ml fraction. The purified NNV preparation was a pool of the 2 x 0.5 ml fractions collected at the 45% - 60% sucrose interface in each tube.

The protein concentration and yield of the 2 aliquots of purified NNV used for animal inoculation are shown in Table 10. Protein concentration was determined using 60% sucrose w/v in TEN to blank the spectrophotometer. Although the yields were low, the purified NNV preparations contained sufficient capsid protein for use as antigen for inoculation of sheep and rabbits to generate anti-NNV antibodies.

Table 10. Yield of purified NNV from culture in SSN-1 cells in NUNC cell factories

| Sample | Protein concentration** mg/ml | Volume Ml | Yield* Mg |
|---|---|---|---|
| 1201/08-1 | 0.25 | 1.20 | 0.30 |
| 1201/08-2 | 0.19 | 1.51 | 0.29 |

* this is the total amount of purified NNV obtained from SSN-1 cell culture in one 10 stack cell factory

In electron microscopy the purified NNV preparations contained 120 particles/grid square, 80% of which were intact; no cell debris was evident (Figure 5).

In SDS PAGE the purified NNV appeared as a double band at approximately 38 and 40 kDa, which is consistent with the NNV capsid protein (Hegde et al., 2002) (Figure 6). No other protein was evident. A single band at approximately 60 - 70 kDa was evident in the clean SSN-1 cell preparation; possibly this was albumin from the foetal bovine serum used in the culture medium. Thus, SDS PAGE was not sufficiently sensitive to detect the SSN-1 cell proteins in the quantities likely to contaminate purified virus preparations. There was no evidence on SDS PAGE of sheep serum proteins in the purified NNV. Samples collected after immuno-precipitation contained several protein bands which matched those seen in sheep serum, but the heavy and light chain IgG proteins were removed.

Figure 5. Negative contrast electron microscope image of purified betanodavirus particles (1201/08-1). Scale bar = 100 nm.

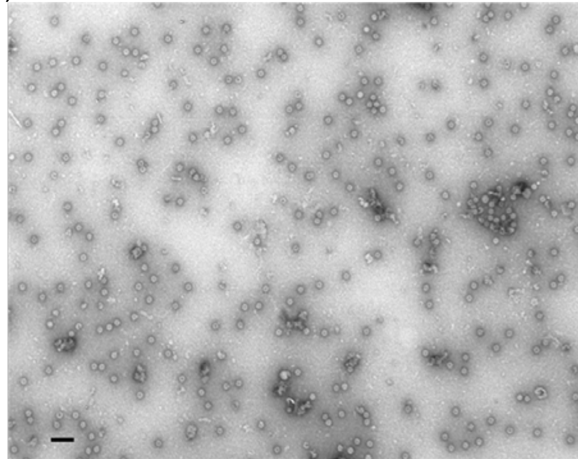
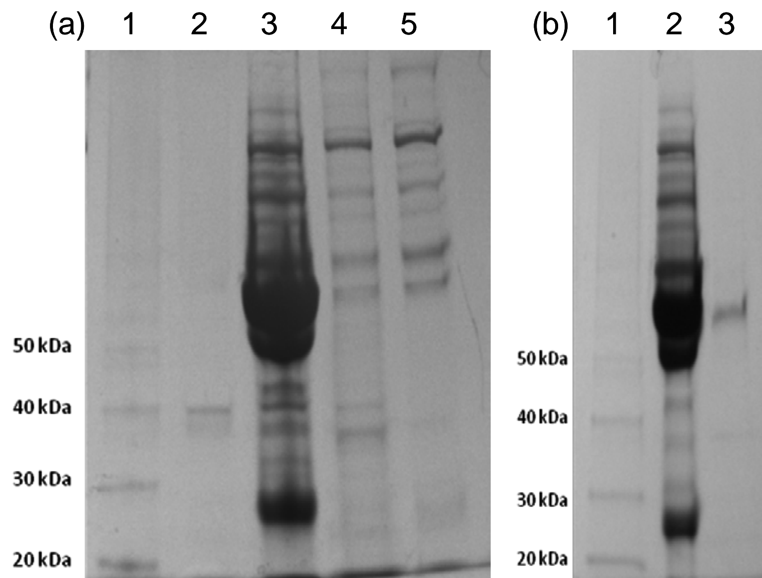


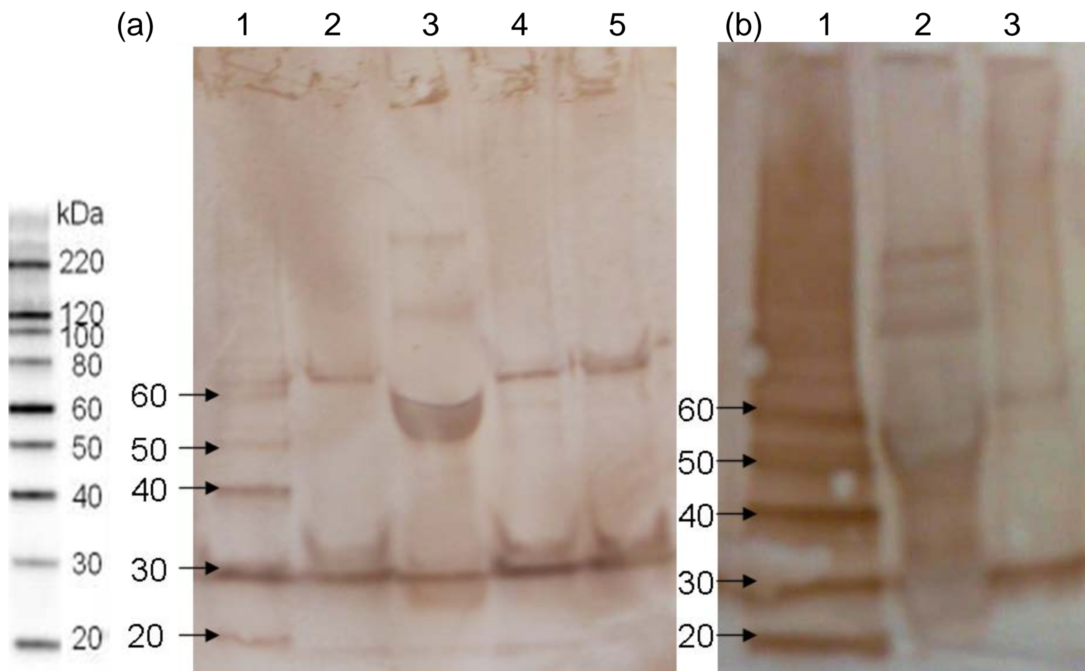
Figure 6. SDS PAGE gel images. The purified virus preparation (1201/08-1) in lane 2 shows only the 38/40 kDa capsid protein. (a) Lane 1: Protein mass standard with sizes indicated; Lane 2: purified virus preparation (25 µl of 1201/08-1); Lane 3: virus preparation mixed with sheep anti-SSN-1 serum before affinity chromatography (Step 7); Lane 4: virus preparation with sheep serum after affinity chromatography, before sucrose gradient centrifugation (flow-through from Step 8); Lane 5: as for lane 4, but from a fraction collected in step 6 which was not used to produce the purified NNV. (b) Lane 1: protein size marker; Lane 2: anti-SSN-1 sheep serum (1 µl); Lane 3: NNV free SSN-1 cell preparation from negative control cells as in step 2 (25 µl).



Two bands were identified in a Western blot of the purified virus preparation at approximately 32 and 70 kDa (Figure 7a, lane 2). Bands of similar size were evident in the uninfected SSN-1 cell preparation (Figure 7b, lane 3). This may indicate incomplete purification of NNV. Alternatively, the antigens in the purified NNV preparation may be from the sheep serum used in immuno-precipitation or proteins in the bovine serum used in the culture medium that were recognised directly by the secondary antibody (donkey anti-sheep conjugate). A number of bands were stained in a Western blot containing only sheep serum (Figure 7b, lane 2). Further Western blotting experiments are required to determine the nature of these proteins.

It is possible that other SSN-1 cell proteins may be present in the purified virus preparation which were not detected by the Western blot. Additional purification efforts were not pursued because they reduced the yield of virus, and such contamination was not likely to reduce the fitness of the preparation for the intended purpose.

Figure 7. Western blot with sheep anti-SSN-1 serum used as the primary antibody, donkey anti-sheep IgG antibody used as the conjugate and developed with the Dako chromogen kit. (a) Lane 1: protein size markers; Lane 2: purified betanodavirus preparation; (25 µl of 1201/08-1); Lane 3: virus preparation with sheep anti-SSN-1 serum before affinity chromatography (Step 7); Lanes 4 and 5: virus preparation with sheep serum after affinity chromatography, before sucrose gradient centrifugation (flow-through from Step 8); Lane 5: as for lane 4, but from a fraction collected in step 6 which was not used to produce the purified NNV.
(b) Lane 1: protein size marker; Lane 2: anti-SSN-1 sheep serum; Lane 3: betanodavirus free SSN-1 cell preparation, as for figure 3b.




**ii) Production and screening of antisera against the rCP and native virus antigens of betanodavirus in mammals and fish**

The specificity of mammalian antibodies generated against a purified NNV antigen preparation requires careful evaluation in ELISA and in other immunological assays. Provided that the viral preparation was sufficiently free of cellular and other irrelevant antigens, anti-sera raised in sheep should be specific for NNV antigens. However, anti-sera raised in species such as rabbit may contain antibodies against sheep serum components in addition to antibodies against NNV.

*Recombinant coat protein (rCP)*
The aim of this study was to produce high titred antiserum in rabbits and sheep against the rCP of NNV and to produce control positive and negative serum samples for antibody ELISA in fish.

A vaccine was prepared by mixing #R753 column refolded rCP (360 ug/ml) with Montanide ISA50V adjuvant 60:40 as an oil in water emulsion with thiomersal preservative 0.013%. Vaccine was stored at 4°C until required. The dose of rCP was 144 ug per 1 ml dose for rabbit and sheep and 43 ug per 0.3ml dose for fish.

Blood samples were collected from two rabbits and two sheep prior to immunisation. Each animal was given two doses of vaccine (sheep subcutaneously; rabbits intramuscularly) with one month between doses. Blood samples were collected two weeks after the second dose.

Five barramundi (190-240 mm TL; 78-141g) and two Australian bass (265-340 mmTL, 287-400g) were anaesthetized in benozocaine 40mg/L and given 0.3ml of vaccine intraperitoneally and this was repeated one month later. Blood samples were collected from the fish at approximately 2 week intervals.

Blood was allowed to clot for 2 hours then centrifuged; serum was harvested and stored at -20°C. Duplicate fish serum samples were also pre-diluted 1:10 in TSGM and stored at -20°C.

*Purified NNV*
A stable emulsion of Montanide ISA50V adjuvant and purified NNV (lot 1201/08-1 in PBS) in the ratio 60:40 was prepared. Unused vaccine was stored for one month at 4°C until required for the booster dose. Each 1 ml dose of vaccine contained 30 ug of NNV protein. This preparation was used for the first and second doses. Two further doses were prepared as above using purified NNV (lot 1201/08-2 in PBS) and each 1 ml dose of vaccine contained 27 ug of NNV protein. This preparation was used for the third and fourth doses. Two 4 month old New Zealand white rabbits and two 14 month old Merino sheep were immunised. Each animal was given a 1ml dose of vaccine subcutaneously; this was repeated for a total of 4 doses. A blood sample was collected from all animals prior to the first vaccine, 2 weeks after the second dose, and then 2 weeks after the fourth dose.

Blood was collected from the ear vein of rabbits and the jugular vein of sheep, allowed to clot for at least 1 hour, centrifuged at 2000g for 15 mins and serum was poured off, placed in 5 ml screw capped vials and stored at -20°C.

For Australian Bass, the vaccine was prepared in a similar fashion. The first dose contained approximately 40 µg of heat inactivated purified NNV (60° C for 60 min) as described above. The booster dose had the same amount of chemically

inactivated (Beta propiolactone) virus. The antigen in an aqueous form was emulsified in a 2:3 ratio with Montanide ISA50v for a final volume of 0.3 ml per fish. Seven fish were immunized intraperitoneally with the antigen while 3 fish were immunized with the adjuvant emulsified with PBS using the same ratio, volume and protocol. Blood was collected from the caudal vein and dispensed in 0.8 ml Z serum separation MiniCollect ® tubes. After 2 hours at room temperature, tubes were centrifuged on a bench centrifuge at 2000g for 20 min. Serum was recovered with a pipette and stored in 1 ml screw tubes at -20°C.

### iii) Development of ELISA for detection of anti-NNV antibodies in fish

The aim of this work was to develop ELISA protocols for the detection of antibodies produced against NNV as a result of infection of fish; the target sample was serum.

The work was divided into two stages:

1. Development of a method for capture and binding of antigen onto a solid phase (the ELISA plate)

2. Development of an indirect ELISA for detection of antibodies in fish serum which bind to the antigen.

Many reagents were used in this study in order to assess their suitability for use in immunoassay. They are described in table 11.

Table 11. Summary of anti-NNV antibodies used in this study

| Antigen used in immunisation | Batch number | Origin | Bleed date |
|---|---|---|---|
| **Rabbit** | | | |
| Purified nodavirus | FAH 1202 | France[1] | Unknown |
| **Sheep** | | | |
| NNV recombinant coat protein | Q146 | FRDC project 2001-626 supplied by DPIF QLD | |
| NNV recombinant coat protein | PS01 | FRDC project[3], SVC 10/206 | 26/12/2010 |
| NNV recombinant coat protein | PS02 | FRDC project[3], SVC 10/206 | 26/12/2010 |
| NNV recombinant coat protein | Q357 | PhD study[2], SVC 07/021 | 18/06/2007 |
| NNV recombinant coat protein | Q358 | PhD study[2], SVC 07/021 | 18/06/2007 |
| Purified NNV from CSCI gradient | Q359 | PhD study[2], SVC 07/021 | 18/06/2007 |
| Purified NNV | V092 | FRDC project[3] SVC 10/236 | 29/03/2011 |
| Purified NNV | V093 | FRDC project[3] SVC 10/236 | 29/03/2011 |
| **Fish** | | | |
| Barramundi | | | |
| Crude extract of fish homogenate | | PhD study[2], SVC 04/01, 04/02, 04/03, 04/04 | 06/2003 |
| Pre-bleed | | FRDC project[3] SVC10/178-2 | 2010 |
| NNV recombinant coat protein | | FRDC project[3] SVC 10/208 | 01/11/2010 |
| Australian bass | | | |
| Purified NNV | | FRDC project[3] SVC 12/043 | 05/2012 |

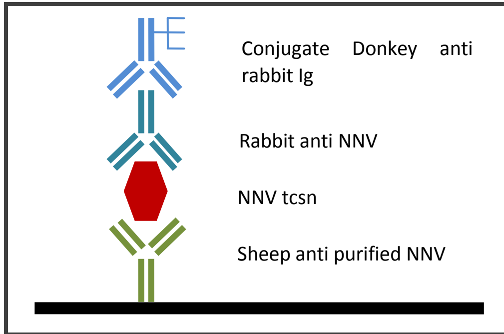
[1] supplied by Dr. Jeannette Castric, Agence Francaise de securite Sanitaire des Aliments (AFSSA)
[2] supplied by Kylie Gore
[3] this FRDC project 2008/041

*Development of a method for capture and binding of antigen onto a solid phase (the ELISA plate)*

*General assessment of anti-NNV antibodies in an indirect sandwich ELISA.* In the general method wells of a 96-well polystyrene Immulon® plate were coated with 100 ul of one of the sheep or rabbit antibodies diluted in borate coating buffer and incubated overnight at 4$^0$C. After washing 5 times with milli-Q purified water plus 0.05% v/v Tween 20, a tissue culture supernatant containing infectious NNV was added to each well and incubated for 60 min at RT. The plate was washed manually to remove live NNV, then, washed as described above. The manipulation of the live NNV antigen was carried out in a class 2 cabinet. The plate was blocked with phosphate buffered saline with 0.1% v/v Tween 20 (PBST) and 1% w/v gelatin for 30 min at RT. The second antibody diluted in PBST with 0.1% w/v gelatin (PBSTG) was added and incubated for 60 min at RT. After washing, a conjugate (either KPL donkey anti sheep- or swine anti-rabbit – HRP) diluted 1:1500 in PBSTG was added and incubated for 60 min at RT. After washing, ABTS chromogen was added and incubated for 20 min. The reaction was stopped by the addition of 50 ul/well of stop solution and OD was read in a Thermo™ microplate photometer with a 405 nm filter. The format is shown in Figure 8.

It was found that sera raised in rabbits at the University of Sydney had poor reactivity against NNV regardless of the antigen used in the immunization. Antibodies produced by Dr. Castric in France (FAH 1202) on the other hand, generated high OD signal when tested with the positive control, although high OD readings were also found in negative control wells. The French reagent was therefore used in initial experiments on the capture system. There were additional problems with the rabbit antibodies raised at the University of Sydney. Numerous experiments were conducted with appropriate controls and it was determined that the rabbit antibodies were binding to primary sheep antibodies. The reaction of the rabbit antibody to the sheep antibody could be titrated, and it was abolished by adsorption of rabbit antibody solution with 100 ug/ ml of affinity purified sheep Ig. Furthermore, no difference in reactivity was revealed between different antigen controls (live virus, negative SSN-1 supernatant, no antigen) suggesting low reactivity of rabbit antisera for NNV. This was attributed to the use of sheep serum in the viral purification process (it was included with the purpose of adsorbing the SSN-1 cell debris that was present). The remaining immunoglobulin that was not captured in the affinity purification process, or other sheep serum proteins, were part of the purified NNV antigen, and appear to have acted as stronger antigens than NNV when administered to the rabbits. They would have been recognized as “self” by the sheep which were immunized with the same preparation, and therefore sheep were able to respond to the NNV antigen component. This explains both the reactivity of the sheep antisera to NNV and the lack of reactivity of the rabbit antisera to NNV.

Figure 8. ELISA format to evaluate capture antibodies




*Choice of capture antibody.* Antibodies against NNV raised in sheep had good performance in ELISA as capture antibodies. There was no observable difference in the antigenicity of the different inocula used in this study, i.e. sheep immunised with either rCP or purified NNV produced antibodies with suitable affinity for use in ELISA (Figure 9). However, the antibody produced within FRDC Project 2001/626 (Q146), where the antibodies raised in sheep against NNV recombinant protein, showed low reactivity when used in the capture ELISA format despite being successfully used in other immunoassays like IHC and IFAT. Determining the reason for this was beyond the scope of this study.

A comparison of the reactivity towards the virus and other relevant antigens in the reaction was carried out using two different negative controls: wells lacking antigen and wells containing uninfected SSN-1 cell culture supernatant. The difference in OD between positive and negative controls was sufficient to predict the presence of the virus (Figure 10).

Figure 9. Representative results for sheep anti NNV reagents in the capture ELISA format. The dilution of the second (detection) antibody is displayed in the legend (FAH#1202 rabbit αNNV).

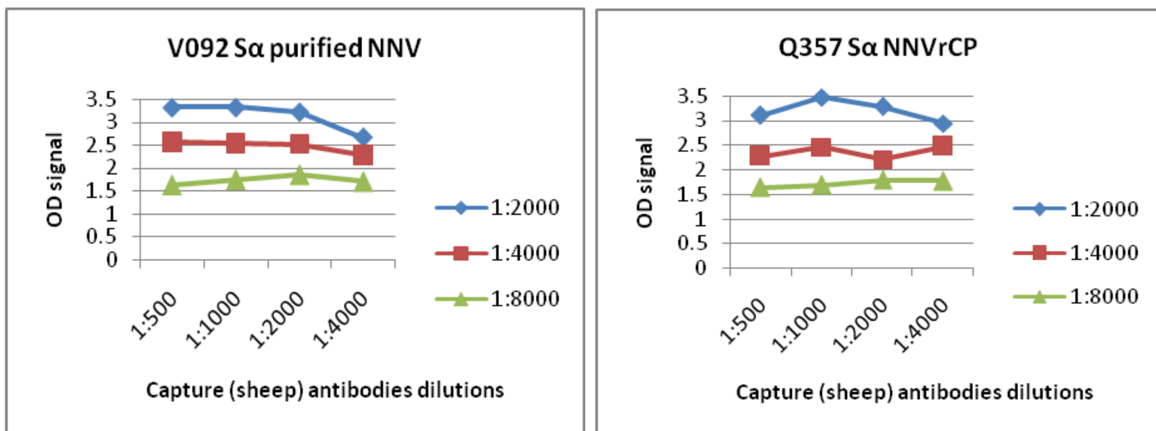
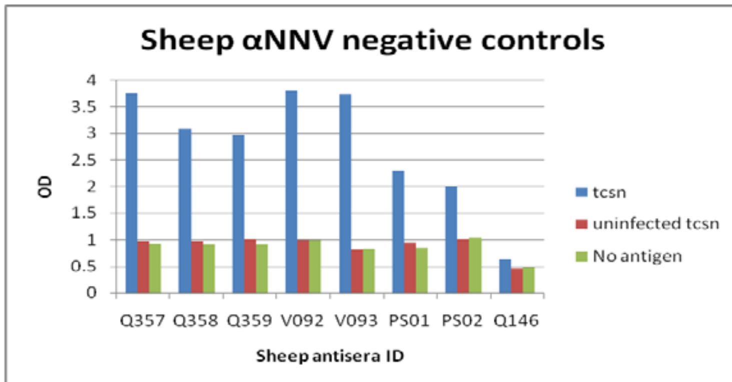
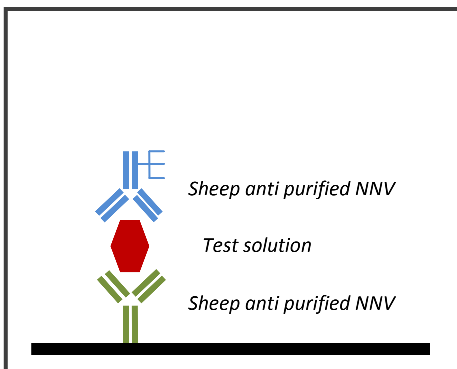


Figure 10. Performance of sheep anti NNV antibodies in the capture ELISA format tested with different controls; TSCN is the positive control while uninfected TCSN and no antigen are the negative controls




*Simplification of the ELISA format - monospecies sandwich ELISA.* Addressing the situation where a large amount of sheep NNV antiserum of high titre and specificity was available, and where non-specific binding was detected in the sheep-rabbit format described above, a monospecies sandwich ELISA was developed in order to optimize the antigen detection system. The format for this assay is shown in Figure 11.

Figure 11. Monospecies sandwich ELISA format for antigen detection




Wells of a 96-well polystyrene Immulon® plate were coated with 100 ul of affinity purified sheep anti-NNV antibodies (batch #V092) diluted in borate coating buffer and incubated overnight at 4$^{0}$C. After washing 5 times with milli-Q purified water plus 0.05% v/v Tween 20, a tissue culture supernatant containing infectious NNV was added to each well and incubated for 60 min at RT. The plate was washed manually to remove live NNV in a class 2 cabinet and washed again as described above. The plate was then blocked with phosphate buffered saline with 0.1% v/v Tween 20 (PBST) and 1% w/v gelatin for 30 min at RT. Sheep anti-NNV antibodies (batch V093) labelled with HRP diluted in PBST with 0.1% w/v gelatin (PBSTG) was added and incubated for 60 min at RT. After washing, ABTS

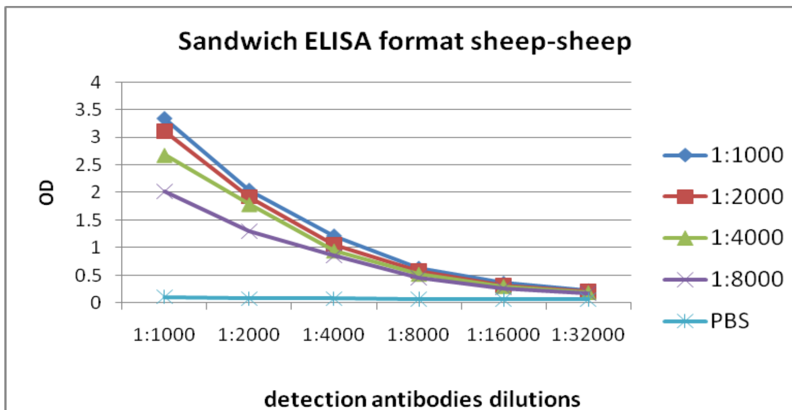
chromogen was added and incubated for 20 min. The reaction was stopped by the addition of 50 ul/well of stop solution and OD was read in a Thermo™ microplate photometer with a 405 nm filter.

Purification of serum immunoglobulins (Ig) was carried out on an AKTA purifier fast performance liquid chromatography system (GE Healthcare) using a 5 ml Hi-Trap protein A for rabbit serum or protein G column for sheep serum (GE Healthcare).

Conjugation of affinity purified sheep anti NNV antibodies was carried out using the Lightning-Link™ HRP conjugation kit (Innova Biosciences) following the protocol suggested by the manufacturer.

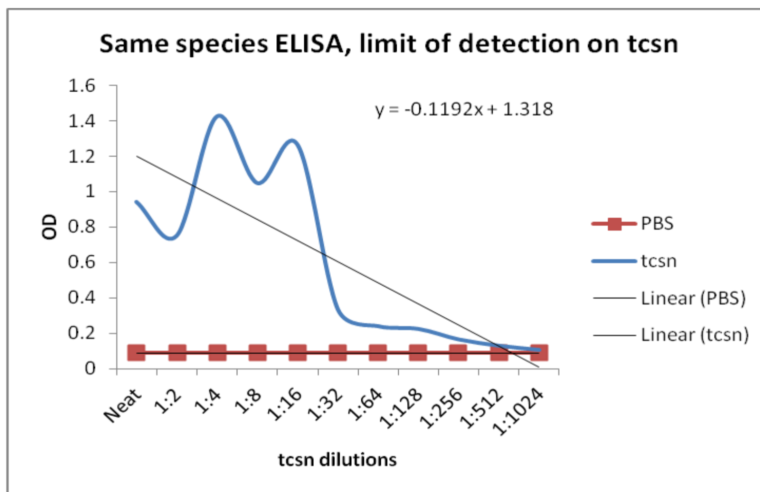
The results indicated that this assay was simpler and faster than the format with sheep and rabbit antibodies and a separate conjugate described above, and improved specificity was observed. OD readings of the negative control wells were always below 0.116 despite a low dilution of the antibodies. In contrast, OD was very high in the presence of the virus, with values of up to 3.34. Little change was observed in readings when capture antibodies were diluted 2 fold, however the signal declined rapidly with increasing dilutions of the detection antibodies (Figure 12).

Figure 12. Titration of sheep anti-NNV conjugate in the monospecies sheep-sheep NNV sandwich ELISA format. Capture antibody (V092) dilutions are displayed in the legend



*Analytical sensitivity of the monospecies sandwich ELISA as an antigen capture ELISA.* The monospecies sandwich ELISA may have potential as a diagnostic screening test. Although not an objective of the project, further analysis was carried out in order to assess the antigen capture ELISA for direct detection of NNV. Analytical sensitivity of the monospecies ELISA was established using two fold dilutions of SSN-1 TCSN infected with a concentration of $10^8$ $TCID_{50}$/ml. The limit of detection of the assay was established to be 7.8x $10^4$ $TCID_{50}$ using an arbitrary cut off point of an OD of 0.22 (Figure 13). This is consistent with the sensitivity of another antigen detection ELISA for a piscine virus in TCSN (Whittington and Steiner, 1993). Analytical specificity on the other hand, was tested using uninfected SSN-1 TCSN and EHNV BF-2 cell TCSN. As results were negative for both analytical specificity appeared to be high.

Figure 13. Limit of detection. SSN-1 TCSN dilutions. Capture and detection antibodies were used at a dilution of 1:1500




*Development of an indirect ELISA for detection of antibodies in fish serum which bind to the antigen*

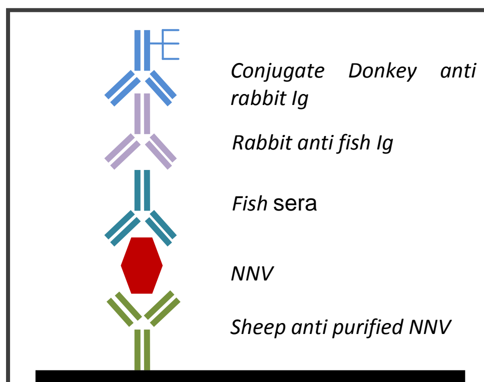
It was concluded from the work described above that an efficient antigen capture system had been developed. This system would form the basis for an indirect antibody detection ELISA for fish serum. The ELISA format shown in Figure 14 was used.

