RAPID DETECTION OF BETANODAVIRUS PRESENCE IN SEA BASS (LATES CALCRIFER) BASED ON RT-PCR (REVERSE TRANSCRIPTASE PCR)

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ABSTRACT

Betanodavirus are the causative agents of viral nervous necrosis (VNN) in cultured marine fish, as Sea bass (Lates calcarifer). The aims of current study toward in establishing the rapid method to detect the presence of Betanodavirus in Sea bass. A total of 30 death aquarium Sea bass, which were collected from Ben Tre province, Vung Tau province and positive control which confirmed within viral nervous necrosis, were enrolled into present study. Initially, we were successful in primers designed, which were also confirmed the specificity to Betanodavirus and sensitivity which reached at least 10^3 copies/ml. Finally, we successfully adopted rapid detection of Betanodavirus presence based on RT-PCR. As the results, 3 of 30 cases were positive to Betanodavirus. Then, the results were confirmed by sequencing and the similarity was showed. Therefore, in further study, it will be continuous in detecting the presence of Betanodavirus in various kinds of fishery samples.

Keywords: *Betanodavirus; Sea bass; viral nervous necrosis; RT-PCR.*

1. Introduction

In Vietnam, aquaculture, especially in Sea bass cultured (Fig. 1), has been recognized as one of the fasting growing industry and expected to supply of fishery products. However, rapid growing, expansion and intensification of its cultured has led to disease outbreaks, of which infection caused by *Betanodavirus* is a major concerning. *Betanodavirus* infected has emerged as an alarming constraint to Sea bass fish culture, which is associated with high mortality particularly in larvae and juvenile fish (Skliris *et al.,* 2001; Shetty *et al.,* 2012).

Figure 1. Sea bass (*Lates calcarifer*)

Betanodavirus, a single stranded RNA (ssRNA), is the causative agents of viral nervous necrosis (VNN) in a variety of cultured marine fish, such as Sea bass (*Lates calcarifer),* worldwide, including South and East Asia, Oceania, the Mediterranean, the UK, Scandinavia, and North America (Chi *et al.*, 2003; Gomez *et al.*, 2004; Gomez *et al.*, 2006; Shetty *et al.*, 2012). Necrosis, vacuolation of central nervous tissues (brain and spinal cord), eye retina is the most characteristic lesion of VNN, and affected fish shows abnormal swimming behavior (Gomez *et al.*, 2006). Thus, this disease infected by *Betanodavirus* could cause mass mortality in population of marine aquaculture fish production, and the mortality rate has been increased rapidly during the past several years due to their higher market demand and

economic value. Therefore, paying attention to this disease, current study has carried out to adopt an approach to detect *Betanodavirus* based on RT-PCR (Reversed transcription PCR).

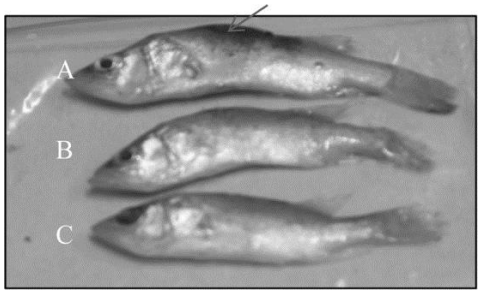


Figure 2. Asian Sea bass (*L. calcarifer*) juveniles infected with nodavirus. A: Arrow shows dark coloration on the body surface. B & C: Highly emaciated infected fish (Shetty *et al.*, 2012).

2. Materials and methods

Samples collection

Sea bass (*L. calcarifer*) was confirmed as positive with viral nervous necrosis, collected in Ben Tre province, Vietnam, served as positive control in present study. For experiments, the dead Sea bass samples, that doubted in caused by viral nervous necrosis, collected in Vung Tau province and Ben Tre province, Vietnam.

RNA isolation and RT-PCR assay

Fish sample brain or eyes was collected, then, total of RNA was isolated by Trizol method (pH = 4). Total of RNA was enrolled into RT-PCR assay to convert to cDNA. RT-PCR was carried out in a total of 25 µl containing 10 µl RNA, which isolated from positive or doubt VNN, and 15 µl RT solution (containing dNTPs, Reversed transcriptase enzyme, RT buffer) (Viet A Cooperation). RT-PCR assay was subjected to initial incubation at 25^{o}C for 5 minutes, followed by 42^{o}C for 30 minutes and 85^{o}C for 5 minutes, terminated and stored at 4^{o}C.

