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Evaluation of the Draft Australian and New Zealand Standard Diagnostic Procedure (ANZSDP) for PCR-detection of Betanodaviruses

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ABSTRACT—Several Australian betanodaviruses (NNVs) and other, exotic NNVs were used in a study of the performance of a nested PCR test in common use in Australia and New Zealand. This test for the detection of NNVs, designated the ANZSDP VNN Nested PCR, was compared with a currently available commercial PCR kit for NNV detection. With respect to the endemic viruses, the ANZSDP was pan-specific for the NNVs tested, while the commercial PCR kit failed to detect the South Australian strain of barramundi *Lates calcarifer* NNV. With respect to the relative sensitivities of the two tests, in general, the ANZSDP appeared more sensitive than the commercial test. The exception was the Australian bass *Macquaria novemaculeata* NNV isolate, which was detected at lower levels by the commercial test. Among the exotic NNVs, both tests detected red-spotted grouper nervous necrosis virus and barfin flounder nervous necrosis virus, but neither test detected striped jack nervous necrosis virus (SJNNV). Further investigation revealed mismatches between SJNNV sequences and the nested primers used in the ANZSDP.

Key words: betanodavirus, viral nervous necrosis, viral encephalopathy and retinopathy, PCR, VNN

Viral encephalopathy and retinopathy or viral nervous necrosis was first recognized in Australia in the late 1980s (Glazebrook *et al*., 1990); disease outbreaks occurred in hatchery-reared barramundi *Lates calcarifer* larvae in which it was demonstrated that virus (now known to be betanodavirus) was associated with typical histological lesions present in the brain and retina. Since then, within Australia, nodavirus infections have been reported from the Northern Territory, Queensland, South Australia, Tasmania and Western Australia in a number of both freshwater and marine finfish species (Munday *et al*., 2002). While detection and identification of the causative virus in diseased fish are relatively straightforward (OIE, 2006), of major concern is the lack of a fully validated and rapid diagnostic test for the detection of asymptomatic carriers, for example, broodstock fish. In an attempt to enhance Australia's diagnostic capability for detection and identification of betanodaviruses, a draft Australia and New Zealand Standard Diagnostic Procedure (ANZSDP) was developed (Moody *et al*., 2004), that includes both immunodiagnostic and molecular diagnostic (reverse-transcriptase polymerase chain reaction, RT-PCR) tests.

Here, we describe a study that compares the performance of the draft ANZSDP VNN Nested PCR (also known as the ANZSDP VNN PCR) for the detection of betanodaviruses with a currently available commercial PCR kit for betanodavirus detection. Preliminary information on a nodavirus that was detected, with the ANZSDP VNN Nested PCR, in Australian bass *Macquaria novemaculeata* is also presented.

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Materials and Methods

Virus isolates

Samples of fish nodaviruses used for the comparative testing are described in Table 1. The samples of known endemic isolates were either original tissue homogenates from infected juvenile fish (confirmed positive by previous testing using histology, immunohistochemistry, nested PCR and sequencing of amplicons) or cell culture supernatants (confirmed as nodavirus-positive by nested PCR and sequencing of amplicons). These endemic isolates had been identified as members of the red-spotted grouper nervous necrosis virus (RGNNV) genotype after phylogenetic analysis of the partial T2 or T4 coat protein gene sequences (Moody *et al*., unpublished data). In addition, a previously unknown nodavirus has been isolated from Australian bass using the E-11 cell line (Iwamoto *et al*., 2000) and has been designated, tentatively, Australian bass nervous necrosis virus (ABNNV). The exotic betanodaviruses, striped jack nervous necrosis virus (SJNNV, strain SJNag93), barfin flounder nervous necrosis virus (BFNNV, strain JFIwa98) and RGNNV (strain SGWak97), were used for comparison with the endemic betanodaviruses (Table 1). Other endemic and exotic fish-pathogenic viruses used as negative controls to assess specificity of the two tests are shown in Table 1. Testing of endemic isolates was carried out at the Tropical and Aquatic Animal Health Laboratory (TAAHL), Townsville, Queensland and testing of exotic isolates was undertaken at the Australian Animal Health Laboratory Fish Diseases Laboratory (AFDL), CSIRO Livestock Industries, Geelong, Victoria.