Various blocking solutions in different concentrations as well as different concentrations of capture and detection antibodies and incubation protocols were tested in the preliminary stages of this study. The best results were obtained with the following conditions. A volume of 100ul of affinity purified sheep anti-NNV antibodies (batch# V092) diluted in borate coating buffer was added to the wells of a 96-well polystyrene Immulon® plate and incubated overnight at 4°C. The following steps were carried out at RT. After washing 5 times with RO water plus 0.05% v/v Tween 20, a solution of semi-purified NNV diluted 1:300 was added to

testing wells and PBS solution was added to control wells in a 100ul volume for both. The plate was then incubated for 60 min and washed manually in a class 2 cabinet in order to remove unbound infectious virus. The wells were blocked with a 100ul volume of PBST plus 0.1% w/v skim milk and incubated for 30 min. After washing as described above, fish antibodies were added and incubated for 60 min, a washing step followed and rabbit anti Australian Bass antibodies diluted 1:1500 were incubated in the wells for 60 min. These antibodies were shown to be broadly cross reactive with barramundi and so were used for both species. After the subsequent wash step, the conjugate donkey anti rabbit Ig-HRP (KPL) diluted 1:1500 was added and incubated for 60 min before the final wash. ABTS was added in a 100ul volume/well and incubated for 20 min. The reaction was finally stopped with 50 ul/well of stopping solution and OD was established with a Thermo™ microplate photometer with a 405 nm filter.

It is recommended that semi purified NNV antigen be used rather than NNV infected cell culture supernatant. The method of preparation of this antigen is as follows. The contents of a SSN-1 cell factory infected with NNV were freeze thawed at -20°C, then centrifuged at 3000 x *g* for 30min and clarified TCSN was decanted and retained. Cell pellets were pooled in 5ml clarified TCSN and sonicated (5 sec, setting 9, 3 pulses: 5 sec setting 6, 2 pulses). Sonicated cells were centrifuged at 5000 xg for 30min; supernatants were added to clarified TCSN and the pellets were discarded. Pooled supernatants were centrifuged at 10000 x *g* for 17 hours, decanted and a total of 10 ml of pelleted virus was stored at -80°C in aliquots of 20ul, which is the amount required for one ELISA plate (5.5 ml of diluted antigen). Alternatively 20ul of the antigen can be diluted in 5.5 ml PBS and frozen at -20°C without loss of activity (during this study).

Figure 14. NNV Indirect ELISA Format for fish antibody detection. Semi purified NNV obtained from tissue culture supernatant is used as antigen.



Numerous optimization experiments were conducted to reduce non-specific reactivity of fish serum components. Optimization was achieved initially by assessing different proteins in blocking solutions. Results showed that the best protein to be used in the blocking solution was skim milk, creating a 12 fold difference between average of positive and negative wells in the system with barramundi. Background OD readings could be reduced to 0.2. Use of semi-purified NNV antigen diluted in PBS instead of the cell culture supernatant also reduced background reactivity. No reactivity was observed with negative control SSN-1 cell culture supernatant.

*Results obtained using positive control fish sera.* OD values obtained from barramundi serum samples taken before immunisation with NNV rCP differed significantly from those taken after immunisation ($p$<0.001). There were two Australian bass included in this study and they had two very different responses to the vaccine. Both fish showed a clear difference between pre-bleed and immune bleed values. In the presence of the virus, readings were around 0.5 for prebleeds compared to 1 and 1.9 for immune bleeds of fish T24 and T26, respectively, at a serum dilution of 1:1600. However, only fish T26 displayed values in immune bleeds where wells with virus could be distinguished from well without virus (difference up to 6 fold). In other words, only antibodies from fish T26 were specific to NNV virus.

**iv) Evaluation of alternative test formats: competitive ELISA and serum neutralisation test**

The indirect antibody detection sandwich ELISA developed in this project may require a species specific anti-fish reagent for use in other species, although fortuitously one reagent was suitable for both barramundi and Australian bass. To avoid this potential problem two assays were trialled that do not require a species specific anti-fish reagent: competitive ELISA and serum neutralisation test. In theory these tests could be used in any species of fish after validation.

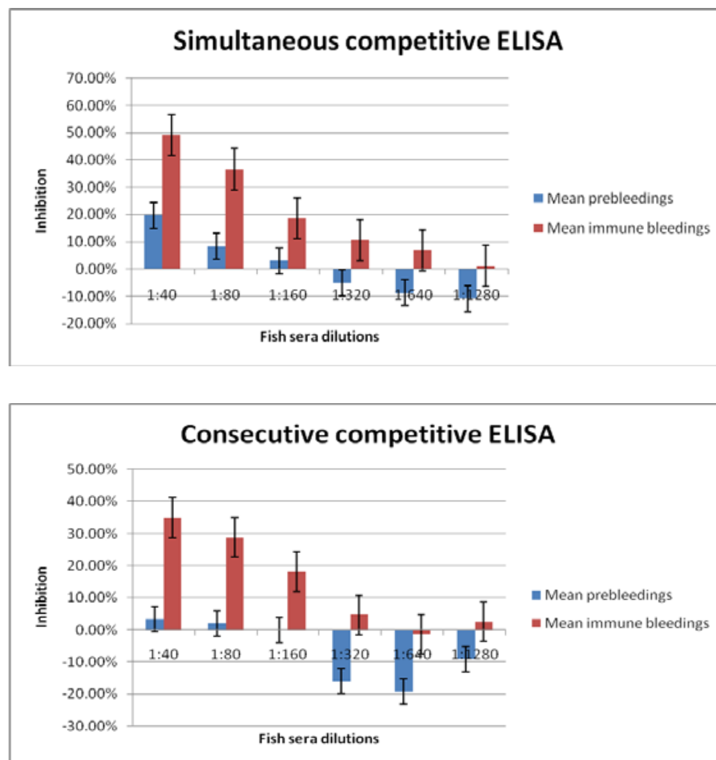
*Competitive ELISA*
Competitive ELISA was carried out in two different ways, a simultaneous or competitive format and a consecutive or inhibition (blocking) format. Although both formats included the same components, the order of reagents was different. In the inhibition format the fish serum was incubated for an hour and washed before the addition of labelled antibodies, whereas in the competitive format the fish serum and labelled antibodies were incubated simultaneously. Reagents and concentrations were optimized in order to obtain a target OD of approximately 1.5 in the absence of inhibition.

Immulon ® plate wells were coated with 100 ul of affinity purified sheep anti NNV (batch #V092) diluted 1:2000 in borate coating buffer and incubated overnight at 4°C. The following steps were carried out at RT. Plates were washed 5 times with RO water plus 0.05% v/v Tween 20, a solution of 1:300 of semi-purified NNV was added to testing wells, and PBS was added to "blank wells". The plate was incubated for an hour and washed manually in a class 2 cabinet in order to avoid possible live virus release. The subsequent blocking step was performed with PBST plus 1% w/v skim milk and 30 min incubation followed by a washing step as described before. Fish sera were diluted in PBST + 0.1% w/v skim milk and added in different concentrations. A row of wells was left without the fish antibodies in order to assess OD readings of the ELISA system without inhibition. Labelled affinity purified sheep anti NNV (batch # V093) was added at a concentration of 1:1000 diluted in the same diluent as the fish serum according to the protocol established for each format. After washing, 100 ul of ABTS substrate were added and incubated for 20 min. OD readings were obtained with a Thermo™ microplate photometer using the 405 nm filter.

The results indicated that inhibition of up to 79% was obtained with antisera from Barramundi (sample ID IB2), at a dilution of 1:40. However at this concentration, inhibition was generally high for all sera tested regardless of the immune status. At a dilution of 1:80 a greater difference could be observed between prebleed and immune sera. For immune bleeds, different degrees of response could be detected, being high inhibition for some samples and low inhibition for others, a pattern which corresponded to the antibody detection ELISA results. Thus, samples with high OD difference (positive-negative wells) in the sandwich ELISA format had the greatest percentage inhibition in the competitive test. Sample IB7 for instance, from Australian Bass (T24), with very high non-specific OD values in the sandwich ELISA had a very low level of inhibition in the competitive format. Although inhibition of immune sera was greater in the competitive protocol, a greater difference between pre-bleeds and immune-bleeds was observed using the blocking protocol (Figure 15).

A total of 91 barramundi brood stock sera were analyzed in the competitive ELISA format. The correlation of these results with those from the indirect antibody sandwich ELISA was very poor (-0.36), and the latter was found to provide consistent outcomes between replicates, and results were correlated with qPCR (see below). It was concluded that the competitive ELISA was of lower utility than the indirect antibody detection sandwich ELISA.

Figure 15. Competitive ELISA, simultaneous and consecutive protocols showing differential inhibition between pre-bleed and immune bleed samples in fish immunised with rCP of NNV.




*Serum neutralisation test*

In a serum neutralisation test, antibodies in the sample are allowed to react with live virus, causing the virus to be neutralized, which means that it is unable to replicate in a cell monolayer. Studies were undertaken at EMAI to identify optimal conditions for the detection of anti-nodavirus antibodies by use of a serum neutralisation test. Due to the limited availability of fish serum at the time, the initial experiments used sheep anti-NNV antiserum prepared at the University of Sydney by immunising sheep with Australian bass nodavirus that had been cultured and purified at EMAI. A comparison was made of virus detection in the assay by microscopic detection of cytopathology and by immunoperoxidase staining (Moody et al., 2004a). Virus serum mixtures were incubated prior to the addition of SSN-1 cells. In the prototype assay the sheep NNV antisera V093 and V094 had high neutralising titres (≥1280) against 100 $TCID_{50}$ Australian bass nodavirus.

Based on several experiments with fish serum, the neutralisation test appeared to be unsatisfactory because of the large volume of serum required. Therefore it is unlikely to have practical application.

**v) Validation of the ELISA using serum samples from exposed/infected and control fish**

*Indirect sandwich ELISA*
A volume of 100ul of affinity purified sheep anti-NNV antibodies (batch# T238.2, a freeze dried then reconstituted batch of V092) diluted 1:2000 in borate coating buffer was added to the wells of a 96-well polystyrene Immulon® plate and incubated overnight at 4°C. The following steps were carried out at RT. After washing 5 times with RO water plus 0.05% v/v Tween 20, 100ul of a solution of semi-purified NNV diluted 1:300 in PBS was added to testing wells and PBS alone was added to blank wells. The plate was incubated for 60 min and washed manually in a Class 2 Biosafety cabinet in order to remove unbound live virus. The wells were blocked with a 100ul volume of PBST plus 1% w/v skim milk and incubated for 30 min. After washing as described above, fish serum was added at a dilution of 1:150 in PBST 0.1% skim milk diluent; positive and negative controls were also added; sera were incubated for 60 min. A washing step followed and rabbit anti Australian bass immunoglobulin antiserum diluted 1:1500 was incubated in the wells for 60 min. After the subsequent wash step, the conjugate donkey anti rabbit Ig-HRP (KPL) diluted 1:1500 was added and incubated for 60 min before the final wash. The chromogen ABTS was added at 100ul per well and incubated for 20 min. The reaction was stopped with 50 ul/well of stop solution and OD was measured with a Thermo™ microplate photometer using a 405 nm filter. The OD for the positive control was used for standardization purposes.

Due to the “stickiness” or low specificity associated with fish serum, all samples were tested in duplicate on the same plate in the presence and absence of NNV antigen (“testing” and “blank” wells). The mean of the duplicates was calculated and the ratio between the means of the testing and blank wells was established as the OD ratio, being the number of times that the OD of wells with NNV was higher than the OD of wells with PBS.

*Decision limit quality control*
Results were considered valid only when the OD ratio for the positive control was 7.8 or above, otherwise the assay was repeated.

*Positive-negative cut-points*
Samples were considered positive only when they met two criteria, both OD and OD ratio above defined levels. These levels were defined arbitrarily based on the exposure history of the sample panel, parallel qPCR results and expected sensitivity and specificity. For barramundi the OD cutoff point was established at 0.7 and the OD ratio cutoff point at 2.85. Hence samples were positive when their OD was higher than 0.7 and the OD ratio for testing wells was 2.85 times higher than the OD for blank wells (Figure 16a). For Australian bass the cut-off point was established using the same criteria but the final limits were lower due to lower overall reactivity and higher specificity of results. The cut-off point for the

OD was 0.4 and for the OD ratio was 2.5. (Figure 16b). The cut-off points chosen affect both sensitivity and specificity. To illustrate the principle, the impact of these cut-point levels on sensitivity and specificity in barramundi is shown in Figure 16c.

Figure 16a. Overall ELISA results for barramundi brood stock. The cut-off points for OD and OD ratio are expressed as intersecting lines. Results in the top right quadrant are considered positive.

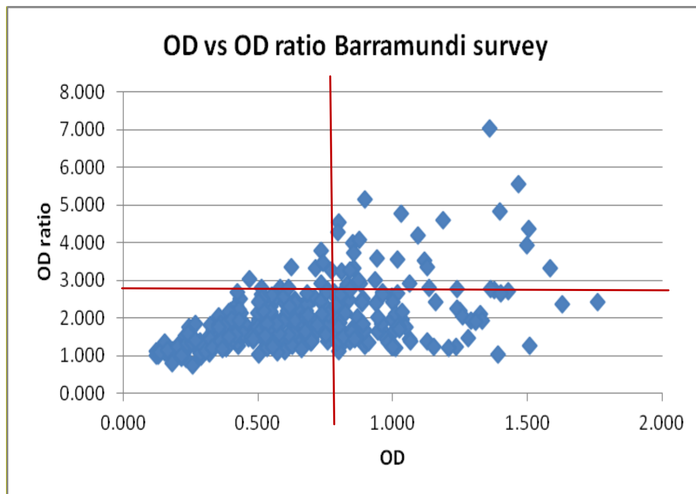



Figure 16b. Magnitude of antibody levels in a persistently infected population of Australia bass. The cut-off points for OD and OD ratio are expressed as intersecting lines. Results in the top right quadrant are considered positive.

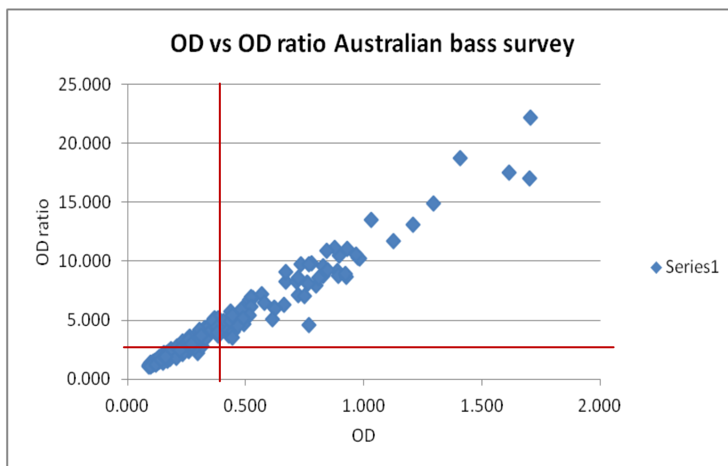
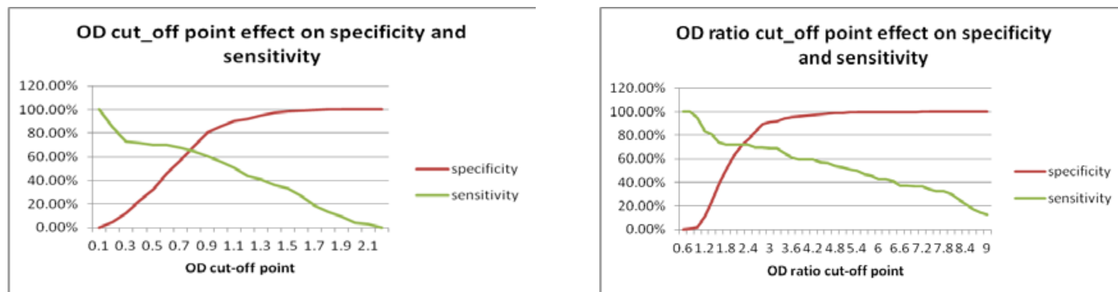


Figure 16c. The effect of cut-point on assay sensitivity and specificity. The data are for all unknown samples in barramundi assuming that they come from non-exposed populations




*Positive and negative control sera*

Control sera were obtained from barramundi included in an immunization trial with NNV rCP. Aliquots of sample SVC 10/178-2 were used as the negative control and aliquots of SVC 10/208-2 were used as the positive control. For Australian bass, samples from the immunization trial with purified NNV were used as control, SVC 12/043-2 as the negative controls and SVC12/043-17 as the positive control.

*Definition of serum samples used to evaluate the ELISA*

Serum samples were available from 726 fish which were classified using objective criteria (Table 12):

- Immunized fish: these were fish which were deliberately exposed to viral antigens by intraperitoneal injection. The antigens were either rCP, crude fish homogenate extract and purified NNV(as described above).
- Naturally exposed fish: these were fish which had survived an outbreak of NNV infection between 2007 and 2010 at Darwin. The cause of the outbreak was confirmed using virus isolation and/or qPCR.
- Fish of unknown status: these were apparently healthy fish. There was no other history except that they did not come from populations which were known to be infected with NNV. They include brood stock and pre-immunisation samples from fish that were later used in immunization trials. The populations of origin were in regions of Australia were NNV may occur.

Table 12. Classification of serum samples used to evaluate antibody detection ELISA

| *Exposure status of population* | *Location* | *Species* | *Number* |
|---|---|---|---|
| *Immunized* | *NT* | *Barramundi* | *33* |
| | *NSW* | *Australian bass* | *28* |
| | | *Barramundi* | *16* |
| *Naturally Exposed* | *NT* | *Barramundi* | *96* |
| | *NSW* | *Australian bass* | *170* |
| *Unknown* | *NT* | *Barramundi* | *224* |
| | *QLD* | *Barramundi* | *99* |
| | | *Grouper (E. coioides and E. lanceolatus)* | *40** |
| | *NSW* | *Australian bass* | *10* |
| *Total* | | | *716* |

*10 samples additional were not tested

**Results**

The results from each of the three different types of fish population suggest that the ELISA provides biologically meaningful test outcomes.

***Immunized fish***

The OD values from sera from 5 immunised barramundi were significantly greater than those from the same fish collected prior to immunisation with NNV rCP ($p$<0.001) (Figure 17). Only one fish retained an identification tag throughout the study and its response is shown in Figure 18. Similar results were obtained in an experiment in barramundi which was conducted at the Darwin Aquaculture Centre by Kylie Gore in 2003 (Figure 19). Barramundi were immunised with crude purified heat inactivated fish homogenate obtained from NNV-infected barramundi. These sera had been archived at the University of Sydney for 9 years prior to testing in this project.

Figure 17. Mean ELISA OD values for barramundi (3 fish at prebleeding and weeks 1-2; 3 remaining fish at weeks 3-4) which were tested before and after immunisation with rCP from NNV.

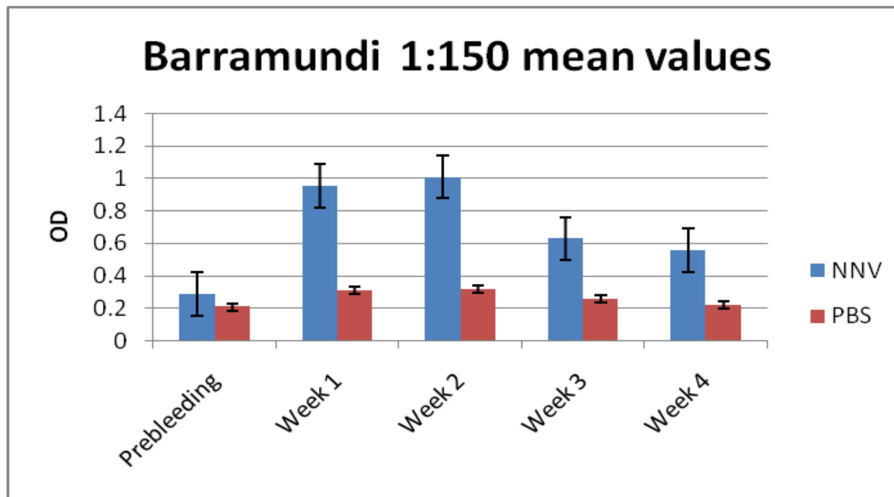



Figure 18. ELISA OD levels in an individual barramundi (A186) before and after immunisation with rCP from NNV

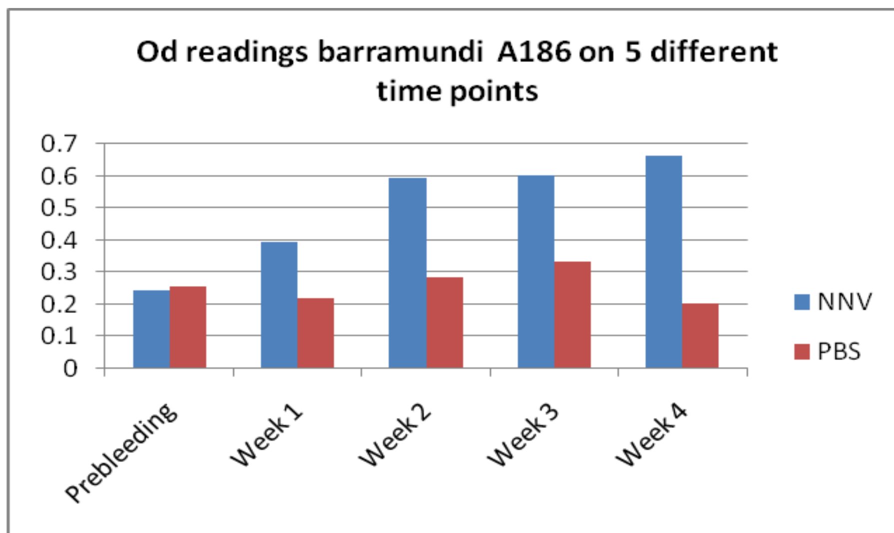
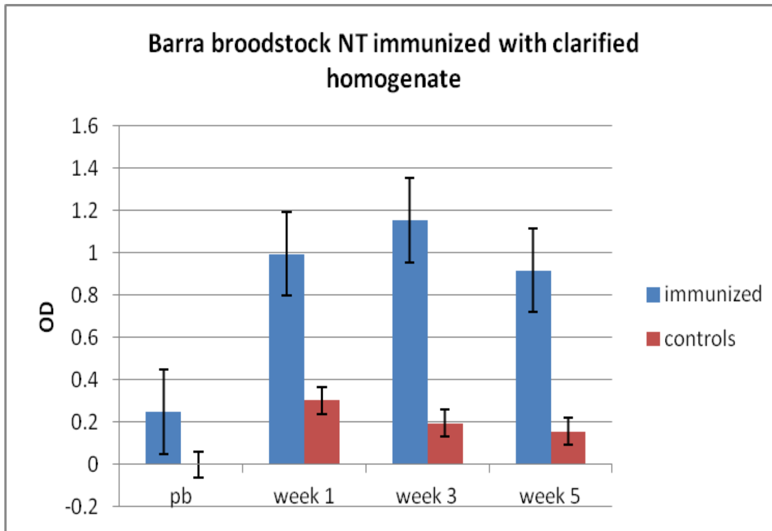
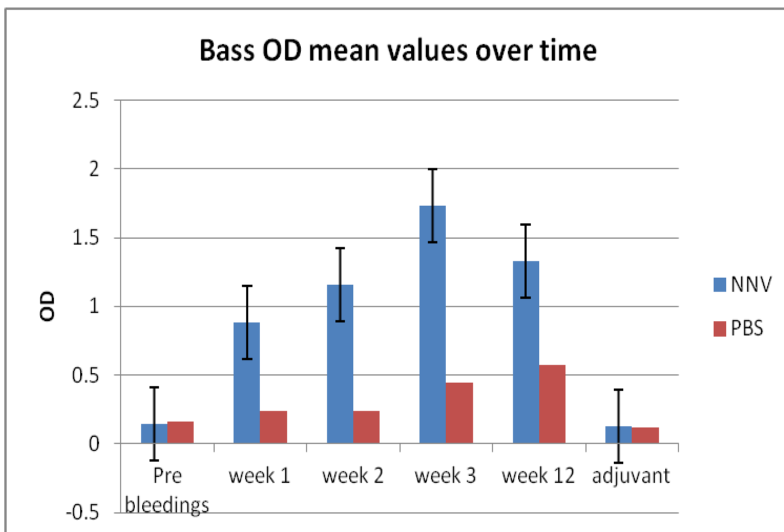


Figure 19. Mean ELISA OD values for 33 barramundi which were tested before and after immunisation with inactivated clarified homogenate prepared from barramundi larvae presenting with clinical NNV infection.




The ELISA detected an increase in antibody levels 6 Australian bass which had been immunised with purified NNV (Figure 20).

Figure 20. Mean ELISA OD values for 6 Australian bass which had been immunised with purified NNV in adjuvant and 3 which were given PBS and adjuvant.




The conclusions from these results are that there was a specific adaptive immune response after immunisation of barramundi and Australian bass that can be assessed by ELISA, and that the antibody levels were very strong. The OD

signal decreased overtime as did the specificity of the reaction in Australian bass. This is obvious in Figure 20 were the OD in wells without NNV antigen increased over time. Another interesting observation was that serum samples are highly stable over time when stored at -20 ° C. Some of the samples had been stored 9 years prior to analysis but still revealed a typical post vaccinal humoral immune response.

*Fish of unknown status*

*Barramundi.* Detailed results of serology (ELISA) tests on 79 brood stock held at DAC are presented in Appendix 4. Of these fish, 43 did not participate in spawning during the study. Eleven of the 36 brood stock which did participate in spawning during the study were associated with infected batches of larvae/juveniles. None of the gonadal fluid or blood clot samples from any of the brood stock tested positive in qPCR. Ignoring the time of testing, seropositive (i.e. ELISA positive) brood stock were associated with both infected and uninfected cohorts, but too few brood stock were tested prior to a cohort becoming infected to assess whether ELISA status was associated with risk of infection. However, the prevalence of ELISA positive fish increased over time, with none of 38 fish tested being positive in 2010, 3 of 48 positive in 2011 and 13 of 59 positive in 2012. This is consistent with progressive exposure of these fish to NNV. Furthermore, repeated sampling of the same fish over time enabled assessment of seroconversion: this was observed in fish numbers 7, 11, 16, 17, 23, 27, 32, 62, 67, 68.

In addition, 99 samples from barramundi spawners at Queensland hatcheries which were collected during March-April 2012 were analyzed with the antibody detection ELISA (Table 13). The arbitrary cut-off point of 0.7 was used for the optical density (OD) and 2.85 for the OD ratio.

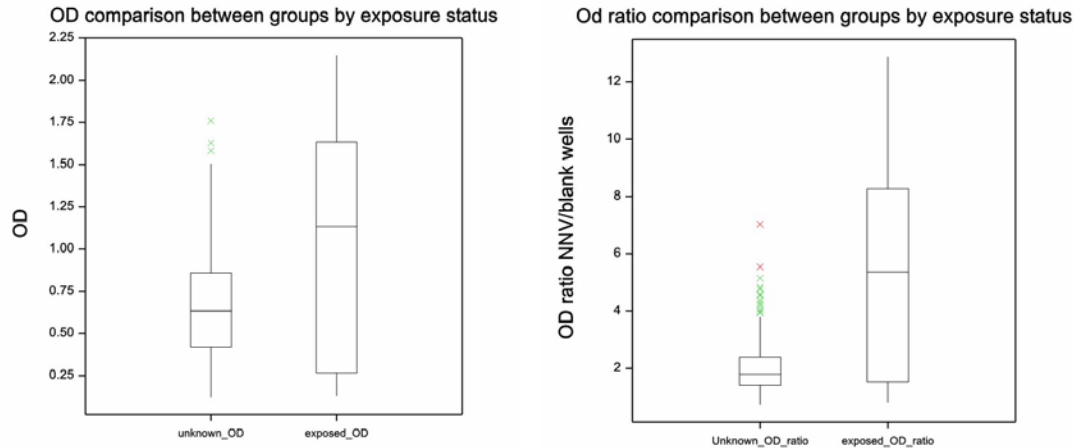
Table 13. Results of ELISA of serum samples from 99 brood stock in hatcheries in far north Queensland

| Hatchery | Species | OD mean | OD ratio mean | ELISA positives/no. tested |
|---|---|---|---|---|
| 1 | Barramundi | 0.65 | 1.94 | 0/7 |
| 2 | Barramundi | 0.58 | 2.21 | 2/13 |
| 3 | Barramundi | 0.74 | 2.12 | 6/79 |

*Naturally exposed fish*

*Barramundi.* The antibody levels in barramundi which were known to have been exposed to NNV were significantly higher than those in fish of unknown history ($P<0.001$) (Figure 21). The exposed fish were all survivors of outbreaks and most were about 1 year old whereas the unknown fish were mostly brood stock and pre-immunisation samples from fish that were later used in immunization trials, but otherwise their locations and ages were not known.

Figure 21. Antibody levels in barramundi which were known to have been exposed to NNV or which were of unknown history.




The serum samples were collected from three affected cohorts of barramundi, two of which pre-date the present study. Tissue samples from two cohorts were tested by qPCR, while blood samples from all three were tested by ELISA (Table 14). Tissue and blood samples were matched to individuals. The 96 fish were survivors of outbreaks and samples were taken 3-10 months after the outbreak. Of these samples, 47 sera were tested in ELISA in parallel with qPCR on nervous tissue. Of these 47 fish, 45 were positive on qPCR while 37 were positive in ELISA (Table 14). Thus there was a moderate level of agreement between the two tests in this population.

