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EFFECTIVENESS OF A RECOMBINANT VACCINE BASED ON RNA2 CAPSID PROTEIN AGAINST NERVOUS NECROSIS VIRUS IN HYBRID GROUPER

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ABSTRACT

Nervous Necrosis Virus (NNV) is a devastating viral disease in marine aquaculture, causing significant economic losses worldwide, including in Indonesia. The virus mainly infects larvae and juveniles of marine fishes. This study aimed to determine the effectiveness of a recombinant vaccine from betanodavirus coat proteins expressed in *Escherichia coli* fish against NNV infection in hybrid grouper. An RNA2 capsid protein was selected and used as the recombinant vaccine. NNV-RNA2 gene was inserted into the protein expression system vector of pET SUMO and cloned in cells of bacteria *Escherichia coli* strain BL-21. The results of blast homology analysis exhibited that the amino acid sequence of the NNV-RNA2 showed high similarity with *Lates calcarifer* encephalitis viral coat protein gene. *E. coli* expressing NNV-RNA2 protein was inactivated using 0.03% formalin and mechanically inactivated by freeze-thaw and sonication methods. The inactivated recombinant *E. coli* vaccine was then injected intramuscularly into hybrid grouper juveniles (single vaccine). Subsequently, the juveniles were challenged with NNV at 7, 14, and 21 dpv (days post-vaccination). Injection of 0.1 mL sterile PBS served as the control. Single vaccine applications using formalin-inactivated vaccines resulted in higher antibody titers than those of mechanically-inactivated vaccines. Both vaccines were only able to increase antibody titer up to 7 dpv. Therefore, re-vaccination (booster vaccine) was done on day-10 after the first vaccination using a formalin-inactivated vaccine. The booster vaccine could protect hybrid grouper against NNV ($P<0.05$) at four weeks post-vaccination. However, the mortality of vaccinated and control fish was not significantly different ($P>0.05$) after challenged with NNV for six weeks after. This recombinant vaccine has the potential to be developed into a polyvalent vaccine by combining viral and other bacterial vaccines in future research.

KEYWORDS: antibody titer; hybrid grouper; NNV-RNA2; recombinant protein vaccine; RPS

INTRODUCTION

Over the past decade, aquaculture has grown at an average annual growth rate of approximately 6% worldwide despite many challenges. Viral diseases are one of the major challenges that are threatening the sustainable growth of finfish farming globally (Dhar *et al*., 2013). The spread of viral diseases through international trade is now a proven threat to aquaculture. Movement of animals showing no apparent clinical signs or carrying sub-clinical infection, asymptomatic carrier hosts, and shipment of contaminated/infected eggs are threats to the sustainable growth of finfish farming (Rodgers *et al*., 2011). Viral nervous necrosis (VNN) is one of the viral diseases caused by Nervous necrosis virus (NNV). It is classified as a major pathogen for several economically important fish species worldwide (Lin *et al.*, 2007). NNV belongs to the family Nodaviridae and the genus Betanodavirus. NNV is a small non-enveloped icosahedral virus with sizes ranging between 25-30 nm and contains bi-segmented single-strand positive RNA as the genetic material. RNA1 (approximately 3.1 kb in length) encodes an RNA-dependent RNA polymerase for viral replication, whereas RNA2 (1.4 kb) encrypts a viral capsid protein (Iwamoto *et al.,* 2004; Sommerset & Nerland, 2004). Betanodaviruses are divided into four genogroups based on the partial sequences of RNA2: barfin flounder nervous necrosis (BFNNV), tiger puffer nervous necrosis (TPNNV), striped jack nervous necrosis (SJNNV) and red-spotted grouper nervous necrosis (RGNNV) (OIE, 2018).

In Indonesia, mass mortalities caused by NNV were reported from various cultured marine fish such as sea bass (*Lates calcarifer*), humpback grouper

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(*Cromileptes altivelis*), tiger grouper (*Epinephelus fuscoguttatus*), marbled grouper (*E. polyphekadion*), coral trout grouper (*Plectropomus leopardus*), and hybrid grouper. The clinical signs of NNV are abnormal swimming behaviors, including erratic, spiral or twirling, and belly-up swimming patterns with black body color. These signs occurred on 1-2 days from initial symptoms before the fish become weak because of the decrease in appetite (Mahardika *et al*., 2015).