Sample preparation

At the time of collection of samples derived from finfish in Australia, tissue samples were homogenized in viral transport medium (VTM; Medium 199, supplemented with 500 IU/mL penicillin G, 500 μg/mL streptomycin sulphate and 2 μg/mL amphotericin B) and stored in 0.9 mL aliquots at –80°C. For samples derived from cell cultures, culture supernatants were harvested from infected cell cultures and stored in 0.9 mL aliquots at –80°C. For comparative testing, samples were thawed, clarified by centrifugation at 10,000 $\times$ *g* for 10 min at 5°C, and 10-fold dilution series (neat to 10^{-9}) were prepared in VTM from the supernatants. Replicate aliquots of each set of serial dilutions were stored at –80°C until required.

Sample testing

The draft ANZSDP VNN Nested PCR (Moody *et al*., 2004) is based on a primary RT-PCR using the R3 (5′-CGAGTCAACACGGGTGAAGA-3′) and F2 (5′-CGTGTCAGTCATGTGTCGCT-3′) primers, that target the SJNNV coat protein gene, and RT-PCR cycling conditions described by Nishizawa *et al*. (1994) to produce a 426 bp amplicon. This is followed by the use of the nested primers NR3 (5′-GGATTTGACGGGGCTGCTCA-3′) and NF2 (5′-GTTCCCTGTACAACGATTCC-3′), which were designed based on coat protein gene sequences from sea bass (*Dicentrarchus labrax*) nodavirus, and the nested PCR cycling conditions described by Thiéry *et al*. (1999) to produce a 294 bp amplicon. The procedure uses the High Pure Viral RNA Extraction Kit (Roche) for the RNA extraction and StrataScript RT (Stratagene) for reverse transcription and Hot StarTaq Master Mix (QIAGEN) for PCR amplification.

Samples were tested using the ANZSDP VNN Nested PCR according to Moody *et al*. (2004), and by the IQ2000™ VNN Kit (Farming IntelliGene Technology

Table 1. Betanodaviruses and other viruses used in this study

Viruses	Source
Betanodaviruses	
Australian bass nervous necrosis virus (ABNNV)	New South Wales (NSW)
Barramundi nervous necrosis virus (BNNV-QLD)	Queensland (QLD)
Barramundi nervous necrosis virus (BNNV-SA)	South Australia (SA)
Sleepy cod nervous necrosis virus (SCNNV)	QLD
Barramundi cod nervous necrosis virus (BCNNV)	QLD
Estuary cod nervous necrosis virus (ECNNV)	QLD
Striped jack nervous necrosis virus (SJNNV, strain SJNag93)	Japan, provided by T. Nakai
Barfin flounder nervous necrosis virus (BFNNV, strain JFIwa98)	Japan, provided by T. Nakai
Red-spotted grouper nervous necrosis virus (RGNNV, strain SGWak97)	Japan, provided by T. Nakai
Other fish viruses	
Tasmanian aquabirnavirus	Tasmania
Tasmanian Atlantic salmon reovirus	Tasmania
Bohle iridovirus (BIV-QLD)	QLD
Swordtail aquabirnavirus (StAB-QLD)	QLD
Viral haemorrhagic septicaemia virus (isolate 23.75)	France, provided by P. de Kinkelin
Infectious haematopoietic necrosis virus (WRAC strain)	USA, provided by J. Winton
Infectious salmon anaemia virus (reference strain Glesvaer/2/90)	Norway, provided by B. Dannevig

Corporation, Taiwan) according to the manufacturer's instructions, with one exception. The sample preparation protocol of the IQ2000™ Kit required the head, brain or eye to be homogenized in the RNA Extraction Solution. Since the samples had already been homogenised, a volume of 200 μL of the stored supernatant was used for RNA extraction.

In addition, a modified version of the ANZSDP VNN Nested PCR, in which the annealing temperature was shifted from 60°C to 50°C and a new reverse primer ABNR3 (5′-AGTAAGCCACGCCATCTG-3′) was used, was evaluated to see if either test could differentiate barramundi nervous necrosis virus (BNNV) from ABNNV.