Table 14. The results of testing samples from 96 fish from cohorts of barramundi which were infected with NNV at Darwin

| *Cohort* | *Disease* | *Age* | *No. fish* | *No. qPCR positive* | *No. ELISA positive* |
|---|---|---|---|---|---|
| *10/07** | *Survivors of clinical outbreak* | *3 mths* | *31* | *26* | *20* |
| *09/07*** | *Subclinical infection* | *3 mths* | *46* | *nt* | *27* |
| *10/09**** | *Survivors of clinical outbreak* | *10 mths* | *19* | *19* | *17* |

* collected by Paul Hick; ** collected by John Humphrey; *** this study

*Australian bass.* These fish were persistently infected with NNV after exposure as fingerlings at Port Stephens Fisheries Centre. More details about these fish are provided in a later section of this report. Fish were culled from the group over time and blood samples were collected. High antibody levels were detected in some fish, and antibody levels generally declined over time (Figures 22 and 23). Using the arbitrary positive-negative cut-offs described in the methods, the prevalence of antibody-positive fish in 2010 when fish were 3 years old was 43%; it declined to 35% in 2012 when the fish were 5 years old.

Figure 22. Antibody levels in random samples of Australian bass in a naturally infected population over time.

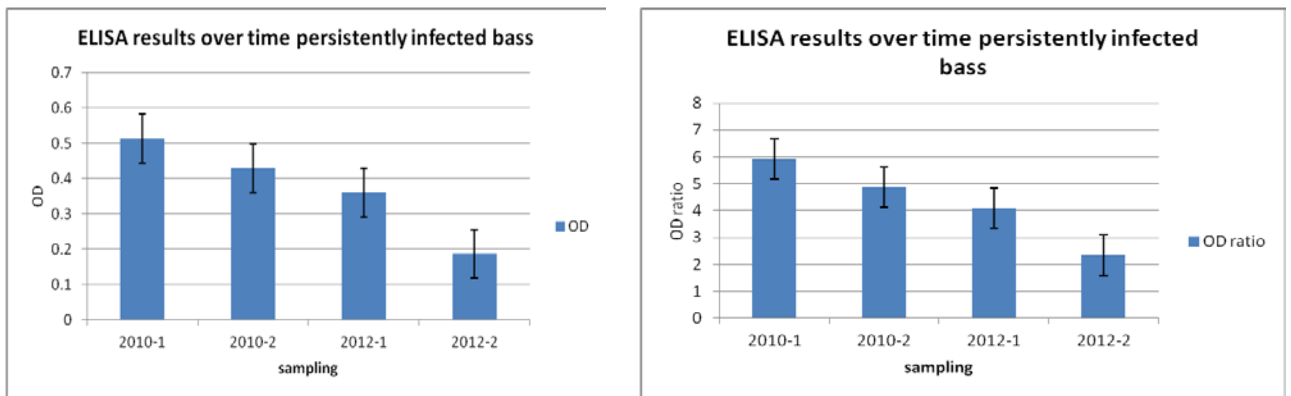
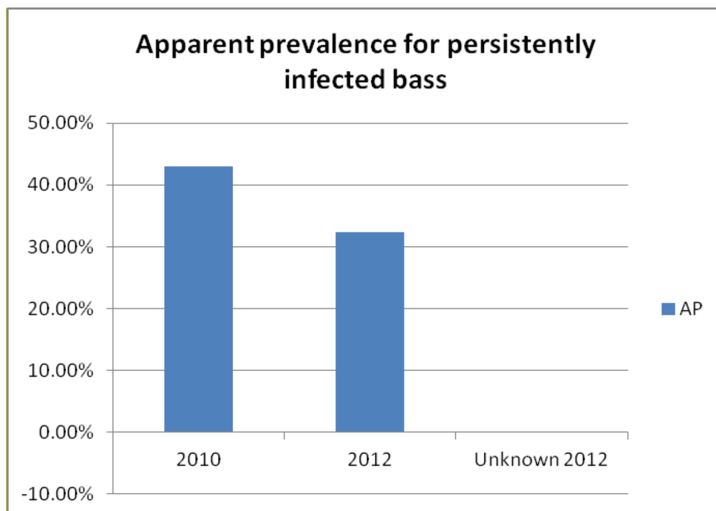



Figure 23. Apparent prevalence of persistently infected bass analysed overtime from 2010 (n=100) to 2012 (n=70) compared to results for samples taken from fish with unknown exposure status (n=10).



*Other species of fish.* In addition, serum samples from 18 gold-spot cod (*Ephinephelus coioides)* and 22 Queensland grouper were examined in antibody detection ELISA. Although the results cannot be evaluated without known positive and negative control sera, some fish had specific antibody levels (indicated by substantially high ELISA OD in wells with antigen compared to wells without antigen). Reactivity in the presence of the viral antigen was 2.5 times greater control wells (OD ratio) for 50% of the gold-spot cod and 36% of the Queensland grouper.

## Objective 4

## To provide a basis for development of a national proficiency testing scheme for the detection and identification of betanodaviruses

National proficiency testing requires two key components – standardized samples which can be shipped and tested at each laboratory, and a standard protocol with which to conduct the test. Both components were addressed in this project.

### Interlaboratory tests

Soon after this project commenced in 2010 the Australian National Quality Assurance Program (ANQAP) initiated a national ring test for NNV. This activity superseded some of the activity of this project. FRDC agreed to revise the project milestone to advise ANQAP on the best procedures for NNV ring testing if problems were identified with their current procedure; this advice would be limited to the types of samples to be circulated and methods for stabilising material for shipment. Note that ANQAP uses the term "VER" for NNV.

Ring testing for detection of NNV was conducted again under the auspices of ANQAP in 2011 (VER PCR; test code 11/46; October 2011). All participating laboratories (except EMAI) used the test that was transferred as part of this FRDC project. ANQAP advised that not all of the participating laboratories passed satisfactorily.

The project team observed that positive samples had Cts in the range 26.5 to 31.6 (lab with lowest Cts) to 36.5 to 41.2 (lab with highest Cts), and generally Cts were above 33. Such high Cts indicated that the majority of samples contained very low levels of NNV nucleic acid. Stability of the nucleic acid is an important consideration given that these samples are sent to other laboratories for testing, with unknown effects on nucleic acid integrity. A small degree of sample deterioration could render these samples negative, and such effects are likely not to be uniform as material is shipped around Australia on various transport corridors at room temperature.

The project team advised ANQAP that data from this FRDC project suggested that NNV material is not very stable. A 1 log reduction in activity in qPCR associated with storage of infectious virus in RLT buffer under ideal conditions (eg -80°C) associated with time at that temperature or freeze-thaw had been observed. There were greater losses in samples that reached 37°C. Therefore samples that test positive initially could be rendered negative by storage. ANQAP advised that a consensus interpretation is used to assess results from participating labs, and confirmed that this would not indicate that there is a problem with the samples themselves. It was of concern to ANQAP that the results for this FRDC project showed a lack of stability of NNV in control materials. ANQAP follows ISO/IEC guidelines as required as part of its NATA

accreditation. This requires one stability test per year, which occurred in January 2011 for the VER samples.

ANQAP advised that it had been storing the samples in the laboratory at room temperature. The samples consisted of TCSN suspended in AVL buffer, derived from virus obtained from Queensland barramundi and grown on SSN-1 E-11 cells. In December 2011, by visual observation, they were "out of solution" with a gel like crystalline appearance on the bottom of the ampoule. On receipt of the samples in July 2010, ANQAP was required to dilute the stock into specified dilutions and store them at room temperature. ANQAP had received reports from participating labs of crystallisation which has resulted in some reproducibility problems if the crystals are not dissolved via slight heating. Labs had not recorded any other condition or problems with the samples.

ANQAP advised that as these samples and this test are relatively new in the proficiency testing program, there are no historical data to show what the correct procedures, storage conditions, duration of storage or frequency of quality/stability testing should be. Therefore ANQAP relies on the yearly stability testing.

As a result of interaction with this project ANQAP resolved to monitor these samples more closely.

**Australian and New Zealand Standard Diagnostic Test Procedure (ANZSDP)**

Standard diagnostic test methods have been prepared for a number of aquatic animal pathogens, including NNV (Betanodavirus infections of finfish). However, the new tests which were developed or validated in this project are not yet included in the current approved ANZSDP.

During this project a new ANZSDP was drafted and is ready for technical review by nodavirus researchers and diagnosticians. This will be followed by formal submission and technical review which is conducted under the auspices of SCAHLS. The draft document is provided as Appendix 3.

## Objective 5

## To provide recommendations for improved biosecurity protocols in relation to nodavirus infection and fish translocation

### Introduction

Objective 5 is “to provide a basis for improved biosecurity protocols in relation to nodavirus infection and fish translocation”. Fundamentally the question that needs to be answered is whether nodavirus is transmitted to larvae from the brood stock or does the virus enter the larval culture system via contaminated seawater or other means, i.e. is vertical or horizontal transmission the source of virus for clinical outbreaks of VNN. To answer this question the tests that were developed and described in earlier sections of this report were applied in three studies: i) epidemiology of NNV in barramundi in the Northern Territory ii) epidemiology of NNV infection in north Queensland, and iii) epidemiology of NNV infection in Australian bass in NSW.

### Terminology and production stages

The terminology used for life history stages of barramundi at Darwin Aquaculture Centre is provided in Table 14a. This nomenclature may not be accepted by all stakeholders.

Table 14a. Life stage terminology for barramundi at Darwin Aquaculture Centre

| | |
|---|---|
| *Egg* | *0 day* |
| *Larvae* | *1-14 days of age* |
| *Fry* | *15 days of age and older. Barramundi metamorphose from larvae to fry from 13-17 days of age (depending on temperature etc). From 15 days of age salinity can be reduced to freshwater* |
| *Fingerling* | *21 days of age and older. Fry are moved from the larval rearing system to the nursery system* |
| *Juvenile* | *Once fish are in grow out systems* |
| *Advanced juveniles or sub-adults* | *Immature gonad is apparent* |

**Hypothesis testing – vertical or horizontal infection**

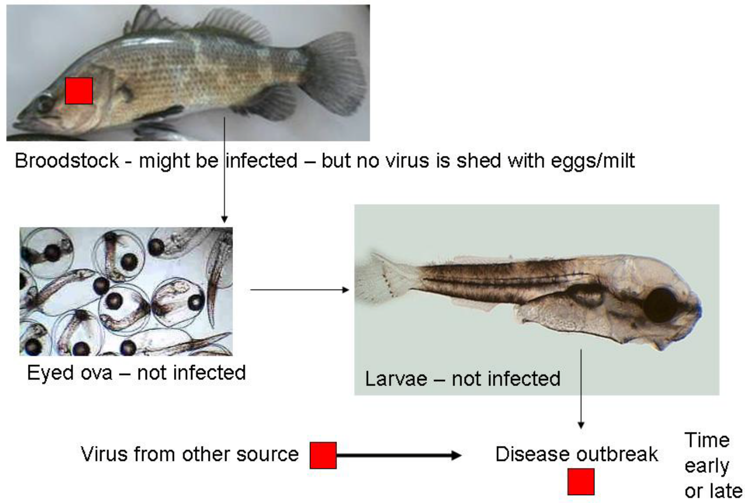
The assessment of whether larvae and fry become infected with NNV vertically or horizontally was based on a repeated cross-sectional survey approach, using qPCR tests of gonad, eggs, larvae, fry and fingerlings. The interpretation of test results is shown in Table 15 and illustrated in Figure 24.

Table 15. Criteria for vertical or horizontal transmission

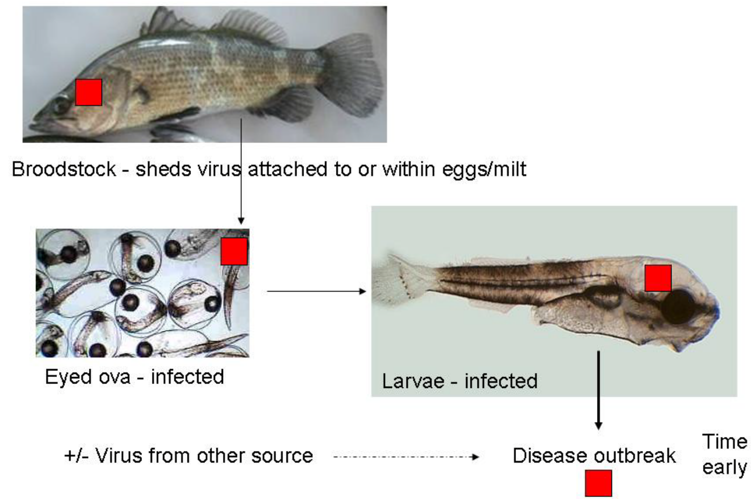
| *Life history stage* | *Vertical* | *Horizontal* |
|---|---|---|
| *Broodstock (PCR, ELISA)* | *+ve* | *+ve or –ve* |
| *Eggs (PCR)* | *+ve* | *-ve* |
| *Early larvae (PCR)* | *+ve* | *-ve* |
| *Outbreak (PCR)* | *+ve* | *+ve* |

Figure 24. Criteria for horizontal and vertical transmission of NNV

## Horizontal transmission criteria




## Vertical transmission criteria



**Studies in barramundi at Darwin Aquaculture Centre, Northern Territory**

There were three components of this study:
1. Broodstock testing and evaluation
2. Longitudinal study of juvenile barramundi including disease investigation in fish from grow-out farms
3. Opportunistic sampling of wild barramundi

It is important to note that PCR tests conducted before 2009 were the conventional nested PCR test while qPCR (qR2T) tests were conducted after 2009 as published (Hick and Whittington, 2010).

*Broodstock study*
The only site holding barramundi brood stock in the Northern Territory is the Darwin Aquaculture Centre. All current barramundi brood stock in the hatchery were tested by qPCR on gonad material (or reproductive tract fluid). Individual brood stock were microchipped during quarantine within 2 months of arrival at DAC so that subsequent testing of their progeny could be correlated to brood stock test status. Cohort larvae can be matched generally to 1 female and 2 male brood stock. The sampling of brood stock was opportunistic due to their high value. It was not possible to obtain tissue samples at necropsy as fish were clinically healthy and were re-homed at the completion of their breeding life.

Broodstock were anaesthetized in Aqui-S® (an equal-part mixture of eugenol and polysorbate 80) at a concentration of about 40 ppm. Disinfected catheters and syringes were used to withdraw reproductive tract fluid or gonad material, generally about 0.1 to 0.2 ml. This was placed into a sterile vial, held chilled in an esky with ice bricks then frozen at -20$^0$C and stored at -80$^0$C if not tested the same day. Blood was collected from the caudal vein using a sterile syringe and needle and placed into serum separating tubes, allowed to clot overnight under refrigeration and serum was separated from clots by centrifugation at 2000 x *g* for 15 minutes. Serum was dispensed in 0.5 ml to 1 ml aliquots and stored at -80$^0$C. Blood clots were retained in the original blood tube and stored at -80$^0$C for qPCR.

During the period of study, a total of 79 brood stock animals at the DAC were sampled, including resident and new replacement brood stock. Of these, 36 animals were used for barramundi fingerling production included in this study.

Of the 79 brood stock held at DAC, 43 did not participate in spawning during the study. Eleven of the 36 brood stock which did participate in spawning during the study were associated with infected batches of larvae/juveniles (Appendix 4: fish numbers 3, 14, 15, 21, 25, 29, 37, 38, 40, 41, 44). As discussed in the section dealing with Objective 2, none of the gonadal fluid or blood clot samples from any of the brood stock at DAC tested positive in qPCR (Appendix 4). Eight of the 11 brood stock associated with infected cohorts also produced uninfected cohorts (numbers 3, 15, 21, 25, 37, 38, 40, 41); the other 3 fish (numbers 14, 29, 44) were used only for cohort 10/09, which was detected to be infected at day 34.

These three fish were retained and tested in 2012 (negative). Ignoring the time of testing, seropositive (i.e. ELISA positive) brood stock were associated with both infected and uninfected cohorts, but too few brood stock were tested prior to a cohort becoming infected to assess whether ELISA status was associated with risk of infection. However, the prevalence of ELISA positive fish increased over time, with none of 38 fish tested being positive in 2010, 3 of 48 positive in 2011 and 13 of 59 positive in 2012. Furthermore, 10 fish seroconverted during captivity at the hatchery (fish numbers 7, 11, 16, 17, 23, 27, 32, 62, 67, 68). This is consistent with progressive exposure of these fish to NNV.

*Longitudinal study of juvenile barramundi including disease investigation in fish from grow-out farms*

The age at which fish come into contact with potentially contaminated water is important for horizontal transmission. The intensively tank reared larvae at the DAC are stocked into indoor tanks with ultra-filtered and UV treated seawater. Live-feed are maintained in the same water. Fish are weaned to commercial dry feed at around 15-day-old. After 21-day-old, fingerlings are moved into the nursery system where there is reticulation of sand-filtered seawater with no UV treatment.

A sampling strategy was devised which would include ideally pre-ozone and post-ozone treated fertilized eggs, larvae at 5 day intervals and 19-21 day-old fry before their relocation to the nursery facility then every five days until 40-day-old. The proposed minimum sampling level and the pooling rate assumed a prevalence of >5% in an infected population, and no negative effect of pooling on test sensitivity. The number of samples to be collected was specified to be not less than 150 eggs, 150 larvae (1 to 10-day-old), 60 fry or fingerlings (15 to 40-day-old) (Table 16). Fish were collected randomly, placed in pools in sterile sample bottles or new zip-lock bags (e.g. Glad®). Other than the eggs and 1 day old larvae, all fish were euthanased by overdose of Aqui-S® prior to freezing. All samples were frozen immediately at -20°C and transferred to -80°C upon receipt at the laboratory.

In addition to the targeted NNV testing of all batches of barramundi fingerlings at the DAC, VNN testing by qPCR was also included in general disease outbreak investigation in juvenile and sub-adult barramundi at DAC and local farms. Farmers identified fish submitted to the Berrimah Veterinary Laboratories as the corresponding DAC cohort, to enable continual longitudinal study. Brain and retina samples were collected from individual fish for disease investigation and stored at -80 ° C if testing could not be conducted immediately. Selected samples of brain and eye were referred to the University of Sydney for further testing.

Table 16. Indicative schedule of sampling for a batch of fish at Darwin Aquaculture Centre

| Age/class | Number of animals in a pool of sample for laboratory submission | Number of qPCR tests to be conducted (based on 0.1 g tissue per pool) |
|---|---|---|
| Pre-ozone eggs | 1 pool of 150 per pool | 1 |
| Post-ozone eggs | 1 pool of 150 per pool | 1 |
| 1-2 day-old larvae | 1 pool of 150 per pool | 1 |
| 5 day-old larvae | 2 pools of 75 fish per pool | 2 |
| 10 day-old larvae | 6 pools of 25 fish per pool | 6 |
| 15 day-old fry | 6 pools of 10 fish per pool | 6 |
| 19-21 day-old fry | 6 pools of 10 fish per pool | 6 |
| 25-day-old fingerlings | 12 pools of 5 fish per pool | 12 |
| 30-day-old fingerlings | 60 fish in pools of 4 | 15 |
| 35-day-old fingerlings | 60 fish in pools of 2 | 30 |
| 40-day-old fingerlings | 60 fish in pools of 2 | 30 |

The history of outbreaks of nodavirus infection at DAC since 2002 is summarised in Table 17. This study covers the period from 2008, with the first longitudinal sampling commencing in 2009. Prior to 2008 there were infection events in 4 of the 7 years, but only a few batches of fish were affected in any one year. A detailed investigation was conducted in 2007 (Hick et al., 2011a), when there was one clinically affected batch of larvae and one subclinically infected batch of fingerlings present at DAC concurrently.

Table 17. History of nervous necrosis virus infection in batches of barramundi produced at the Darwin Aquaculture Centre since 2002. Each batch is derived from a separate spawning event

| *Year* | *No. batches affected/ No. produced* |
|---|---|
| *2002* | *2/7* |
| *2003* | *0/8* |
| *2004* | *1/8* |
| *2005* | *2/7* |
| *2006* | *0/6* |
| *2007* | *2/5* |
| *2008* | *0/5* |
| *2009* | *2/4** |
| *2010* | *0/4* |
| *2011* | *1/4* |
| *2012* | *0/1* |

*there was another batch that became infected subclinically at two grow-out farms (see Table 18a, cohort 04/09)

In 2008, nodavirus infection was not detected in batches of fish at DAC in routine health certification tests of samples of 21 to 23 day old fish. The sample size of 30 fish was sufficient to detect prevalence as low as 10% with 95% confidence, assuming that there was no effect of pooling samples on test sensitivity.

In 2009, three batches of fish from DAC were shown to be infected but as one batch was infected after transfer to two grow-out farms (Farm A and Farm B), it is not shown as an infected batch at DAC in Table 18. This batch (04/09) tested negative at 19 days of age in routine health certification. The sample size of 30 fish was sufficient to detect prevalence as low as 10% with 95% confidence, assuming that there was no effect of pooling samples on test sensitivity. The other two infected batches in 2009 at DAC had clinical signs. The onset of clinical disease for cohort 09/09 was at 18 days of age and that for cohort 10/09 was at 34 days of age (Table 18a). Although there were inconclusive qPCR results at 2 days of age in the 09/09 batch, there was no conclusive evidence of infection prior to 5 days of age in the 09/09 batch and prior to 34 days of age in the 10/09 batch, hence suggesting horizontal infection in both cases. Sample sizes were sufficient to detect prevalence of <2% with 95% confidence assuming that there was no effect of pooling samples on test sensitivity. Persistent infection in the survivors of the 10/09 batch was detected for 2 years at three different grow-out farms (Farm A, Farm B and Farm C). However, re-infection of these fish at the farm level cannot be excluded.

In 2010, nodavirus infection was not detected in 4 cohorts of fish at DAC based on routine health certification tests and targeted surveillance for this project. The sample size of 60 fish was sufficient to detect prevalence as low as 5% with 95% confidence, assuming that there was no effect of pooling samples on test sensitivity.

In 2011, NNV infection was detected in 1 of 4 batches of fish at DAC. The first indication of the infection was in a batch of fingerlings, cohort 04/11, which had been transferred to a grow out farm, Farm C, at 50 days of age with subsequent clinical signs of NNV detected in fish at the farm at 61 days of age. The infection was confirmed by qPCR and histopathology. However, the fish from this batch which remained at DAC were clinically normal. These fish were tested at 64 days of age and were subsequently confirmed to be also infected with NNV by qPCR and histopathology. All prior tests of this batch at DAC were negative for NNV (Table 18a). The sample size was sufficient to detect prevalence as low as 5% with 95% confidence, assuming that there was no effect of pooling samples on test sensitivity. A subset of this batch was transferred at 37 days of age from DAC to grow-out at an aquaculture facility at a local high school and these fish remained healthy and were confirmed to be NNV negative at 69 days of age by qPCR and histopathology. Samples of blood and gonad fluid from three brood stock were tested 8 months prior and two months after the relevant spawning event for this batch (fish numbers 25, 37, 38 in Appendix 4) and all results were

negative. The results are consistent with horizontal infection of the batch between 37 and 61 days of age.

In 2012, nodavirus infection was not detected in routine health certification tests of samples of 20 day old fish at DAC. The sample size of 60 fish was sufficient to detect prevalence as low as 5% with 95% confidence, assuming that there was no effect of pooling samples on test sensitivity.

More detailed results of the laboratory tests for each cohort in this study are provided in Appendix 5.

Table 18a. Results of qPCR tests on infected batches of fish in the longitudinal study at DAC in 2009 and 2011. The number of fish tested during this study and pool size varied according to the age and size of the fish with the target tissue pooling rate being 0.1 g.

| ***Batch*** | ***Age infection first detected*** | ***Location of fish*** | ***Age (no. fish tested)*** | ***qPCR results*** | ***Age at onset of clinical disease*** | ***Comments*** |
|---|---|---|---|---|---|---|
| *04/09* | *13 mths* | *DAC* | *19 days (30)* | *Negative 3/3 pools* | *N/A* | *NNV genome detected in fish during other disease investigations in two grow-out farms, Farm A & B.* |
| | | *Growout farms* | *13 -16 mths (5)* | *Positive 4/5 fish; Ct 23-32* | | |
| *09/09* | *5 days* | *DAC* | *Eggs (160-320)* | *Negative 16/16 pools* | *18 days* | *Clinical NNV confirmed at DAC. Batch terminated day 23* |
| | | | *2-days (160)* | *Inconclusive 7/20 pools; Ct >33* | | |
| | | | *5-days (160)* | *Positive 2/20 pools; Ct 24-31* | | |
| | | | *15-days (110)* | *Positive 1/14 pools; Ct 29* | | |
| | | | *18-days (60)* | *Positive 8/8 pools clinically normal fish; Ct 7-30* | | |
| | | | *18-22 days (110)* | *Positive 20/20 pools clinically affected fish; Ct 6-12* | | |
| *10/09* | *34 days* | *DAC* | *10-days (550)* | *Negative 23/23 pools* | *34 days* | *Clinical NNV confirmed at DAC, survivors were sent to three grow-out farms, Farm A, B & C.* |
| | | *DAC* | *12-days (360)* | *Negative 15/15 pools* | | |
| | | *DAC* | *21-days (150)* | *Negative 11/11 pools* | | |
| | | *DAC* | *34-days (70)* | *Positive 7/7 pools clinically affected fish; Ct 12-15* | | |
| | | *DAC* | *54-days (60)* | *Positive 7/12 pools clinically healthy fish; Ct 25-31* | | |
| | | *DAC* | *69-94 (8)* | *Positive 8/8 pools clinically healthy fish; Ct 20-28* | | |
| | | *Growout farms* | *9-25 mths (26)* | *Positive 44/52 brain or retina; Ct 15 - >33* | | |
| *04/11* | *61 days* | *DAC* | *5 days (20-25)* | *Negative 1/1 pool* | *61 days* | *Clinical NNV confirmed in only one grow-out farm, Farm C.* |
| | | *DAC* | *10 days (30)* | *Negative 2/2 pools* | | |
| | | *DAC* | *21 days (60)* | *Negative 6/6 pools* | | |
| | | *DAC* | *27 days (30)* | *Negative 3/3 pools* | | |
| | | *Growout farm* | *61 days (16)* | *Positive 8/8 pools; Ct 6-12* | | |
| | | *DAC* | *64 days (10)* | *Positive 5/5pools; Ct 10-16* | | |
| | | *High school* | *69 days (10)* | *Negative 5/5 pools* | | |

*Opportunistic sampling of wild barramundi*

There are currently eight disease control zones (zone 1 to zone 8) for barramundi in the Northern Territory. The zoning strategy was based on factors including geographic regions, natural distribution of barramundi, historic movement records, population dynamics, genetic factors, and epidemiology of pathogen infection records.

DAC and barramundi farms in the Northern Territory are all located in zone 1. DAC replacement brood stock fish are currently sourced only from zone 1, although previously also from zone 2 where there was an operating sea-cage barramundi farm. During the period of this study, opportunistically collected samples from wild barramundi from zone 1 and several other zones were submitted for testing. Samples were tested using the qPCR using individual brain and retina samples. New replacement barramundi brood stock caught from the wild or from a grow-out farm were tested by qPCR during quarantine at DAC, using blood clot or gonad material as for resident brood stock. All samples were stored at -80°C if testing could not be conducted immediately.

Table 18b shows the serology and qPCR results of clinical and post-mortem samples of these fish. NNV was not detected by qPCR and all of the fish tested were negative in ELISA.

Table 18b. Results of testing of wild caught barramundi and newly caught brood stock

| Collection date | Sample ID | Sample type | No. of tests | Pool size | Nodavirus qPCR result | Serology | Comments |
|---|---|---|---|---|---|---|---|
| 22/07/2010 | New brood stock | Reproductive fluid | 15 | 1 | Neg (BVL) | N/A | Fish from Farm C (zone 1), was DAC cohort 04/08, same fish as 2010-0755 |
| 22/07/2010 | New brood stock | Blood | 16 | 1 | Neg (Usyd) | Neg (Usyd) | Fish from Farm C (zone 1), was DAC cohort 04/08, same fish as 2010-0751 |
| 22/10/2010 | New brood stock | Reproductive fluid & blood clot | 5 | 1 | Neg (BVL) | N/A | Fish caught locally (Zone 1) in Aug 2010 |
| 22/10/2010 | New brood stock | Blood | 5 | 1 | Neg (Usyd) | Neg (Usyd) | Fish caught locally (Zone 1) in Aug 2010 |
| 24/11/2010 | Wild caught | Fish head | 38 | 1 (brain, retina separately) | Neg (Usyd) | N/A | Fish caught at Roper River catchment (Zone 6), by commerical wild barramundi fishers on 10/10/2010; age structure analysis confirmed fish were 3 to 12 years old |
| 11/08/2011 | Wild dead | Fish | 2 | 0.1 g each tissue (brain, retina) | Neg (BVL) | N/A | One dead fish found near sewage treatment plant in Darwin (Zone 1), 50 cm total length possibly sub-adult, multiple parasitic granulomas in multiple organs |
| 13/01/2012 | Wild caught | Fish | 8 | 0.1 g each tissue (brain, retina) | Neg (BVL) | N/A | Fish caught for toxicology testing in relation to chemical spillage from a derailed train in Edith River in Katherine (Zone 4), four wild barra caught, total length 54 cm to 57 cm possibly subadult. |
| 17/02/2012 | New brood stock | Blood | 2 | 0.1 g (blood clot) | Neg (BVL) | Neg (Usyd) | Fish caught locally (Zone 1) in Aug 2011 |

## Epidemiological study in north Queensland

Three surveys were planned for this project, a brood stock survey, a longitudinal survey of juvenile barramundi and a study of reef finfish brood stock.