The infection route of NNV is arguably through vertical transmission from infected broodstock to their offspring, and horizontal transmission from sick fish to healthy fish (Breuil *et al.,* 2002). Prevention of the disease during larval and juvenile stages may be possible through broodstock management, disinfection of hatchery water or biosecurity measures. In addition to controlling the disease, vaccination may prove to be useful. Protective immunity has been obtained in several fish species by using recombinant betanodavirus coat proteins expressed in *Escherichia coli* (Húsgaro *et al.,* 2001; Tanaka *et al*., 2001; Yuasa *et al.*, 2002; Sommerset *et al*., 2005), and recombinant betanodavirus coat proteins expressed in baculoviruses (Thiery *et al*., 2006). However, the availability of recombinant vaccine of betanodavirus coat proteins expressed in *Escherichia coli* derived from local Indonesian virus genes is limited. Cloning and sequence analysis of the gene of betanodavirus protein coat and its vaccine production have been done by Murwantoko *&* Nuraini (2008). The efficacy of the VNN-DNA capsid vaccine was studied by Andriyani (2012). However, the vaccines have not widely known or distributed to fish farmers. Currently, the research on a recombinant vaccine based on betanodavirus coat proteins using local isolate from Bali has been done at the Institute for Mariculture Research and Fisheries Extension, Gondol. The present study was the extended research to determine the effectiveness of the recombinant vaccine from betanodavirus coat proteins expressed in *Escherichia coli* against VNN infection in hybrid grouper.

MATERIALS AND METHODS

Virus Stock

Viruses were obtained from the eyes and brains of NNV naturally infected fish. Eye and brain samples were separately homogenized in PBS (phosphate buffer saline) at a ratio of 1: 5 v/v and centrifuged at 5,000 rpm for 10 min. The supernatant obtained was filtered with a microfilter (0.45 μm^2). The obtained filtrate was stored at -80°C and used as both a DNA source and a challenge test.

Amplification of Target Gene

Genom of the virus stock was extracted with RNA lysis buffer according to the manufacture protocol (IQ 2000™). The primary designs for RNA2 were obtained from several RNA sequence blasts: SJNNV (AB025018.1), *Dicentrarchus labrax* NNV (JN189936.1), *Solea senegalensis* betanodavirus (JN189919.1), Senegalese sole Liberian betanodavirus (JNFJ803923.1), and SJNNV gene for coat protein (AB056572.1). The primary sequences are presented in Table 1. Gene amplification was performed by reversing the RNA2 gene into DNA using the M-MLV Reverse Transcriptase (Solis-Biodyene) kit at 50°C for 1 hour and 94 °C for 7 minutes, followed by DNA amplification in 40 cycles (one cycle consists of denaturation temperature at 94°C for 45 seconds, annealing temperature at 52°C for 45 seconds and extension temperature at 72°C for 90 seconds), and the final extension at 72°C for 5 minutes. The amplification results were visualized in agar gel.

Cloning and Gene Expression

The construction of a recombinant *Escherichia coli* expressing the coat protein was previously described in detail by Mahardika & Mastuti (2015) for GSDIV (grouper sleepy disease iridovirus). The same procedure was applied to obtain a recombinant *Escherichia coli* containing the coat protein gene of a betanodavirus from diseased hybrid grouper "Cantik" (*E. fuscoguttatus* X *E. polyphekadion*) with slight modifications. Briefly, results of the PCR amplification were purified using a PCR purification kit and visualized in agar gel to obtain a single band. The purification results were then ligated on a vector of pET SUMO (Champion™ pET SUMO, Invitrogen). The ligation results were transformed into the *E. coli* strain Mach1™-T1R. The transformation process was done by heat shock. The transformed bacteria were grown on LB agar medium containing 50 ìg/mL of kanamycin. Each growing colony was taken and re-cultured on a 10 mL liquid LB medium containing 50 ìg/mL of kanamycin to be used for DNA analysis and plasmid stock. The colonies growing on the liquid LB were extracted using a prepease minispin plasmid kit (USB, Affimetrix), and its plasmid was amplified using the target gene and SUMO primers. The known plasmids carrying the target genes were then re-transformed into *E. coli* strain BL-21 for expression purposes. The transformants were grown in LB agar medium containing kanamycin. Each growing colony was re-cultured on a 10 mL liquid LB medium containing kanamycin and 0.1 mM IPTG (isopropyl-thio-b-D-galactoside) to induce proteins. The presence of protein