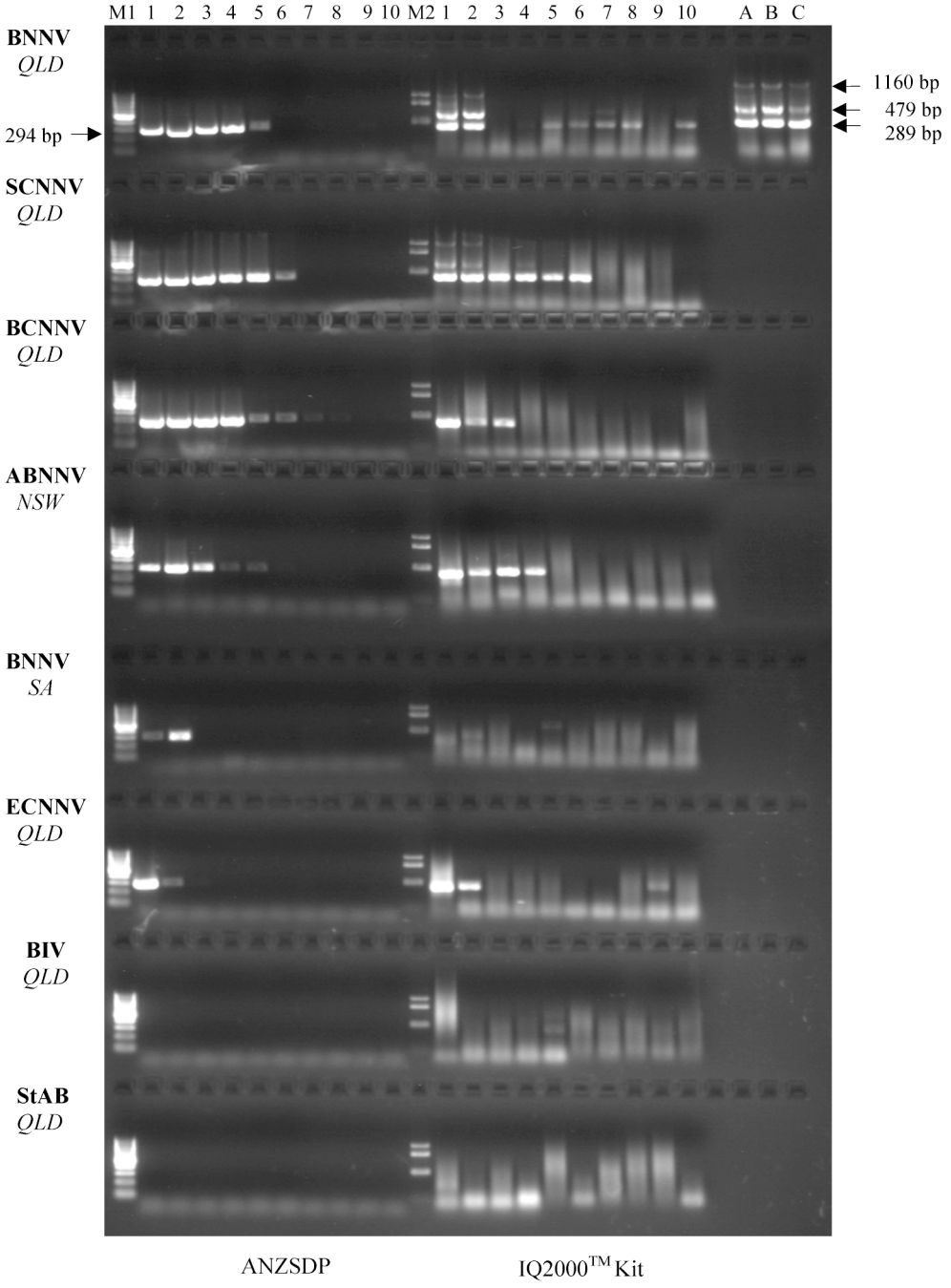

Fig. 1. Detection of endemic betanodaviruses and other viruses with the ANZSDP VNN Nested PCR and IQ2000™ VNN Kit. Betanodavirus samples are BNNV-QLD, SCNNV-QLD, BCNNV-QLD, ABNNV-NSW, and BNNV-SA, and other viruses are BIV-QLD and StAB-QLD (see Table 1). Lanes 1–10 are serial 10-fold dilutions from undiluted (Lane 1) to 10^{-9} (Lane 10). A, B and C correspond to 10^{-1}, 10^{-2} and 10^{-3} dilution of the positive control material for the IQ2000™ VNN Kit. Positive bands are 294 bp for the ANZSDP test and 289 (main band), 479, or 1160 bp for the IQ2000 test. M1: 100 bp MW ladder marker; M2: IQ2000™ Kit marker

