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Development of a Nested *rt*-PCR Assay for Diagnosis of Acute as well as Asymptomatic Betanodavirus Infection in Fishes

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Abstract

Betanodavirus is a bipartite single stranded RNA virus infecting fishes, causing the disease viral nervous necrosis. The virus infects the central nervous system and other neuronal tissues like retina of the eye. Mortality happens soon after the onset of the disease which often results in complete loss of crop within a few days. When the number of virions is low, the virus may cause asymptomatic infection. Correct diagnosis at this stage can prevent a potential disease outbreak and save the fish stock from a major loss. Unfortunately, such an early diagnostic system is lacking in the industry. In this scenario, a nested *rt*-PCR based diagnostic assay was developed for betanodavirus infection in fishes. The assay has been validated against acute as well as asymptomatic infection in Asian Seabass. This assay could detect both acute as well as asymptomatic infection.

Keywords: Betanodavirus; Nested *rt*-PCR; VNN; Acute infection; Asymptomatic infection

1. Introduction

Viral Nervous Necrosis (VNN) is a globally emerging disease reported in more than 100 fish species including farmed, wild as well as ornamental fish species Maltese and Bovo, 2007; Doan *et al*., 2016; OIE, 2018). Larval and juvenile finfishes are affected the most with characteristic clinical signs. The disease causes high mortality in fishes which often reaches up to 100%. Clinical signs include abnormal behavior such as cork-screw like swimming and non-spontaneous responses. Necrosis and vacuolation in the brain and retinal tissues are characteristic. The disease is caused by members of the genus *Betanodavirus*, family Nodaviridae. The virus is non-enveloped, icosahedral (25 to 30 nm in diameter) and contains two segments (RNA1 and RNA2) of positive sense single-stranded RNA (ssRNA). In India, the disease has been reported in cultured Asian Seabass (Azad *et al*., 2005; Parameswaran *et al*., 2008). Apart from acute infection, the virus causes asymptomatic infection too, which lacks any clinical manifestations. Asymptomatic infection naturally transmits infective virus in to healthy fishes (Castric *et al*., 2001; Barker *et al*., 2002; Chi *et al*., 2003; Johansen *et al*., 2003; Korsnes *et al*., 2012). An asymptomatic host may develop acute disease when it is stressed (Binesh, 2014).

Molecular diagnostics being highly reliable and reproducible are getting much importance currently. Its advantages over other methods are specificity and high sensitivity. Different molecular technologies were developed for diagnosis of betanodavirus which include reverse transcription PCR (rt-PCR) (Grotmol *et al*., 2000), quantitative rt-PCR (q-rt-PCR) (Grove et al., 2006; Hick and Whittington, 2010; Hodneland et al., 2011), rt-NASBA (Starkey et al., 2004), reverse transcription loop mediated isothermal amplification (rt-LAMP) (Hwang *et al*., 2016; Suebsing, Oh, and Kim, 2012; Sung and Lu, 2009; Xu *et al*., 2010) and real time rt-LAMP (Mekata *et al*., 2014). Among these methods, rt-PCR is the most widely used technique which involves conversion of viral RNA into cDNA (reverse transcription) and subsequent amplification of a short fragment of it (PCR). Nested PCR format improves sensitivity and specificity of the test substantially, in which another round of PCR is performed with an internal pair of nested primers (Dalla Valle *et al*., 2000; Thiery *et al*., 1999). The first PCR based molecular diagnostic test was developed for betanodavirus based on T4 variable region of the coat protein gene of SNNV (Nishizawa *et al*., 1994). This method is recommended for confirmation of acute VNN by the world organization for animal health, OIE (OIE, 2018), though amplification failure of this protocol was reported rarely (Nishizawa *et al*., 1996). Many other protocols have been developed since but, most of them were designed to confirm acute infection with VNN. They were not validated to diagnose asymptomatic infection.

In India, asymptomatic infection with fish nodavirus has been detected with an overall prevalence of 19.7% in farmed and wild fishes (unpublished data). A tool to detect such infections will be of great advantage to aid in general surveillance, hatchery operations and broodstock development programmes. Early detection of the virus could substantially reduce the risk of casualties and consequential economic losses in aquaculture facilities. In this context, new betanodavirus specific nested rt-PCR primers were designed and their performance was evaluated in PCRs with RNAs extracted from VNN positive clinical samples and samples of asymptomatic infection and a vector construct of betanodavirus RNA2. The best performing primer pair was then compared to a commercially available assay for VNN.