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Table 1. The primary sequences used to amplify the targeted genes

Genomic region		Oligonucleotide sequence (5' to 3' direction)	Expected size of amplicons
RNA2	ORFRNA2 -26F	ATGGTACGCAAAGGTGATAAGAAAWTGRCA	1,300 bp
	R1VNNRNA2	TTAGTTTTCCGAGTCAACMCKGGTG	

expression in *E. coli* strain BL21 was confirmed by SDS-PAGE analysis following the method, which was previously described by Sommerset *et al*. (2005).

DNA Sequence Analysis

Twelve samples of PCR products from plasmids on transformants *E. coli* strain Mach1 containing both of the four samples of amplified ~1300 bp fragment (RNA2-VNN primers) and eight samples of amplified ~1450 bp fragment (SUMO primers) were purified using the Qiaquick PCR purification kit (Qiagen) according to the manufacturer's protocol. The purified DNA was sequenced using a DNA sequencer (Macrogen) using two pair oligonucleotide primers, ORFRNA2-26F/R1VNNRNA2 and SUMO F/R for sequence determination. The forward and reverse DNA sequences generated in this study were then aligned using BioEdit v7.1 software (Hall, 2011). DNA sequences were analyzed using the Basic Local Alignment Search Tool (BLAST) to know its homology and ORF Finder for the prediction of nucleotide acids.

Inactive Vaccine

A 50 μg/mL colonies of induced *E. coli* strain BL-21 was inactivated by adding formalin to the final concentration of 0.03% and kept for a night, or mechanically activated by fresh-taw method (at the temperature of -20°C and 42°C, respectively for 10 minutes with 3 repetitions) and sonication (4 x 15 minutes). Formalin was removed by centrifugation at 6000 rpm for 5 min. Furthermore, the pellet was taken and re-diluted to obtain the density of the bacteria equivalent to the initial density (10^6 CFU/mL). Inactive vaccines were stored at -20°C until further use.

Vaccination Test

Vaccination tests were performed on juveniles of hybrid grouper "Cantik" with an average total length of 12.52 ± 2.45 cm. Vaccines were administered by intramuscular injection with a dose of 0.1 mL/fish. Vaccination test was done through two trials: the first trial was aimed to know when the best time to do booster vaccine. In the first trial, 525 of hybrid grouper fish were injected with the recombinant protein NNV vaccine (A. NNV RNA2-6 with mechanical inactivation, B. NNV RNA2-6 with formalin inactivation, C. NNV RNA2-10 with mechanical inactivation, D. NNV RNA2-10 with formalin inactivation), and E. injected with PBS as the control. The fish were kept in a net cage (0.5x0.5x1 m/hole) floating in a fiber tank with a volume of 4 m^3. A total of five fish per treatment were sampled at 5, 10, and 15 days after vaccination to analyze their antibody titers (OD). The other 10 and 20 fish (total 30 fish) per treatment were sampled at 7, 14, and 21 days after vaccination for challenge tests against NNV inocula. The Relative Percent Survival (RPS) of fish during the virus challenge test was calculated using the formula (Kai & Chi, 2008):

$$RPS = \frac{1-(V)}{C} \times 100$$

where:
V = percent cumulative mortality of vaccinated group
C = percent cumulative mortality of the control group

A total of 270 healthy hybrid "Cantik" grouper were used on the second trial. Ninety fish from each group were injected with recombinant protein NNV vaccine isolates of NNV RNA2-6, isolates of NNV RNA2-10 with mechanical or formalin inactivation vaccine according to the percentage of RPS on the first trial, and PBS as a control. The booster was done at the time according to antibody titer test results at the first trial. The fish were kept in a net cage (0.5 × 0.5 × 1 m/hole) floating in a fiber tank with a volume of 4 m^3. The 30 fish were subsequently challenged with NNV inocula on the two, four, and six weeks after the booster vaccine. The other five fish per each treatment were sampled at 2 and 4 weeks after vaccination to analyze their antibody titers. The mortality of fish during the challenge test was statistically calculated by the Anova test.