*History of NNV infection at hatcheries in far north Queensland*
Although a larger number of hatcheries were approached for this study, samples were able to be collected only from four hatcheries in far north Queensland. The history of these hatcheries was obtained and is summarized below.

Hatchery 1. There were repeated tank larval rearing runs in 2011 and 2012. The only outbreak of VNN was in May 2011 in barramundi larvae, which were supplied by Hatchery 2. There was poor initial survival when the fish arrived as 1/2 day-old larvae, and this was followed by increasing mortalities in 8 to 11 day-old larvae, when only 10% of the original 650,000 larvae remained. Viral nervous necrosis was diagnosed based on the presence of typical histopathological lesions in the brain and retinas. There had been no previous outbreaks recorded at Hatchery 1 and it was assumed that this outbreak was linked to the introduced larvae. All larval rearing is done in an intensive clear-water larval rearing system, where rearing tanks receive filtered and UV treated seawater.

Hatchery 2. The last outbreak of VNN was in February 2011 in pond-reared fry. An increased mortality rate and lack of vigour at feeding was noted in these pond-reared 21 day-old barramundi fry. Viral nervous necrosis was diagnosed based on the presence of typical histopathological lesions in the brain and retinas in the submitted fry. Since then there have been no further outbreaks at Hatchery 2. All larval rearing is now done following an intensive clear-water larval rearing system, where rearing tanks receive filtered and UV treated seawater.

Hatchery 3. There have been no recorded VNN outbreaks since the hatchery opened more than 8 years ago. All larval rearing is done following an intensive clear-water larval rearing method, where rearing tanks receive filtered and UV treated seawater.

Hatchery 4. This is a grouper hatchery which rears two species, Queensland groper and gold-spot cod. The only VNN outbreak in Queensland groper was in February 2012 in 112 day-old tank-reared juveniles. These fish were being fed artificial feed in a tank nursery system receiving coarsely filtered seawater. However the outbreak was mild as less than 10 clinically abnormal fish were seen in a population of several thousand fish. The last VNN outbreak in gold-spot cod was in May 2012 in 52 day-old tank-reared juveniles. These fish were also in the tank nursery system receiving coarsely filtered seawater. Approximately 200 of 34,000 fish were affected. The hatchery has reared progeny of both species since these outbreaks with no VNN detected. This hatchery has a strict biosecurity protocol where fertilised eggs are disinfected

with ozone prior to hatching in a hatchery section that receives UV sterilised water. VNN outbreaks have been observed only after the fish have left the hatchery section and been moved to the nursery tanks. The nursery tanks receive seawater from Trinity Inlet that has only been through sand-filters. Therefore it is suspected that betanodavirus infections are introduced into the nursery system via intake water.

*Broodstock study*

Initial industry information indicated that there were five sites holding barramundi brood stock located from Bowen in the south to the Atherton Tablelands in the north. All hatcheries which maintained captive barramundi brood stock from Bowen north were offered a real time PCR test on gonad material (or reproductive tract fluid) and permission to take a blood sample from the caudal vein of brood stock was requested. Hatcheries were asked to tag the brood stock (if they had not done so already) so that subsequent testing of their progeny could be correlated to brood stock test status. Broodstock were anaesthetised and the hatchery technician catheterised the reproductive tract with a sterile silicone tube. Reproductive tract material was withdrawn by aspiration. If no gonad material was present, reproductive tract fluid was the sample collected. Samples were held on ice and returned the same day to the laboratory for processing. In some situations, due to the distance from the laboratory, the samples were frozen or placed in RNA stabilizer until returned to the laboratory. If possible, while the fish were anaesthetised, a blood sample was collected from the caudal vein. Blood was allowed to clot and centrifuged at 2000 x *g*. Serum was collected, some was diluted 1:10 in TSGM and stored at -20ºC, while the remainder was stored at -20$^0$C undiluted. Clots were stored for qPCR at -20$^0$C. An initial blood sample was collected into anti-coagulant (citrate-EDTA [0.45M NaCl, 0.1M glucose, 30mM tri-sodium citrate, 26mM citric acid and 2mM EDTA] in equal volume with blood) to ensure free-flowing blood could be collected without anti-coagulant. This sample was retained at -20$^0$C as an emergency reserve.

Gonad/reproductive tract and blood clot samples were able to be collected from brood stock from four hatcheries in north Queensland during the survey. These were tested using qPCR with negative results as shown in Tables 19 and 20.

Table 19. Results of qPCR tests of gonad/reproductive tract samples from 96 brood stock from hatcheries in far north Queensland. All qPCR assays were performed using 5µL of template then repeated using a template volume of 8.5µL as this was thought to be a means of increasing sensitivity.

| Hatchery | Species (no.) | qPCR result |
|---|---|---|
| 1 | Barramundi | Negative 7/7 |
| 2 | Barramundi | Negative 14/14 |
| 3 | Barramundi | Negative 75/75 |

Table 20. Results of qPCR tests of blood clot samples from 96 brood stock in hatcheries in far north Queensland

| Hatchery | Species | qPCR result |
|---|---|---|
| 1 | Barramundi | Negative 7/7 |
| 2 | Barramundi | Negative 13/13 |
| 3 | Barramundi | Negative 76/76 |

In addition, 99 samples from barramundi spawners at Queensland hatcheries which were collected during March-April 2012 were analyzed with the antibody detection ELISA. The arbitrary cut-off point of 0.7 was used for the optical density (OD) and 2.85 for the OD ratio. A small proportion of fish tested positive for antibody against NNV (<10%) (Table 21).

Table 21. Results of ELISA of serum samples from 99 brood stock in hatcheries in far north Queensland

| Hatchery | Species | OD mean | OD ratio mean | ELISA positives/no. tested |
|---|---|---|---|---|
| 1 | Barramundi | 0.65 | 1.94 | 0/7 |
| 2 | Barramundi | 0.58 | 2.21 | 2/13 |
| 3 | Barramundi | 0.74 | 2.12 | 6/79 |

*Longitudinal study of juvenile barramundi*

All hatcheries were offered real time PCR testing on 1 day-old larvae and 21-28 day-old fry at harvest. Three hatcheries were requested to provide multiple samples of larvae and fry at approximately 5 day intervals. The proposed minimum sampling level and the pooling rate assume prevalence of >10% in an infected population, and no negative effect of pooling on test sensitivity (Table 22).

Table 22. Proposed sampling rates for the survey in far north Queensland.

| Age/class | Number of animals in a sample | Number of qPCR tests per sample |
|---|---|---|
| 1-2 day-old larvae | 150 per pool | 1 |
| 5 day-old larvae | 150 per pool | 1 |
| 10 day-old larvae | 30 larvae in 3 pools of 10 | 3 |
| 15 day-old fry | 30 heads in 6 pools of 5 | 6 |
| 16-20 day-old fry | 32 heads in 8 pools of 4* | 8 |
| 21+ day-old fry | 32 dorsal heads in 8 pools of 4 | 8 |
| Total | | 27 |

Fish were collected randomly, placed in zip-lock bags (Glad) in pools in lots of about 150 for 1 day old to 5 day-old larvae; lots of 10 for 10 day-old; lots of 5 for 15 day-old or older. Other than the eggs and 1 day old larvae, all larvae and fry were euthanased in benzocaine prior to freezing. All samples were frozen immediately at -20$^0$C. Alternatively fish were collected randomly then packed in water with oxygen for transport to the laboratory.

VNN outbreaks in barramundi were monitored opportunistically through routine diagnostic testing by BQ-DAFF.

Samples of whole fish were able to be collected from 3 hatcheries in far north Queensland during the survey, and these were tested using qPCR. The results are shown in Table 23. NNV infection was not detected.

Table 23. Results of qPCR tests on barramundi samples collected from hatchery fish in far north Queensland in 2012

| *Hatchery* | *Age infection first detected* | *Rearing method* | *Age (no. tested)* | *qPCR results* | *Age at onset of clinical disease* |
|---|---|---|---|---|---|
| *1* | *N/A* | *Intensive, clear-water larval rearing* | *1-day (150)* | *Negative 1/1 pool* | *N/A* |
| *2* | *N/A* | *Intensive, clear-water larval rearing* | *21-days (32)* | *Negative 8/8 pools* | *N/A* |
| *3* | *N/A* | *Intensive, clear-water larval rearing* | *29-days (32)* | *Negative 8/8 pools* | *N/A* |

*Reef finfish brood stock*

At the time the research was planned (July 2010) there were 90 brood stock held at the Northern Fisheries Centre, Cairns; these included 40 coral trout, 30 Queensland groupers (*E. lanceolatus*) and 20 gold-spot cod (*Epinephelus coioides*). These captive, wild-caught tropical marine finfish were available to be sampled. At the time of sampling in 2012 this involved nine gold-spot cod and five Queensland groupers. Blood samples from 18 gold-spot cod and 32 Queensland grouper were also collected for antibody ELISA and qPCR.

Table 24. Results of qPCR tests of gonad/reproductive tract samples from brood stock from Northern Fisheries Centre in north Queensland. All qPCR assays were performed using 5µL of template then repeated using a template volume of 8.5µL as this was thought to be a means of increasing sensitivity.

| *Hatchery* | *Species (no.)* | *qPCR result* |
|---|---|---|
| *4* | *Epinephelus coioides* | *9/9 fish negative* |
| | *Epinephelus lanceolatus* | *5/5 fish negative* |

Serum samples from 18 gold-spot cod (*Ephinephelus coioides)* and 22 Queensland grouper (10 samples could not be tested) were examined in antibody detection ELISA. Although the results cannot be evaluated without known positive and negative control sera, some fish had specific antibody levels (indicated by substantially high ELISA OD in wells with antigen compared to wells without antigen). Reactivity in the presence of the viral antigen was 2.5 times greater than in control wells (OD ratio) for 50% of the gold-spot cod and 36% of the Queensland grouper.

Insufficient data were obtained to make any conclusions about the epidemiology of NNV infection in marine species and further research is therefore required.

## **Epidemiological study of betanodavirus in Australian Bass (*Macquaria novemaculeata)* in NSW**

The main methods of control of betanodavirus infection in farmed fish are by endeavouring to exclude spawning from infected brood stock, disinfection of eggs by ozone, screening larvae and prevention of horizontal transmission. To this end, detection of infected brood stock and rapid and accurate diagnosis of infected juveniles are of great importance.

The aim of the study was to determine how long betanodavirus persisted in naturally infected Australian bass (*Macquaria novemaculeata)* and whether the virus was transferred vertically from infected brood stock to fertilised eggs and larvae. The study involved hatchery-produced, Australian bass that became naturally infected with betanodavirus in 2006 between 3 weeks and 3 months of age and reached sexual maturity in 2012. These fish are currently housed at EMAI.

*Overview of study*

*Origin of fish*. In 2006, betanodavirus was identified from a sample of 3 month old larvae clinically/apparently healthy larvae at Port Stephens Fisheries Institute (PSFI). There were no clinical signs of disease in the fish and no betanodavirus had been detected in the routine testing of larvae collected at 21 days of age.

*Housing of fish*. Fish were held in a tank at the hatchery in seawater until 19 months of age (40.5 ± 14.5 g) when approximately 700 fish were transferred into freshwater in 4 x 500L tanks operating as two 1000L tank systems at the University of Sydney, Camden, NSW. At 53 months of age the remaining 120 fish were transferred from the University of Sydney into 2 x 1000L tanks operating as a single freshwater system at EMAI, Menangle.

*Testing*. Sampling of fish occurred at regular intervals and tissue samples collected from the fish were tested by a real time RT PCR (qRT PCR) for betanodavirus and virus isolation was attempted on tissue samples that were positive in the qRT PCR.

*Spawning*. In July 2012 at approximately 71 months of age, the fish were sexually mature; ova from12 females and milt from 12 males were used to produce fertilised ova and larvae that were tested by qRT PCR to detect betanodavirus.

*Methods*

*Samples.* Samples of fish from the infected batch were collected at regular intervals throughout the study (Table 25). Where possible, blood was collected prior to euthanasia and tissue samples were collected immediately or the fish were stored at -80°C until processing. Five hundred larvae were collected at 3 months of age, 100 fish at 4 and 6 months, 100 fish at monthly

intervals from 4 to 18 months, 30 fish prior to transfer from the hatchery to the university, 28 fish two weeks after transfer (mortalities), 15 fish from 22 months to 36 months at approximately 3-monthly intervals, 30 fish at 45m, 16 at 48m, 8 fish within 2m of transfer to EMAI at 53m (mortalities), 3 mortalities over the next year, 15 at 69m (pre-spawning) and 12 females that were induced to spawn at 71m. When the fish were small, the head was homogenised and tested in pools of up to 5 fish. From 6 months individual fish were processed. Brain and eyes, with as much tissue removed as possible apart from the retina, and blood (where possible) were collected. From 22 to 47.5 months of age, the tissues collected were blood, brain, eye, anterior kidney, liver, spleen, and gonad when this was discernable. At 71m brain, retinae, ovary and dorsal fin clips were collected.

*Spawning.* The spawning was done at EMAI by experienced staff from PSFI. Twelve rotund females (142-400g) and 12 males (102 -216g) were injected with Human Chorionic Gonadotrophin and held separately in two 200L tanks of warmed artificial seawater maintained at 18 °C (WASW). After approximately 36 hours, the eggs from each female were stripped manually into a separate wide-mouthed container and the milt from each male was collected into a syringe. The milt from the 12 males was pooled and 1mL was added to approximately 20ml of eggs from each female, stirred and then emptied into a bucket of aerated WASW. After 1.5 hours the proportion of fertilised eggs was estimated using a dissecting microscope and batches where there were no fertilised eggs were discarded. Samples of eggs, fertilised eggs, developing embryos and larvae and milt (individual and pooled), were collected for betanodavirus detection by qRT PCR. The female fish used in the spawning were euthanased to collect samples of brains, retinae, dorsal fin clips and gonads to test for betanodavirus by qRT PCR.

Table 25. Longitudinal study of betanodavirus-infected Australian Bass – ages and numbers of fish sampled

| Age at sampling | Number of fish Sampled |
|---|---|
| 21 days | 150 |
| 3 months | 500 |
| 4 months | 100 |
| 6 months | 100 |
| 11 to 18 months | 100 per month |
| 19 months (pre transfer to the University of Sydney, Camden) | 30 |
| 19.5 months (mortalities post transfer) | 28 |
| 22 to 36 months | 15 at ~3 monthly intervals |
| 45 months | 30 |
| 48 months | 16 |
| 53 to 65months (mortalities post transfer to EMAI and sporadic) | 11 |
| 69 months (prespawning) | 15 |
| 71months (females from spawning trial) | 12 |

*Preparation of fish tissues for detection of virus.* At the start of the project, tissues were homogenised with disposable pellet pestles (Edwards Instrument Co) in approximately 1mL of serum free tissue culture medium (MEM, Gibco) supplemented with antibiotics (1% 10,000IU penicillin sodium and 10,000µg streptomycin) and centrifuged at 4 °C at 2500 x *g* for 15 minutes. From early 2009, tissues were mechanically disrupted by micro-bead beating (Hick *et al*, 2010) using 1mm zirconia beads for one or two 45 sec pulses in a Minibeadbeater (Biospec products) and centrifuged at 4 °C at 9500 x *g* for 10min. The clarified supernatant was stored short-term at $4^{0}$C, used for both the PCR and virus isolation and then stored at -80 °C. Blood was treated as described (Moody et al., 2004b) before analysis.

*PCR.* Initially, the larvae/fry were tested using a nested RT-PCR (nPCR) for betanodavirus as described (Moody et al., 2004b) with the modifications for detecting betanodavirus in Australian bass as described (Crane et al., 2007). However, from mid-2007 a real time RT PCR (qRT PCR) was used and earlier samples were retested with the qRT PCR.

All tissues sampled were analysed using a real-time RT-PCR (qPCR) assay with betanodavirus specific primers and probe developed in the Virology Laboratory at EMAI in 2007. Total nucleic acid was extracted from samples using a magnetic bead based kit (Mag-MAX 96 viral RNA Isolation Kit, Life Technologies) on a semi automated magnetic particle handling system (Kingfisher 96, Thermo Fisher Scientific, Finland). The assay utilised a

TaqMan MGB probe (Applied Biosystems) and one-step amplification (45 cycles) was performed on an Applied Biosystems 7500 Fast Real-Time PCR System. A commercial master mix was used for the PCR (AgPath-ID One-Step RT-PCR kit, Life Technologies) and reactions were run under the standard cycling conditions recommended by the manufacturer. The assay was based on conventional TaqMan technology using primer sequences that were conserved across 8 betanodavirus sequences lodged in Genbank and from sequences of 3 betanodavirus isolates from barramundi and bass in Australia. The qRT PCR assay had been validated for use as a diagnostic assay using a large collection of known betanodavirus-infected and betanodavirus-free tissue homogenates and cell cultures of Australian bass and barramundi betanodavirus isolates previously assayed by a nested PCR (Moody *et al*, 2004) and by virus isolation in SSN-1 cells with and without immunoperoxidase staining (Moody et al., 2004b). This assay differs from the validated nodavirus qRT PCR (qR2T assay) which was used elsewhere in this project (Hick and Whittington, 2010).

*Virus isolation.* Clarified, homogenised tissue supernatant (0.1mL) was inoculated into the medium on nearly confluent SSN-1 cell cultures in tubes in duplicate. The tubes contained 2mL of Leibovitz L-15 culture medium (Gibco) supplemented with 5% foetal bovine serum and 1% antimicrobial solution (10,000 IU /ml penicillin sodium, 10,000 µg/ml streptomycin and 25 µg/ml amphotericin B in 0.85% saline; antibiotic–antimycotic 100X, Gibco) and the cultures were incubated at 25°C. The cultures were observed daily for CPE typical of betanodavirus and if no CPE was present by 7d post inoculation, the cultures were blind passaged onto fresh SSN-1 monolayers. Each sample underwent 1 primary culture and up to 4 blind passages although the majority had 2 blind passages. The cell culture fluid from the first passage showing CPE or the last blind passage was tested by qRT PCR to detect evidence of nodavirus RNA.

*ELISA.* Blood samples were collected from the fish over time and tested in ELISA for antibodies against NNV as described in the section on objective 2.

*Results*

The qRT PCR and VI results for tissues that gave a positive result in qRT PCR are shown in Table 26. Betanodavirus was detected by both qRT PCR and virus isolation from 100% of the samples until 6 months of age. After this age, the proportion of samples in which betanodavirus was detected declined for both assays, although the qPCR detected more positive samples at every age tested. The decline was not constant over time, particularly from 19.5 months when fewer fish were assayed at each sampling. The lowest proportion of qPCR positive brains was 44% at 48 months and varied between 53 and 100% at other times. Virus was isolated from brain between 4 and 25 months and at 53 months (mortalities). Virus isolation from eyes/retinae was undertaken at 12 months and 22 to 71 months of age and virus was isolated from fish at 12 months (28%), 25 months (7%), 31 months (13%), 36 months (60%) from apparently healthy fish and from fish that died

from unknown causes at 53 months (36%) (Table 26 and Figure 25). Virus was isolated from whole larvae, heads or brains without blind passage until 6 months of age but only by passaging of cultures from 11-71 months. Virus was detected by qRT PCR and isolated from 1 rudimentary gonad at 47.5 months. Betanodavirus was not detected in the other tissues assayed either by qPCR or virus isolation at any age. These tissues included blood, anterior kidney, spleen, liver, heart, at 36 – 69 months also intestines and gonad and at 71 months, dorsal fin clip.

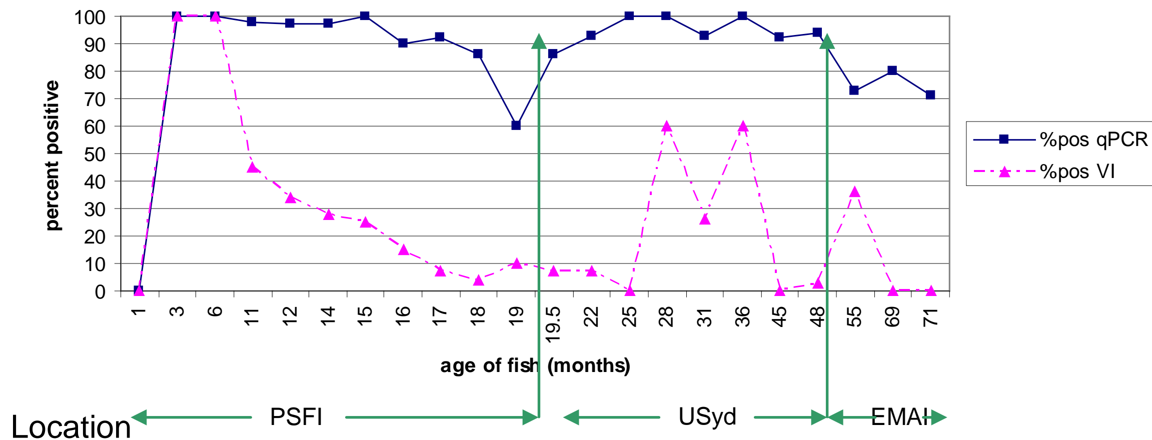
The mean number of cycles to threshold (Ct) for the qRT PCR positive samples is shown in Table 26. The mean Cts for samples from fish 3-6 months old were <25 and virus was isolated from these samples without passaging. From 11 – 71 month old the mean Cts were > 25 and at some ages >35. From 11-28 months old the mean Cts were closer to 30 and from 31-69 months old were closer to 35. Generally, the higher the mean Ct, the lower the proportion of fish from which virus could be isolated and more blind passages were required to isolate it. However, whether a virus could be isolated from a sample could not be predicted from either the mean or the individual Ct value as the fish got older e.g. for 71 months old positive eye samples, the mean Ct was 33.5 $\pm$ 1.7 and no virus was isolated after 3 blind passages in SSN 1 cell cultures while for 36 months old positive eyes the mean Ct was 33.7 $\pm$ 2.3 and virus was isolated from 60% of the qRT PCR positive eyes.

Table 26. Detection of betanodavirus by qPCR and virus isolation from 21 days to 71 months old

| Age (months) | Tissue | No. of fish tested | No. samples tested | PCR No. samples pos | PCR % samples pos | PCR Ct ± s.d. | VI No. samples pos | VI % samples pos |
|---|---|---|---|---|---|---|---|---|
| 0.7 | Pools of 15 larvae | 150 | 10 | 0 | 0 | Nd | nt | n/a |
| 3 | pools of 5 larvae | 500 | 100 | 100 | 100 | 13.9± 3.2 | 100 | 100 |
| 4 | Heads | 100 | 100 | 100 | 100 | 15.5± 4.2 | 100 | 100 |
| 6 | Brain | 100 | 100 | 100 | 100 | 24.6± 2.4 | 100 | 100 |
| 11 | Brain | 100 | 100 | 98 | 98 | 32.6± 2 | 45 | 45 |
| 12 | Brain | 100 | 100 | 88 | 88 | 35.3± 2.3 | 17 | 17 |
| 12 | Eye | 99 | 99 | 99 | 100 | 28.8± 2.7 | 28 | 28 |
| 14 | Brain | 100 | 100 | 97 | 97 | 31.1± 4 | 29 | 29 |
| 15 | Brain | 100 | 100 | 100 | 100 | 32.4± 2.9 | 25 | 25 |
| 16 | Brain | 100 | 100 | 90 | 90 | 30.6± 2.9 | 15 | 15 |
| 17 | Brain | 100 | 100 | 92 | 92 | 35.2± 3.6 | 7 | 7 |
| 18 | Brain | 100 | 100 | 86 | 86 | 37.2±4.1 | 4 | 4 |
| 19 | Brain | 30 | 30 | 18 | 60 | 36.4± 3.3 | 3 | 10 |
| 19.5 | Brain | 28 | 28 | 24 | 86 | 34.6± 2.6 | 2 | 7 |
| 22 | Brain | 15 | 15 | 10 | 67 | 35.2± 2.2 | 1 | 7 |
| 22 | Eye | 15 | 15 | 11 | 93 | 31.4± 2.2 | 0 | 0 |
| 25 | Brain | 15 | 15 | 12 | 80 | 33.5± 2.5 | 9 | 60 |
| 25 | Eye | 15 | 15 | 15 | 100 | 30.5± 2 | 1 | 7 |
| 28 | Brain | 15 | 15 | 13 | 87 | 30.4± 2.3 | 0 | 0 |
| 28 | Eye | 15 | 15 | 15 | 100 | 34.2± 3 | 0 | 0 |
| 31.5 | Brain | 15 | 15 | 8 | 53 | 35.3± 2.7 | 2 | 13 |
| 31.5 | Eye | 15 | 15 | 14 | 93 | 35.3± 1.9 | 2 | 13 |
| 36 | Brain | 15 | 15 | 11 | 73 | 36.5± 2.5 | 0 | 0 |
| 36 | Eye | 15 | 15 | 15 | 100 | 33.7± 2.3 | 9 | 60 |
| 45 | Brain | 30 | 60 | 19 | 63 | 35.9± 2.1 | 0 | 0 |
| 45 | Eye | 30 | 60 | 55 | 92 | 34.9± 1.8 | 0 | 0 |
| 48 | Brain | 16 | 16 | 7 | 44 | 35.9± 1.1 | 0 | 0 |
| 48 | Eye | 16 | 31 | 29 | 94 | 36.5±1.3 | 1 | 3 |
| 48 | rudimentary gonad | 16 | 16 | 1 | 6 | 35.9 | 1 | 6 |
| 53-60 | Brain | 11 | 11 | 8 | 73 | 36.5± 1.5 | 2 | 18 |
| 53-60 | Eye | 11 | 11 | 8 | 73 | 32.1±1.1 | 4 | 36 |
| 69 | Brain | 15 | 15 | 9 | 60 | 35.7±1.1 | 0 | 0 |
| 69 | Eye | 15 | 15 | 14 | 93 | 33.3±2.8 | 0 | 0 |
| 71 | Brain | 12 | 12 | 7 | 58 | 34.5± 2 | 0 | 0 |
| 71 | Eye | 12 | 12 | 9 | 75 | 33.5± 1.7 | 0 | 0 |

Nd not detected

Figure 25. Proportion of sampled fish that were betanodavirus positive (percent) in qPCR and virus isolation as a function of age. The location of fish is shown: PSFI, Port Stephens fisheries Institute; USyd, University of Sydney; EMAI, Elizabeth Macarthur Agricultural Institute




Following spawning, the samples that were collected and the results of a single aliquot of each sample are shown in Table 27. Unfortunately, due to the breakdown of the remaining unfertilised eggs, all larvae had died by 42h post hatch. There was no betanodavirus detected in any of the eggs, fertilised eggs, embryos, or larvae in the experiment. There was also no virus isolated from the adult females that produced the eggs although viral RNA was detected in brain tissue (58% of fish) and retinae (75%) suggesting that there was insufficient complete virus for replication in cell culture.

Table 27. Samples collected and betanodavirus detection results in spawning products, fertilised eggs, larvae and adult females

| Sample | Age | No. of samples | No of qPCR positive samples | No of VI positive samples |
|---|---|---|---|---|
| Eggs | At collection | 12 | 0 | NT |
| Milt –individual | At collection | 12 | 0 | NT |
| Milt - pooled | At collection | 1 | 0 | NT |
| Fertilised eggs | 1.5 h | 12 | 0 | NT |
| Fertilised eggs – embryos | 40h post fertilisation | 10 | 0 | NT |
| Fertilised eggs – embryos | 52h post fertilisation | 10 | 0 | NT |
| Larvae | 4h post hatch | 10 | 0 | NT |
| Embryos and unfertilised eggs | 4h post hatch | 10 | 0 | NT |
| Larvae | 21h post hatch | 10 | 0 | NT |
| Embryos and unfertilised eggs | 21h post hatch | 10 | 0 | NT |
| Larvae | 42h post hatch | 10 | 0 | NT |
| Embryos and unfertilised eggs | 42h post hatch | 10 | 0 | NT |
| Spawned females –brain | 71m | 12 | 7<br>Ct 34.5 $\pm$ 2 | 0 |
| Spawned females –retina | 71m | 12 | 9<br>Ct 33.5 $\pm$ 1.7 | 0 |
| Spawned females –ovary | 71m | 12 | 0 | NT |
| Spawned females – dorsal fin clip | 71m | 12 | 0 | NT |

High antibody levels were detected in some fish, and antibody levels generally declined over time (see results for Objective 2 in this report). The prevalence of antibody-positive fish in 2010 when fish were about 36 months old was 43%; it declined to 35% in 2012 when the fish were about 60 months old.

