

## THE EFFECTS OF CRUDE RECOMBINANT VIRAL PROTEIN VACCINES AGAINST GROUPEE SLEEPY DISEASE IRIDOVIRUS (GSDIV) ON HUMPBACK GROUPEE (*Cromileptes altivelis*)

Ketut Mahardika and Indah Mastuti

Institute for Mariculture Research and Development

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### ABSTRACT

Infection of *Megalocytivirus* cause serious mass mortality in marine fish in South East Asian countries. The aim of this study was to produce recombinant of GSDIV capsid protein and its protection to humpback grouper *Cromileptes altivelis* against grouper sleepy disease iridovirus (GSDIV). A major capsid protein (MCP) was selected for use as a crude subunit vaccines. This gene target (MCP) was inserted to the protein expression system vector of pET SUMO and cloned in cells bacteria *Escherichia coli* strain BL-21. The MCP was succeeded to be induced using 1 mM of IPTG. Results of protein analysis using MALDI TOF-TOF indicated that the MCP has measurement of 49.566 kDa with PI index of 6.00, and contained 453 amino acids. BLAST homology analysis exhibited that the amino acid sequence of the MCP showed high similarity with MCP of Red Sea Bream Iridovirus (RSIV). *E. coli* expressing MCP protein was inactivated using 0.03% formalin overnight and washed using PBS. The inactivated *E. coli* as a crude subunit vaccine was then injected intramuscularly to humpback grouper juveniles. Subsequently, the juveniles were challenged tested with GSDIV. The juveniles vaccinated with the MCP recombinant bacteria showed significantly higher survival rates than control those vaccinated with PBS. Thus, the MCP fusion protein is considered as a potential vaccine against GSDIV infections in grouper.

**KEYWORDS:** humpback grouper, GSDIV, protein recombinant, vaccine

### INTRODUCTION

In Indonesia, *Megalocytivirus* have been reported to infect and caused mass mortality in marine fish such as orange-spotted grouper *Epinephelus coioides* (Koesharyani *et al.*, 2001; Mahardika *et al.*, 2001; 2003), humpback grouper *Cromileptes altivelis* (Mahardika *et al.*, 2004), tiger grouper *E. fuscoguttatus*, malabar grouper *E. polyphkadion*, coral trout grouper *Plectropomus indicus* (Mahardika *et al.*, 2009b), sea bass *Lates calcarifer* (Mahardika & Mastuti, 2010), and cobia *Rachycentron canadum* (Mahardika, personal communication). Recently, this disease is supposed to cause "drop scale disease" in barramundi in Batam island (Sunarto, personal communication). *Megalocytivirus* are also reported to infect freshwater fish such as dwarf gourami *Colisa lalia*, African lampeye *Apocheilichthys normani* cultivated in Sumatra and exported to Japan via Singapore (Sudthongkong *et al.*, 2002a), gourami *Osphronemus gourami* and tilapia *Osteochillus hasselti* (Koesharyani, personal communication).

An effective and efficient drug for treatment of viral diseases has not been found. Antibiotics and vitamins were used to prevent secondary infections and boost the general immune system of fish. Furthermore, addition of omega interferon and liquid filtration of the spleen from diseased fish on RSIV pure virus (*in vitro*) has not been able to inhibit the proliferation of the virus in cells (Mahardika & Miyazaki, 2009). Therefore, an early prevention using a specific vaccine for the *Megalocytivirus* disease is required. Prevention of *Megalocytivirus* infections has been successfully carried out by administration of inactivated vaccine and DNA-RSIV vaccine (Nakajima *et al.*, 1997; 1999; Caipang *et al.*, 2006). Vaccination has also been successfully performed on humpback grouper in preventing GSDIV infection by commercial vaccine (Biken, Japan) (Mahardika *et al.*, 2008). However, the price of the vaccine is very expensive which is imported from Japan. Vaccinations using inactivated GSDIV virus which isolated from diseased fish organs or virus resulting from proliferation in cultured Grunt Fin (GF) cells have been successful in reducing the mortality of grouper to GSDIV viral infections in a laboratory (Mahardika *et al.*, 2010; unpublished data). However, the crude GSDIV virus from

# Correspondence: Institute for Mariculture Research and Development. Jl. Br. Gondol, Kec. Gerokgak, Kab. Buleleng Kotak Pos 140, Singaraja-Bali 81101, Indonesia.  
Phone: + (0362) 92272  
E-mail: [info.gondol@gmail.com](mailto:info.gondol@gmail.com)

diseased fish organs are not reliable continuously in large quantities and or GSDIV virus cannot develop optimally on GF cells characterized by the virus titer that is still very unstable. Therefore, it is required to apply recombinant technology methods for mass supply of GSDIV vaccine.