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2. Materials and Methods

2.1 Primers Used

Primers for nested rt-PCR were designed through GenScript online PCR primers design tool (www.genscript.com/tools/pcr-primers-designer/ standard). Nucleotide sequence of the coat protein gene of *Lates calcarifer* nervous necrosis virus (BVN4, GenBank accession no: GU953669) previously isolated in India from an asymptomatic, farmed Asian Seabass (Binesh, 2013) was used as the template. Optimum values for primer size, Tm and product size input in the primer design software were 20bp, 58.0 ° C and of 500bp respectively.

Total 5 pairs of primers were designed such that four pairs were positioned within an outermost pair. Reverse primer (BKR1) of the outer most pair was used for reverse transcription while the pair itself (BKF1/BKR1) was used for primary PCR producing an amplicon of 917 bp. Four internal primer pairs BVNF2/BVNR1, BKF3/BKR3, BKF4/BKR4 and BKF5/BKR5 when used in parallel nested PCRs produced amplicons of size 482, 269, 215 and 242 bp respectively. Designed primers were synthesized and procured from IDT (USA). Comparative positions and properties of the designed primers are illustrated in Fig. 1; Table 1.

2.2 Plasmid construct of RNA2 as positive control

PCR amplification and cloning of full length RNA2 of a fish nodavirus isolate was performed previously (Binesh, 2013). This was available as a vector construct (PBVN4) to be used in this study as a positive control.

2.3 Samples used

Total RNA was extracted from the samples using Nucleospin RNAII (MACHEREY NAGEL) following the manufacturer's instructions. Extracted RNA was reconstituted in nuclease free water and stored at -70 °C until used.

Reverse transcription of extracted total RNA was performed with reverse transcription primer. Briefly, 2 µL extracted total RNA was incubated in a 20 µL of reaction mixture containing 1X reaction buffer with 0.5 mM of each dNTPs, 10 mM DTT, 40 U of RNase Inhibitor,0.5 pmols of reverse primer and 200 U M-MLV reverse transcriptase. The reaction set up was incubated at 37 °C for 1 hour followed by inactivation of reverse transcriptase at 85 °C for 15 minutes. Primary PCR was performed in 20 µL total reaction volume containing 2.5 mM dNTPs, 1 pmol each of forward and reverse primers,

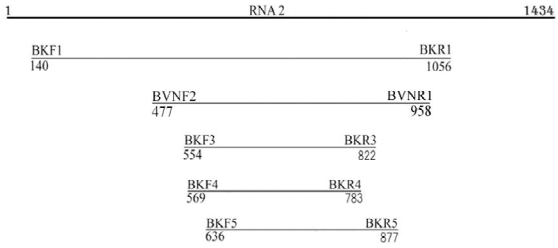


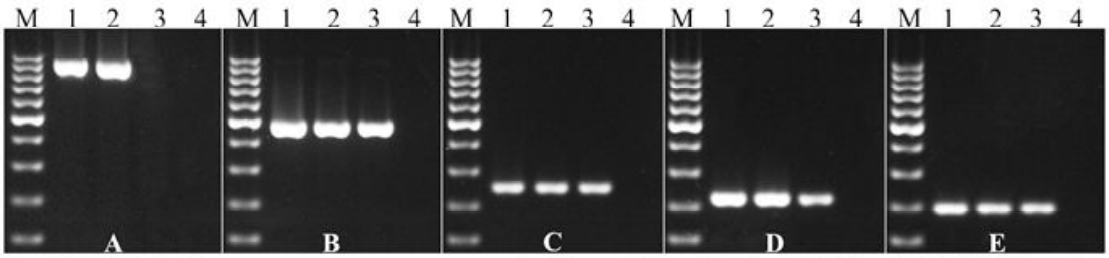
Fig. 1. Comparative position of the new primers on RNA2 of *Lates calcarifer* nervous necrosis virus (BVN4, GenBank accession no: GU953669)