*Discussion*

The longitudinal study of Australian bass, *M. noveamaculata*, that were found to be naturally infected with betanodavirus at 3 months of age, has demonstrated that the virus or viral RNA can persist in subclinically infected fish to at least 71 months of age (females 142 – 400 g, males 102 – 216 g). It was detected in 75% of the sampled fish by qRT PCR at 71 months old. The virus was shown to be viable by virus isolation in a few fish (3%) at 48 months and only from 2 fish that died from unknown causes at 53 months old. Virus was isolated from 1 rudimentary gonad (6%) at 48 months and thus warranted further exploration of the possibility of vertical transmission of virus in this species of fish that is slow to reach sexual maturity.

At no time were there any clinical signs of betanodavirus infection and the fish appeared to grow normally which suggests that Australian bass may not be clinically affected by betanodavirus infection when they are infected at some time after 3 weeks of age.

The proportion of fish from which betanodavirus could be isolated declined over time whilst the fish were maintained at the hatchery in estuarine water even though there was a slower (insignificant) decline in the proportion of fish in which betanodavirus was detected by qPCR indicating that there was insufficient complete virus to be detected by this method and suggests that this qRT PCR is a more sensitive method of detection of infection than virus isolation. The viral load in tissues, expressed as Cts, also declined over time but could not be used to predict whether viable virus was present or detectable, although generally where Ct was <30, virus could be isolated. This is of great importance for management of stocking of infection-free fish into an environment of unknown status.

Subclinical betanodavirus infection was described in commercially farmed halibut (*Hippoglossus hippoglossus)* in Norway that were survivors of a natural outbreak of viral nervous necrosis (VNN) and the virus was shown to be still viable in these fish 9 -12 months after hatching (Johansen et al., 2004). This study demonstrated that virus was still viable, in some of the fish, at least 4 years after natural infection.

Virus was not detected in blood samples or other tissues, including anterior kidney in this study and has not been detected in blood or reproductive fluids from Australian bass brood stock in commercial hatcheries over a 3 year period (unpublished data) which indicates that screening these fluids or tissues would not provide a warranty that the juveniles were uninfected.

Others have observed increased virus levels in infected cod receiving prednisolone-acetate, an immunosuppressor used to simulate stress (Korsnes et al., 2012). These authors concluded that periods of immunosuppression such as sexual maturation, spawning, handling or suboptimal environmental conditions may promote virus replication and thus cod with persistent NNV infections may represent a risk of spreading NNV virus in aquaculture. In a spawning study in this project, there was no betanodavirus detected in

samples of eggs, milt, embryos or larvae to 42 hours post hatching, although virus was detected by qRT PCR in brain and/or eye tissue from most of the females used in the trial. However, no virus was isolated in SSN1 cell cultures from any of the qRT PCR positive tissue samples from the females which suggests that there was no, or insufficient complete virus present in these tissues to initiate cell infection. Although there was no apparent vertical transmission of virus in this instance, this trial has neither proved nor disproved the hypothesis that betanodavirus can be vertically transmitted to offspring in either Australian bass and further research is required. Further research is also required to determine whether these fish can be re-infected with betanodavirus as their antibody levels decline.

Suitable preventive and prophylactic health management strategies are required to ensure that hatchery reared Australian bass (*Macquaria novemaculeata*) are betanodavirus free before they are released into river systems and freshwater impoundments where the fish have unknown betanodavirus status.

## Overall final discussion

*Development and transfer of a new real time PCR method (Objectives 1 and 3)*

A reverse transcriptase quantitative real time PCR was successfully transferred to five aquatic animal health diagnostic laboratories in Australia during the project. Each laboratory was able to establish and run the assay according to specifications, and then participated in the Australian National Quality Assurance Program (ANQAP) for NNV. A plasmid standard and a heat inactivated tissue culture supernatant containing NNV were identified as robust standards for inclusion in the assay. The new qPCR assay was confirmed to detect all of the known genotypes of NNV. One laboratory (EMAI) maintained an in house qPCR protocol during the project, and continues to offer this alternative test. The involvement of so many laboratories enabled technical limitations to be identified and overcome, resulting in a robust protocol with some potential for flexibility in choice of commercial reagents. This reduces the risk that the protocol becomes too dependent on reagents from any one commercial supplier.

*Development and evaluation of a serological test (Objective 2)*

An enzyme-linked immunosorbent assay was developed during this study. The test measures the level of antibody against NNV in blood samples. It was a superior test compared to virus neutralisation and competitive ELISA. The test was applied successfully in epidemiological studies in both barramundi and Australian bass. The results indicated that both species of fish respond to infection with NNV with an antibody response, assuming that fish survive the infection. In both species, individuals were identified that had both antibody in blood samples and NNV present in brain/retina. Over time (months to years), the proportion of fish with antibodies in blood and virus in nervous tissue decreased, as did the levels of both antibodies and virus. During this project it was not possible to obtain enough samples to develop a full understanding of the potential of the ELISA test. Further research is needed to demonstrate if it may be a useful test to identify exposure of populations of fish to NNV.

*Development of national proficiency testing for NNV (Objective 4)*

During this project the Australian National Quality Assurance Program (ANQAP) commenced proficiency testing (ring testing) for NNV. As mentioned above, the laboratories that participated in this project also participated in ANQAP. The availability of current research data from the project significantly influenced the way ANQAP viewed control samples and “unknown” samples that were sent to the participants in the QA tests. Specifically, data from the project enabled the researchers to question assumptions about the stability of NNV in samples that were circulated to participating laboratories. Enhanced assessment and assurance of sample stability during ring tests will ensure that programs such as ANQAP continue to enjoy strong support from participating laboratories. The QA program is underpinned by a standard protocol, (Australian and New Zealand Standard Diagnostic Test Procedure, ANZSDP) the content of which was revised by researchers based on the findings of this FRDC project. This is another benefit of contemporary research for routine activities in Australian aquatic animal health laboratories.

*Recommendations for improved biosecurity protocols in relation to nodavirus infection and fish translocation (Objective 5)*

Objective 5 contained the on-farm and experimental epidemiological research that would lead to practical outcomes for the Australian aquaculture, wild fishery and recreational fishing sectors. The main findings from the epidemiological investigations conducted in barramundi and Australian bass for objective 5 were:

i) Barramundi brood stock did not appear to be a source of infection for cohorts of larvae
   a. although barramundi brood stock at DAC (25% in 2012) and at 2 of 3 hatcheries in north Queensland (7% in 2012) had been exposed to NNV, based on the presence of specific antibodies in blood which were detected with the new ELISA, there was no evidence of an active, vertically transmissible form of NNV infection based on qPCR tests of gonad and blood clot samples (176 brood stock were tested, 80 fish from DAC and 96 from the three hatcheries in north Queensland).
   b. At DAC, where observations were made over several years and where outbreaks of NNV infection were recorded during the study, there was no apparent relationship between infected batches of larvae and the test results of brood stock. That is, brood stock with negative qPCR and negative ELISA were associated with 3 of the 4 infected cohorts, while the fourth infected cohort was derived from qPCR negative brood stock, several of which were ELISA positive at a later time indicating exposure to NNV. In north Queensland, outbreaks of NNV were recorded in 2011 and 2012, but all brood stock were gonad/blood qPCR negative and all those at one of two affected hatcheries were also ELISA negative. Rationalisation of the brood stock test results obtained in this project using the new qPCR with those obtained in a previous FRDC project (2002-043) which appeared to show that up to 10% of barramundi brood stock could shed NNV in gonadal fluid cannot be achieved because the previous project used a test which has been superceded and which may not have had the same specificity as the new test.

ii) Both barramundi and Australian bass may carry NNV in brain for at least several years, but may not transmit the infection to their progeny because:
   a. Barramundi which survived NNV infection could be successfully grown out and retained NNV in brain for at least 2 years, but by this time the viral load was low. However, this indicates a potential for persistent infection in fish which may become recruited to hatcheries as brood stock. Some of these fish were also positive in ELISA.
   b. A longitudinal study in Australian bass confirmed persistent infection at low viral load in brain of adult survivors of an NNV infection acquired as juvenile fish. Some of these fish were

positive in ELISA. However, there was no evidence of transmission of infection when these brood stock were spawned.

iii) All four cohorts of NNV-infected larval barramundi which were examined in a longitudinal study at DAC became infected during larval rearing or at a grow out farm because:
   a. There was no evidence of NNV infection in eggs
   b. The first confirmed qPCR positive samples were detected at 5 days-old or later.

iv) Circumstantial evidence from north Queensland suggested a reduction in the incidence of NNV outbreaks when barramundi were reared in intensive “clearwater” systems using UV treated water compared to pond-based “greenwater” culture using untreated seawater. This transition of culture method occurred during the project, following industry observations of repeated NNV outbreaks in extensive larval rearing ponds and this has been supported by the extension of the findings from an earlier study (Hick et al., 2011a).

v) The hypothesis of horizontal transmission was confirmed because:
   a. The infection status of brood stock was variable
   b. There was no evidence of infection of egg stage
   c. The first detectable infection occurred at or after 5 days-old

vi) The findings of this study were in agreement with a prior study at DAC in which horizontal transmission was confirmed in two cohorts in 2007 (Hick et al., 2011a).

The conclusion that NNV infection in larval barramundi is due to horizontal transmission leads to several logical recommendations to prevent outbreaks and translocation of NNV:

i) Emphasis should be placed on protecting larval fish from exposure to NNV rather than brood stock testing.

ii) While brood stock tests have been considered to be important in the past the results of this study suggest that they should be a secondary precaution. Specifically, qPCR test positive (gonadal fluid) brood stock could be excluded from spawning. However, it was not possible to evaluate whether test positive brood stock pose a risk because none were identified in hatcheries in this study. There are insufficient data to recommend testing blood samples from brood stock using ELISA. Therefore further research is warranted on the infection status of brood stock using both qPCR and ELISA.

iii) The most likely source of horizontal infection is the water supply to the hatchery. Larval fish should be provided with UV-treated, filtered water to reduce the likelihood of exposure to NNV. Clean water should be provided for as long as possible before transfer of fish to grow out because it is uncertain at what age barramundi and Australian bass become resistant to the clinical effects of NNV infection. This question requires research. As UV treatment of large volumes of water is expensive and may not be uniformly effective (for example in turbid water), research is required to find a more cost effective and efficient system to provide NNV-free water for

young fish. Research is also needed to identify the exact source of infection in the environment, i.e. the means by which intake water becomes contaminated by NNV. Presumably it is from an environmental reservoir host fish.

iv) Hatcheries should adopt all-in-all-out batch culture to avoid a situation where a batch of fish with sub-clinical infection acts as a reservoir of high loads of NNV at a time when younger fish are present. This is because it is difficult to prevent horizontal transmission from one batch to another within a hatchery (Hick et al., 2011a).

v) Hatcheries should develop biosecurity protocols to reduce the risk of horizontal transmission of NNV associated with introduction of brood stock, live feed, personnel movements, visitors and other factors. Protocols will need to be developed specifically for each hatchery as circumstances vary from hatchery to hatchery.

vi) Protocols to prevent translocation of NNV with movements of live fish should recognise:

   a. There is a high risk that the survivors of NNV outbreaks will be sub-clinical carriers (barramundi and Australian bass)
   b. The prevalence of carrier fish, the viral load in carriers and hence the level of risk of transmission of NNV with carriers decreases with the age of fish
   c. Outbreaks of NNV can be subclinical even in young fish. This was observed in both barramundi and Australian bass. While histopathology can be used in young fish to detect active NNV infection causing brain and eye lesions, qPCR is the only objective test able to detect the presence of subclinical NNV infection. Therefore the qPCR test is recommended for screening fish populations for NNV infection.
   d. Some fish in populations which have been exposed to NNV and which contain carriers have specific antibodies for NNV which can be detected by ELISA (barramundi and Australian bass). ELISA requires blood samples which can be obtained without killing fish, so it may be applicable to high value species/individuals. Further research is required to determine whether naturally exposed ELISA positive fish are actively infected, and whether ELISA can be used for practical purposes in individuals or populations for surveillance. However, based on observations at grow out stage of both barramundi and Australian bass suggest that the ELISA is a potential test for exposure to NNV infection at population level.

## BENEFITS AND ADOPTION

The benefits and beneficiaries of this research project align with those identified in the project application, which was based on national consultation coordinated by FRDC. The national aquatic animal health laboratory network has benefited by technology transfer and has adopted the new qPCR test and control reagents, and has also benefited from nationally coordinated interlaboratory quality assurance testing for NNV under the Australian National Quality Assurance Program (ANQAP), which adopted new information about NNV sample stability.

Although it is too soon after the discovery phase to expect adoption of all biosecurity recommendations, circumstantial evidence from north Queensland suggests a reduction in the incidence of NNV outbreaks in barramundi which are now reared in intensive “clearwater” systems using UV treated water compared to previous pond-based “greenwater” culture using untreated seawater. This transition of culture method occurred during the project, following industry observations of repeated NNV outbreaks in extensive larval rearing ponds and this has been supported by the extension of the findings from an earlier study (Hick et al., 2011a).

The wild fisheries and recreational fishing sectors have benefited from the adoption by aquatic animal health laboratories of improved testing methods for NNV which have far greater sensitivity than previous methods. This has helped reduce the chance that NNV is spread through movement of fish from aquaculture into natural ecosystems.

## FURTHER DEVELOPMENT

At the completion of this project there remained a number of key knowledge gaps, that if closed, would provide greater confidence in recommendations to control NNV infection in finfish. Recommendations for further development include:

1. The ELISA blood test. This test has an advantage over other tests in that it requires blood samples which can be obtained without killing fish, so just like sampling gonadal fluid it may be applicable to high value species/individuals. Further research is required to determine whether naturally exposed ELISA positive fish are actively infected with NNV, and whether ELISA can be used for practical purposes in individuals or populations for surveillance. However, based on observations at grow out stage of both barramundi and Australian bass suggest that the ELISA is a potential test for exposure to NNV infection at population level. This is justification for further observational research, including longitudinal studies.
2. The qPCR. The biological significance of test positive outcomes with high Ct, that is, low number of viral copies, requires investigation because it is possible that regulatory action such as destruction of batches of fish or quarantine could be imposed following reporting of such results. The results of this project suggest that the likelihood of infectious virus being present in fish with high Ct positive test outcomes is low, based on mostly negative virus isolation results from barramundi or Australian bass with such qPCR results. However, it is already known that virus isolation is less sensitive than qPCR (Hick et al., 2011b; Hick and Whittington, 2010), meaning that some fish with high Ct may in fact carry live virus. The infectivity of such fish for susceptible fish could be tested experimentally by cohabitation. The group of Australian bass which are currently held at EMAI are suitable for this type of experiment.
3. The effect of pooling tissues from a number of small fish on qPCR test sensitivity is not known. It is currently necessary to pool tissues from small fish to obtain a tissue mass of sufficient size for extraction of DNA, but there is potential for reduced sensitivity.
4. The identification of horizontal transmission as being the most likely means by which young fish become infected with NNV in hatcheries in Australia raises a question – what is the reservoir of infection? Most probably NNV enters the hatchery via the water supply, suggesting that infected wild fish exist in the local environment. Developing better understanding of the distribution and abundance of wild fish with NNV infection would be useful to predict risk for hatcheries. It appears that horizontal transmission to hatchery fish is episodic rather than continuous, because some batches of fish can be healthy and others infected, but other than that observation there are no data. Transmission could coincide with for example seasonal finfish spawning events in nature which may provide a large population of susceptible hosts in which NNV could amplify prior to moving with water onto a hatchery.

5. Methods for disinfecting seawater to remove infectious NNV need to be developed so that hatcheries can provide clean water to young fish for as long as possible. The efficacy of UV and other treatments against NNV is uncertain. Other methods for treating large volumes of water require evaluation, such as ozone and chlorine.
6. Aligned to the provision of clean water is a need to understand the period of susceptibility of young fish to NNV. Observations in this project suggest that once fish reach a certain (but unknown) age, they may become infected with NNV, and NNV may spread to infect most fish, but there will not be any sign of disease. It is important to prevent infection until this threshold age at which fish become resistant to clinical disease. At older ages it is possible that fish may become resistant to infection per se. Research is required using experimental transmission of NNV under controlled conditions to determine the threshold age of resistance.
7. Methods for disinfecting eggs, while unlikely to provide a major benefit for NNV given horizontal transmission, may be an added precaution against NNV and may be useful against other diseases. Further investigation is required of ozone egg disinfection including the dose and the method for different fish species, and how it could be applied in an integrated biosecurity program. Ozonation needs to be investigated from the perspectives of both viral disinfection and impact on the viability of eggs. Ozonation may have a benefit in reducing horizontal transmission as brood stock typically are held in non-UV treated water which may lead to contamination of eggs during spawning with NNV present in the water supply.
8. With respect to restocking waters for recreational fishing, and based on current knowledge, the risks of transfer of NNV between regions through movement of fish from aquaculture can be addressed by testing batches of fish using qPCR, and allowing only test negative batches to be translocated for restocking purposes. Histopathology will not detect subclinically infected fish and should not be used for certification purposes as outlined above. However, pathogen pollution from aquaculture is relevant only if the destination waters are free of NNV. Surveys of wild to confirm this are necessary, and should be based on qPCR tests of wild fish. As this test requires destruction of fish, consideration should also be given to using ELISA, as part of further investigation of the potential of the ELISA blood test to be used for this purpose. If infection is detected at population level, the virus needs to be isolated and identified at molecular level because there are several distinct strains of NNV and it could be important not to super-infect wild fish populations with a different strain of NNV originating from aquaculture.
9. There was only a limited opportunity to study NNV in marine species during this project. Insufficient data were obtained to make any conclusions about the epidemiology of NNV infection in marine species and further research is therefore required. The methods developed in this project, including the new tests and epidemiological study design, can be applied to marine finfish.

The main data from this project, comprising the animal identification and the results of molecular assays, virus isolation and ELISA are summarised in appendices to this report. Raw data are stored at the Faculty of Veterinary Science University of Sydney in electronic form and may be accessed by contacting the author. Owner details for most samples from Queensland are blinded for reasons of confidentiality.

## PLANNED OUTCOMES

The overall planned outcome of this project was to ensure the sustainability and profitability of the aquatic industry and the health of natural resources by providing industry and governments with improved tools to make sound and risk-based decisions for VNN management and control and for animal movements. This was in addition to a broader responsibility towards the Australian community to ensure the sustainability of Australian aquatic natural resources.

This overall outcome was achieved through the development and extension of improved diagnostic capability for NNV to five aquatic animal health laboratories in Australia, improvements to national ring testing for NNV, and elucidation of the main origin of NNV infection for hatchery fish. This will lead to disease prevention strategies being developed specific for each hatchery. Improved testing protocols that do not rely on histopathology but instead on the new qPCR test will improve the level of confidence that batches of fish produced through aquaculture are NNV-free and suitable for use in grow out or restocking programs in NNV-free areas. This will protect both recreational and commercial fisheries in these areas.

Extension of R&D results to industry was achieved through presentations to the Australian Barramundi Farmers Association conference, the Australasian Aquaculture Conference and through industry meetings that were convened by state jurisdictions. Extension of the most recent findings of the project, namely confirmation that horizontal transmission is the main route for infection of fish in hatcheries, will be ongoing. How this will be achieved with the recently announced closure of the Oonoonba laboratory remains to be determined as staff from this laboratory have been critical in extension activities to prevent diseases in aquaculture in north Queensland.

## CONCLUSION

As a result of this project a reverse transcriptase quantitative real time PCR (qPCR) to detect NNV and suitable assay controls were successfully transferred to five aquatic animal health diagnostic laboratories in Australia, established and run according to specifications. The laboratories have since participated in the Australian National Quality Assurance Program (ANQAP) for NNV with good results, and as a result of this project the assessment of sample stability during ANQAP ring tests was improved. The program is underpinned by an Australian and New Zealand Standard Diagnostic Test Procedure which was revised by researchers based on the findings of this project. The new qPCR test was shown to detect all of the known NNV genotypes.

The new qPCR test requires samples of brain/eye to be taken from dead fish. An enzyme-linked immunosorbent assay (ELISA) which is based on blood samples collected from live fish (brood stock and grow out fish) was also developed during this study. The test measures the level of antibody against NNV. It was applied successfully in epidemiological studies in barramundi and Australian bass and the results indicated that both species respond to infection with NNV with an antibody response. Individual fish were identified that had both antibody in blood samples and NNV present in brain/retina. Over time (months to years), the proportion of fish with antibodies in blood and virus in nervous tissue decreased, as did the levels of both antibodies and virus. Further research is needed to demonstrate whether the ELISA test may be useful to identify NNV infected populations.

The main finding from the epidemiological investigations conducted in barramundi and Australian bass was that brood stock did not appear to be a source of infection for cohorts of larvae, even though some had been exposed to NNV, based on their history and/or on the presence of specific antibodies in blood which were detected with the new ELISA. There was no evidence of an active, vertically transmissible form of NNV infection based on qPCR tests of gonad and blood clot samples. There was no apparent relationship between infected batches of larvae and the test results of brood stock. However, adults of both barramundi and Australian bass may carry NNV in brain for at least several years. Four batches of NNV-infected larval barramundi were examined in a longitudinal study at DAC, and they were found to have become infected during larval rearing or at a grow out farm. In summary, the hypothesis of horizontal transmission of NNV was confirmed because the infection status of brood stock was variable, there was no evidence of infection of egg stage and the first detectable infection occurred at or after 5 days-old. The findings of this study were in agreement with a prior study at DAC in which horizontal transmission was confirmed in two cohorts in 2007 (Hick et al., 2011a).

Recommendations were developed to prevent outbreaks of NNV on hatcheries and to prevent translocation of NNV through aquaculture. Emphasis should be placed on protecting larval fish from exposure to NNV rather than brood stock testing. The most likely source of horizontal infection is the water supply to the hatchery. Larval fish should be provided with UV-

treated, filtered water to reduce the likelihood of exposure to NNV. Clean water should be provided for as long as possible before transfer of fish to grow out. Hatcheries should adopt all-in-all-out batch culture to avoid a situation where a batch of fish with sub-clinical infection acts as a reservoir of high loads of NNV at a time when younger fish are present. Hatcheries should develop biosecurity protocols to reduce the risk of horizontal transmission of NNV associated with introduction of brood stock, live feed, personnel movements, visitors and other factors. Protocols will need to be developed specifically for each hatchery.

Regardless of whether fish are required for grow out or for restocking, protocols to prevent translocation of NNV with movements of live fish from aquaculture, should recognise that there is a high risk that the survivors of NNV outbreaks will be sub-clinical carriers. The prevalence of carrier fish, the viral load in carriers and hence the level of risk of transmission of NNV with carriers decreases with the age of fish. Furthermore, outbreaks of NNV can be subclinical even in young fish. Therefore objective laboratory tests must be used to screen batches of fish for NNV infection. Histopathology can no longer be recommended as the sole screening test and qPCR should be used instead.

Recommendations were made for further development to determine whether naturally exposed ELISA positive fish are actively infected with NNV, and whether the ELISA can be used for practical purposes in individuals or populations for surveillance because the ELISA is a potential test for exposure to NNV infection at population level. The biological significance of qPCR test positive outcomes with high Ct (low number of viral copies) also requires further investigation because it is possible that regulatory action such as destruction of batches of fish or quarantine could be imposed following reporting of such results. The results of this project suggest that the likelihood of live virus being present in fish with high Ct positive test outcomes is low, however, virus isolation is less sensitive than qPCR (Hick et al., 2011b; Hick and Whittington, 2010).

Developing better understanding of the distribution and abundance of wild fish with NNV infection would be useful to predict risk of horizontal transmission for hatcheries. Methods for disinfecting seawater to remove viable NNV need to be developed so that hatcheries can provide clean water to young fish for as long as possible. There is also a need to understand the period of susceptibility of young fish to NNV. It is important to prevent infection until this threshold age at which fish become resistant to clinical disease. Methods for disinfecting eggs may be an added precaution against NNV and may be useful against other diseases.

With respect to restocking waters for recreational fishing, it is important to conduct surveys of wild fish to confirm that NNV is not already present.

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## APPENDIX 1: INTELLECTUAL PROPERTY

This project has not developed any intellectual property that requires legal protection.

## APPENDIX 2: STAFF

Many people contributed to the research described in this report.

The core project team included Richard Whittington (University of Sydney), Kitman Dyrting (Berrimah Veterinary Laboratory, Northern Territory), Ian Anderson (Oonoonbah Veterinary Laboratory Queensland), Diana Jaramillo (University of Sydney), Alison Tweedie (University of Sydney), Jane Frances (New South Wales DPI), Peter Kirkland (EMAI, New South Wales), Edla Arzey (EMAI, New South Wales), Paul Hick (EMAI, New South Wales), Mark Crane (CSIRO AAHL) and Nick Moody (CSIRO AAHL). This report was prepared by Richard Whittington using results, data and other information contributed by the team members, who also commented on a draft of the report.

Additional acknowledgements are required for specific sections of the research project. The following list may not be complete, and if so apologies are extended from the core project team for any omissions. The transfer of PCR technology to other laboratories was successful thanks to the efforts of Susan Walsh (Northern Territory) and Kelly Condon (Queensland). Virus purification was undertaken by Kylie Gore, Paul Hick, Edla Arzey, Elizabeth Moane, Sylvia Vrankovic, Nicole Joel, Mukesh Srivastava and Melinda Frost. The longitudinal studies in barramundi and Australian bass were conducted with assistance from Stewart Fielder, Luke Cheviot, Luke Vandenberg, Damon Gore and other staff from the Darwin Aquaculture Centre and Port Stephens Fisheries Institute. Additional laboratory support at EMAI was provided by Yogini Lele, Katherine King, Scott Petterson, Rod Davis and Glenda Macnamara.

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Special thanks are due to the many barramundi farmers who provided samples and practical advice throughout this project.

## APPENDIX 3: DRAFT AUSTRALIAN AND NEW ZEALAND STANDARD DIAGNOSTIC TEST PROCEDURE FOR NNV

## *Betanodavirus Infections of Finfish*


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## *Part 1  Diagnostic Overview*

### *Summary*


Viral Nervous Necrosis (VNN), syn Viral Encephalopathy and Retinopathy (VER), is a serious viral disease of finfish caused by a betanodavirus. Disease has been reported from over 40 fish species from tropical to temperate climates from most continents around the world, with the exception of Africa, and most reports of disease have been associated with species in aquaculture facilities. Clinical disease is most commonly observed in larval and juvenile finfish, and this is the case in Australia, although clinical disease in adult fish is increasingly being reported. Mortality rates of up to 100% are most commonly seen in larval fish and tend to decrease as the size of the infected fish increases. Fish surviving infection can become asymptomatic carriers and while the most common mode of transmission appears to be vertically from sub-clinically infected broodstock to progeny during spawning, horizontal transmission can occur. Betanodavirus infection has a significant economic, social and environmental impact in Australia through direct losses and inhibition of trade for established and emerging aquaculture industries, restriction on locations suitable for aquaculture expansion and suspension of fish restocking programs due to concerns on the impact of native fish species due to translocation of the virus with stock. Tests have been developed to detect the infection in fish and for health certification for regulatory purposes. Due to insufficient knowledge regarding the immune response in infected fish all tests are based on detection of the virus. Virus

isolation, real-time Reverse-Transcriptase Polymerase Chain Reaction (RT-qPCR) and nested RT-PCR (RT-nPCR) are used to detect viral sequence in fresh material and microscopic examination is used for disease diagnosis in fixed material. Immunological tests (Immunocytochemistry Test; ICCT or Indirect Immunofluorescent Antibody Test; IFAT), RT-qPCR or RT-nPCR are used to confirm the identity of viruses isolated in cell culture as *Betanodavirus* with sequencing of amplicons produced by conventional RT-PCR used to confirmation of strain identity. Immunohistochemistry Test (IHCT) or IFAT are used to confirm or exclude nodavirus from fixed histological tissue sections where the nature of lesions may be ambiguous.

**1. Aetiology**

The virus causing Viral Nervous Necrosis (VNN) is a member of the genus *Betanodavirus* of the family *Nodaviridae* It is non-enveloped and icosahedral in shape with a diameter of approximately 25 to 30 nm. Virions contain two segments (RNA1 and RNA2) of positive sense single-stranded RNA (ssRNA) with the RNA2 segment containing the sequence for the viral coat protein.[1] The RNA2 segment is highly conserved among isolates and is the target for the detection of viral RNA by molecular methods and viral coat protein by immunological methods. There are four recognised genotypes: striped jack nervous necrosis virus (SJNNV); tiger puffer nervous necrosis virus (TPNNV); barfin flounder nervous necrosis virus (BFNNV) and red-spotted grouper nervous necrosis virus (RGNNV).[2] A possible fifth genotype; turbot betanodavirus (TNV) has recently been proposed.[3] All Australian betanodavirus isolates have been identified as members of the RGNNV genogroup, where they occur in two distinct clusters.[24] Betanodavirus has not been detected in New Zealand.