Production of vaccine is currently supported by the technology of recombinant proteins, by inserting the virus genes to be expressed in the host cell. This technology provides several advantages i.e. it does not require viral culture, the possibility to select specific proteins, and economically of mass-scale production. Institute for Mariculture Research and Development (IMRAD), Gondol was obtained a DNA library GSDIV capsid protein gene, and constructed this gene to the expression vector. The targetted gene was chosen based on previous study that the MCP gene is conserved region and plays a role in pathogenicity of the megalocytivirus virus (Sudthongkong *et al.*, 2002b; Shimmoto *et al.*, 2010). The aim of this study was to produce a recombinant capsid protein GSDIV and its protection to hump-back grouper against GSDIV infection.

## MATERIALS AND METHODS

### *E. coli* Strain, Virus Stock and DNA Preparation

*Escherichia coli* strains MACH1™ -T1R and BL21(DE3) were obtained commercially (Invitrogen, Cat. No. C8620-03 and C6000-03). The pET SUMO vector (Invitrogen, Lot. no. 841075) has been described previously (Lee *et al.*, 2008). *E. coli* manipulations were performed according to the manufacturers instructions. The standard DNA and protein analysis were carried out as described by Sambrook *et al.* (1989).

The virus was obtained from the spleen and kidneys of naturally infected fish. Spleen and kidneys were homogenized in nine times of the volume of EMEM-2 and centrifuged at 3,000 rpm for 15 minutes. The supernatant was filtered at 0.45 µm<sup>2</sup>. The filtrate is stored in -80°C and used as a source of the DNA template, vaccine trials, and propagation of the virus in healthy fish.

### Construction of Bacterial Expression Vector

Viral DNA was extracted from the virus pellet using a lysis buffer according to the protocol (IQ2000™ kit). Amplification of genomic DNA using Go Taq PCR Core System I kit (Promega, Cat. M7660) with the primer design of DNA sequences capsid protein MCP RSIV (AB666334.1) with sequences 5' ATGTCTGMRATCTCAGGTGCRAACG'3 (MCPBP), and 5' TTACAGGATAGGGAAGCCTGCAGCG '3 (MCPFP) with a weight of 1,362 bp. The MCP gene amplifica-

tion was carried out in 30 cycles (denaturation temperature of 94°C for 1 minute, annealing temperature of 55°C for 1 minute and extension temperature of 72°C for 1 minute for one cycle), followed by denaturation temperature of 94°C for 1 minute and 72°C during the final extension for 7 minutes, and the cooling temperature of 4°C for 5 minutes. The resulting amplicons were visualized in agarose gel 1.5 % in TAE 1x.

PCR product was purified using a PCR purification kit (Roche, Cat. No. 11732668001) and visualized in agarose gel (1.5 % in TAE 1x) to get a purify and single band. The purified DNA was then ligated to the vector pET SUMO (Champion™ pET SUMO, Invitrogen) according to the protocol, briefly 2 mL of PCR product was mixed with 1 mL of 10x ligase buffer, 2 mL of vector pET SUMO, 1 mL of T4 DNA ligase and sterile water to a total volume of 10 mL, and then incubated at 15°C overnight. The ligation product was transformed in bacteria *Escherichia coli* strain Mach1™ -T1R.