The Enzyme-Linked Immunosorbent Assay (ELISA)

Blood samples were left overnight at 4°C to coagulate, centrifuged (6000 rpm for 5 min), and sera collected, aliquoted, and stored at -20°C. Elisa analysis was performed following the procedures previously developed by Jaramillo *et al.* (2016) and procedures developed by Aquatic Diasnotic Ltd. Briefly, rNNV RNA-6 or rNNV RNA-10 was diluted in carbonate-bicarbonate buffer (15 mM Na_2CO_3, 35 mM $NaHCO_3$, pH 9.6) to 10 μg/ mL, and 96-well microtitre

plates (Nunc, Roskilde, Denmark) were coated with 150 μL/well overnight at 22°C. The plates were post-coated with 100 μL of 1% PBS-BSA and incubated at 22°C for three hours. A 50 uL of serum sample was then added to each well and incubated at 22°C for one night (one sample made in duplo). All wells were washed three times with 100 μL PBS-Tween. Then, 50 μL anti-Asian sea bass (*Lates calcarifer*) IgM monoclonal antibody labeled with horseradish peroxidase (Aquatic Diagnostics Ltd) was added in each well and incubated at 22°C for one hour. After washing three times with PBS-Tween, the binding was visualized using 50 μL TMB-ELISA (Serva). The reaction was stopped after 10 min by the addition of 50 μL 2M H_2SO_4. After equilibration for 30 min at 4°C, the optical density (OD) was measured at 450 nm in an ELx800™ spectrophotometer.

RESULTS AND DISCUSSION

Target Gene Amplification

NNV genes obtained from the diseased fish's eye and brain were amplified with designed primers based on the SJNNV gene in the region of the coat protein. The optimization of amplification conditions produced amplifiers with the correct size (1,300 bp) (Figure 1).

Some researchers also used the RNA2 gene as the target gene for the vaccine (Húsgaro *et al*., 2001; Thiery *et al*., 2006; Murwantoko & Nuraini, 2008; Kim *et al.,* 2015).

Ligation and Transformation of NNV RNA2 Gene

The ligation of the NNV-RNA2 gene in the vector of pET SUMO, and then transformed into the cell *E. coli* strain Mach1 produced several colonies, one of which (the isolate *E. coli* Mach1-D1) was taken for re-transformed into the cell of *E. coli* strain BL21. One colony of *E. coli* strain Mach1 and four colonies of *E. coli* strain BL21 (colonies 6, 7, 9, and 10) were found to carry recombinant plasmids by insertion of NNV-RNA2 genes with the correct orientation. Insertion of recombinant plasmid was indicated by a thick band of 1,450 bp in PCR analysis using a combination of forward/reverse primers of the pET SUMO and the NNV-RNA2 gene (Figure 2) which we had successfully developed in our previous research for the GSDIV-MCP gene (Mahardika & Mastuti, 2015).

Coat Protein Expression

SDS-PAGE analysis of the purified *E. coli*-expressed inclusion bodies showed a dominating band of approximately 58 kDa (Figure 3), which was 10 kDa higher than the molecular weight of recAHNV-C (including an N-terminal His-Tag) (Sommerset *et al*., 2005), and relatively lower than the molecular weight of GSDIV-MCP with target DNA of 1,362 bp (Mahardika & Mastuti, 2015).