Cell culture

The cloned cell line, E-11, derived from the SSN-1 cell line (Iwamoto *et al*., 2000) was provided by Professor T. Nakai (Hiroshima University, Japan) and used to generate and titrate the nodavirus stocks used in this study. Stock cultures of E-11 cells were maintained at 25°C using L-15 Leibovitz culture medium supplemented with 2 mM glutamine, 100 IU penicillin/mL, 100 μg/mL streptomycin and 10% (v/v) fetal bovine serum (FBS). For viral replication and titration, the FBS concentration was reduced to 2% (v/v) and the incubation temperatures were 20°C for SJNNV and BFNNV and 25°C for RGNNV, BNNV and ABNNV. To determine viral titres, a 10-fold dilution series of each virus was established in 96-well plate cultures of E-11 cells which were then incubated at the appropriate temperatures for 14 days. The $TCID_{50}$/mL was determined for each virus using the method of Reed and Muench (1938).

To ensure that the BNNV and ABNNV stocks were pure, representative isolates of these viruses were clone-purified three times using the limiting dilution method in E-11 cell cultures. Sequence analyses were undertaken using cloned viral isolates.

Results

Betanodaviruses tested at TAAHL

Results from testing samples using both the PCR protocols on the duplicated serial 10-fold dilutions of the endemic viruses are presented in Fig. 1. With respect to specificity, the ANZSDP test detected all of the nodaviruses tested, while the IQ2000 test detected all the nodaviruses, except for the South Australian barramundi isolate (BNNV-SA). Neither test recognized any of the non-nodavirus isolates used. With respect to the relative sensitivity of the two tests, in general, the ANZSDP test appeared more sensitive than the IQ2000 test. The exception was ABNNV (Australian bass isolate) which was detected by the IQ2000 test in one further dilution than the ANZSDP test. The IQ2000™ Kit was designed to produce up to three amplicons, 289, 479 and 1160 bp in size, depending on the intensity of infection, but this feature did not appear to be consistent in the results obtained under the conditions used in this study.