The process of transformation was done by heat-shock method, i.e. incubating 2 mL of mixture ligation and 50 mL of competent cells in crushed ice for 20 minutes, then heated in a water bath temperature of 42°C for 40 seconds and then quickly moved in ice to be added to 250 mL of growth medium SOC (super optimal catabolite expression). The transformation solution was grown for 1 hour at 37°C with a speed of 200 rpm in automatic incubator shaker. Result of the growth of these cells was cultured in LB agar medium containing kanamycin 50 mg/mL. Subsequently, any colonies that grew were taken to be grown in 10 ml LB broth media containing kanamycin 50 mg/mL. As a stock, a total of 0.85 mL of LB broth containing bacteria was mixed with 0.15 mL of sterile glycerin and stored at -80°C. Colonies that can grow in LB broth containing kanamycin 50 mg/mL indicated that the competent cells carrying a recombinant plasmid. To determine the orientation of insertion of the targetted gene was done by PCR and gene sequence analysis. The recombinant plasmid was extracted using a plasmid minispin prepease kit (USB, Lot. 1104/001). The PCR amplification to determine the orientation of the insertion of genes MCP (SUMO primer pairs forward and MCPBP) was conducted in 30 cycles (denaturation temperature of 94°C for 1 min., annealing temperature of 55°C for 1 min. and extension temperature of 72°C for 1 min. for one cycle), continued to the denaturation temperature of 94°C for 1 min, and a final extension of 72°C for 7 min., followed by storage temperature of 4°C for 5 min. The correct orientation will result in DNA amplicon size of 1,465 bp (according to criteria in pET SUMO kit).

### Protein Expression

Plasmid which has been known to carry the targeted gene with the correct orientation was then transformed into competent cells of *E. coli* strain BL21. Transformation by heat-shock method was performed by incubating the recombinant plasmid and 50 mL BL21 in crushed ice for 30 minutes, then heated in a water bath with temperature of 42°C for 40 sec. and quickly moved into the ice to be added to 250 mL of SOC growth medium. Result of the transformation was grown for 1 hour at 37°C with a speed of 200 rpm. Transformant was grown in LB agar medium containing kanamycin. Any colonies that grew were cultured in 10 mL of LB broth media containing kanamycin for the purpose of analysis and stock.

Expression of the protein was done by inducing BL21 culture that has reached the density of  $10^8$  cfu/mL which determined by the absorbance value at OD<sub>600</sub> to 1.5. A total of 10 µL stock BL21 cultures were grown in 70 mL of LB broth containing kanamycin of 50 mg/mL with shaking of 125 rpm at 37°C. Sufficient density was achieved after 48 hours. Then, 0.1 mM of IPTG was added into cultured BL21 to induce protein. Induction conducted overnight. Induction results were divided into 20 mL and 50 mL to protein analysis and used to crude inactivated subunit vaccine.

The presence of protein expression in BL21 was confirmed by SDS-page. A total of 20 mL cultured BL21 that had been induced with IPTG was centrifuged at 6,000 rpm for 10 min. Pellets were then lysed enzymatically with lysozyme and mechanically with freeze-thawing method and sonication. Enzymatic lysis was performed by incubating 100 µL of pellet with 150 µL of 200 mM lysozyme at 37°C for 5 min. Then, these cells were lysed with freeze-thawing and sonication (freeze at -80°C and melt at a temperature of 42°C for 30 times, and then sonicated for 4 x 15 minutes). The cell solution was centrifuged at maximum speed for 1 min. A 10 µL of pellet and 25 µL of supernatant were taken for the SDS-page analysis.

SDS-page sample was prepared by adding 1x sample buffer (1.25 mL of 0.5 M Tris-CL pH 6.8, 1 mL of 100% glycerol, 0.2 mL of beta-merkaptoethanol, 0.01 g bromo phenol blue, 2 g SDS, and sterile distilled water to a total volume of 10 mL) into pellet and 2x sample buffer into the supernatant with an equivalent volume. The mixture solution was heated in boiling water for 5 min. and cooled at room temperature. The sample was then injected into 12% of porous gel polyacrylamide that had been prepared. On one hole of gel was injected with 10-200 kDa protein marker (Bio Basic Inc., Lot. 1203SH00072277R2) to determine the molecular weight of the protein. Elec-

trophoresis was performed in a buffer containing glycine for 45 min. at a voltage of 200 volts. Gel electrophoresis was stained with comassie brilliant blue (CBB) for one hour. The excess of gel color was reduced by soaking in the decolorizer solution (7 mL of glacial acetic acid, 7 mL methanol, and 86 mL of distilled water) for 30 min. with a shaker and subsequently allowed to stand until one night.