**2. Clinical Signs**

Clinical signs are most commonly observed in larvae and fry and are due to damage to the nervous tissue of the spinal cord, brain and retina caused by the virus. Typically, affected fish display abnormal behaviour, including spiral swimming and rapid uncoordinated darting movement, with mass mortalities occurring over a short period of time.[4,5] Colour changes (lightening or darkening of diseased fish), cessation of feeding and increased susceptibility to cannibalism may also be observed. Gross lesions are uncommon, but overinflation of the swimbladder in infected sevenband grouper [6] and red drum[7] has been reported.

**3. Epidemiology**

VNN is an acute infectious disease of primarily finfish larvae and fry cultured in seawater. Disease has been described in more than 37 fish species from all continents with the exception of Africa.[4,8] Mortalities of up to 100% are most common in larvae with susceptibility generally decreasing as the age of the fish increases.[8,4,5,9] However, significant mortalities of some species of fish at harvest size[6,10] and of some species of fish cultured in freshwater have been reported.[11] In Australia, nodavirus has been detected in Australian bass (*Macquaria novemaculata*), barramundi (*Lates calacrifer*), barramundi cod (*Cromileptes altilevis*), goldspotted rockcod (*Epinephelus coioides*), flowery cod (*Epinephelus fuscoguttatus*)

and striped trumpeter (*Latris lineata*) from marine aquaculture facilities in New South Wales, Northern Territory, Queensland, South Australia and Tasmania and from sleepy cod (*Oxyeleotris lineolatus*) from a freshwater aquaculture facility in Queensland.[24] As other species are evaluated for aquaculture potential, the range of species found to be susceptible to infection is likely to increase. Nodaviruses have been detected in juvenile fish surviving experimental and natural infection,[5,12,13,25] and while the length of viral persistence is unknown, virus has been detected in halibut and Australian bass surviving acute infection for at least 12 months after the initial disease outbreak.[9,25] Virus has also been detected in healthy adult fish of known susceptible species,[6,12,13,25,26] and from healthy fish of species in which disease has not been observed previously.[14,15] Exposure to the virus, or recombinant coat protein of the virus, can induce a protective neutralising antibody response in susceptible fish[16] although duration of this response is unknown. Antibodies have been detected by ELISA in broodstock,[17] although ELISA-positive fish have still produced VNN virus-positive offspring.[18] The relationship between immune status and presence of infectious virus within the fish is still to be determined. The most common mode of transmission is still thought to be vertically from infected broodstock to progeny [4,8] however horizontal transmission does occur.[4,27]

**4. Occurrence and Distribution**

Betanodavirus infections have been reported from finfish in Queensland, the Northern Territory, New South Wales, Tasmania, South Australia and Western Australia.[24] No betanodavirus infections have been reported in New Zealand. While no phylogenetic information is available for Western Australian isolates, all the other studied Australian isolates have been identified as members of the RGNNV genotype.[24] However, the isolates form two distinct groups within this genotype; one group comprising isolates from New South Wales and South Australia and a second group comprising isolates from Queensland, the Northern Territory and Tasmania. As the effect of the isolates on native finfish species is unknown, strict controls are in place, to reduce the risk of translocation of virus with commercial stock or stock used for restocking programs and to reduce the risk of escape of virus from aquaculture facilities into the environment. Exclusion of the virus from aquaculture premises, good hygiene in these premises and reduced stocking densities have contributed to decreasing the incidence of VNN outbreaks. Screening by nested RT-PCR and use of only NNV-negative broodstock has also reduced the occurrence of disease in larvae.[18,19,20]

**5. Lesions**

VNN is characterised by vacuolating encephalopathy and retinopathy.[21] Lesions are usually less severe in older fish than juvenile fish and depending on the age of the fish severity of the vacuolation can range from one or two affected cells to necrosis of entire regions of the brain and retina. In general, vacuolation occurs more often in the optic tectum and cerebellum of barramundi than in the telencephalon and medulla oblongata. Focal pyknosis and karyorrhexis of the neural cells, granularity of the neuropil and accumulation of eosinophilic material in macrophages may also be observed.[21] Descriptions of microscopic changes have been published.[4,5,21]

## 6. Diagnostic Tests and Specimens

Clinical signs in larvae and fry are an indication of nodavirus infection, but definitive diagnosis requires observation of vacuoles in tissue sections, detection of the viral antigen in fresh or fixed tissue, or virus isolation followed by confirmation of Betanodavirus by immunological or molecular methods. Only antigen detection tests have been validated for use; antibody detection tests have not been validated.

### 6.1 Fixed material

Diagnosis can be made on the observation of vacuoles in the nervous tissue of the spinal cord, brain and retina, which have been fixed in formalin or Bouin's fixative and stained with H&E using light microscopy. If clinical signs have been seen, especially in larvae or fry, then observation of vacuolation is enough for diagnosis. However, if there is any doubt, confirmation of the presence of virus in the tissue sections by transmission electron microscopy (TEM), immunohistochemistry test (IHCT) or indirect fluorescent antibody test (IFAT) is required. While both the IHCT and IFAT perform equally well when lesions are observed in H&E stained sections by light microscopy the IHCT is more sensitive at detecting nodavirus in sub-clinically infected fish than the IFAT, which in turn is more sensitive than histology.[12] Comparisons of TEM with the IHCT and IFAT are not available, however, as TEM requires specialised equipment and expertise that is not always readily available, the IHCT is recommended for routine confirmation of infection in fixed material.

The RT-qPCR is the most appropriate test for molecular detection of viral RNA in tissue fixed in 80 to 95% analytical grade ethanol or RNAlater (or equivalent) as this assay is 10-fold to 1000-fold more sensitive than the RT-nPCR, depending on the isolates being tested.[25] Confirmation of RT-qPCR results by conventional RT-PCR and sequencing of representative amplicons should be undertaken to confirm the positive result. This is especially important when no clinical signs have been observed or the disease is suspected in a new species or in a new geographic location.

### 6.2 Unfixed material

Detection of the virus in samples of unfixed tissue is undertaken by virus isolation in susceptible cell cultures[8], RT-qPCR[25] or RT-nPCR.[12,24,28] Virus isolation can be undertaken in either SSN-1 or E-11 cell cultures with confirmation of the identity of viruses isolated in cell culture undertaken by RT-qPCR, RT-nPCR, IHCT or IFAT.

The RT-qPCR is the most appropriate test for molecular detection of viral RNA in unfixed tissue as this assay is 10-fold to 1000-fold more sensitive than the RT-nPCR, depending on the isolates being tested.[25] Confirmation of RT-qPCR results by conventional RT-PCR and sequencing of representative amplicons should be undertaken to confirm the positive result. This is especially important when no clinical signs have been observed or the disease is suspected in a new species or in a new geographic location.

Testing of broodstock eggs, sperm and blood can be undertaken by RT-qPCR or RT-PCR testing, although optimal sample and sample collection, preparation and testing protocols are still to be determined. While positive results of valid tests can be accepted, negative results may be false-negative results.

6.3 Specimen Storage

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

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

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

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## *Betanodavirus Infections of Finfish*

### *Part 2: Diagnostic Test Methods*

*1. Detection of Betanodavirus*

*1.1 Immunohistochemistry Test for fixed material*

#### 1.1.1 Principle of the test

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

#### 1.1.2 Reagents

- Polyclonal antibody

The primary sheep anti-BNNV rCP or sheep anti-SCNNV rCP polyclonal antibody is available from the suppliers described in Part 3: Reagents and Kits of this document.

- Peroxidase conjugated secondary antibody

Any commercial anti-sheep IgG [H+L] horseradish peroxidise conjugate can be used. New batches should be tested using positive and negative control slides.

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

| | |
|---|---|
| Tris | 9.68 g |
| NaCl | 116.9 g |
| Deionised water | 4 L |

- 0.1% trypsin in TBS

| | |
|---|---|
| Trypsin (1:250) | 0.2 g |
| TBS | 4 L |

- 3% $H_2O_2$ in methanol

| | |
|---|---|
| $H_2O_2$ | 6.0 mL |
| Methanol | 200 mL |

- 5% bovine serum albumin (BSA) in TBS

| | |
|---|---|
| BSA | 2.5 g |
| TBS | 50 mL |

- 2.5% BSA in TBS

| | |
|---|---|
| BSA | 1.25 g |
| TBS | 50 mL |

- ImmunoPure® Metal Enhanced DAB Substrate Kit (Pierce, USA).

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

- Deionised water

- Mayer's haematoxylin

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

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

- Lithium carbonate

| | |
|---|---|
| Lithium carbonate | 2.8 g |
| Deionised water | 200 mL |

- Mounting medium

### 1.1.3 Equipment

- Deparaffinised, rehydrated tissue sections mounted on positively charged glass histology slides.
- Hydrophobic marker
- Humidified 37°C chamber
- Compound microscope

### *1.1.4 Test Procedure*

a) Circle the tissue sections with a hydrophobic marker. Tissue sections should not be allowed to dry at any stage

b) Add 1 mL 0.1% trypsin to each tissue section and incubate at 37°C for 30 minutes in a humidified chamber.

c) Wash three times with TBS.

d) Block endogenous peroxidase by immersing the tissue sections in 3% $H_2O_2$ in methanol at room temperature for 20 minutes.

e) Wash three times with TBS.

f) Block non-specific binding sites by incubating each tissue section with 1 mL 5% BSA in TBS in a humidified chamber at 37°C for 20 minutes.

g) Wash three times with TBS.

h) Add 1 mL Sheep $\alpha$-NNV rCP polyclonal antibody, diluted 1/1000 in 2.5% BSA in TBS, to each tissue section and incubate in a humidified chamber at 37°C for 60 minutes.

i) Wash three times with TBS.

j) Add 1 mL of Rabbit $\alpha$-Sheep IgG [H+L] HRP conjugate, diluted 1/1000 in 2.5% BSA in TBS, to each tissue section and incubate in a humidified chamber at 37°C for 60 minutes.

k) Wash three times with TBS.

l) Prepare the ImmunoPure® Metal Enhanced DAB Substrate Kit according to the manufacturer’s instructions. Add 1 mL to each tissue section and incubate for 10 minutes at room temperature. Stop development by immersing the slides in deionised water.

m) Counterstain tissue sections with Mayer’s haematoxylin for 60 seconds, rinse in tap water for 60 seconds, blue in lithium carbonate for 60 seconds and rinse in tap water for 60 seconds.

n) Mount tissue sections under a coverslip using an aqueous mounting medium and examine with a compound microscope.

### 1.1.5 Quality Control Aspects

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

1.1.6 Interpretation

Any positive staining indicates the presence of the betanodavirus coat protein and the fish is considered to be undergoing an active infection. When a single fish from a larger group is positive in an IHCT, that is sufficient evidence to consider the entire group is infected.

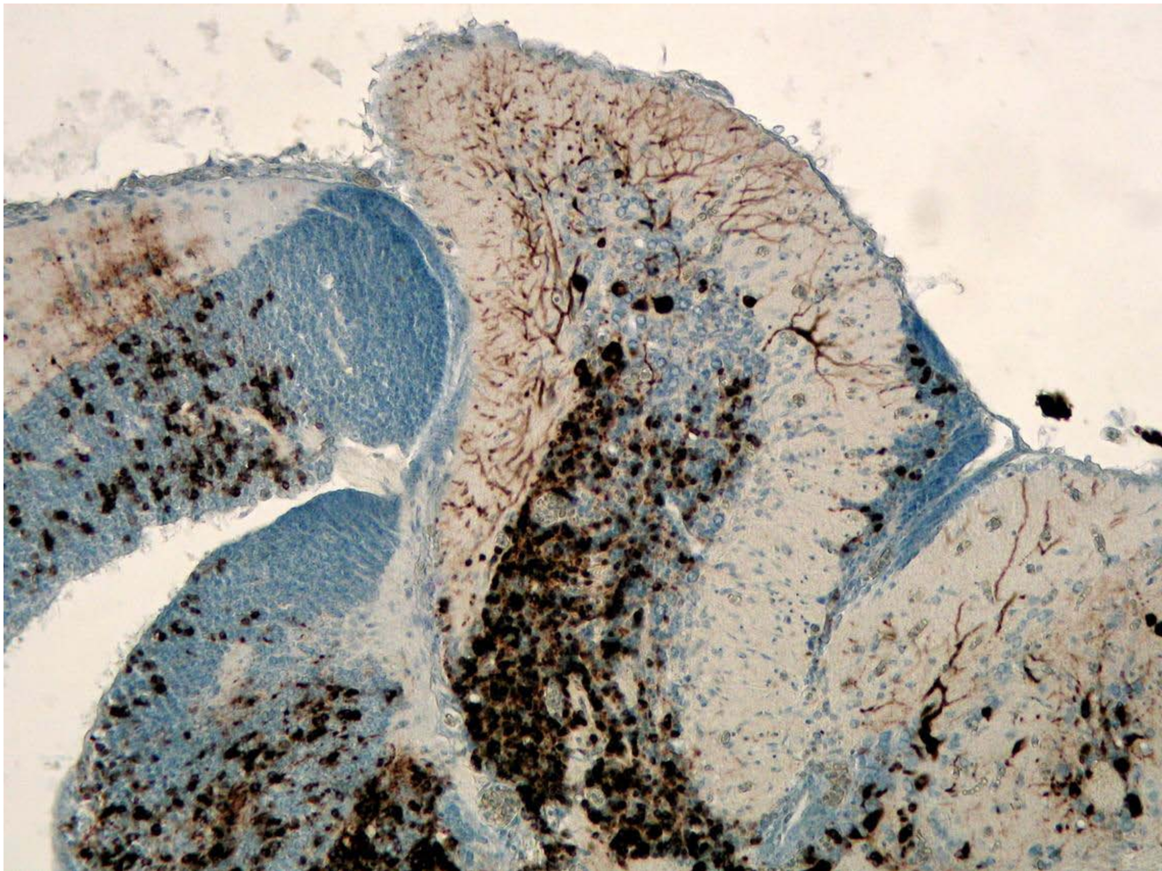


Figure 1 **Positive NNV IHCT in the brain of a heavily infected barramundi, indicated by the dark brown/black spots. x200**

## *1.2 Indirect Fluorescent Antibody Test for fixed material*

**1.2.1 Principle of the test**

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

### 1.2.2 Reagents

- Polyclonal antibody

The primary sheep anti-BNNV rCP or sheep anti-SCNNV rCP polyclonal antibody is available from the suppliers described in Part 3: Reagents and Kits of this document.

- Fluorescent conjugated secondary antibody

Any commercially-available anti-sheep IgG [H+L] fluorescent conjugate can be used. Cyanine 2 (Cy2™) is more intense and less prone to photo bleaching than fluorescein isothiocyanate (FITC). New batches should be tested using positive and negative control slides.

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

| | |
|---|---|
| Tris | 9.68 g |
| NaCl | 116.9 g |
| Deionised water | 4 L |

- 0.1% trypsin in TBS

| | |
|---|---|
| Trypsin (1:250) | 0.2 g |
| TBS | 4 L |

- 5% bovine serum albumin (BSA) in TBS

| | |
|---|---|
| BSA | 2.5 g |
| TBS | 50 mL |

- 2.5% BSA in TBS

| | |
|---|---|
| BSA | 1.25 g |
| TBS | 50 mL |

- Aqueous mounting medium

### 1.2.3 Equipment

- Deparaffinised, rehydrated tissue sections mounted on positively charged glass histology slides.
- Hydrophobic marker
- Humidified 37°C chamber
- Fluorescent microscope

### *1.2.4 Procedure*

a) Circle the tissue sections with a hydrophobic marker. Tissue sections should not be allowed to dry at any stage

b) Block non-specific binding sites by incubating each tissue section with 1 mL 5% BSA in TBS in a humidified chamber at 37°C for 60 minutes.

c) Wash three times with TBS.

d) Add 1 mL Sheep $\alpha$-NNV rCP polyclonal antibody, diluted 1/1000 in 2.5% BSA in TBS, to each tissue section and incubate in a humidified chamber at 37°C for 60 minutes.

e) Wash three times with TBS.

f) Add 1 mL of Rabbit $\alpha$-Sheep IgG [H+L] Cy2™ conjugate, diluted 1/1000 in 2.5% BSA in TBS), to each tissue section and incubate in a humidified chamber at 37°C for 60 minutes.

g) Wash three times with TBS.

h) Mount the tissue sections under a coverslip using an aqueous mounting medium and examine with a fluorescent microscope.

### 1.2.5 Quality Control Aspects

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

### 1.2.6 Interpretation

Any positive staining indicates the presence of the betanodavirus coat protein and the fish is considered to be undergoing an active infection. When a single fish from a larger group is positive in an IFAT, that is sufficient evidence to consider the entire group is infected.

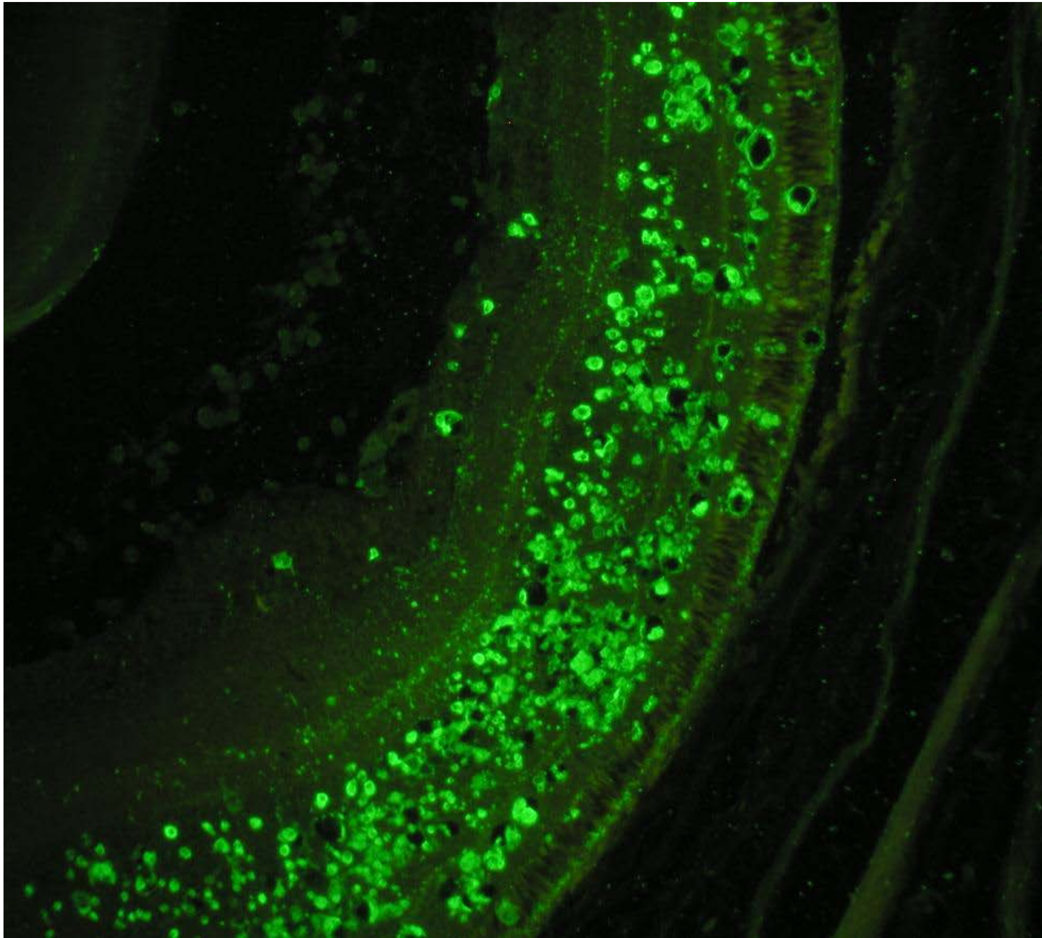


Figure 2 **Positive NNV IFAT in the retina of a heavily infected barramundi, indicated by the bright green spots. x200**

## *1.3 Real-time RT-PCR (RT-nPCR)*

**1.3.1 Principle of the test**

Real-time RT-PCR is used to amplify a specific sequence from an RNA target. Firstly, reverse transcription (RT) converts the specific target on the viral RNA into complementary DNA (cDNA). Incorporation of a sequence-specific probe with a fluorescent dye at the 5’ end and a quencher at the 3’ end increases the specificity of the assay, as this probe must also bind to the target sequence with the primers during the annealing stage. As the PCR amplifies the specific sequence from this cDNA to produce multiple copies, the 5’ to 3’ exonuclease activity of the *Taq* polymerase releases the fluorophore from the bound probe. Thereof, as the effect of the quencher has been eliminated, the fluorescence emission can be detected. As the amount of target sequence is increased by the PCR, the amount of fluorescence increases and the PCR continues. The primers, probe and cycling conditions used in these procedures are fully described elsewhere.[25] The protocol described here is the modification validated for use through equivalence testing at the AAHL Fish Diseases Laboratory.

**Table 1** Primer and probe sequences used in the NNV RT-qPCR test

| Primer | Sequence |
|---|---|
| qR2T-F | 5'- CTT CCT GCC TGA TCC AAC TG -3' |
| qR2T-R | 5'- GTT CTG CTT TCC CAC CAT TTG -3' |
| **Probe** | |
| qR2Tprobe | 5'- **6FAM** CAA CGA CTG CAC CAC GAG TTG **TAMRA** -3' |

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

**1.3.2 Reagents**

- Transportation Medium (TM)

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

- QIAamp Viral RNA Mini Kit (Qiagen, USA) or MagMAX™-96 Viral RNA Isolation Kit (Applied Biosystems, USA)

The QIAamp Viral RNA Mini Kit has been validated for use by AFDL for RNA and DNA extractions (generally used if ≤24 samples). The MagMAX™-96 Viral RNA Isolation Kit has been validated for use by AFDL for RNA and DNA extractions from (generally used if ≥24 samples). Alternative kits should be validated for use.

- TaqMan One-Step RT-PCR Master Mix Reagents (Applied Biosystems, USA).

The TaqMan One-Step RT-PCR Master Mix is specifically mentioned as this reagent is routinely used for RT-qPCR assays by AFDL. The AgPath-ID One-Step RT-PCR Kit (Applied Biosystems, USA) and QuantiTect Probe RT-PCR Kit (Qiagen, USA) have also been evaluated during equivalence testing with no significant differences in assay performance observed. Alternative kits should be validated for use.

- Primers

- RNase-free deionised water

**1.3.3 Equipment**

- Real-time PCR instrument (Applied Biosystems 7500 Fast)

Any real-time PCR instrument can be used after validation for use.

### 1.3.4 Test Procedure

#### 1.3.4.1 Sample preparation

a) Fish or organ samples are homogenised in TM. The tissue required is dependent on the size of the fish (Table 2) with a 1:10 (w/v) tissue suspension in TM required for subsequent analysis. Homogenisation can be achieved using a mortar and pestle, bead beater[25] or stomacher bag and 2lb hammer.[12]

**Table 2** Tissue samples required

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

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

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

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

#### 1.3.4.2 Real-time RT-PCR procedure

a) RNA is extracted according to the manufacturer's instructions.

b) Two microlitres of template is added to replicate wells of Master Mix containing 12.5 µL TaqMan One-step RT-PCR Master Mix, 0.625 µL 40× Multiscribe and RNase inhibitor mix, 0.9 µM each primer, 0.25 µM probe and water to 23 µL. Negative (e.g. no template control; NTC), positive and internal controls must be included with each test run.

c) Reverse transcription and PCR amplification are conducted in a thermal cycler programmed with the following cycles: 1 cycle of 50°C for 30 minutes, 1 cycle of 95°C for 10 minutes, 45 cycles of 95°C for 15 seconds and 60°C for 60 seconds.

d) Results are recorded and analysed using the software provided by the manufacturer of the real-time PCR instrument.

1.3.5 Quality Control Aspects

For the assay and test results to be accepted the following criteria must be fulfilled:

a. The NTC and/or negative sample control must have no evidence of specific amplification curves.
b. Each positive control must yield a specific amplification curve and mean Ct values within the acceptable range according to accumulated quality control data.
c. Test samples with characteristic amplification curves and Ct values are considered test positive.

1.3.6 Interpretation

A positive RT-qPCR result is indicative of the presence of nodavirus RNA in the sample. However, a positive RT-qPCR does not indicate whether the sample is infectious. Samples which are test positive from species or geographic locations where betanodavirus infections have not been recorded previously should be amplified by conventional RT-PCR and sequenced to confirm the result. This is especially important when no other diagnostic test has been used. A negative result from finfish tissue is indicative of the absence of nodavirus RNA in the sample.

*1.4 Nested RT-PCR (RT-nPCR)*

**1.4.1 Principle of the test**

Nested RT-PCR is used to amplify a specific sequence from an RNA target. Firstly, reverse transcription (RT) converts the specific target on the viral RNA into complementary DNA (cDNA). The primary PCR then amplifies the specific sequence from this cDNA to produce multiple copies. To achieve even greater sensitivity a second, or "nested", PCR, which targets a specific DNA sequence within the primary PCR amplicon, is used. The NNV nested RT-PCR test is based on the R3-F2 primers (Table 3) and RT-PCR cycling conditions to amplify a 426bp sequence and the nested primers NR'3-NF'2 (Table 3) and nested PCR cycling conditions to amplify a 294bp sequence from the T4 region of the betanodavirus coat protein gene. The primers and cycling conditions used in these procedures,[22,23] as modified, are fully described elsewhere.[12, 24] One significant modification exists for testing of blood, where BSA is added at 1 μg/μL to the primary PCR.

**Table 3** Primers used in the NNV nested RT-PCR test

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

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

**1.4.2 Reagents**

- Transport Medium (TM)

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

- QIAamp Viral RNA Mini Kit (Qiagen, USA) or MagMAX™-96 Viral RNA Isolation Kit (Applied Biosystems, USA)

The QIAamp Viral RNA Mini Kit has been validated for use by AFDL for RNA and DNA extractions (generally used if ≤24 samples). The MagMAX™-96 Viral RNA Isolation Kit has been validated for use by AFDL for RNA and DNA extractions from (generally used if ≥24 samples). Alternative kits should be validated for use.

- StrataScript™ RT (Stratagene) , StrataScript™ buffer (Stratagene, USA)

Any RT could be used, however not all RTs perform to the same standard[12] and comparative testing should be undertaken if reagents are changed. StrataScript™ RT is specifically mentioned as this reagent was used for the optimisation and validation work for the NNV nested RT-PCR.

- 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)

Any Taq could be used, however not all Taqs perform to the same standard[12] and comparative testing should be undertaken if reagents are changed. The HotStarTaq™ Master Mix Kit is specifically mentioned as this reagent was used for the optimisation and validation work for the NNV nested RT-PCR and the hot start format reduces the risk of reagent degradation due to temperature fluctuations and contamination due to a reduction in the number of components the operator must add.

- 10mg/mL Bovine Serum Albumin (for testing blood)

- DNA ladder and loading dye

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

- Agarose

- Dye (e.g. ethidium bromide, SybrSafe)

**1.4.3 Equipment**

- Thermal Cycler (Eppendorf MasterCycler)

- Gel electrophoresis system

- Gel documentation system

**1.4.4 Test Procedure**

**1.4.4.1 Sample preparation**

a) Fish or organ samples are homogenised in TM. The tissue required is dependent on the size of the fish (Table 4) with a 1:10 (w/v) tissue suspension in TM required for subsequent analysis. Homogenisation can be achieved using a mortar and pestle, bead beater[25] or stomacher bag and 2lb hammer.[12]

**Table 4** Tissue samples required

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

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

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

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

**1.4.4.2 Nested RT-PCR procedure**

e) RNA is extracted according to the manufacturer's instructions.

f) RNA is transcribed in a 20 µL reaction mix, containing 9.5 µL 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.

g) Primary PCR amplification is carried out in a 50 μL reaction mix, containing 5 μL of the RT reaction, 0.2μM of each primer (R3 and F2), 2.5mM $MgCl_2$, 25 μL of HotStarTaq™ Master Mix and deionised water. If blood is being tested, then add BSA at 1 μg/μL. Reactions are conducted in a 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.

h) Nested PCR amplification is carried out in a 50μl reaction mix, containing 1μl of the primary PCR reaction, 1.0μM of each primer (NR'3 and NF'2), 25μl of HotStarTaq™ Master Mix and deionised water. Reactions are conducted in a 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.

i) Reaction products are analysed after electrophoresis through a 1.5% to 2% agarose gel containing 10 mg/mL ethidium bromide. Amplicons are visualised using a UV transilluminator

1.4.5 Quality Control Aspects

a) The NTC and/or negative sample control must have no evidence of specific amplicons.

b) A positive reaction for the primary RT-PCR results in the production of one discrete 426bp amplicon and for the nested RT-PCR results in the production of one discrete 294bp amplicon (Figure 3).

