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# Cloning and Sequence Analysis of Coat Protein Gene of Betanodavirus, the Causative Agent of Viral Nervous Necrosis of Grouper

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*Abstract*

Viral nervous necrosis (VNN) caused highly destructive disease in hatchery reared larvae and juveniles of marine fishes, including grouper. Betanodavirus, the causative agent of VNN is unenveloped, icosahedral, and the genome is composed of bipartite of single stranded, positive sense RNA. The RNA2 fragment encodes the coat protein. The objective of this research is to clone coat protein gene of betanodavirus. Total RNA from VNN infected grouper was used to amplify coat protein gene with RT-PCR method using designed primers. PCR products were cloned into pBSKSII and sequenced. In this study, coat protein gene of betanodavirus was successfully and firstly cloned in Indonesia. The open reading of the gene was composed by 1017 nucleotides and encoded 338 amino acids. This betanodavirus isolate was belonged to Redspotted grouper nervous necrosis virus group and showed high homology with Dragon grouper nervous necrosis virus which isolated from China.

Keywords : cloning, sequence analysis, coat protein gene, betanodavirus, grouper

## Introduction

Fish disease is one of the limiting factors in aquaculture. Two diseases have been identified cause severely affect on the grouper culture are bacterial vibriosis caused by *Vibrio* sp and viral nervous necrosis (VNN) (Yuasa *et al*., 2000). VNN also referred to viral encephalopathy and retinopathy (VER), or fish encephalitis due to the disease is characterized by the development of a vacuolating encephalopathy and retinopathy associated with arrays of virus-like particles in infected neurons and degeneration of neurons throughout the central nervous system including the retina. These symptoms suggest that the viruses, once they infect a neuron, replicate there and are transported across a synapse to the next neurons (Ikenaga *et al*., 2002). VNN causes highly destructive disease in hatchery reared larvae and juveniles of marine fishes. VNN disease has spread to 30 or more marine fish species of 14 families (Iwamoto *et al*., 2004). In several of these species, VNN-associated mortalities approaching 100% have been reported (Munday & Nakai 1997), and in Indonesia this disease caused 100% mortality on grouper, especially during larva and juvenile stages (Zafran *et al*., 2000). VNN also could infect tilapia, *Oreochromis mossambicus*, an important species for freshwater and marine aquaculture (Skliris and Richard, 1999), moreover the virus also have been identified in guppy, *Poicelia reticulata*, an ornamental freshwater fish (Hedge *et al*., 2003). Using experiment in fection, Furusawa *et al* (2006) showed that medaka (*Oryzias latipes*) exhibited disease

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symptoms following inoculation with betanodavirus. This disease has spread to the Indo-Pacific region, the Mediterranean region, Scandinavia, and North America (Iwamoto *et al*., 2004). Those reasons imply that VNN represents a significant barrier to commercial marine aquaculture activities, which also possible to affect freshwater fish aquaculture.

VNN is caused by a member of Betanodaviruses which is an unenveloped, icosahedral capsids (25–30 nm in diameter), and the genome is composed of bipartite, single-stranded, positive sense RNA molecules. The larger genomic segment, RNA1 (3.1 kb), encodes an RNA-dependent RNA polymerase (called protein A). The smaller genomic segment, RNA2 (1.4 kb), encodes the coat protein (CP). Subgenomic RNA3 (378 nucleotides) was characterized as an alternative splicing of RNA1 that encodes protein B (Iwamoto *et al*., 2004). Protein B has function as suppressor for post transcriptional gene silencing (Iwamoto *et al*., 2005). Based on the similarity of the partial RNA2 sequences encoding the C-terminal halves of the coat protein, betanodaviruses can be classified into four groups, designated striped jack nervous necrosis virus (SJNNV), berfin flounder nervous necrosis virus (BFNNV), tiger puffer nervous necrosis virus (TPNNV), and red spotted grouper nervous necrosis virus (RGNNV), (Nishizawa *et al*, 1997). The host ranges of SJNNV and TPNNV are limited to striped jack (*Pseudocaranx dentex*) and tiger puffer (*Takifugu rubripes*), respectively, whereas BFNNV has been isolated from some cold water species, such as barfin flounder (*Verasper moseri*) and Pacific cod (*Gadus macrocephalus*).

In this study we cloned, sequenced and analyzed coat protein gene (RNA2) from brown-marbled grouper which cultured in Indonesia.

## Materials and Methods

### *Fish sample and VNN detection*

The brown-marbled groupers (*Epinephelus fuscoguttatus*) were collected from hatchery which showing VNN infection signs such as loss of equilibrium, abnormal swimming, and mortality in Situbondo, East Java. Total RNA was extracted from eyes and brains using Isogen (Wako pure chemicals, Japan) according to the manual. The present of betanodavirus was confirmed by IQ2000 VNN detection kit (Farming Inteligent, Taiwan).