### DNA Sequence Analysis

DNA sequencing was conducted to determine the DNA sequence of the gene MCP-GSDIV virus. The samples used were the result of amplification of vectors that have been inserted by MCP gene in the correct orientation. PCR product was then purified by PCR purification kit (Roche). Samples were sequenced using the services of 1st BASE provider (Singapore). DNA sequences were analyzed by BLAST to know its homology, and ORF Finder for prediction of nucleotide acids.

### Protein Analysis

The function of the target gene in expressing the protein was confirmed by sequenced of protein production. The sample used for the sequencing was a piece of band in gel electrophoresis-polyacrilamide that showed over-expression. Protein sequencing through a service provider (proteomics International Pty Ltd, Australia) which uses the technique of protein digestion with trypsin, and the resulting peptides were analyzed by MALDI TOF-TOF (matrix-assisted laser desorption/ionization mass spectrometry time of flight-time of flight) tandem mass spectrophotometer (4,800 proteomics analyzer, AB Sciex). Protein identification using Mascot software based on data Ludwig NR. Results obtained from the provider reanalyzed by BLAST to know its homology.

### Crude Protein Subunit Vaccine Preparation

A 50 µg/mL cultured of *E. coli* strain BL21 that had been induced and known to express proteins (by SDS-page) was inactivated by adding formalin to a final concentration of 0.03%. Formalin inactivation was carried out for one night at room temperature. Formalin was cleared by centrifugation at 6,000 rpm for 5 min. and the pellet was lysed by sonication (4 x 15 min.). The pellet was diluted into obtain the density of bacteria that was equivalent to the initial density ( $10^8$  CFU/mL).

### Vaccination Trials

Vaccine trials were done using juveniles of hump-back grouper with total length of 6-8 cm. The vaccine was given by intramuscular injection with a dose of 0.1 mL/fish, while control group was given PBS



(negative control) or CAT (positive/protein control). Vaccine trials conducted in laboratory scale (15-20 fish in aquarium with volume of 100 L) and a semi-mass scale (100 and 150 fish in a floating net at 1 m x 1 m x 0.75 m, which floated in concrete tank). The vaccine is given as a single and booster with intervals of 7 days. GSDIV virus challenge test was done after 14, 20, 30 days from the last vaccination. Effectiveness of vaccine was determined by the value of the relative percentage survival (RPS) after challenged with the GSDIV virus which has a 100% lethal dose. The data of challenge test results were presented in the form of percent mortality and RPS (percent survival ratio) with the formula:

$$RPS = \frac{(1 - \% \text{ mortality of treatment})}{\% \text{ mortality of control}} \times 100$$

## RESULTS AND DISCUSSION

### Amplification of Gene Target

Target genes which obtained from the spleen of diseased fish could be amplified with primers that were designed based on the gene RSIV (red sea bream iridovirus) in the region of the MCP. Optimization of amplification conditions could produce amplicon with the correct size of 1,362 bp (Figure 1). This result indicated the gen GSDIV in the region of the MCP have similarity with the gen RSIV. The result is resembled with a previous research which claimed that the ATPase gene and the MCP gene of SBIV (sea bass iridovirus), RSIV (red sea bream iridovirus), GSDIV (grouper sleepy disease iridovirus), ALIV (African lampeye iridovirus), and DGIV (dwarf gourami

iridovirus) were highly homologous (> 95.8%, > 94.9% identity) (Sudthongkong *et al.*, 2002b).

### Cloning and Gene Expression

Some colonies of *E. coli* strain Mach1 were known to carry the recombinant plasmid with the insertion of targetted gene into the vector of pET SUMO. It was shown from the result of a target gene amplification using primer vector and primer target gene. The amplicons exhibited 150 base pairs (total 1,512 bp) longer than the target genes due to the use of primary vector (Figure 2).

Recombinant plasmid was expressed the protein in cells *E. coli* strain BL-21 with induction of 0.1 mM of IPTG. Results of electrophoresis with gel SDS-page indicated that molecular weight of the recombinant MCP was 60 kDa (Figure 3). MCP is comprised of the outer capsid proteins with a molecular weight of ~ 50 kDa. The MCP gene sequence is conserved regions in members of family *Iridovirus*. It could be used in basic detection of viral particles in the family of *Iridovirus* (Chincar *et al.*, 2011).