0.5 U of DyNAzyme II DNA polymerase (Fermentas, USA) and 2 µL cDNA as the template. Each parallel nested reactions were performed in 20 µL total reaction volume which contained 2.5 mM dNTPs1 pmol each of forward and reverse nested primers, 0.5 U of DyNAzyme II DNA polymerase (Fermentas, USA) and 2 µL primary PCR product as the template. PCR program used was same for all reactions. Briefly, it included one cycle of initial denaturation at 94 °C for 3 minutes, 35 cycles of denaturation at 94 °C for 30 seconds, primer annealing at 58 °C for 40 seconds and primer extension at 72 °C for 1 minute and one cycle of final extension at 72 °C for 10 minutes. By the end, PCR products were resolved on 1.5% TBE agarose gel stained with 0.5 µg/ml ethidium bromide. Detection efficiency of the best performing primers was then compared with IQ2000 VNN Detection and Prevention System (GeneReach Biotechnology Corp., Taiwan) which is a commercially available assay for VNN. The kit was used as per the directions provided by the manufacturer except that the vector construct PBVN4 was used as positive control. The IQ2000 kit has been claimed to be semi quantitative and capable of differentiating 3 different levels of infection. By design, it is expected to produce three bands with severe infection, two bands with light infection and a single band if the sample is negative. To compare the newly developed assay with IQ2000 assay, four samples each of acute and asymptomatic infections were used. The vector construct PBVN4 was used as positive control and the negative control had no template DNA.

DNA quantity of the vector construct PBVN4 was estimated using a spectrophotometer. For sensitivity estimation assay, the vector construct was serially diluted so as to get a final concentration of the order of 10^{9} to 10^{-2} copies per reaction.

Table 1. Details of primers designed in this study for early diagnosis of VNN.

Name	Sequence (5'...3')	Position	Annealing temp. (°C)	Amplicon size (bp)
BKF1	TGTGTCTAAGGCCTCGACTG	140-159	58	917
BKR1	CCGGATGACCCGGTTAGTTT	1037-1056		
BVNF2	AAATGGTGGGAAAGCAGAAC	477-496	58	482
BVNR1	CATCTGCGAAACGTCTTGTT	939-958		
BKF3	TCTCACGTCACCTGGTCG	554-571	58	269
BKR3	CAGCTGGAAGACTGCTCCAT	803-822		
BKF4	TCGGCTGATACTCCTGTGTG	569-588	58	215
BKR4	TGTGGATCCTAGGAGGATGG	764-783		
BKF5	AGTGTTCGACTGAGCGTTCC	636-655	58	242
BKR5	CAGCTCGATCAACATCTCCA	858-877		



Lane M: 100 bp ladder
Lane 1 : Laboratory strain of *Betanodavirus*
Lane 2 : Sample of acute infection
Lane 3 : Sample of asymptomatic infection
Lane 4 : No template control

A: Primary PCR with primers BKF1 and BKR1 (917 bases)
B: Nested PCR with primers BVNF2 and BVNR1 (482 bases)
C: Nested PCR with primers BKF3 and BKR3 (269 bases)
D: Nested PCR with primes BKF5 and BKR5 (242 bases)
E: Nested PCR with primers BKF4 and BKR4 (215 bases)