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

1.4.6 Interpretation

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

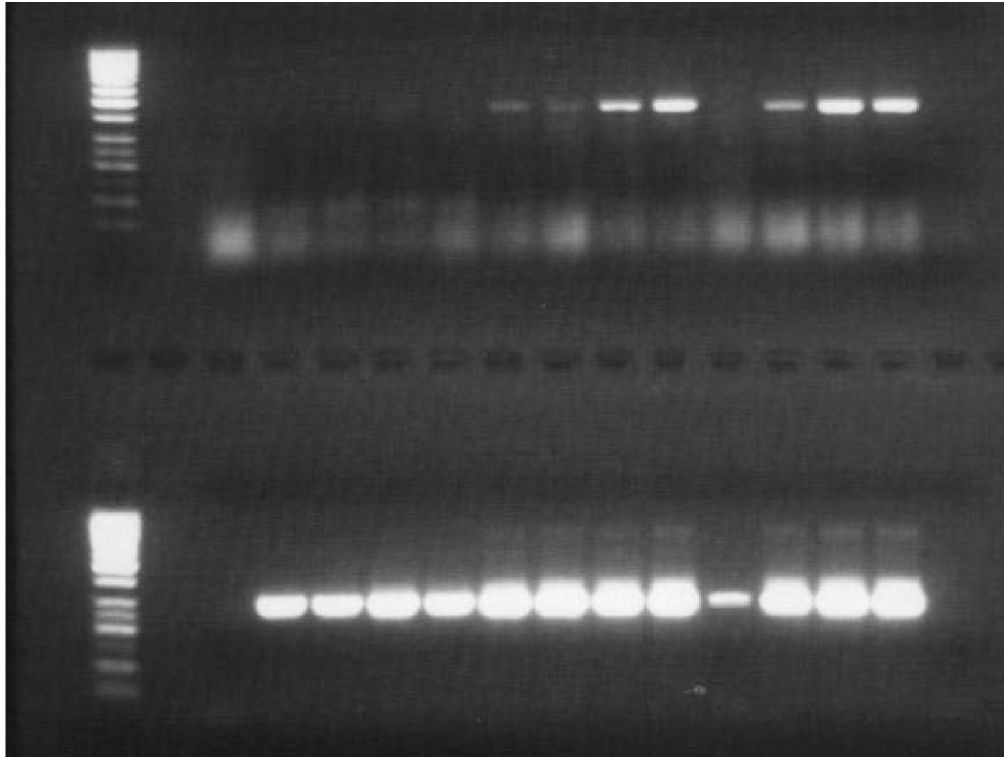


Figure 3 **Positive nested RT-PCR results. Upper gel: Primary RT-PCR – positive amplicon of 426bp. Lower gel: nested RT-PCR – positive amplicon of 294bp.**

1.5 Virus Isolation

**1.5.1 Principal of the test**

Virus isolation in susceptible cell cultures is the "gold standard" for investigation of the presence of infectious virus in tissue. By testing samples using susceptible cell culture, the presence of infectious can be determined.

**1.5.2 Reagents**

- SSN-1 and/or E11 cells.
- Growth medium: Leibovitz's L-15 medium supplemented with 10% foetal bovine serum, 2mM Glutamine, 100 IU/mL penicillin and 100ug/mL streptomycin sulphate.
- Maintenance medium: Leibovitz's L-15 medium supplemented with 2% foetal bovine serum, 2mM Glutamine, 100 IU/mL penicillin and 100ug/mL streptomycin sulphate.

**1.5.3 Equipment**

- Equipment required for the establishment and maintenance of a fish cell culture laboratory has been discussed previously.[29]

**1.5.4 Test Procedure**

**1.5.4.1 Sample preparation**

a) Fish or organ samples are homogenised in TM and should be kept chilled (4°C to 10°C) during processing. Sample preparation and virus isolation are ideally undertaken on the day of sample receipt. If this is not possible, samples should be stored at -80°C.
b) All manipulations are undertaken in a Class II Biological Safety Cabinet using aseptic technique and sterile equipment and reagents.
c) The sample/tissue required is dependent on the size of the fish (Table 5).

**Table 5** Tissue samples required

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

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

1.5.4.2 Virus isolation

**a)** The procedure described is for virus isolation in SSN-1 or E11 cells cultured in 24-well tissue culture plates. If different culture vessels are used (e.g. 96-well plates, 25cm² tissue culture flasks) volumes are adjusted proportionally.

b) Cells are seeded at a density of $2 \times 10^5$ cells/mL with 1.5mL of cell suspension and incubated at 22°C. Cultures should be less than 24 hours old when inoculated.

c) On the day of inoculation of the samples, examine cell cultures to be used by inverted light microscopy. Ensure that they are approximately 70% to 80% confluent, free from overt microbial contamination and mitotic figures are visible (that is, they are actively dividing). Any problems should be noted and, if necessary, fresh cultures prepared for use on the next day.

d) Discard the cell culture medium from the wells of the SSN-1 or E-11 tissue culture plate.

e) Inoculate duplicate cultures with 150uL of each sample dilution (1/10 and 1/100). One set (column) of four well-cultures on each 24-well tissue culture plate should be used as negative controls, which are inoculated with 150uL TM.

g) Incubate the cultures at 22°C for 1 h.

h) Following adsorption, add 1.5mL maintenance to each culture (negative controls first) yielding final sample dilutions of 1/100 and 1/1000. Place culture plates to the 22°C incubator.

i) On the day following inoculation, and every 1-3 days thereafter, examine the cultures, by inverted light microscopy, for any microbial contamination, tissue sample cytotoxicity and viral cytopathic effect (CPE).

**1.5.4.3 Subculturing**

a) At 8-12 days after inoculation, the test cultures not showing CPE should be passaged. For each tissue sample, the contents of each of the four replicate cultures, irrespective of dilution, are pooled into sterile centrifuge tubes.

b) If tissue sample cytotoxicity or bacterial/fungal contamination has been observed during the initial culture period for each pool, the pooled contents should be filtered, using 0.45µm or 0.22µm filter, into the sterile centrifuge tube. A 0.45µm is preferred to reduce the likelihood of eliminating viruses. Alternatively, samples could be microcentrifuged at 10000 x *g* for 10 minutes at 5°C.

c) Each pooled sample is used to make a further 1/10 dilution of each pool by pipetting 0.5mL of the supernatant into the corresponding 1/100 test tubes containing 4.5mL TM.

d) Without decanting the cell culture medium, inoculate duplicate fresh cell cultures in 24-well culture plates, prepared as described above, with 150uL of the pooled supernatant and the 150uL of the 1/10 dilution of the pooled supernatant. Place culture plates to the 22°C incubator.

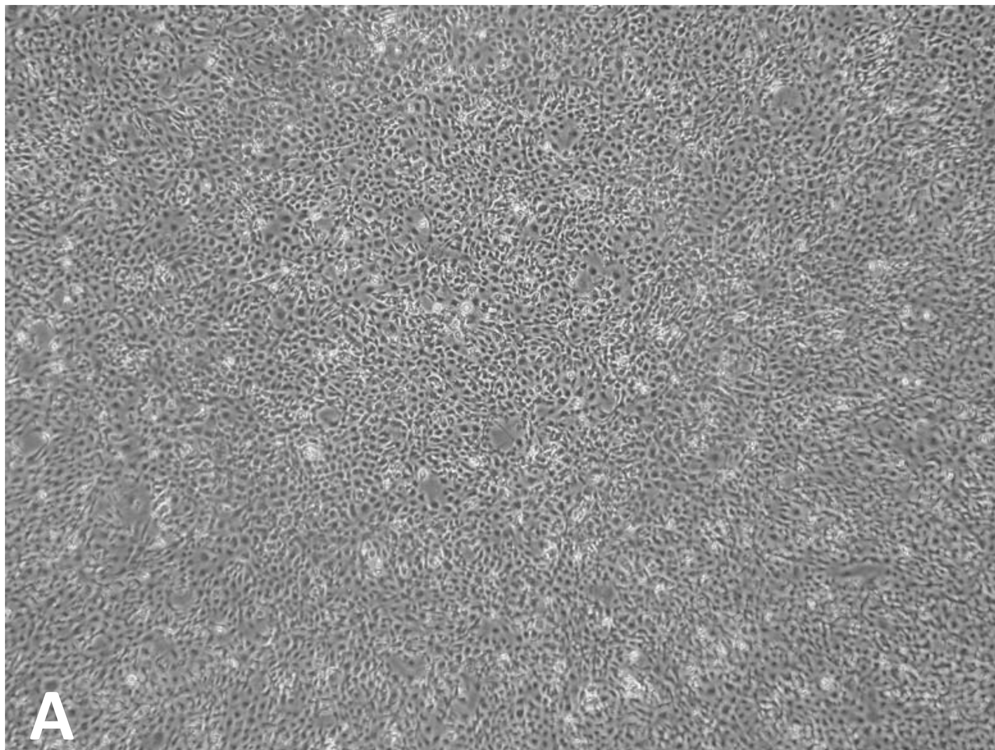
e) Observe these cultures for at least 9-13 days and record observations. Irrespective of the time at which the passage occurred, cell cultures should be observed for a total of at least 21 days for completion of the assay.

**1.5.5 Quality Control Aspects**

a) The test is valid if the negative control cultures retain normal cellular morphology for the full period of incubation in the absence of bacterial contamination.
b) To ensure cell lines, used on a routine basis, are susceptible to the viruses of concern, virus titrations on each of the cell lines should be carried out on a regular basis (every 3-6 months preferably just prior to the onset of export certification testing).

**1.5.6 Interpretation of results**

a) The test is valid if the negative control cultures retain normal cellular morphology for the full period of incubation.
b) The test sample is negative if the inoculated cell cultures retain normal cellular morphology similar to the negative control cultures and the cellular monolayer retains normal integrity. If virus isolation is the only test performed, confirmation by ICCT or IFAT should be undertaken to avoid false-negative results.
c) If any of the cell cultures inoculated with test samples demonstrate any abnormalities, such as increased intracellular vacuolation, or monolayer destruction (Figure 4), further investigation is required, such as examination by electron microscopy, further sub-culturing or confirmation of the presence of betanodavirus by molecular methods as described above, or by ICCT or IFAT.



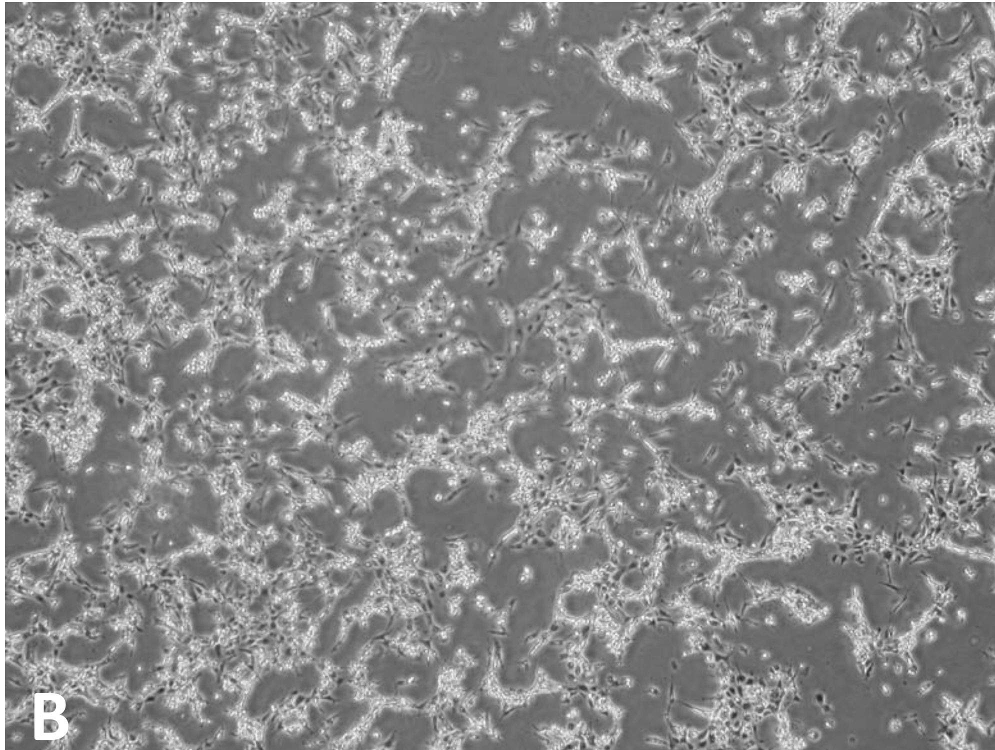



Figure 4a **Uninfected SSN-1 cell culture, 4 days post seeding**

Figure 4b **BNNV-infected SSN-1 cell culture, 4 days post seeding, 3 days post inoculation**

1.6 ICCT and IFAT of cell cultures

**1.6.1 Principal of the test**

This test uses polyclonal antibodies raised in sheep against the recombinant coat protein of a barramundi or sleepy cod betanodavirus isolate and an anti-sheep IgG horseradish peroxidase conjugated secondary antibody, to localise viral coat protein in fixed tissue cultures containing nervous tissue of finfish. The test is used to confirm or exclude betanodavirus as the agent causing CPE or to exclude betanodavirus infection in cell cultures not showing CPE.

**1.6.2 Reagents**

- See 1.1.2 above

**1.6.3 Equipment**

- Slide staining rack
- Fluorescent inverted microscope

### 1.6.4 Test Procedure

#### 1.6.4.1 Fixation

a) Drain cells. Dispose of supernatant according to standard procedures. Wash the cells gently in TBS.
b) Fix the cells in 50% acetone/50% methanol. Add 1mL per well for 24-well plates or 3mL per flask for 25cm² flasks. Incubate for 5 minutes at room temperature, with gentle agitation.
c) Remove solvent and rinse with TBS.

#### 1.6.4.1 ICCT

a) Block endogenous peroxidase by adding 3% $H_2O_2$ in methanol. Add 1mL per well for 24-well plates or 3mL per flask for 25cm² flasks. Incubate for 20 minutes at room temperature.
b) Wash each well three times with TBS.
c) Block non-specific binding sites by 5% BSA in TBS. Add 1mL per well for 24-well plates or 3mL per flask for 25cm² flasks and incubate for 30 minutes at 37°C.
d) Wash each well three times with TBS.
e) Immediately before use dilute Sheep α-NNV rCP polyclonal antibody 1/1000 in 2.5% BSA in TBS.
f) Add diluted 1° Ab to the cell culture. Add 500μl to each well for 24-well plates or 3mL per flask for 25cm² flasks. Incubate at 37°C for 60 minutes.
g) Wash each well three times with TBS.
h) Immediately before use dilute Rabbit α-Sheep IgG [H+L] HRP conjugate 1/1000 in 2.5% BSA in TBS.
i) Add conjugate to the cell culture. Add 500μl to each well for 24-well plates or 3mL per flask for 25cm² flasks. Incubate at 37°C for 60 minutes.
j) Prepare the HRP Chromogen, according to the manufacturer's instructions. Add 1mL per well for 24-well plates or 3mL per flask for $25cm^2$ flasks. Stop development after 10 minutes by aspirating the HRP Chromogen and replacing with Milli-Q water.
k) Counterstain in Mayer's haematoxylin for 60 seconds, rinse in Milli-Q water for 60 seconds and blue in lithium carbonate for 60 seconds. Add 1mL per well for 24-well plates or 3mL per flask for 25cm² flasks.
l) Rinse in Milli-Q water, wash each slide well three times with TBS and observe under the inverted microscope.

1.6.4.2 IFAT

a) Block non-specific binding sites by adding 1mL 5% (w/v) BSA in TBS to each well and incubate for 30 minutes at 37°C.
b) Wash each well three times with TBS.
c) Immediately before use dilute Sheep α-NNV rCP polyclonal antibody 1/1000 in 2.5% BSA (w/v in TBS).
d) Add diluted 1° Ab to the cell culture. Use 500μl to each well for 24-well plates or 3mL per flask for 25cm2 flask. Incubate at 37°C for 60 minutes.
e) Wash each well three times with TBS.

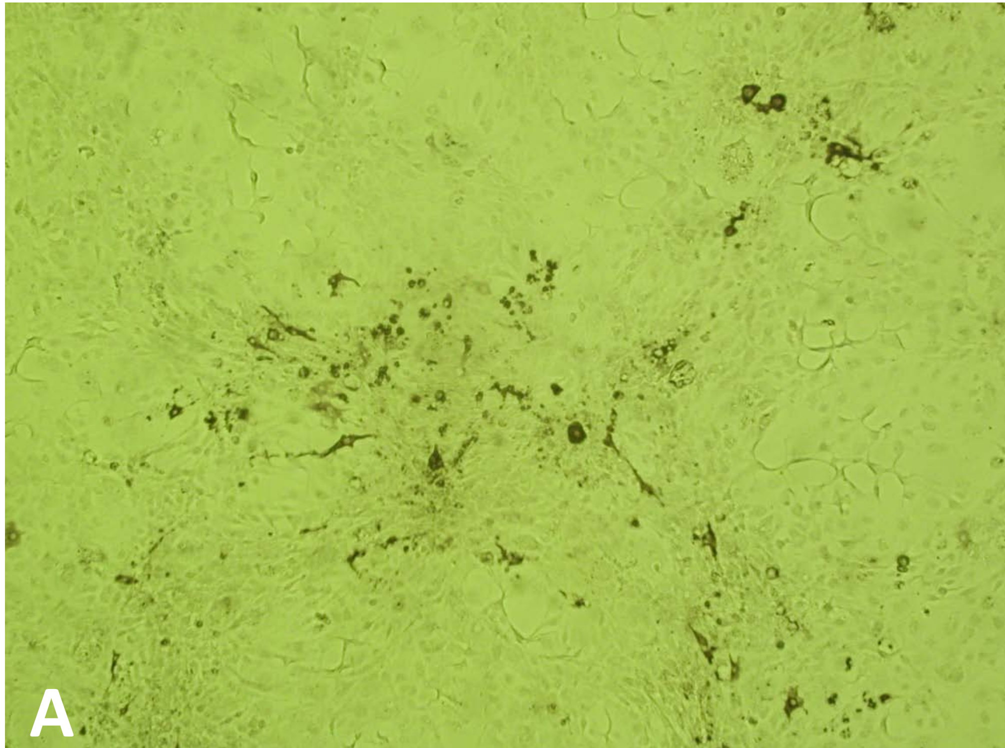
f) Immediately before use dilute Rabbit $\alpha$-Sheep IgG [H+L] Cy2™ conjugate 1/1000 in 2.5% BSA (w/v in TBS).
g) Add Cy2 conjugate to the cell culture. Use 500$\mu$l to each well for 24-well plates or 3mL per flask for 25cm2 flask. Incubate at 37°C for 60 minutes.
h) Turn on the mercury burner of the fluorescent microscope 20 minutes prior to use.
i) Wash each well three times with TBS and observe under the fluorescent microscope.

### 1.6.5 Quality Control Aspects

Positive and negative control tissue cultures must be included in every test. Ideally, a positive cell culture exhibiting a low level of infection should also be included. For the ICCT to be valid, dark brown or black staining cytoplasm of cells must be observed in the positive control cultures (Figure 5). For the IFAT to be valid, bright green fluorescence in the cytoplasm must be observed in the positive control cultures (Figure 6). No specific staining should be seen in the negative control cultures.

### 1.6.6 Interpretation

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



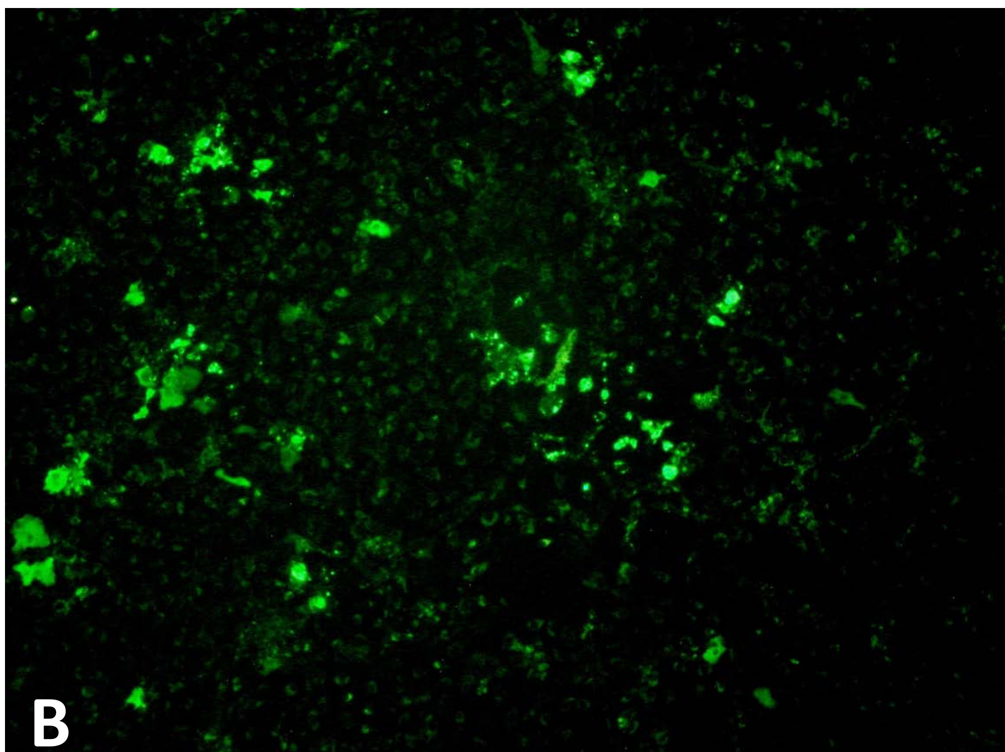




Figure 5a **ICCT positive BNNV-infected SSN-1 cell culture**

Figure 5b **IFAT positive BNNV-infected SSN-1 cell culture**

## *Part 3: Reagents and Kits*

**3.1 Betanodavirus polyclonal antibody**
Polyclonal antibodies, raised in sheep against the recombinant coat proteins from barramundi and sleepy cod betanodavirus isolates are available from the authors or the Tropical and Aquatic Animal Health laboratory (Section 4.1 below).

**3.2 Betanodavirus-infected finfish for positive control slides for IHCT**
Tissue from betanodavirus infected finfish on histological slides is available from the address of the authors.

**3.3 Betanodavirus-infected finfish homogenate and Betanodavirus-infected SSN-1 cell culture supernatant for positive control for RT-qPCR and RT-nPCR**
Homogenates from betanodavirus infected finfish are available from the authors or the Tropical and Aquatic Animal Health laboratory (Section 4.1 below).

## Part 4: Suppliers of Reagents and Kits

**4.1 Australia:**

**Tropical and Aquatic Animal Health Laboratory – NNV polyclonal antibodies, positive control tissue and positive control homogenates.**
Virology Laboratory
Tropical and Aquatic Animal Health Laboratory
Department of Primary Industries and Fisheries
PO Box 1085
Townsville QLD 4810
Tel (07) 4722 2603
Fax (07) 4778 4307

**Pierce - ImmunoPure® Metal Enhanced DAB Substrate Kit.**
www.piercenet.com
Quantum Scientific Pty Ltd
30 Finchley St,
Paddington, QLD 4064
Tel 1800 777 168
Fax 1800 625 547

**ROCHE - High Pure Viral RNA Extraction Kit**
www.roche-applied-science.com
Roche Diagnostics Australaia Pty Ltd
Southbank Corporate Park
Logan House
25 Donkin St,
West End, QLD 4101
Tel 1800 802 409
Fax 1800 066 598

**Stratagene - StrataScript™ RT**
www.stratagene.com
Integrated Sciences
2 McCabe Pl,
Willoughby, NSW 2068
Tel (02) 9417 7866
Fax (02) 6417 5066

**QIAGEN - HotStarTaq™ Master Mix Kit**

*www.qiagen.com*
PO Box 641,
Doncaster, VIC 3108
Tel 1800 243 800
Fax (03) 9840 9888

**Applied Biosystems - TaqMan One-Step RT-PCR Master Mix Reagents**
www.applied biosystems.com.au
Head Office (Melbourne)
30-32 Compark Circuit
Mulgrave VIC 3170
Tel 1800 636 327
Fax  1800 143 363

**4.2 New Zealand:**

**Tropical and Aquatic Animal Health Laboratory – NNV polyclonal antibodies, positive control tissue and positive control homogenates.**
There is no source in New Zealand, and supplies would need to be obtained from the Australian source at the above address.

**Pierce - ImmunoPure® Metal Enhanced DAB Substrate Kit.**
www.piercenet.com
Global Science & Technology Ltd
241 Bush Rd,
Albany, Auckland
Tel (09) 443 5867
Fax (09) 444 7314

**ROCHE - High Pure Viral RNA Extraction Kit**
www.roche-applied-science.com
Roche Diagnostics N.Z. Ltd
15 Rakino Way,
Mt Wellington,
Auckland
Tel (09) 276 4157
Fax (09) 276 9817

**Stratagene - StrataScript™ RT**
www.stratagene.com
Global Science & Technology Ltd
241 Bush Rd,
Albany, Auckland
Tel (09) 443 5867
Fax (09) 444 7314

**QIAGEN - HotStarTaq™ Master Mix Kit**

*www.qiagen.com*
Biolab Ltd
244 Buch Rd,
Albany, Auckland
Tel 0800 933 966
Fax (09) 980 6788

**Applied Biosystems - TaqMan One-Step RT-PCR Master Mix Reagents**
www.applied biosystems.com.au
Tel 0800 636 327
Fax 0800 664 887

## APPENDIX 4: RESULTS OF BROOD STOCK TESTING AT DARWIN AQUACULTURE CENTRE

Results of broodstock testing each year at Darwin Aquaculture Centre. Cohorts of larvae in bold were infected with NNV. ELISA results in bold are positive. All qPCR results were negative.