### *Amplification of coat protein gene*

A primers pair was designed to amplify full length of coat protein (CP) gene based on the homology of sequences RNA2 from various genotypes of betanodavirus. The restriction site of *Eco*RI was added on the 5'-end of the forward primer, *Hind*III was added on the 5'-end of the reverse primer. The sequence primers are forward primer CPNNV-N-F TTGCGAATTCAAATGGTACGCAAAGG T, reverse primer CPNNV-C-R ATACAAGCTTGGGATCCGGATCACCC GGT. The primers are synthesized by Hokkaido System Science (Tokyo, Japan).

For reverse transcription, the total RNA extracted from VNN positive samples were used as template to produce first stand cDNA using random primers and other chemicals from First Strand cDNA synthesis kit (Takara) following the manual procedures. The PCR reaction to amplify CP encoded gene was performed using first stand cDNA as template, specific primers as described above and PCR kit (Boehringer). PCR was run on an automatic thermal cycler (Perkin-Elmer 480) for one cycle a 95°C for 5 minutes, then 25 cycles at 95°C for 30 sec, 55°C for 30 sec, 72°C for 90 sec and one cycle at 72°C for 5 minutes. After amplification, the PCR products were electrophoreted on a 1.5% agarose-TAE (40mM Tris-Acetate pH 8.3, 1

mM EDTA) gel and stained with ethidium bromide.

*Cloning and sequensing*

The PCR products were purified using phenol extraction and followed by digestion with *Eco*RI and *Hind*III. After digestion, the PCR products were agarose electrophoreted and DNA bands were recovered from agarose using glass powder method (Murwantoko *et al*. 2004). Purified PCR fragments were ligated into pBSKSII (Strategene) which digested with same enzymes using T4 DNA ligase (Toyobo) at 16°C for over night. Ligation mixtures were transformed into *Escherichia coli* DH5á using heat shock on 42°C for 90 sec followed incubation on ice. The bacteria were cultured on LB agar plate containing 50mg/l amphicilin for over night. The growth colonies were cultured in LB broth containing 50mg/l amphicilin at 37°C for over night. To verify the present of recombinant, the plasmids from bacteria were purified using lysis alkali minipreparation method (Sambrook and Russel, 2001) and digested with restriction enzyme and electrophoreted.

The recombinant plasmids were purified using PEG method (Murwantoko *et al*, 2004), briefly the plasmids were treated with RNAse at 37°C for 30 minutes. The solution containing 50% PEG, 1.6 M NaCl was added on same volume and incubated on RT for 15 minutes. The plasmids were precipitated using centrifugation and washed with 70% ethanol. The purity of plasmids were evaluated using UV spectrophotometer. The high quality plasmids were sequenced using Big DyeTM terminator (PE biosystem, USA), T3 and T7 primers and performed on automatic thermal cycler Perkin-Elmer 480 and analyzed on an ABI310 DNA auto-sequencer (Perkins Elmer).

Genotype analysis

The sequence result from sequencer was edited and analyzed by Genetyx program. Homology of the sequence with data on gene bank was analyzed using BLAST method (Altschul *et al*., 1990) and homology with selected data was performed with Clustal (Thomson *et al*., 1994).

Results and Discussion

Multiple alignment of several RNA2 sequences of betanodavirus from groupers which commonly cultured in Indonesia such as sevenband grouper (AY324870.1), *Epinephelus coioides* (AF534998.3), dragon grouper (AF245004.1) and redspotted grouper (AY744705.1) revealed that the sequences share high homology between 98 to 99% with a half part on 5' end show higher homology than other half on 3' end (data not shown). However the sequences around start and stop codons share high homology, and based on those sequences, the primers which used in this experiment are designed. The *Eco*RI and *Hind*III sites were not recognized on those sequences, so those restriction site sequences were added into primer in order to make more easy during cloning.

Confirmation of the present of betanodavirus to the total RNA samples showed that a sample gave positively result. Then we used those RNA to amplify coat protein gene by reverse transcriptase PCR (RT-PCR) using designed primers. Checking of PCR product on agarose electrophoresis showed that a single band with size approximately 1000 bp was amplified (data not shown). That size of PCR product is same with the prediction.

The PCR products were digested with restriction enzymes and ligated into pBSKSII. Transformation of ligation mixtures into *E. coli* DH5a produced some colonies appear in LB agar containing amphicilin. Plasmids from growth colonies

were digested with *Bam*HI to check the present of DNA insert. Digestion of three plasmids produced 3 DNA bands, approximately on 2800, 700 and 300 bps (Figure 1). The 2800 bp band was came form the plasmid and other two bands were came from DNA insert. This result showed that the CP gene was successfully cloned and also described that the *Bam*HI restriction site was present in the CP gene.