DNA Sequence of NNV RNA2

NNV RNA2 gene sequencing was obtained throgh partial nucleotide sequence (partial cds), i.e. 1,113 of 1,300 nucleotides (Figure 4). BLAST homologous analysis showed that the NNV-RNA2 genes have similarity up to 98% with the partial sequence of NNV: *Lates calcarifer* encephalitis viral coat protein gene (EU380202.1), complete genome sequence of dragon grouper nervous necrosis coat protein gene (AY721615.1), complete genome sequence of red-spotted grouper nervous necrosis virus isolate GMNNV-Korea coat protein mRNA (DQ116038.1), complete genome sequence of red-spotted grouper nervous necrosis virus isolate SpPm-IAusc1586 10 capsid protein gene (KC896562.1), complete genome sequence of tiger grouper nervous necrosis virus isolate TGNNV0809-Tuaran-Malaysia-1 coat protein (Cp)

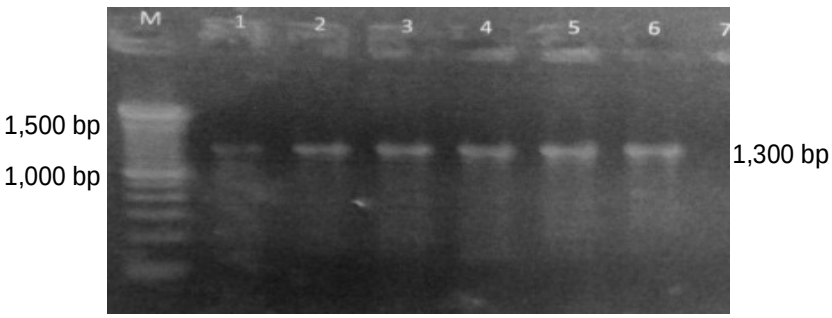


Figure 1. Agarose gel visualization of the NNV RNA2 gene shows the expected amplicon size of 1,300 bp. M: 100 bp DNA ladder, Line 1-6: samples of eye and brain of diseased fish suffering VNN infection.

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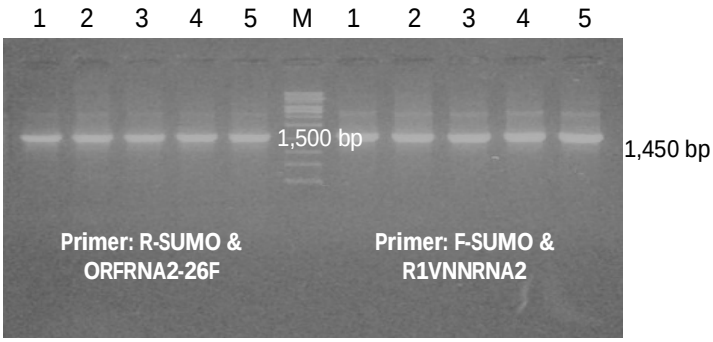


Figure 2. PCR confirmation of the recombinant plasmid for correct orientation using combination primer of pET SUMO and primer of NNV-RNA2. Line 1-4: plasmids of *E. coli* strain BL2- isolate number of 6, 7, 9, and 10, Line 5: plasmid of *E. coli* strain Mach1-isolate D1.

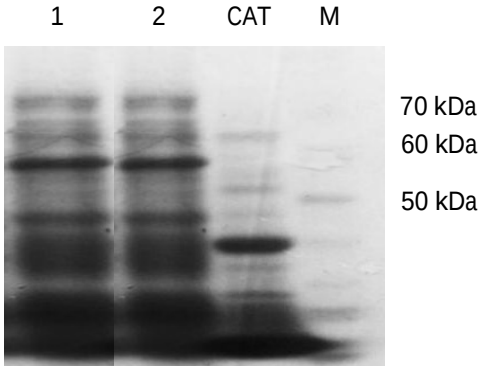


Figure 3. *Escherichia coli*-expressed recombinant NNV-RNA2 coat protein analyzed by SDS-PAGE. M: molecular weight marker (Broad Range precision protein standard); lane 1, purified rNNV RNA2-10, lane 2: purified rNNV RNA2-6, CAT: purified rCAT SUMO.

mRNA (HQ859945.1), and complete genome sequence of tiger grouper nervous necrosis virus isolate TGNNV0708-Gondol-Indonesia-2coat protein (Cp) mRNA (HQ859972.1).

Vaccination Test

The first vaccination test indicated that formalin-inactivated crude RNA2 recombinant protein had the potential to induce fish immunity against VNN infection. Result of the challenge test with NNV having a lethal dose of 80% showed that the recombinant protein vaccine (NNV RNA2-isolates 6 and 10) inactivated with formalin had a relative percent survival value (RPS) of 40.0% and 53.33% higher than that of the recombinant protein vaccine that was mechanically-inactivated and the control treatment in the first-week post-vaccination. However, the vaccine's ability to maintain the specific immunity of hybrid grouper fish decreased dramatically in the second and third weeks after vaccination indicated by a low RPS value (Table 2).