PCR assay for *Betanodavirus* detection

The amplification was carried out in a total of 25 µl containing 2.5 µl cDNA, 0.5 µl each forward and reverse primer (Table 1), in which, the characteristic of both primers were evaluated by IDT oligo analyzer (https://www.idtdna.com/calc/analyzer), BioEdit v7.2.5, 12.5 µl master mix and 9.0 µl nuclease-free water. PCR assay was subjected to initial incubation at 95^{o}C for 5 minutes, followed by 40 cycles at 95^{o}C for 30s, 50^{o}C for 30s, 72^{o}C for 30s and 72^{o}C for 6 min for final incubation. Each PCR product was directly loaded onto a 2.0% agarose gel, stained with ethidium bromide, and directly visualized under UV illumination. Then, PCR products were sequencing to confirm the specificity of primers and target gene amplification.

Table 1. Sequencing of primer used in current study

Primer	Sequence (5'-3')
Forward primer	5'- GGAGTGTTCGAYTGAGCGT - 3'
Reversed primer	5'- CGAAACCAGCCTGCAGGT - 3'

Note: Y indicated C or T.

3. Results and discussion

Characteristics of primers

Characteristics, including length, %GC, T_m (Melting temperature), Gibbs free energy for hairpin loop, homodimer and heterodimer structure formations, were accessed by IDT oligo analyzer (Table 2).

Table 2. Characteristics of primers used in present study

Primer	T_m (^oC)	L (bp)	%GC	(1) (Kcal/mole)	(2) (Kcal/mole)	(3) (Kcal/mole)	P (bp)
F.P	55.1	19	55.3	-0.11	-6.76	-7.13	283
	56.1						
	57.1						
R.P	58.4	18	61.1	-0.65	-16.38		

Note: F.P: forward primer; R.P: reverse primer; Tm: melting temperature; Gibbs free energy for (1) hairpin loop, (2) homodimer and (3) heterodimer structure formations; P: PCR product.

Proper primer design is important for application in several molecular techniques, such as PCR, DNA sequencing, hybridization, etc., thus, it is necessary to evaluate primer characteristics. According to table 2, forward primer's melting temperature was ranked from 55.1^{o}C to 57.1^{o}C, average in 56.1^{o}C, due to the Y contained in primer sequence. However, temperature of both primers, and other characteristics, length, %GC, T_m (Melting temperature), Gibbs free energy for hairpin loop, homodimer and heterodimer structure formations, in which excepted reversed primer (ΔG = -16.38 Kcal/mole), were according to guideline of primer designed.

The specific of primers were evaluated by Bioedit and BLAST (NCBI). Both forward and reversed primers were specific to *Lates calcarfier* nervous virus: accession number HM485328 (Forward primer: Max score = 36.2, Query = 100%, E-value = 0.94; and Reversed primer: Max score = 35.4; Query = 100%, E-value = 1.6). and amplified product yield to 283 bp (Fig. 3).

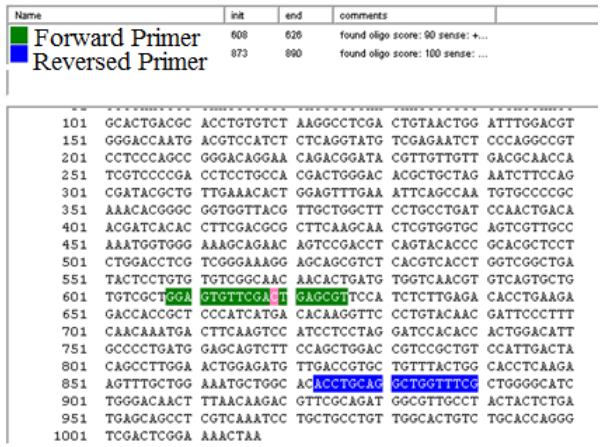


Figure 3. The alignment of both primers with *Lates calcarfier* nervous virus, accession number HM485328.

Evaluation of primer and PCR condition for analysis

Initially, RT-PCR and PCR were carried out within two groups (1) *Piscine nodavirus* was used as positive; and (2) negative control within non-nodavirus were used to evaluate the amplification of primers. Then, the PCR product was directly loaded onto a 2.0% agarose gel, stained with ethidium bromide, and directly visualized under UV illumination,

shown in Fig. 4.