Betanodaviruses tested at AFDL

1) Specificity of PCR

Three exotic nodavirus isolates and two endemic

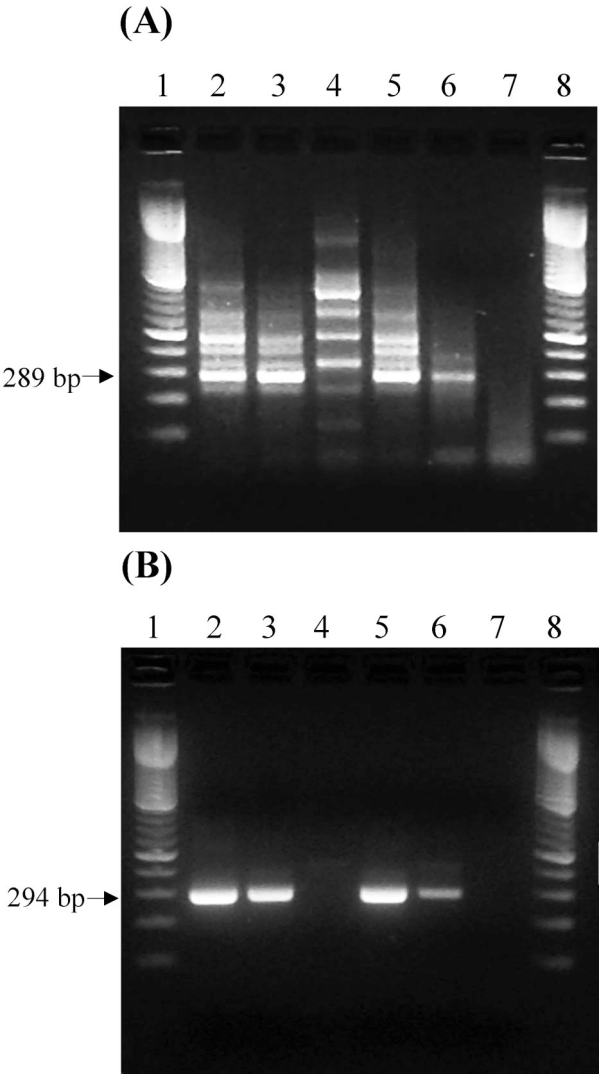

Fig. 2. Detection of endemic and exotic betanodaviruses with (A) the IQ2000™ VNN Kit and (B) the ANZSDP VNN Nested PCR. Lanes 1 and 8: 100 bp MW ladder marker; lane 2: BNNV; lane 3: ABNNV; lane 4: SJNNV; lane 5: RGNNV; lane 6: BFNNV; lane 7: water (negative control). Positive results in (A) and (B) are indicated by the presence of 289 and 294 bp bands, respectively.

Forward primer (NF2)	5′	-	G	T	T	C	C	C	T	G	T	A	C	A	A	C	G	A	T	T	C	C	-	3′
SJNNV (D30814)	5′	-	C	G	C	C	A	C	T	C	C	A	C	A	A	C	G	A	T	T	C	C	-	3′
SJNNV (AB056572)	5′	-	C	G	C	C	A	C	T	C	C	A	C	A	A	C	G	A	T	T	C	C	-	3′
SJNNV (AFDL)	5′	-	C	G	C	C	A	C	T	C	C	A	C	A	A	C	G	A	T	T	C	C	-	3′

Reverse primer (NR3)	5′	-	G	G	A	T	T	T	G	A	C	G	G	G	G	C	T	G	C	T	C	A	-	3′
SJNNV (D30814)	5′	-	A	G	A	T	T	T	G	C	C	G	T	G	G	T	T	G	C	T	G	G	-	3′
SJNNV (AB056572)	5′	-	A	G	A	T	T	T	G	C	C	G	T	G	G	T	T	G	C	T	G	G	-	3′
SJNNV (AFDL)	5′	-	A	G	A	T	T	T	G	C	C	G	T	G	G	T	T	G	C	T	G	G	-	3′

Fig. 3. Sequence comparison between the nested primers (NF2 and NR3) for the ANZSDP VNN Nested PCR and the primer annealing site on the SJNNV isolates. D30814 and AB056572 were retrieved from GenBank, and SJNNV (AFDL) was the isolate used in the present study. Nucleotides that were identical in all the sequences are shaded.