Results of the challenge test with the NNV virus were similar to the results of the antibody titer shown in Figure 5. The absorbance value of blood serum of the hybrid grouper 'Cantik' vaccinated with NNV-RNA2 isolates 6 and 10, which were inactivated either formalin or mechanically methods were higher than that of the control. However, these antibody titers decreased after 14 days post-vaccination. Formalin-inactivated vaccine gave higher antibody titers than that mechanically inactivated vaccines. Therefore, it was necessary to booster/re-vaccination at 14 days after vaccination. The timing of this booster was based on the antibody titer results, whereby the antibody titer peaks at 10 days after the vaccination and was low at day 14 dpv. The booster vaccine was given in the event of a decrease in the antibody titer. The booster was expected to increase the antibody titer and maintain it for an extended period.

In the second experiment, re-vaccination (booster) was done at 14 days after the first vaccination using

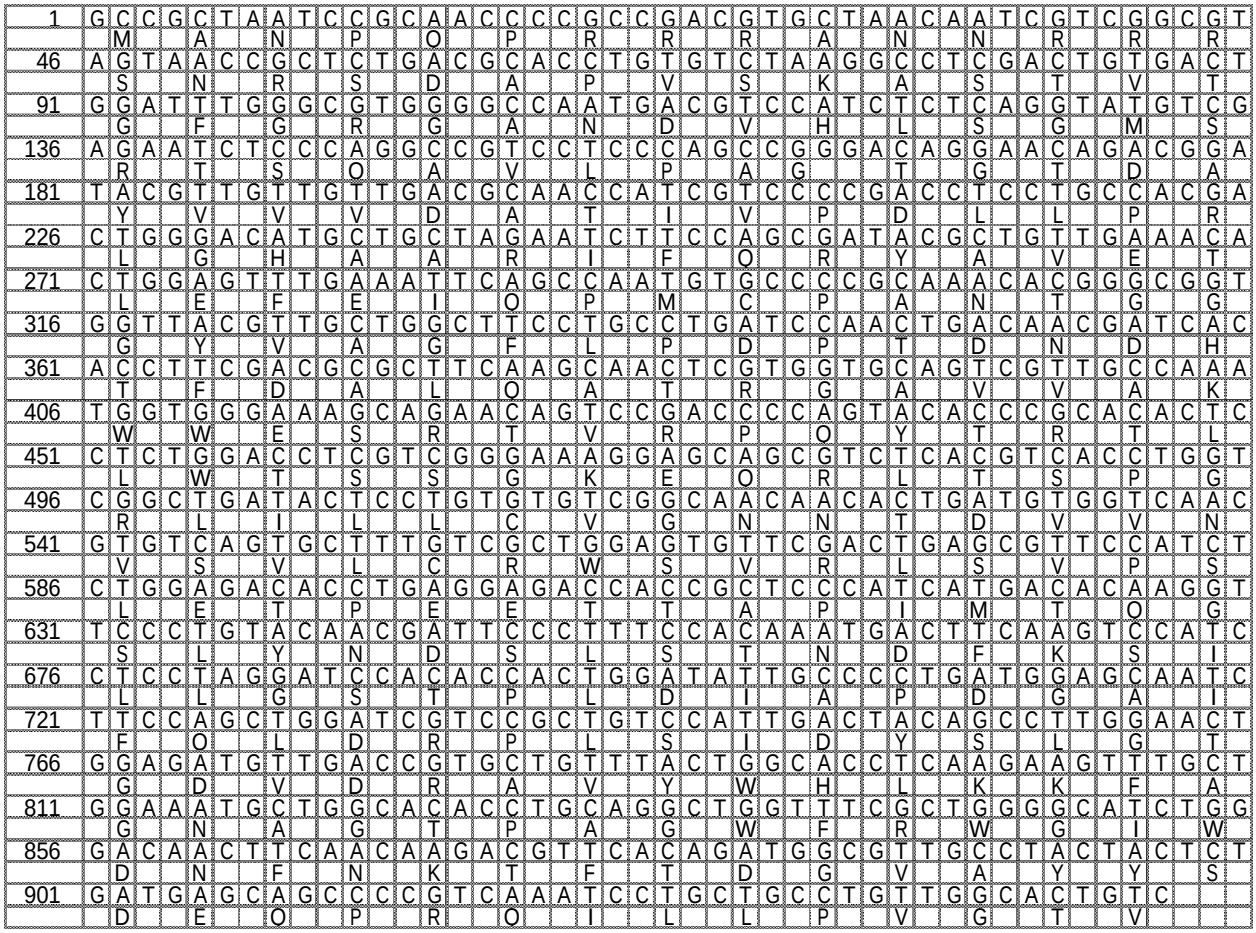


Figure 4. Partial coding sequence (partial cds) of gene encoding NNV-RNA2.

Table 2. RPS value of vaccinated-hybrid grouper "Cantik" after challenged with NNV-inocula

Treatments	Method of inactivation	Number of trial	Seven days post-vaccination			14 days post-vaccination			21 days post-vaccination		