| Fish no. | ID | Origin | Cohorts of larvae | ELISA | | | | | | qPCR* | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | 2010 | | 2011 | | 2012 | | 2010 | 2011 | 2012 |
| | | | | OD | OD ratio | OD | OD ratio | OD | OD ratio | | | |
| 1 | 01C728D9 | record not found | N/A | | | | | | | | | |
| 2 | 01C8F901 | F1 of cohort 11/01, Dec 2003 | 09/08 | | | 0.65 | 2.23 | 0.87 | 3.00 | | B | |
| 3 | 01C90BC8 | F1 of cohort 11/01, Dec 2003 | **10/09**<br>08/10 | | | | | **0.77** | **3.25** | GF | GF | |
| 4 | 01C91A38 | F1 of cohort 11/01, Dec 2003 | N/A | | | | | | | GF | | |
| 5 | 01FA6E0E | Channel Island Middle Arm, Mar 2008 | 11/11<br>03/12 | | | 0.58 | 2.80 | 0.51 | 2.81 | GF | GF, B | |
| 6 | 01FAB749 | Channel Island Middle Arm, Mar 2008 | 05/10 | 0.82 | 2.44 | 0.70 | 1.83 | | | B | B | |
| 7 | 01FBA90A | Channel Island Middle Arm, Mar 2008 | 02/10 | 0.46 | 1.62 | **0.85** | **3.73** | 0.60 | 2.57 | B | B | |
| 8 | 06021DC9 | Channel Island Middle Arm, Mar 2008 | 09/11 | | | 0.42 | 1.40 | | | | B | |
| 9 | 0658B402 | Channel Island Middle Arm, Mar 2010 | N/A | | | 0.58 | 1.47 | 0.78 | 1.51 | | B | |
| 10 | 0658B7CE | Channel Island Middle Arm, Sept 2007 | 11/08 | | | 1.07 | 1.37 | | | GF | B | |
| 11 | 0658B9D7 | Channel Island Middle Arm, Mar 2008 | 02/10<br>05/10 | 1.33 | 1.94 | **0.85** | **3.98** | **0.90** | **5.15** | B | B | |

| Fish no. | ID | Origin | Cohorts of larvae | ELISA | | | | | | qPCR* | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | 2010 | | 2011 | | 2012 | | 2010 | 2011 | 2012 |
| | | | | OD | OD ratio | OD | OD ratio | OD | OD ratio | | | |
| 12 | 0658B9EA | Channel Island Middle Arm, Mar 2008 | N/A | 0.74 | 2.34 | 0.51 | 2.45 | 0.60 | 2.39 | B | B | |
| 13 | 0658BF3B | Channel Island Middle Arm, Mar 2008 | 02/10 01/11 | 0.80 | 2.03 | | | **1.36** | **7.02** | B | GF | |
| 14 | 0658C2EA | Channel Island Middle Arm, Mar 2008 | **10/09** | | | 0.50 | 1.83 | 0.40 | 1.37 | | B | |
| 15 | 0658C4DD | Channel Island Middle Arm, Sept 2007 | **09/09** 08/10 | | | | | 0.42 | 2.33 | | | |
| 16 | 0658C579 | F1 of cohort 04/08; May 2009 | N/A | 0.96 | 1.67 | **0.88** | **2.92** | | | GF,B | B | |
| 17 | 0658CF54 | F1 of cohort 04/08; May 2010 | N/A | 1.01 | 1.21 | 0.78 | 1.45 | **0.94** | **3.59** | GF,B | B | |
| 18 | 0658D85B | F1 of cohort 04/08; May 2010 | 02/10 | 0.68 | 2.18 | 1.00 | 2.42 | | | B | B | |
| 19 | 0658D8B7 | Channel Island Middle Arm, Mar 2008 | 09/08 | | | | | **0.88** | **4.07** | | GF | |
| 20 | 0658DA81 | Channel Island Middle Arm, Sept 2007 | N/A | 1.01 | 1.67 | 1.16 | 2.43 | 0.61 | 2.78 | GF, B | B | |
| 21 | 0658E6CB | F1 of cohort 04/08; May 2010 | **04/09** 02/10 03/10 | 0.75 | 2.25 | 0.84 | 2.49 | 0.57 | 1.94 | B | B | |
| 22 | 065D7424 | Channel Island Middle Arm, Mar 2008 | N/A | 0.87 | 1.48 | 1.13 | 2.80 | 0.41 | 1.81 | B | B | |
| 23 | 065D7C90 | Channel Island Middle Arm, Mar 2008 | 03/12 | 0.62 | 2.11 | | | **1.09** | **4.20** | B | GF | |
| 24 | 065D7D06 | Channel Island Middle Arm, Mar 2008 | 03/08 | | | | | **0.80** | **4.28** | | GF | |
| 25 | 065D81C7 | Channel Island Middle Arm, Mar 2008 | **04/11** 03/12 | 0.37 | 1.81 | 0.40 | 2.20 | | | B | B | |
| 26 | 065D8387 | F1 of cohort 04/08; May 2010 | N/A | 1.13 | 1.38 | 0.78 | 1.81 | 1.21 | 1.21 | GF, B | | |

| Fish no. | ID | Origin | Cohorts of larvae | ELISA | | | | | | qPCR* | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | 2010 | | 2011 | | 2012 | | 2010 | 2011 | 2012 |
| | | | | OD | OD ratio | OD | OD ratio | OD | OD ratio | | | |
| 27 | 065D845E | Channel Island Middle Arm, Mar 2008 | N/A | 0.89 | 1.68 | 1.03 | 1.94 | **0.71** | **3.32** | B | B | |
| 28 | 065D84FE | Elizabeth River, Feb 2005 | N/A | 0.50 | 2.12 | | | 0.35 | 2.04 | GF, B | | |
| 29 | 065D865E | Channel Island Middle Arm, Sept 2007 | **10/09** | | | | | 0.43 | 2.50 | | GF | |
| 30 | 065D8B78 | F1 of cohort 04/08; May 2010 | 11/11 | 0.51 | 1.52 | | | 0.66 | 1.90 | GF | GF | |
| 31 | 065D8D56 | Channel Island Middle Arm, Mar 2008 | N/A | | | | | 0.94 | 2.39 | | | |
| 32 | 065D99CF | Elizabeth River, Sept 2004 | 03/08 | | | 0.42 | 2.29 | **0.73** | **3.80** | GF | B | |
| 33 | 065DA1AB | Channel Island Middle Arm, Mar 2008 | N/A | | | 0.57 | 1.94 | | | GF | | |
| 34 | 065DA7FO | F1 of cohort 04/08; May 2010 | N/A | 1.00 | 1.22 | 0.54 | 1.67 | 0.55 | 2.18 | GF,B | B | |
| 35 | 065DAD56 | Channel Island Middle Arm, Mar 2008 | 01/11 | | | 0.80 | 1.87 | | | GF | | |
| 36 | 065DADEO | F1 of cohort 04/08; May 2010 | N/A | 1.00 | 2.55 | 0.55 | 2.30 | 0.36 | 1.81 | GF,B | B | |
| 37 | 065DB5E7 | Elizabeth River, Feb 2005 | 11/08<br>02/09<br>**04/09**<br>**09/09**<br>02/10<br>**04/11** | 0.76 | 1.97 | 0.96 | 1.84 | 0.70 | 2.52 | B | B | |
| 38 | 065DB601 | Channel Island Middle Arm, Mar 2008 | **04/09**<br>02/10<br>03/10<br>01/11<br>**04/11** | 1.00 | 1.95 | 1.02 | 2.65 | 0.62 | 3.36 | B | B | |

| Fish no. | ID | Origin | Cohorts of larvae | ELISA | | | | | | qPCR* | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | 2010 | | 2011 | | 2012 | | 2010 | 2011 | 2012 |
| | | | | OD | OD ratio | OD | OD ratio | OD | OD ratio | | | |
| 39 | 065DB840 | F1 of cohort 04/08; May 2010 | N/A | 1.51 | 1.27 | | | | | GF,B | GF | |
| 40 | 065DB991 | Channel Island Middle Arm, Mar 2008 | **04/09** 05/10 | 0.43 | 1.59 | 0.48 | 1.41 | 0.25 | 1.35 | B | B | |
| 41 | 065DC568 | Channel Island Middle Arm, Oct 2005 | 09/08 **09/09** | | | 0.78 | 2.11 | 0.55 | 2.59 | | B | |
| 42 | 065DC697 | Elizabeth River, Feb 2005 | N/A | | | | | 0.68 | 2.38 | GF | GF | |
| 43 | 065DD45D | Channel Island Middle Arm, Mar 2008 | N/A | 0.56 | 1.67 | | | 0.40 | 2.11 | B | GF | |
| 44 | 065DD96C | Channel Island Middle Arm, Sept 2007 | **10/09** | | | | | 0.40 | 1.62 | GF | GF | |
| 45 | 065DD9F6 | Channel Island Middle Arm, Sept 2007 | N/A | | | | | | | | GF | |
| 46 | 065DF5FO | Channel Island Middle Arm, Mar 2008 | N/A | 0.76 | 1.77 | 1.29 | 1.94 | | | | | |
| 47 | 065E64CO | F1 of cohort 04/08; May 2010 | N/A | 1.06 | 1.43 | 0.70 | 1.36 | 0.36 | 1.84 | GF,B | B | |
| 48 | 065E6830 | F1 of cohort 04/08; May 2010 | N/A | 0.95 | 1.64 | 0.57 | 1.35 | 0.64 | 2.40 | GF,B | B | |
| 49 | 065E9495 | F1 of cohort 04/08; May 2010 | N/A | 0.97 | 1.43 | 0.60 | 1.13 | 0.57 | 1.94 | GF,B | B | |
| 50 | 065E9CE6 | Channel Island Middle Arm, Mar 2008 | N/A | | | 0.64 | 2.49 | 0.96 | 2.69 | GF | B | |
| 51 | 065EA637 | F1 of cohort 04/08; May 2010 | N/A | 1.33 | 2.11 | | | | | GF,B | GF | |
| 52 | 065EA763 | F1 of cohort 04/08; May 2010 | N/A | 0.89 | 1.82 | 0.50 | 1.97 | 0.57 | 2.54 | GF,B | B | |
| 53 | 065EAA31 | F1 of cohort 04/08; May 2010 | N/A | 0.84 | 1.37 | 0.75 | 1.52 | 0.81 | 2.63 | GF,B | B | |
| 54 | 065EAAA1 | Channel Island Middle Arm, Mar 2008 | N/A | 0.90 | 1.27 | 0.66 | 1.40 | 0.44 | 1.65 | B | B | |
| 55 | 065EACOE | F1 of cohort 04/08; May 2010 | N/A | 0.81 | 1.32 | | | 0.60 | 2.07 | B | GF | |
| 56 | 065EAF45 | Channel Island Middle Arm, Sept | 08/10 | | | | | 0.43 | 1.55 | | GF | |

| Fish no. | ID | Origin | Cohorts of larvae | ELISA | | | | | | qPCR* | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | 2010 | | 2011 | | 2012 | | 2010 | 2011 | 2012 |
| | | | | OD | OD ratio | OD | OD ratio | OD | OD ratio | | | |
| | | 2007 | | | | | | | | | | |
| 57 | 065EB50F | Channel Island Middle Arm, Mar 2008 | N/A | | | | | **0.93** | **3.00** | GF | GF | |
| 58 | 065EB768 | Channel Island Middle Arm, Mar 2008 | N/A | 0.78 | 2.16 | 1.26 | 2.06 | | | B | B | |
| 59 | 065EBA11 | Elizabeth River, Feb 2005 | 04/08<br>11/08<br>02/09 | | | 0.43 | 1.28 | 0.51 | 2.61 | GF | | |
| 60 | 065EBB02 | Channel Island Middle Arm, Aug 2004 | N/A | | | | | 0.79 | 2.51 | | GF | |
| 61 | 065EBE3A | Channel Island Middle Arm, Mar 2008 | 02/09 | | | 0.53 | 1.22 | 0.34 | 1.43 | GF | B | |
| 62 | 065EC469 | F1 of cohort 04/08; May 2010 | N/A | 1.25 | 2.18 | 0.78 | 2.69 | **0.86** | **3.33** | GF,B | B | |
| 63 | 065EC762 | Channel Island Middle Arm, Oct 2005 | 01/08 | | | | | | | GF | GF | |
| 64 | 065EC8B1 | F1 of cohort 11/01, Dec 2003 | 04/08 | | | 0.53 | 1.28 | | | GF | | |
| 65 | 065ECC5F | Channel Island Middle Arm, Mar 2008 | N/A | 0.40 | 1.75 | 0.51 | 1.72 | 0.32 | 1.82 | B | B | |
| 66 | 065ECE78 | Channel Island Middle Arm, Aug 2004 | 01/08<br>04/08<br>03/10 | 0.63 | 1.84 | 0.39 | 1.48 | 0.82 | 2.64 | B | B | |
| 67 | 065ECFA7 | Channel Island Middle Arm, Mar 2008 | N/A | 1.24 | 2.26 | | | **1.02** | **3.56** | B | GF | |
| 68 | 065ED1FC | Channel Island Middle Arm, Mar 2008 | N/A | | | 0.45 | 1.85 | **0.81** | **3.23** | GF | | |
| 69 | 065ED4C5 | Elizabeth River, Feb 2005 | 01/08<br>03/08 | | | | | 0.27 | 1.84 | GF | GF | |

| Fish no. | ID | Origin | Cohorts of larvae | ELISA | | | | | | qPCR* | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | 2010 | | 2011 | | 2012 | | 2010 | 2011 | 2012 |
| | | | | OD | OD ratio | OD | OD ratio | OD | OD ratio | | | |
| 70 | 06E21B5B | Channel Island Middle Arm, Aug 2010 | 09/11 | | | 0.45 | 1.75 | 0.40 | 1.60 | GF | B | |
| 71 | 06E21BEA | Channel Island Middle Arm, Aug 2010 | N/A | | | 0.79 | 1.34 | 1.00 | 1.23 | GF | B | |
| 72 | 06E21DC9 | Channel Island Middle Arm, Aug 2010 | N/A | | | | | 0.29 | 1.29 | GF | | |
| 73 | 06E221BF | Channel Island Middle Arm, Aug 2010 | 11/11 | | | 0.80 | 1.11 | 0.44 | 1.59 | | B | B |
| 74 | 06E22DB8 | Channel Island Middle Arm, Oct 2011 | N/A | | | | | 0.91 | 1.35 | GF | | |
| 75 | 06E22E2B | Channel Island Middle Arm, May 2011 | N/A | | | | | | | | | B |
| 76 | 06E232E8 | Channel Island Middle Arm, May 2011 | N/A | | | | | | | | | B |
| 77 | 06E23982 | Channel Island Middle Arm, May 2011 | N/A | | | | | | | | | B |
| 78 | 06E24AAC | Channel Island Middle Arm, Oct 2011 | N/A | | | | | 0.76 | 2.10 | | | B |
| 79 | 06E24B9E | Channel Island Middle Arm, Aug 2010 | 11/11 | | | 0.61 | 1.28 | 0.54 | 1.44 | GF | B | |

* B blood clot; GF, gonadal fluid

## APPENDIX 5: RESULTS OF COHORT TESTING AT DARWIN AQUACULTURE CENTRE

| Batch | Date Collected | Broodstock microchip no. | Sample type/age | No. tests | Pool size per test | qPCR | qPCR CT values | Virus isolation |
|---|---|---|---|---|---|---|---|---|
| Cohort 01/08 | 31/01/2008 | N/A | Fish (21-day-old) | 6 | 5 head | nPCR Neg | NA | NA |
| Cohort 03/08 | 26/03/2008 | N/A | Fish (21-day-old) | 6 | 5 head | nPCR Neg | NA | NA |
| Cohort 04/08 | 08/05/2008 | N/A | Fish (21-day-old) | 6 | 5 head | nPCR Neg | NA | NA |
| Cohort 09/08 | 24/09/2008 | N/A | Fish (21-day-old) | 6 | 5 head | nPCR Neg | NA | NA |
| Cohort 11/08 | 01/12/2008 | N/A | Fish (23-day-old) | 6 | 5 head | nPCR Neg | NA | NA |
| Cohort 02/09 | 16/03/2009 | N/A | Fish (19-day-old) | 6 | 10 head | Neg | >45 | NA |
| Cohort 04/09 | 27/04/2009 | N/A | Fish (19 -day-old) | 3 | 10 heads | Neg | >45 | NA |
| | 17/05/2010 | N/A | Fish (13-month-old) | 2 | 1 brain | Pos (2/2) | 28.14, 23.32 | Neg |
| | *Disease investigation of grow-out fish from farm A (Atypical gill disease), no histological evidence of Nodavirus infection, immunohistochemistry on brain and eye blocks also negative.* | | | | | | | |
| | 05/07/2010 | N/A | Fish (16-month-old) | 3 | 1 brain | Pos (2/3) | 32.02, 32.23, >45 | Neg |
| | *Disease investigation of grow-out fish from Farm B (suspect management issues as no common pathology), no histological evidence of Nodavirus infection, immunohistochemistry on brain and eye blocks negative.* | | | | | | | |
| | 29/11/2010 | N/A | Fish (19-month-old) | 4 | 1 brian or 1 retina | Neg | >45 | NA |
| | *Disease investigation of grow-out fish from Farm C (suspect poor water quality), no histological evidence of Nodavirus infection.* | | | | | | | |
| | 03/12/2010 | N/A | Fish (19-month-old) | 6 | 1 brian or 1 retina | Neg | >45 | NA |
| | *Disease investigation of grow-out fish from Farm C (suspect poor water quality), no histological evidence of Nodavirus infection.* | | | | | | | |

| Batch | Date Collected | Broodstock microchip no. | Sample type/age | No. tests | Pool size per test | qPCR | qPCR CT values | Virus isolation |
|---|---|---|---|---|---|---|---|---|
| Cohort 09/09 | 12/09/2009 | N/A | Fertilized eggs (pre-ozone) | 8 | 10-20 eggs | Neg | >45 | NA |
| | 12/09/2009 | N/A | Fertilized eggs (post-ozne) | 8 | 10-20 eggs | Neg | >45 | NA |
| | 15/09/2009 | N/A | Tank 1 fish (2-day-old) | 8 | 5 bodies | Neg | >45 | NA |
| | 15/09/2009 | N/A | Tank 1 fish (2-day-old) | 2 | 20 bodies | Neg | >45 | NA |
| | 15/09/2009 | N/A | Tank 2 fish (2-day-old) | 8 | 5 bodies | Indeterminate | 33.26, 36.56, 38.76, 36.58, 38.56, 34.75, 35.84, >45 | Not done |
| | 15/09/2009 | N/A | Tank 2 fish (2-day-old) | 2 | 20 bodies | Neg | >45 | NA |
| | 18/09/2009 | N/A | Tank 1 Fish (5-day-old) | 8 | 3-5 bodies | Pos (1/8) | 36.87, 24.28, rest >45 | Not done |
| | 18/09/2009 | N/A | Tank 1 Fish (5-day-old) | 2 | 20 bodies | Neg | >45 | NA |
| | 18/09/2009 | N/A | Tank 2 Fish (5-day-old) | 8 | 5 bodies | Pos (1/8) | 38.93, 39.53, 31.16, 39.11, 40.69, rest >45 | Not done |
| | 18/09/2009 | N/A | Tank 2 Fish (5-day-old) | 2 | 20 bodies | Neg | >45 | NA |
| | 28/09/2009 | N/A | Tank 1 Fish (15-day-old) | 5 | 3 bodies | Neg | >45 | NA |
| | 28/09/2009 | N/A | Tank 1 Fish (15-day-old) | 2 | 20 bodies | Neg | >45 | NA |
| | 28/09/2009 | N/A | Tank 2 Fish (15-day-old) | 5 | 3 bodies | Indeterminate | 39.14, 37.39, rest >45 | Not done |
| | 28/09/2009 | N/A | Tank 2 Fish (15-day-old) | 2 | 20 bodies | Pos (1/2) | 36.19, 28.47 | Not done |
| | 01/10/2009 | N/A | Tank 1 Fish (18-day-old) subclinical | 2 | 5 heads | Pos (2/2) | 29.44, 28.65 | Not done |
| | 01/10/2009 | N/A | Tank 1 Fish (18-day-old) subclinical | 2 | 10 heads | Pos (2/2) | 28.16, 27.91 | Not done |
| | 01/10/2009 | N/A | Tank 2 Fish (18-day-old) subclinical | 2 | 5 heads | Pos (2/2) | 7.55, 7.43 | Not done |
| | 01/10/2009 | N/A | Tank 2 Fish (18-day-old) subclinical | 2 | 10 heads | Pos (2/2) | 8.52, 8.46 | Not done |
| | 01/10/2009 | N/A | Tank 2 Fish (18-day-old) sick | 2 | 5 heads | Pos (2/2) | 7.34, 7.25 | NA |
| | 01/10/2009 | N/A | Tank 2 Fish (18-day-old) sick | 2 | 10 heads | Pos (2/2) | 8.04, 7.40 | NA |
| | 02/10/2009 | N/A | Tank 2 Fish (19-day-old) sick | 2 | 2 heads | Pos (2/2) | 9.31, 9.15 | Not done |
| | 04/10/2009 | N/A | Tank 1 Fish (21-day-old) sick | 3 | 2-3 heads | Pos (3/3) | 10.08, 9.34, 13.07 | Not done |
| | 04/10/2009 | N/A | Tank 2 Fish (21-day-old) sick | 3 | 2 heads | Pos (3/3) | 9.07, 9.18, 11.45 | Not done |
| | 05/10/2009 | N/A | Tank 1 Fish (22-day-old) sick | 2 | 5 heads | Pos (2/2) | 7.45, 8.06 | NA |

| Batch | Date Collected | Broodstock microchip no. | Sample type/age | No. tests | Pool size per test | qPCR | qPCR CT values | Virus isolation |
|---|---|---|---|---|---|---|---|---|
| | 05/10/2009 | N/A | Tank 1 Fish (22-day-old) sick | 2 | 10 heads | Pos (2/2) | 7.90, 7.53 | NA |
| | 05/10/2009 | N/A | Tank 2 Fish (22-day-old) sick | 2 | 5 heads | Pos (2/2) | 6.60, 6.72 | NA |
| | 05/10/2009 | N/A | Tank 2 Fish (22-day-old) sick | 2 | 10 heads | Pos (2/2) | 6.81, 6.93 | NA |
| Cohort 10/09 | 26/10/2009 | N/A | Tank 1 Fish (10-day-old) | 8 | 20-25 bodies | Neg | >45 | Not done |
| | 26/10/2009 | N/A | Tank 2 Fish (10-day-old) | 8 | 20-25 bodies | Neg | >45 | Not done |
| | 26/10/2009 | N/A | Tank 1 fish (10-day-old) | 7 | 25 bodies | Neg | >45 | Not done |
| | 28/10/2009 | N/A | Tank 1 Fish (12-day-old) | 7 | 23-25 bodies | Neg | >45 | Not done |
| | 28/10/2009 | N/A | Tank 2 Fish (12-day-old) | 8 | 24 bodies | Neg | >45 | Not done |
| | 06/11/2009 | N/A | Tank 1 Fish (21-day-old) | 5 | 10-15 bodies | Neg | >45 | Not done |
| | 06/11/2009 | N/A | Tank 2 Fish (21-day-old) | 6 | 12-15 bodies | Neg | >45 | Not done |
| | 19/11/2009 | N/A | Fish (34-day-old) (clinical) | 7 | 10 heads | Pos (7/7) | 13.55, 13.13, 14.37, 13.56, 12.05, 13.63, 13.42 | Not done |
| | 23/11/2009 | N/A | Fish (38-days-old) (clinical) | 6 | NA | Pos | NA | Pos |
| | 06/12/2009 | N/A | Fish (51-day-old) (clinical) | NA | NA | NA | NA | NA |
| | 07/12/2009 | N/A | Fish (54-day-old) (subclinical) | 69 | 5 heads | Pos | 28.44, 25.63, 25.38, 31.10, 26.45, 26.79, 25.50, the rest >45 | Pos |
| | 16/12/2009 | N/A | Fish (61-day-old) (healthy) | NA | NA | NA | NA | NA |
| | 17/12/2009 | N/A | Fish (62-day-old) (healthy) | NA | NA | NA | NA | NA |
| | 24/12/2009 | N/A | Fish (69-days-old) (healthy) | 4 | 1 head | NA | 23.66, 20.58, 22.39, 22.81 | Not done |
| | 18/01/2110 | N/A | Fish (94-days-old) (healthy) | 16 | 1 brain & eye pool | Pos (4/4), | 23.13, 27.85, 26.49, 25.65 | NA |
| | 13/07/2010 | N/A | Fish (9-month-old) (healthy) | 2 | 1 brain or 1 retina | Pos (2/2) | Brain- 28.35; Retina- 31.32 | NA |
| | 03/09/2010 | N/A | Fish (10-month-old) | 22 | 1 brain or 1 retina | Pos (22/22) | Brain- 31.18, 28.24, 15.81, 27.59, 29.69, 26.48, 27.50, 30.43, 28.39 ,25.73, 33.99; Retina- 33.28, 35.74, 26.28, 29.25, 29.40, 28.37, 30.58, 38.94, 31.90, 30.48, 32.82 | NA |
| | *Disease investigation of grow-out fish from Farm B pond F1; dark fish but no mortality; survivor of clincial NNV outbreak DAC cohort 10/09* | | | | | | | |
| | 08/10/2010 | N/A | Fish (11-month-old) | 14 | 1 brain or 1 retina | Pos (13/14) | Brain- 32.76, 33.12, 28.23, 31.21, 31.06, 31.94, 30.58; Retina- 35.53, 37.96, 36.68, >45, 34.83, 33.94, 34.56 | Pos |
| | *Grow-out fish from Farm B Pond F2; clinically normal fish; survivor of clincial NNV outbreak* | | | | | | | |

| Batch | Date Collected | Broodstock microchip no. | Sample type/age | No. tests | Pool size per test | qPCR | qPCR CT values | Virus isolation |
|---|---|---|---|---|---|---|---|---|
| | *DAC cohort 10/09* | | | | | | | |
| | 21/10/2010 | N/A | Fish (12-month-old) | 2 | 1 brain or 1 retina | Pos (1/2) | Brain- 25.38; Retina- >45 | NA |
| | *Grow-out fish from Farm A; clinically normal fish, acute vertebral trauma during handling* | | | | | | | |
| | 09/12/2010 | N/A | Fish (14-month-old) | 2 | 1 brain or 1 retina | Pos (2/2) | Brain- 36.1 ; Retina- 34.54 | NA |
| | *Disease investigation of grow-out fish from Farm A Pond 4; some mortality due to gastroenteritis* | | | | | | | |
| | 10/12/2010 | N/A | Fish (14-month-old) | 2 | 1 brain or 1 retina | Pos (2/2) | Brain- 33.48; Retina- 36.35 | NA |
| | *Disease investigation of grow-out fish from Farm A Pond 4; some mortality due to gastroenteritis* | | | | | | | |
| | 12/09/2011 | N/A | Fish (23-month-old) | 2 | 1 brain or 1 retina | Pos (2/2) | Brain- 21.55; Retina- 29.31 | Pos |
| | *Disease investigation of grow-out fish from Farm A Pond 1; Runty fish, sick from possible Heterosignma algal toxicity and possible other stressors* | | | | | | | |
| | 01/12/2011 | N/A | Fish (25-month-old) | 6 | 1 brain or 1 retina | Indeterminate | Brain- >45, 36.96, >45; Retina->45, 42.58, 39.22 | NA |
| | *Filleted fish carcasses with deformed spines from Farm C, no clincal signs, farmer noticed deformity during harvesting.* | | | | | | | |
| Cohort 02/10 | 10/02/2010 | N/A | Fertilized eggs (pre-ozone) | 1 | 0.1 g | Neg | >45 | NA |
| | 10/02/2010 | N/A | Fertilized eggs (post-ozone) | 1 | 0.1 g | Neg | >45 | NA |
| | 10/02/2010 | N/A | Fish (1-day-old) | 1 | 0.1 g | Neg | >45 | NA |
| | 12/02/2010 | N/A | Fish (5-day-old) | 6 | 0.1 g | Indeterminate | 36.13, the rest >45 | NA |
| | 17/02/2010 | N/A | Fish (10-day-old) | 10 | 0.1 g (25 heads) | Neg | >45 | NA |
| | 22/02/2010 | N/A | Fish (15-day-old) | 11 | 0.1 g (10 heads) | Neg | >45 | NA |
| | 26/02/2010 | N/A | Fish (19-day-old) | 6 | 0.1 g (10 heads) | Neg | >45 | NA |
| | 04/03/2010 | N/A | Fish (25-day-old) | 24 | 0.1 g (10 heads) | Neg | >45 | NA |
| | 10/03/2010 | N/A | Fish (31-day-old) | 8 | 0.1 g (10 heads) | Neg | >45 | NA |
| | *Disease investigation* | | | | | | | |
| Cohort 03/10 | batch terrinated for commercial reasons | | | | | | | |
| Cohort 05/10 | 01/06/2010 | N/A | Fish (25-day-old) | 12 | 5 heads | Neg | >45 | NA |

| Batch | Date Collected | Broodstock microchip no. | Sample type/age | No. tests | Pool size per test | qPCR | qPCR CT values | Virus isolation |
|---|---|---|---|---|---|---|---|---|
| | 10/08/2010 | N/A | Fish (3-month-old) | 4 | 0.1 g (retina & brain) | Neg | >45 | NA |
| | *Sick fish from local school* | | | | | | | |
| Cohort 08/10 | 24/08/2010 | 01C90BC8 | egg mass | 1 | NA | Neg | >45 | NA |
| | 25/08/2010 | N/A | Fertilized eggs (pre-ozone) | 4 | 0.1 g | Neg | >45 | NA |
| | 25/08/2010 | N/A | Fertilized eggs (post-ozone) | 3 | 0.1 g | Neg | >45 | NA |
| | 25/08/2010 | N/A | Fish (1-day-old) | 3 | 0.1 g | Neg | >45 | NA |
| | 16/09/2010 | N/A | Fish (21-day-old) | 6 | 0.1 g (10 heads) | Neg | >45 | NA |
| Cohort 01/11 | 14/02/2011 | N/A | Fish (20-day-old) | 15 | 0.1g (heads) | Neg | >45 | NA |
| Cohort 04/11 | 26/04/2011 | N/A | Fish (5-day-old) | 1 | 0.1 g | Neg | >45 | NA |
| | 01/05/2011 | N/A | Fish (10-day-old) | 2 | 0.1 g | Neg | >45 | NA |
| | 12/05/2011 | N/A | Fish (21-day-old) | 6 | 0.1 g | Neg | >45 | NA |
| | 18/05/2011 | N/A | Fish (27-day-old) | 3 | 0.1 g | Neg | >45 | NA |
| | 20/06/2011 | N/A | Fish (61-day-old) | 4 | 0.1 g (heads) | Pos (4/4) | 8.05, 7.17, 7.17, 10.66 | Not done |
| | *Clincial VNN outbreak in Farm C nursery, stressors included translocation, grading, low temperature & lack of feeding.* | | | | | | | |
| | 23/06/2011 | N/A | Fish (64-day-old) | 5 | 0.1 g (heads) | Pos (5/5) | 15.42, 10.95, 11.65, 12.56, 12.64 | Pos |
| | *Subclinical VNN in DAC; Fish were stressed by low DO several days ago with 40/200 dead, but now clinically normal, samples submitted for testing because of outbreak in Farm C* | | | | | | | |
| | 28/06/2011 | N/A | Fish (69-day-old) | 5 | 0.1 g (heads) | Neg | >45 | NA |
| | *Fish were translocated to a local High School at 37-day-old and have been rearing in fresh bore water, clinically normal fish, submitted for testing because outbreak in Farm C* | | | | | | | |
| Cohort 09/11 | 21/09/11 | N/A | Pre- ozone eggs | 1 | 0.1 g | Neg | >45 | NA |
| | 21/09/11 | N/A | Post-ozone eggs | 1 | 0.1 g | Neg | >45 | NA |
| | 21/09/11 | N/A | Fish (1-day-old) | 1 | 0.1 g | Neg | >45 | NA |

| Batch | Date Collected | Broodstock microchip no. | Sample type/age | No. tests | Pool size per test | qPCR | qPCR CT values | Virus isolation |
|---|---|---|---|---|---|---|---|---|
| | 21/09/11 | N/A | Fish (19-day-old) | 6 | 0.1 g (heads) | Neg | >45 | NA |
| | 03/10/11 | N/A | Fish (30-day-old) | 4 | 0.1 g (heads) | Neg | >45 | NA |
| | *Disease investigation of increased fish mortality during grading in DAC hatchery; peritonitis was suspected* | | | | | | | |
| Cohort 11/11 | 25/11/2011 | N/A | Fish (21-day-old) | 6 | 0.1 g (heads) | Neg | >45 | NA |
| Cohort 03/12 | 13/04/2012 | N/A | Fish (20-day-old) | 6 | 0.1 g (heads) | Neg | >45 | NA |

NA not available N/A not applicable