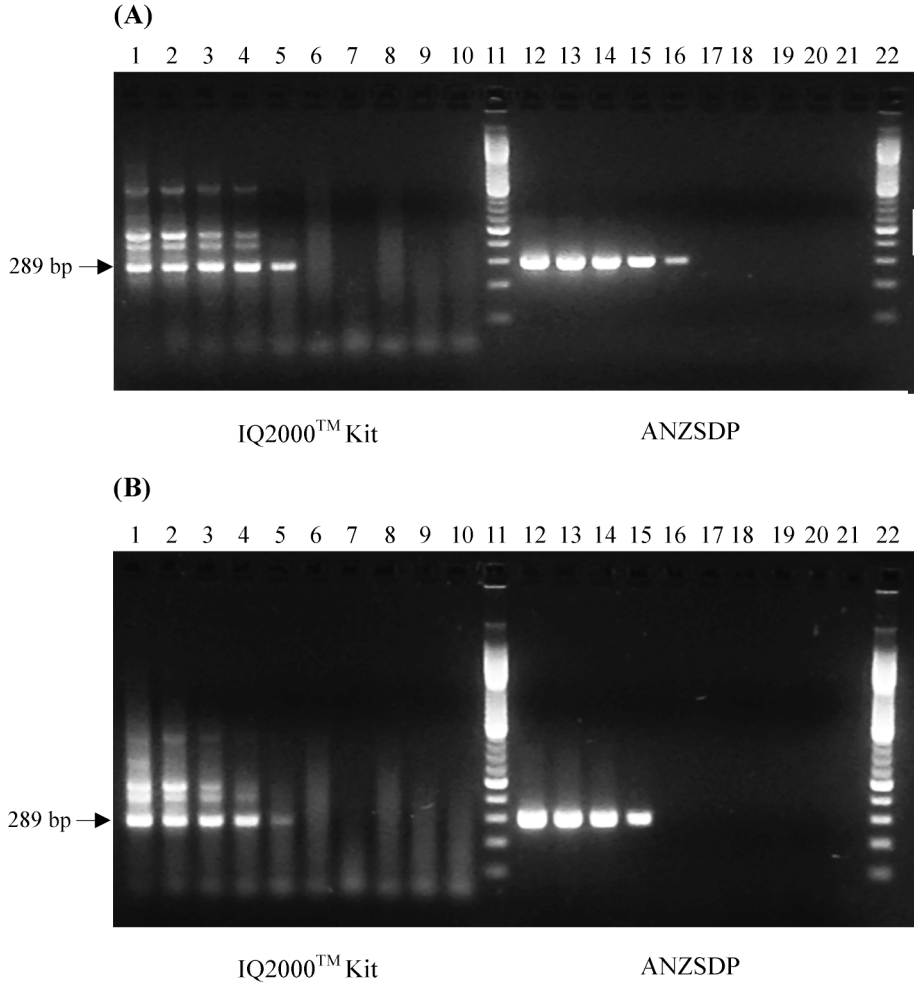

Fig. 4. Detection limit of BNNV (A) and RGNNV (B) by the IQ2000™ VNN Kit and the ANZSDP VNN Nested PCR. Lanes 1 and 12: undiluted tissue culture supernatant fluid; lanes 2 and 13: 10^{-1} dilution; lanes 3 and 14: 10^{-2}; lanes 4 and 15: 10^{-3}; lanes 5 and 16: 10^{-4}; lanes 6 and 17: 10^{-5}; lanes 7 and 18: 10^{-6}; lanes 8 and 19: 10^{-7}; lanes 9 and 20: 10^{-8}; lanes 10 and 21: water (negative control); lanes 11 and 22: 100 bp ladder MW marker. Positive results are indicated by the presence of 289 bp for the IQ2000™ VNN Kit and 294 bp for the ANZSDP VNN Nested PCR.

nodavirus isolates, grown in E-11 cells, were used as targets to compare the IQ2000 and ANZSDP PCR tests. Other non-nodavirus RNA viruses were used as specificity controls. Both the IQ2000 and the ANZSDP PCRs were negative for any of these non-nodavirus isolates (results not shown).

A comparison of the PCR results between the IQ2000 and the ANZSDP is shown in Fig. 2. The results demonstrate that: (1) Both the ANZSDP and the IQ2000 detected 4 of the 5 nodaviruses tested, BNNV, ABNNV, RGNNV and BFNNV; (2) Neither test detected SJNNV, but SJNNV was detected by the primary PCR of the ANZSDP test (results not shown); (3) The ANZSDP yielded much "cleaner" products (i.e., fewer non-specific products) than the IQ2000, making interpretation much easier and allowing a greater degree of confidence in the results.

Since SJNNV was not detected by either nested PCR, the 420 bp amplicon of SJNNV obtained by the primary PCR with the R3 and F2 primers (Nishizawa et al., 1994) was purified from an agarose gel, and sequenced using the same primers. The sequence of the PCR product was found to match closely (98–99%) two other SJNNV sequences in GenBank (D30814 and AB056572). However, there were mismatches between all of the SJNNV sequences and the nested primers used in the ANZSDP test (Fig. 3), in particular the mismatches at the 3′ end of the reverse primer, providing

Table 2. Effect of different annealing temperatures on specificities of ANZSDP and AFDL ABNNV PCR tests

Virus	ANZSDP		AFDL ABNNV*	
	50°C	60°C	50°C	60°C
BNNV	+	+	+	+
ABNNV	+	–	+	+
RGNNV	+	+	+	+

* New primer ABNR3 was used as the reverse primer instead of NR3 in the ANZSDP

Fig. 5. Pair-wise alignment of BNNV and ABNNV sequences. The NF2, NR3 and ABNR3 primers are enclosed in boxes. Sequences that were identical between BNNV and ABNNV are shaded.

an explanation for the negative results in the nested PCR. Due to lack of information about primer sequences, the reason for the failure of the IQ2000 Kit could not be investigated.