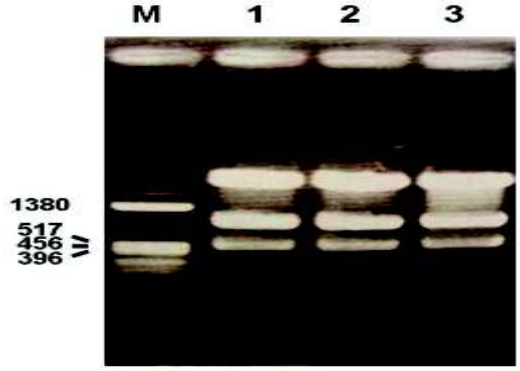


Figure 1. Evaluation of recombinant plasmid by digestion with *Bam*HI. All plasmids contain DNA inserts as shown the present of 3 bands as 2800 bp, 800 bp and 500 bp in size. M = DNA size marker BSM13 / *Hinf*I with indicated size

The positive clones were isolated their plasmid and sequenced using T3 and T7 primers. Sequences from those primers were proceed and combined to determine the full length sequence of coat protein of betanodavirus. The sequence also was analyzed on the prediction of translated amino acids using Genetyx program. The nucleotides and amino acids sequence of coat protein was presented in Figure 2.

Analysis using Genetyx from the sequences showed some restriction sites were present in the sequences such as *Alu*I (781), *Bam*HI (738), *Bsm*I (869), *Nae*I (94), *Pst*I (883), *Pvu*II (780), *Hae*III (56, 131, 203), *Hinc*II (246, 591, 828, 1008). The ORF size is 1017 encoded 338 amino acids which start codon located on nucleotide 9-11, stop codon on 1023-1025. The Researches on betanodavirus in Indonesia have been done

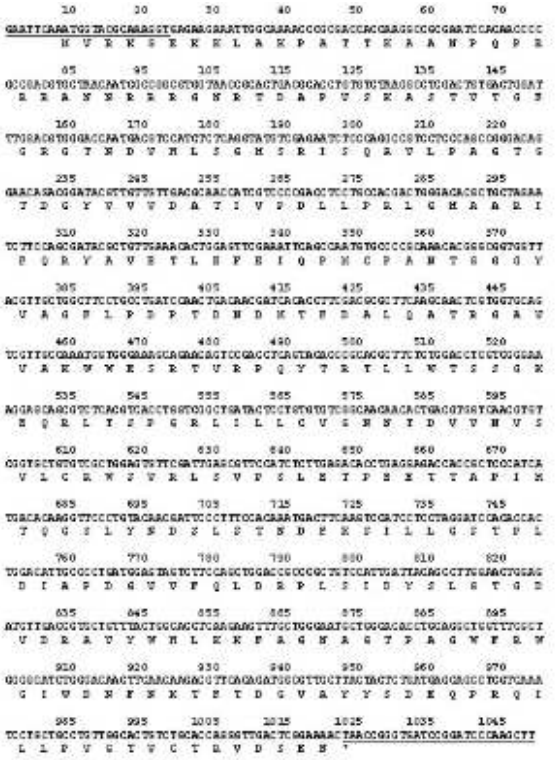

Figure 2. Sequences and translation of coat protein gene from cloned of betanodavirus. Sequences with underline indicate of primer sequences used in this study.

in several fields. Those researches such as pathogenicity on various stages of humback grouper (Zafran *et al*., 2000); detection of VNN using RT-PCR (Susanto *et al*., 2003), application of vaccine (Yuasa *et al*., 2002; Mahardika *et al*., 2002; Roza *et al*., 2004). So far authors have, the information of Indonesian betanodavirus on genomic level was analysis restriction fragment length polymorphisms of betaodaviruses from Bali (Yuasa *et al*., 2002). So this study is the first time cloning and sequence analysis of betanodavirus in Indonesia.

Homology searching of the ORF cloned using Blast method (www.ncbi.nih.gov./blast) showed the highest homology with Dragon grouper nervous necrosis virus coat protein gene which isolated from China (AY721615) with identities 1009 / 1017 (99%) or the different

between two sequences is 8 nucleotides (Figure 3). The Blast result also show high homology with *Lates calcarifer* encephalitis virus isolate LC-121100-IL coat protein gene (AY284974), Redspotted grouper nervous necrosis virus isolate SGNNV-Korea coat protein mRNA (DQ116036), *Epinephelus aeneus* encephalitis virus isolate EA-150102-IL coat protein (AY284967), Sevenband grouper nervous necrosis virus strain SGWak97 coat protein gene (AY324870), Redspotted grouper nervous necrosis virus coat protein mRNA which show identity as 1003 / 1017 (98%); 1000 / 1017 (98%); 999 / 1017 (98%); 998 / 1017 (98%); 998 / 1017 (98%) and 997 / 1017% (98%) respectively.