The results of proteins electrophoresis of gel polyacrylamide was known recombinant proteins (marked with thick band/over expression) parallel to the 60 kDa (Figure 3). This was the effect of the fusion protein SUMO (small ubiquitin-like modifier). This was based on the results of the digestion of proteins from the process of protein analysis by MALDI TOF-TOF which proves that overexpression of MCP protein carrying the 12.1 kDa of protein of *Saccharomyces cerevisiae* and MCP-GSDIV protein (49.566 kDa). The aim of selecting the expression plasmid with

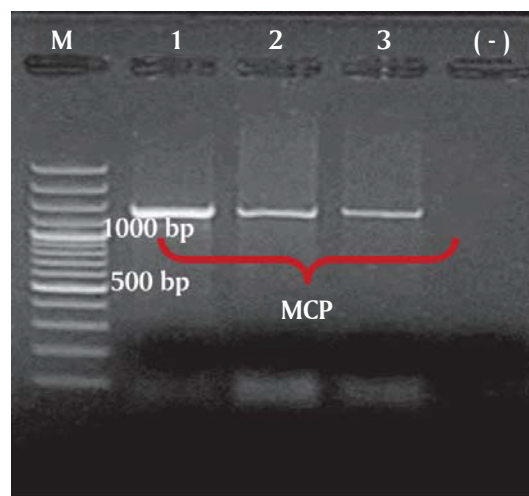


Figure 1. MCP-targetted gene in agarose gel shows the expected amplicon size (1,362 bp.). M: 100 bp DNA ladder, (-): control negative

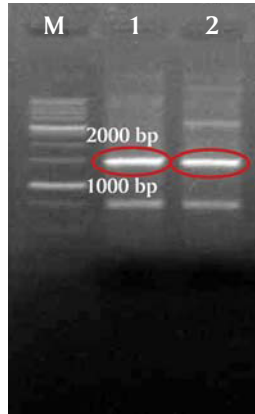


Figure 2. PCR confirmed a recombinant plasmid for correct orientation using plasmid forward primer and targeted gene backward primer. M: 100 bp DNA ladder

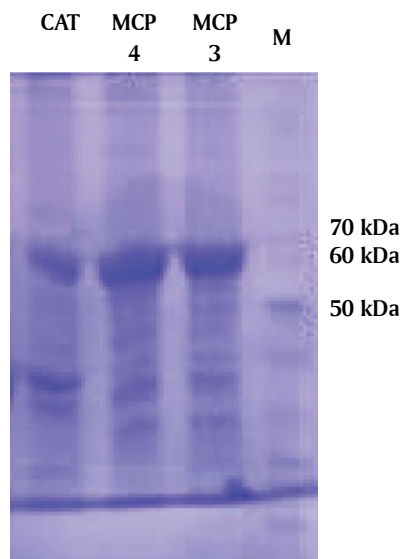


Figure 3. SDS-page gel shows protein expression of MCP gene

SUMO fusion was to improve the solubility of expressed proteins that was easy to be isolated (Der *et al.*, 2008). Further expected protein could be obtained by partially solving of cells through sonication to be used as a crude subunit vaccine.

**Sequence of the DNA Gene Target**

MCP gene sequencing was obtained partial nucleotide sequences (partial cds), i.e. 1,226 of 1,362 nucleotides (Figure 4). BLAST homology analysis showed that the gene MCP-GSDIV have similarity up to 99% with the other isolates *Megalocytivirus* as shown in Table 1. Sequences producing significant alignments:

MCP-encoding gene was known with size of 1,362-1,372 bp and encodes 454 amino acids. The gene revealed a highly identical (> 97.2% identity) with some isolates of *Megalocytivirus* namely SBIV, RSIV, GSDIV, ALIV, DGIV (Sudthongkong *et al.*, 2002b).