2) Sensitivity of PCR

The virus titres of the cell culture supernatants were $2 \times 10^{6.0}$ $TCID_{50}$/mL for BNNV and $2 \times 10^{6.5}$ $TCID_{50}$/mL for RGNNV. The RNAs extracted from each 10-fold dilution were processed by either the IQ2000 or the ANZSDP method. The PCR results for BNNV and RGNNV are shown in Fig. 4. These results demonstrated that: (i) For BNNV, both the IQ2000 and the ANZSDP PCRs performed identically. Each detected BNNV nucleic acid extracted from a 10^{-4} dilution of infected tissue culture supernatant fluid, suggesting that both PCRs could detect 10^2 $TCID_{50}$ of virus. (ii) For RGNNV, the IQ2000 was marginally more sensitive than the ANZSDP. The former detected viral nucleic acid extracted from a 10^{-4} dilution of infected tissue culture supernatant fluid, and the latter detected the same product in a 10^{-3} dilution, this suggesting that the PCRs could detect $10^{2.5}$ and $10^{3.5}$ $TCID_{50}$ of virus, respectively.

3) Differentiation of BNNV and ABNNV

With an annealing temperature of 50°C for the nested PCR, the ANZSDP detected all three tested nodaviruses (BNNV, ABNNV, RGNNV), but ABNNV was negative with an annealing temperature of 60°C for the nested step (Table 2). Sequence analysis of the specific amplicons for BNNV and ABNNV obtained in the primary PCR revealed that there were some differences between the two sequences (Fig. 5). The mismatches between the nested PCR primer sequences and the ABNNV sequence could cause the absence of an amplicon under the higher annealing temperature of 60°C. Thus, the new reverse primer (ABNR3) for the nested reaction was designed, and the nested PCR, designated AFDL ABNNV PCR, with the ABNR3 and NF2 primers allowed amplification of both BNNV and ABNNV targets at 60°C.

Discussion

The ANZSDP and the IQ2000™ Kit PCRs were evaluated at two separate laboratories (TAAHL and AFDL). At TAAHL, most testing was carried out on the tissue-derived nodaviruses that were endemic to Australia. At AFDL, testing was carried out on the tissue culture-derived supernatants of both exotic and endemic nodavirus isolates.

At TAAHL, both the protocols produced positive amplicons for all the tissue-derived endemic betanodaviruses tested, with the exception of the IQ2000™ Kit for the South Australian barramundi sample (BNNV-SA, Fig. 1) where no amplicon was produced. This was considered a false negative result based on repeat testing and sequence analysis (results not shown) of the amplicon yielded by the ANZSDP. The PCR amplicon of the undiluted BNNV-SA sample, when tested by the ANZSDP, was fainter than the band for the next dilution. There are a number of possible reasons for this result, including presence of excessive template in the reaction mixture which is subsequently diluted out,

inhibitors in the sample which are subsequently diluted out, or operator error. The IQ2000™ Kit demonstrated slightly increased sensitivity for the New South Wales Australian bass sample (ABNNV, Fig. 1). The ANZSDP was designed as a simple positive/negative test for the detection of betanodaviruses present in Australia and thus achieved this purpose. The IQ2000™ Kit was designed to produce up to three amplicons depending on the intensity of infection. This was apparent for one or two of the virus samples tested (BNNV-QLD and SCNNV, Fig. 1) but not for others (e.g. ABNNV, Fig. 1) depending on interpretation. The three dilutions (see lanes A, B and C, Fig. 1) of positive control material provided with the kit did not yield the expected results. Determining the intensity of infection from the number of bands produced using the kit could lead to erroneous results. The failure to produce the required number of bands may be due to the RNA extraction procedure, which is not as robust or operator-friendly as the RNA extraction described in the ANZSDP. This may also be the reason for the bands appearing irregularly at higher dilutions for BNNV-QLD (Fig. 1). As the pelleted RNA must be dried, the lids of the RNA extraction tubes need to be kept open, in close proximity to each other, which may have led to cross-contamination. Moreover, in the instances where non-specific bands may occur (Fig. 2) with or without any of the three specific bands, interpretation of the resulting banding pattern could be difficult.