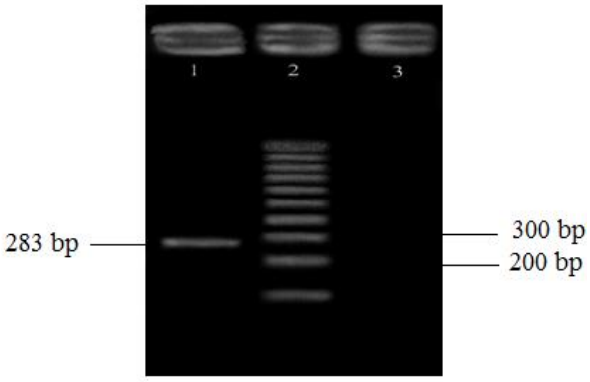


Figure 4. PCR product of (1) positive control: *Piscine nodavirus*; (3) negative control; and (2) 100-bp ladder.

According to Fig. 4, only one band was clearly observed at the length of 283 bp in the case of positive control, whereas no band was observed in the negative control. Based on this result, it could be concluded that primers were able to amplified and detect the presence of *nodavirus* gene, and non-viral infected in negative control. Continuously, the PCR conditions were evaluated, firstly, the temperature of primer annealing and extension were optimized through temperature gradient from 51^{o}C to 58^{o}C. The results of temperature optimized products were observed by electrophoresis, as shown in Fig. 5.

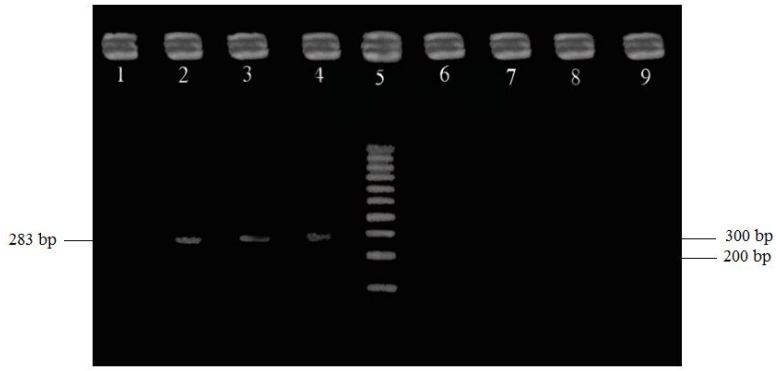


Figure 5. PCR product of temperature gradient test. The temperature gradient was shown in Table 3.

Table 3. The temperature gradient applied in figure 5

Well	Temperature (^{o}C)	Well	Temperature (^{o}C)
1	51	6	55
2	52	7	56
3	53	8	57
4	54	9	58
5	100 bp ladder		

According to Fig. 5, the temperatures, which were from 55^{o}C to 58^{o}C, and 51^{o}C, no band was observed, indicating that those temperatures were not suitable for target gene amplification. Whereas, one band was observed in three wells according to 52^{o}C, 53^{o}C, and 54^{o}C. Remarkably, band in temperature of 52^{o}C was clearer and brighter than the band in temperature of 53^{o}C and 54^{o}C. Thus, 52^{o}C was the best choice in current study for target gene amplification.

Continuously, the specificity of primers was evaluated by PCR assays, which carried out with different primer and organisms, such as *Salmonella*, *Shigella*, *V. chorella*, WSSV, etc. The results were shown in Fig. 6 and Table 4. The results indicated that primers in current study was specific to *Betanodavirus* and shown in well 10.

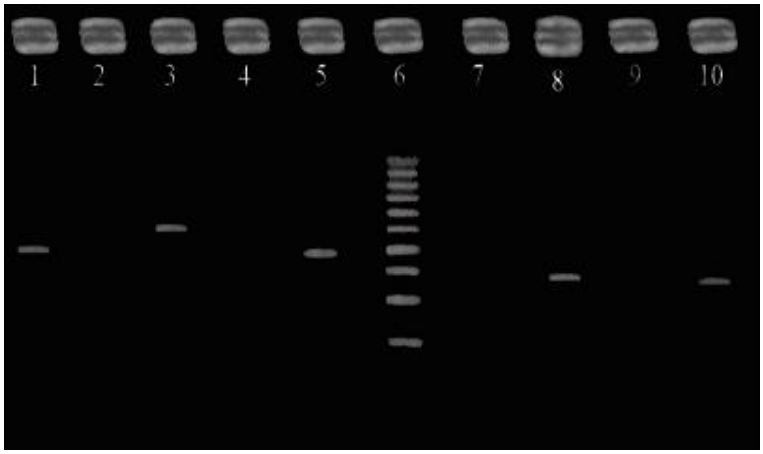


Figure 6. PCR product of temperature gradient test.