Fig. 2. Betanodavirus specific PCR products generated with the new primers

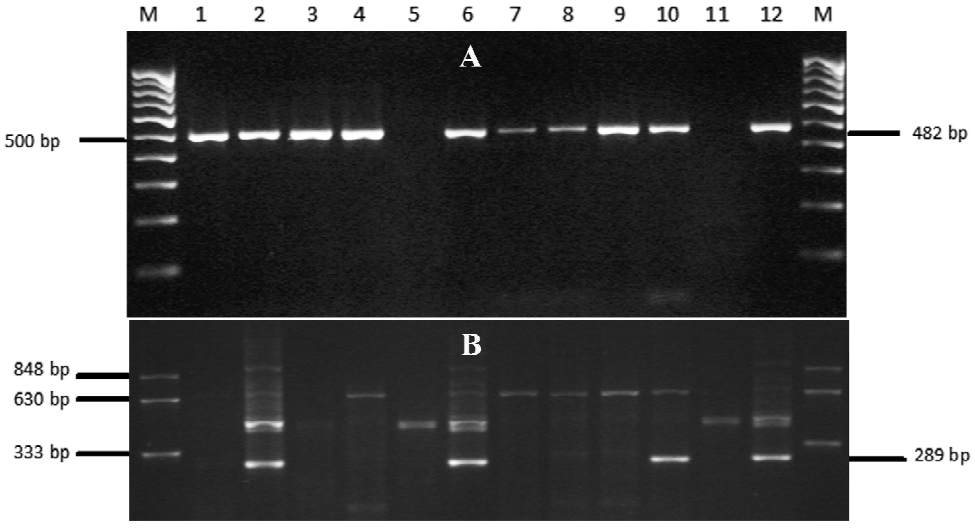
3. Results and Discussion

In the primary PCR, vector construct PBVN4 and the sample of acute infection gave positive band at 917 bp. No amplification was detected for the sample of asymptomatic infection and no template negative control (Fig 2A). All samples, except no template negative control were positive in all the parallel nested reactions (Fig. 2B-E). The primer pair BVNF2/BVNR1 performed best in the reaction with brightest band in agarose gel following electrophoresis.

In comparison, the primer pair BVNF2/BVNR1 could detect all the cases of acute as well as asymptomatic infections (Fig. 3A) while the commercially available assay detected only single case from each group (Fig. 3B).

Sensitivity of the primer pair BVNF2/BVNR1 was high; it could detect up to one copy of the plasmid per reaction (Fig. 4). Good amplification without non-specific bands were observed at $8x10^3$ to 1 copy per reaction; brightest band being at eight copies per reaction. At higher concentration of the plasmid, amplification became less efficient with multiple non-specific bands. No amplification was detected beyond dilution of one copy per reaction.

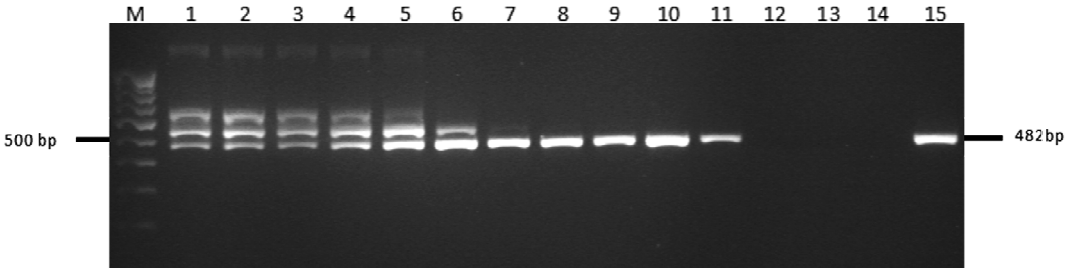
Together, the results indicate that the developed nested rt-PCR assay using BKR1, BKF1/BKR1 and BVNF2/BVNR1 as primers for reverse transcription, primary PCR and nested PCR respectively could be used effectively to detect betanodavirus infection in fishes. It detects both acute as well as asymptomatic infections with high sensitivity. The assay could help aquaculture entrepreneurs to diagnose betanodavirus infection at an early stage when the viral load is low and infection is still asymptomatic. The specificity of the assay is substantially improved by the use of gene specific primers unlike the degenerate primers in other assays (Gomez *et al*., 2004) used to detect asymptomatic infections with betanodavirus.



Gel A: Developed assay in this study
Gel B: Commercially available assay for VNN

Lane M : DNA molecular weight marker
Lanes 1-4 : Clinical cases of *Betanoidavirus* infection
Lanes 7-10: Asymptomatic cases of *Betanoidavirus* infection
Lanes 5&11: Negative controls
Lanes 6&12: Positive controls (P+)

Fig. 3. Comparison of the efficiency of the newly developed assay using the primer BVNF2/BVNR2 and a commercially available assay



Lane M : DNA molecular weight marker
Lane 1 : $8x10^9$ copies/reaction
Lane 2 : $8x10^8$ copies/reaction
Lane 3 : $8x10^7$ copies/reaction
Lane 4 : $8x10^6$ copies/reaction
Lane 5 : $8x10^5$ copies/reaction
Lane 6 : $8x10^4$ copies/reaction
Lane 7 : $8x10^3$ copies/reaction
Lane 8 : $8x10^2$ copies/reaction
Lane 9 : $8x10^1$ copies/reaction
Lane 10: 8 copies/reaction
Lane 11: 1 copy/reaction
Lane 12: $1x10^{-1}$ copies/reaction
Lane 13: $1x10^{-2}$ copies/reaction
Lane 14: Negative control
Lane 15: Positive control ($8x10^2$ copies)

Fig. 4. Sensitivity of the primer BVNF2/BVNR2 in nested rt-PCR with serially diluted vector construct PBVN4

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