Using cell culture as the "gold standard", the threshold of detection of both the PCR tests was slightly less (by at least two orders of magnitude) than that obtained with virus isolation in cell culture, contrary to the conclusions drawn by Dalla Valle *et al*. (2000), and suggesting that cell culture remains the method of choice for the detection of nodaviruses in fish with light infections. However, this should be confirmed using tissue-derived nodavirus preparations since the presence of fish tissues may influence the results of either the PCR test or virus isolation in cell culture. The viruses used at AFDL had all undergone passage in cell culture and it could be argued that they are cell culture-adapted and thus grow well in cell culture compared to viruses freshly isolated from fish tissues.

With respect to specificity, both the ANZSDP and the IQ2000 PCR tests were specific for nodaviruses, but neither test detected all the endemic and exotic nodaviruses tested at AFDL. The results indicate that some optimization is necessary for both the tests. Under the test conditions at AFDL, contrary to expectations, both the tests failed to detect SJNNV, probably due to mismatches in sequence between the primers and templates (Fig. 3), as reported by Dalla Valle *et al*. (2000). For the ANZSDP, these results indicate that nested primer sequences need to be modified to generate a truly generic test for betanodaviruses. This may be a relatively easy exercise for the ANZSDP, given the accessibility of sequence information for both the primers and the relevant viruses. It would not, however, be possible for users to modify the IQ2000 PCR because of the absence of information about this test in the public domain.

Currently, the primers for the ANZSDP VNN Nested PCR need to be modified to detect an exotic betanodavirus, SJNNV. However, the efficacy of the ANZSDP will need to be continually monitored as further exotic and endemic betanodaviruses are described. For betanodaviruses endemic to Australia, the present study demonstrates that the ANZSDP at two different stringencies (50°C and 60°C for primer annealing of the nested PCR) can be used to differentiate BNNV from ABNNV (Table 2). In summary, our results indicate that no matter what PCR test is selected for diagnosis, optimization for local conditions including sensitivity and specificity testing needs to be undertaken.

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References

Dalla Valle, L., L. Zanella, P. Patarnello, L. Paolucci, P. Belvedere and L. Colombo (2000): Development of a sensitive diagnostic assay for fish nervous necrosis virus based on RT-PCR plus nested PCR. *J. Fish Dis.*, **23**, 321–327.

Glazebrook, J. S., M. P. Heasman and S. W. de Beer (1990): Picorna-like viral particles associated with mass mortalities in larval barramundi, *Lates calcarifer* Bloch. *J. Fish Dis.*, **13**, 245–249.

Iwamoto,T., T. Nakai, K. Mori, M. Arimoto and I. Furusawa (2000): Cloning of the fish cell line SSN-1 for piscine nodaviruses. *Dis. Aquat. Org.*, **43**, 81–89.

Moody, N. J., P. F. Horwood and S. McHardy (2004): Aquatic Animal Health Subprogram: development of diagnostic tests for the detection of nodavirus – Final Report. Fisheries Research and Development Corporation Project 2001/626.

Munday, B. L., J. Kwang and N. Moody (2002): Betanodavirus infections of teleost fish: a review. *J. Fish Dis.*, **25**, 127–142.

Nishizawa, T., K.-I. Mori, T. Nakai, I. Furusawa and K. Muroga (1994): Polymerase chain reaction (PCR) amplification of RNA of striped jack nervous necrosis virus (SJNNV). *Dis. Aquat. Org.*, **18**, 103–107.

OIE (2006): Viral encephalopathy and retinopathy, In "OIE Manual of Diagnostic Tests for Aquatic Animals" (fifth edition, 2006). World Organization for Animal Health, Paris, pp.169–175.

Reed, L. J. and H. Muench (1938): A simple method of estimating fifty per cent endpoints. *Am. J. Hyg.*, **27**, 493–497.

Thiéry, R., J.-C. Raymond and J. Castric (1999): Natural outbreak of viral encephalopathy and retinopathy in juvenile sea bass, *Dicentrarchus labrax*: study by nested reverse transcriptase-polymerase chain reaction. *Virus Res.*, **63**, 11–17.