			Number of fish	Mortality (%)	RPS	Number of fish	Mortality (%)	RPS	Number of fish	Mortality (%)	RPS
E. coli contained protein recombinant of RNA2 NNV	Mechanical	1	20	45	40.0	20	60	20.0	20	80	-
		2	10	80	20.0	10	70	-	10	80	-
	Formalin	1	20	35	53.33	20	55	26.67	20	75	6.25
		2	10	60	40.0	10	60	14.29	10	90	(12.5)
E. coli contained protein recombinant of RNA2 NNV	Mechanical	1	20	50	33.33	20	65	13.33	20	80	-
		2	10	80	20.0	10	70	-	10	80	-
	Formalin	1	20	60	20.0	20	60	20.0	20	75	6.25
		2	10	70	30.0	10	60	14.24	10	100	(25.0)
PBS as control	-	1	20	75	-	20	75	-	20	80	-
		2	10	100	-	10	70	-	10	80	-

formalin-inactivated vaccine (Table 3). The percentage of fish mortality on both vaccinated groups after being infected with the NNV at 2 weeks after the booster vaccine was significantly different ($P<0.05$) with control. The percentage of fish mortality in the both of vaccinated groups increased at 4 and 6 weeks after the booster, but it was still significantly different ($P<0.05$) with the control. These results indicated that the booster increased the immunity of hybrid grouper against VNN.

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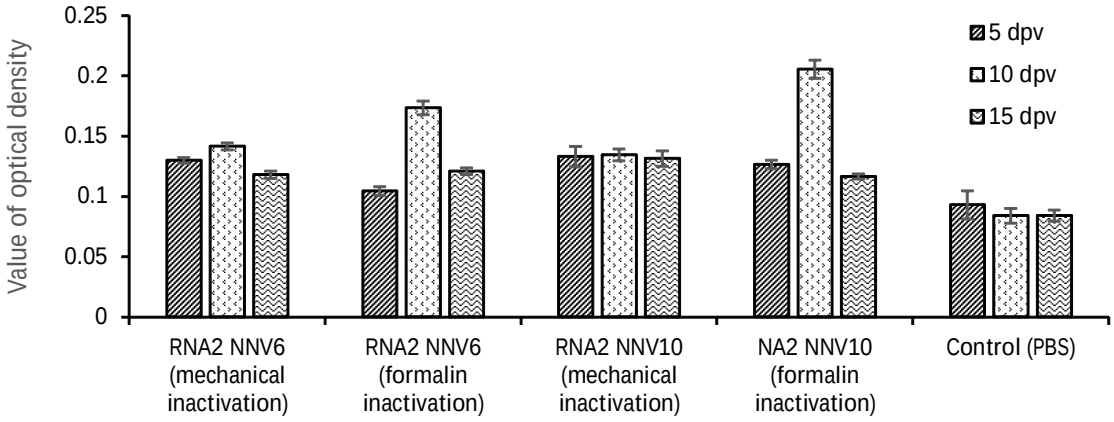


Figure 5. The value of OD (optical density) ELISA for blood serum of vaccinated hybrid grouper "Cantik."