**Sequence of Protein**

Peptide analysis with MALDI-TOF-TOF showed that recombinant MCP-GSDIV expressed a protein fusion MCP and modification protein from vector. The molecular size of MCP was 49.566 kDa with PI value of 6.00, while the modification protein of vector was 12.1 kDa (Figure 5). Peptide identification based on

Table 1. Some isolates *Megalocytivirus* that have similarities with gene MCP-GSDIV

Accession	Description	Maximal score	Total score	Query coverage	E value	Max ident
<a href="#">JQ253372.1</a>	Megalocytivirus Sabah/RAA/2012 strain OSGIV75 major capsid protein gene, complete cds	<u>2254</u>	2254	99%	0.0	99%
<a href="#">HQ317465.1</a>	Infectious spleen and kidney necrosis virus isolate ISKNV-DW major capsid protein (mcp) gene, complete cds	<u>2254</u>	2254	99%	0.0	99%
<a href="#">AF371960.1</a>	Infectious spleen and kidney necrosis virus, complete genome	<u>2254</u>	2254	99%	0.0	99%
<a href="#">AY285745.1</a>	African lamprey iridovirus major capsid protein gene, complete cds	<u>2248</u>	2248	99%	0.0	99%
<a href="#">AB109369.1</a>	Dwarf gourami iridovirus gene for major capsid protein, complete cds	<u>2231</u>	2231	99%	0.0	99%

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11                                     gggtttatcga
22 atctccgcggtttgatgcatggagaccacttgtagcggcgcgac
   I S A F D A M E T H L Y G G D
67 aatgccgtgacctactttgcccgtagaccgtgtagttcctgg
   N A V T Y F A R E T V R S S W
112 tacagcaaaactgcccgtcacctgtcaaacagactggccatgcc
   Y S K L P V T L S K Q T G H A
157 aattttgggcaggagttagtgtagcgggtggcgagggcgcgac
   N F G Q E F S V T V A R G G D
202 tacctattaatgtgtgctgctgtaagatcccctccatcaca
   Y L I N V W L R V K I P S I T
247 tccagcaaggagaacagctacatccgctggtagcaaatctgatg
   S S K E N S Y I R W C D N L M
292 cacaatctagtgaggagggtgctcgggtcattaacgacctggg
   H N L V E E V S V S F N D L V
337 gcacagaccctcaccagcagttcctgactctggaacgcctgc
   A Q T L T S E F L D F W N A C
382 atgatgcccgagcaaacagctctggctacaacaagatgattggc
   M M P G S K Q S G Y N K M I G
427 atgcgacgcacctggtggccggcatcaccaacggccagactatg
   M R S D L V A G I T N G Q T M
472 cccgctctacctaatttggccattcccctcttcttaccctg
   P A V Y L N L P I P L F F T R
517 gacacgggcctggcgttgctaccgtgtctctgccgtacaacgag
   D T G L A L P T V S L P Y N E
562 gtgcatccacttcaagctcggcgctgggaggacctgctcatc
   V R I H F K L R R W E D L L I
607 agccagagcaaccagccgacatggccatcgcacctcacctg
   S Q S N Q A D M A I S T V T L
652 gctaacattggcaatgtagcaccgcactgaccaatgtgtctgtg
   A N I G N V A P A L T N V S V
697 atgggcacttacgctgtactgacaagcaggagcgtgaggtggg
   M G T Y A V L T S E E R E V V
742 gccagctctagctgtagcatgctcattgaacagtgccaggtggcg
   A Q S S R S M L I E Q C Q V A
787 cccgctgtgcccgtcaccgcccagacaattcttggtagctctg
   P R V P V T P A D N S L V H L
832 gacctcagggtcagtcaccccggaagccttgttcttggcagta
   D L R F S H P V K A L F F A V
877 aagaacgtcaccaccgcaacgtgcaagcaactacaccgcgcc
   K N V T H R N V Q S N Y T A A
922 agtcccgtgtatgtcaacaacaaggtgaatctgccattgatggcc
   S P V Y V N N K V N L P L M A
967 accaatccccctgtccgaggtgtcactcatttacgagaacaccct
   T N P L S E V S L I Y E N T P
1012 cggctccaccagatgggagtagactacttcacatctgtcgacccc
   R L H Q M G V D Y F T S V D P
1057 tactactttgcgccagcatgcctgaaatggatgggtttatgacc
   Y Y F A P S M P E M D G V M T
1102 tactgtatatacgttggacatgggcaatatcaacccatgggttca
   Y C Y T L D M G N I N P M G S
1147 accaactacggccgctgtccaacgtccccctggcatgtaagggg
   T N Y G R L S N V P L A C K G
1192 tcggacaatgcaaagaccaccccgggcgggcgggcggaacggc
   S D N A K T T P A G G G G N G
1237 tccggtacacggtggcccaa
   S G T R W P
    