Table 4. The pathogens and primers applied in Figure 6

Well	Pathogens	Primers for detection	Results
1	*Salmonella*	*Salmonella*	+
2	*Salmonella*	*Betanodavirus*	-
3	*Shigella*	*Shigella*	+
4	*Shigella*	*Betanodavirus*	-
5	*WSSV*	*WSSV*	+
6	100 bp ladder		
7	*WSSV*	*Betanodavirus*	-
8	*V. chorella*	*V. chorella*	+
9	*V. chorella*	*Betanodavirus*	-
10	*Betanodavirus*	*Betanodavirus*	+

Note: +: positive; -: negative

Sensitivity of PCR assays were carried out with different copies per milliliter, the results showed that a 283-bp length band was observed in the case of 10^5, 10^4 and 10^3 copies/ml, and no band was observed in others (Fig. 7). Thus, the concentrate of cDNA was detected at least 10^3 copies/ml.

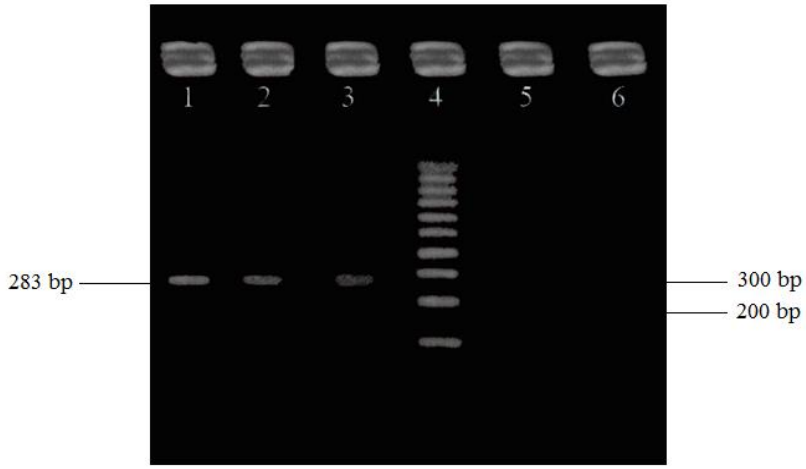


Figure 7. PCR product of sensitivity test. (1), (2), (3), (4), (5) and (6) were 10^5, 10^4, 10^3, 100-bp ladder, 10^2, 10^1, 10^0 copies/ml.

The experimental samples test

Total of 30 dead Sea bass samples, that doubted in caused by viral nervous necrosis, collected in Vung Tau province and Ben Tre province, Vietnam, were enrolled into current study. Total of RNA were isolated and conversed into cDNA, then, PCR assay were carried out with designed primers. As the results, 3 of 30 samples were positive, showing a 283 bp band (Fig. 8).

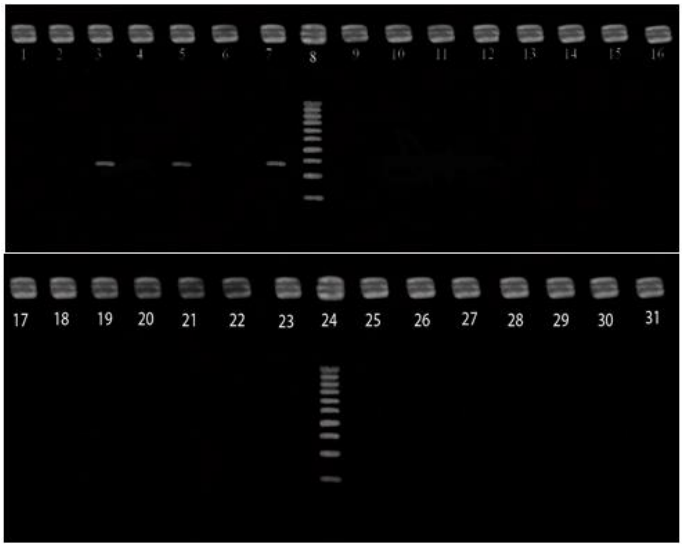
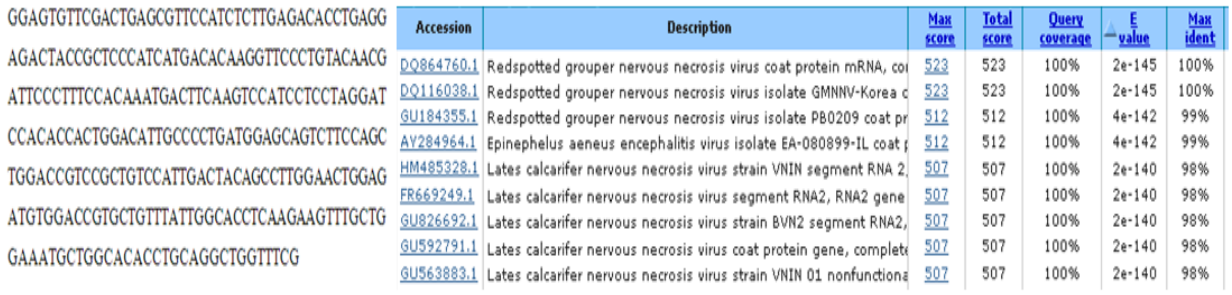