Table 3. Average mortality of vaccinated-hybrid "Cantik" grouper following challenged with NNV at 2, 4, and 6 weeks post booster

Treatments	Two weeks post booster	Four weeks post booster	Six weeks post booster
E. coli contained protein recombinant of RNA2 NNV (isolate 6)	47.62 ± 8.25[a]	66.67 ± 8.25[a]	66.67 ± 8.25[a]
E. coli contained protein recombinant of RNA2 NNV (isolate 10)	57.14 ± 14.29[a]	80.95 ± 8.25[b]	61.90 ± 8.25[a]
PBS (as control)	85.71 ± 14.29[b]	100 ± 0[c]	85.72 ± 0[b]

Note: Values in the same column with the same superscripts are not significantly different (P > 0.05)

This study selected the production of recombinant NNV protein due to the experienced difficulty in producing viral vaccines originating from the results of viral propagation in cell culture and the multiplication of viruses in fish (Mahardika & Mastuti, 2015). Although the initial production costs of making recombinant vaccines are very expensive, the cost of mass production of these types of vaccines is cheaper, and the use of viral proteins is safer than the use of whole-body viruses (Clark & Cassidy-Hanley, 2005; Mahardika *et al.*, 2018). Kim *et al.* (2015) also reported the efficacy of recombinant protein vaccines based on CFPS (Cell-free protein synthesis). This recombinant protein (rNNV-CP) was successfully expressed and purified using CFPS. Administering rNNVCP at a dose of 20 μg/fish successfully protected seven band groupers from NNV infection. The expression of NNV coat protein in tobacco chloroplasts has also been successfully carried out for oral vaccines to induce immunities in seven band grouper. A plant-based vaccine was effectively induced immune response and protected groupers against NNV (Cho *et al*., 2018). The application of recombinant capsid gene NNV, which namely r-FNCP vaccine mixed with complete adjuvant at a dose of 50 μg per a fish, was able to protect Asian sea bass from nodavirus infection resulting on RPS value of 76% (Vimal *et al.,* 2014). Vaccination with an oil-emulsified recombinant SJNNV (rT2) capsid protein could reduce the death of turbot fish against NNV infection with a value of RPS up to 83% (Húsgaro *et al*., 2001). Recombinant proteins (recAHNV-C) vaccine was also reported to provide better protection against NNV virus infections in turbot juvenile fish (RPS up to 67%) than that of pAHNV-C DNA vaccines (Sommerset *et al*., 2005). This recombinant protein vaccine could also be applied together with other recombinant protein vaccines, such as the iridovirus recombinant protein vaccine (Mahardika *et al*., 2018). Both of these recombinant vaccines (bivalent vaccines) were reported to be able to induce MHC class 1 from the larva of the coral trout grouper (*Plectropomus leopardus*). Bivalent vaccines were also reported to be able to increase the anti-NNV antibody serum titer more than one month after the vaccination (Huang *et al*., 2017).

Our results showed that the recombinant protein vaccine could only protect hybrid groupers up to one month after the booster vaccine. It was indicated by the mortality of fish that was not significantly

different ($P > 0.05$) after the challenge test with the NNV virus at 6 weeks. This indicated that the vaccine was not enough to make and maintain a specific immune response until 6 weeks after vaccination. Similar results were also reported by Husgaro *et al.* (2001), where the mortality of vaccinated turbot fish was higher than that of the control group at 5 weeks after vaccination.

CONCLUSIONS

Protein recombinant NNV RNA 2 vaccine has been successfully produced. The application of *E. coli* bacteria carrying recombinant NNV RNA2 protein inactivated using formalin method able to increase the antibody titers resulting in higher RPS values compared to the inactivation of *E. coli* bacteria through the mechanical method. The booster used on day 10 was able to reduce fish mortality ($P < 0.05$) up to 6 weeks after the vaccine administration. Overall, the recombinant NNV RNA2 protein can be used as a vaccine to prevent grouper mortality from NNV virus infection. This recombinant vaccine has the potential to be developed into a bivalent or polyvalent vaccine by combining viral and other bacterial vaccines in future research.

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