| Sequences producing significant alignments: | | Score (Bits) | E Value |
|---|---|---|---|
| gi\|52631437\|gb\|AY721615.1\| | Dragon grouper nervous necrosis vi... | 1953 | 0.0 |
| gi\|33469471\|gb\|AY284974.1\| | Lates calcarifer encephalitis viru... | 1905 | 0.0 |
| gi\|71081827\|gb\|DQ116036.1\| | Redspotted grouper nervous necrosi... | 1881 | 0.0 |
| gi\|33469457\|gb\|AY284967.1\| | Epinephelus aeneus encephalitis vi... | 1873 | 0.0 |
| gi\|37222762\|gb\|AY324870.1\| | Sevenband grouper nervous necrosis... | 1865 | 0.0 |
| gi\|71081831\|gb\|DQ116038.1\| | Redspotted grouper nervous necrosi... | 1865 | 0.0 |
| gi\|53711284\|gb\|AY744705.1\| | Redspotted grouper nervous necrosi... | 1857 | 0.0 |
| gi\|71081829\|gb\|DQ116037.1\| | Redspotted grouper nervous necrosi... | 1857 | 0.0 |
| gi\|71081825\|gb\|DQ116035.1\| | Redspotted grouper nervous necrosi... | 1850 | 0.0 |
| gi\|33113501\|gb\|AF534998.3\| | Epinephelus coioides nervous necro... | 1850 | 0.0 |

Figure 3. The Result of BLAST analysis of coat protein of betanodavirus.

Based on the classification by Nishizawa *et al.* (1997) this betanodavirus was belonged to redspotted grouper nervous necrosis virus (RGNNV). RGNNV is the biggest group which contains 15 isolates. As a comparation the striped jack nervous necrosis virus (SJNNV), berfin flounder nervous necrosis virus (BFNNV) groups only contain 6 and 2 isolates respectively. Phylogenetic analysis on 13 isolates of betanodavirus from Asia and Europe showed that most of isolates (12 isolates) were belonged to RGNNV group and only one isolate was belonged to SJNNV group (Skliris *et al*., 2001). Thiery *et al.* (2004) also found that among the 21 isolates betanodavirus, most of them were belong to RGGNV. RGNNV has a broad host range and causes disease among a variety of warm water fish species, particularly groupers and sea bass (Nishizawa *et al*., 1997). Using RFLP analysis, Yuasa *et al.* (2002) noted that four betanodavirus isolates from Bali were belonged to RGNNV group.

Many species of fish have been infected by VNN. We compare the sequence of this study with the betanodavirus isolates which infected fishes that commonly found in Indonesia. The accession number, description and codes were shown in table 1.

| Code | Description | Acession Number |
|---|---|---|
| Dragon | Dragon grouper nervous necrosis virus coat protein gene | AF318942.1 |
| Greasy | *Epinephelus tauvina* nervous necrosis virus segment RNA2 coat protein mRNA | AF318942.1 |
| Guppy | Guppy nervous necrosis virus coat protein gene | AF499774.1 |
| Sevenband | Sevenband grouper nervous necrosis virus strain SGWak97 coat protein gene | AY324870.1 |
| Coioides | Epinephelus coioides nervous necrosis virus coat protein gene | AF534998.3 |
| Malabar | Malabaricus nervous necrosis virus coat protein gene | AF245003.1 |
| RedSpotted | Redspotted grouper nervous necrosis virus coat protein mRNA | AY744705.1 |
| Stripped-Jack | Striped Jack nervous necrosis virus gene for coat protein | AB056572.1 |

Tabel 1. Code and accession number of sequence which used in the analysis.

Multiple alignment results on those sequences showed that 237 nucleotide positions were different among those sequences. The highest homology as 99% was found between Kloning and Dragon; and the lowest homology as low as 78% was found between Stripped-Jack and Greasy. Generally Stripped-Jack showed the low homology with other sequences with 79% homology. (Data not shown). The phylogram of those sequences was presented in Figure 4. Among those fish species, the stripped jack was not cultured yet in Indonesia, and other species were commonly cultured. The high homology of betanodavirus which infected commonly cultured fish in Indonesia implies that one isolate of betanodavirus can infect and spread into many species. On the other hand in term of disease controlling, the is a benefit that production of vaccine from one isolate might be use to control VNN in many species of fish. However, since there is no data on the genetic variability of betanodavirus in Indonesia, the researches on genetic

variation of betanodavirus base on species and or region in Indonesia are suggested.


Figure 4. Phylogram of betanodavirus coat protein sequences

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