Figure 8. PCR product of dead Sea bass samples with were collected from Ben Tre and Vung Tau province.

Then, the PCR product was confirmed by DNA sequencing, and according to Blast results, the PCR product was similar to sequence of Redspotted grouper nervous necrosis virus with accession number DQ864760, within Total score = 523, Ident = 100% and E-value = 2e-145, Max ident = 100% (Fig. 9). It could be concluded that we were successful in detection of nervous necrosis virus based on PCR assay.



GGAGTGTTCGACTGAGCGTTCCATCTCTTGAGACACCTGAGG
AGACTACCGCTCCCATCATGACACAAGGTTCCCTGTACAACG
ATTCCCTTTCCACAAATGACTTCAAGTCCATCCTCCTAGGAT
CCACACCACTGGACATTGCCCCTGATGGAGCAGTCTTCCAGC
TGGACCGTCCGCTGTCCATTGACTACAGCCTTGGAACTGGAG
ATGTGGACCGTGCTGTTTATTGGCACCTCAAGAAGTTTGCTG
GAAATGCTGGCACACCTGCAGGCTGGTTTCG

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
DQ864760.1	Redspotted grouper nervous necrosis virus coat protein mRNA, co	523	523	100%	2e-145	100%
DQ116038.1	Redspotted grouper nervous necrosis virus isolate GMNNV-Korea c	523	523	100%	2e-145	100%
GU184355.1	Redspotted grouper nervous necrosis virus isolate PB0209 coat pr	512	512	100%	4e-142	99%
AY284964.1	Epinephelus aeneus encephalitis virus isolate EA-080899-IL coat p	512	512	100%	4e-142	99%
HM485328.1	Lates calcarifer nervous necrosis virus strain VNIN segment RNA 2,	507	507	100%	2e-140	98%
FR669249.1	Lates calcarifer nervous necrosis virus segment RNA2, RNA2 gene	507	507	100%	2e-140	98%
GU826692.1	Lates calcarifer nervous necrosis virus strain BVN2 segment RNA2,	507	507	100%	2e-140	98%
GU592791.1	Lates calcarifer nervous necrosis virus coat protein gene, complete	507	507	100%	2e-140	98%
GU563883.1	Lates calcarifer nervous necrosis virus strain VNIN 01 nonfunctiona	507	507	100%	2e-140	98%

(A) (B)

Figure 9. (A) The sequence of PCR product; (B) BLAST result of PCR product showed the similarity to Redspotted grouper nervous necrosis virus.

4. Conclusion

Based on our results, we were successfully in establishment of mRNA Redspotted grouper nervous necrosis virus, which was proved as the major cause of viral nervous necrosis (VNN) in cultured marine fish, sea bass (*Lates calcarifer)*, based on Reversed Transcription PCR. Initially, 3 of 30 case of death Sea bass samples, that doubted in caused by viral nervous necrosis, collected in Vung Tau province and Ben Tre province, Vietnam, were positive to viral nervous necrosis. Moreover, the specificity and sensitivity of primers were also evaluated, as the results, the concentrate of cDNA was detected at least 10^3 copies/ml. In nearby study, it will be continuously carried out on various cultured marine fish.

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