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Figure 4. Partial coding sequence (partial cds) of gene encoding MCP-GSDIV

the its spectra showed that MCP was completely expressed and the peptide sequence of MCP was similar to MCP has MCP-LYCIV (large yellow croake iridovirus) and MCP-GSIV (giant Seaperch iridovirus). This was supported by the results of BLAST analysis that showed 99% similarity with MCP of red sea bream iridovirus (gi | 29,467,056 | BAC66968.1), MCP-infectious spleen and kidney necrosis virus (gi | 315 454 520 | ADU25248.1), MCP-grouper sleepy disease iridovirus (gi | 30,909,113 | AAP37443.1), MCP-infectious spleen and kidney necrosis virus (gi | 57,233,193 | AAW48183.1).

MCP gene was expressed completely, producing peptides with amino acid number of 453. The amino acid sequence was similar to the MCP-RSIV (gi | 29,467,056 | BAC66968.1). These results are resembled with MCP-Iridovirus gene sequences isolated from the South China Sea and South East Asian countries. The MCP-Iridovirus gene consists of 1,362 and 1,372 base pairs and encodes a 454 amino acid (Sudthongkong *et al.*, 2002b). The analysis of the nucleotide and amino acid sequences had visible result of mutual support. Therefore, it can be concluded that the targetted gene expressed the expected protein.

### Vaccination Test on Humbuck Grouper

Vaccine indicated that the MCP gene was potential to induce fish immunity against GSDIV. In a laboratory scale test (Table 2) with challenged by isolates GSDIV that has a 100% lethal dose, a crude subunit vaccine resulted a value of RPS to 54.58%. In a semi-mass scale test (Table 3), GSDIV infection does not occur naturally, thus, GSDIV infection was done experimentally at 20 fish. Results of the challenge test showed that the crude subunit vaccine achieved RPS value of 100% during the 21 days post final vaccination. However, the RPS values decreased after 30 days post final vaccination. This condition was worsened by a bacterial infection that causes tail root (Figure 6). The effects of booster vaccine with an interval of 7 days from the initial vaccine has not shown an increase in the value of RPS after challenge with the virus GSDIV after 30 days post booster vaccine.

The vaccinated fish showed different growth during the vaccination period. All groups were assumed to have same initial weight (8.86 g). The fish vaccinated by the crude subunit vaccine indicated the best growth rate with the average weight of 29.2 g and

Query	1	MSAISGANVTSGFIDISAFDAMETHLYGGDNAVITYFARETVRSSWYSKLPVTLISKQGTGHA	60
Sbjct	1	MSAISGANVTSGFIDISAFDAMETHLYGGDNAVITYFARETVRSSWYSKLPVTLISKQGTGHA	60
Query	61	NFGQEFVSVTVARGGDYLINVWLRVKIIPSITSSKENSYIRWCDNLMHNLVEEVSVSFNDLV	120
Sbjct	61	NFGQEFVSVTVARGGDYLINVWLRVKIIPSITSSKENSYIRWCDNLMHNLVEEVSVSFNDLV	120
Query	121	AQTLTSEFLDFWNACMMPGSKQSGYNKMI GMRSDLVGGITNGQTMPAAYLNLP I PLFFTR	180
Sbjct	121	AQTLTSEFLDFWNACMMPGSKQSGYNKMI GMRSDLVGGITNGQTMPAAYLNLP I PLFFTR	180
Query	181	DTGLALPTVSLPYNEVRIHFKLRRWEDLLISQSTQADMAISTVTVANIGNVTPALTNVSV	240
Sbjct	181	DTGLALPTVSLPYNEVRIHFKLRRWEDLLISQSTQADMAISTVTVANIGNVTPALTNVSV	240
Query	241	MGTYAVLTSEEREVVAQSSRSMLIEQCQVAPRPVPTPVDNSLVHLDLRF SHPVKALFFAV	300
Sbjct	241	MGTYAVLTSEEREVVAQSSRSMLIEQCQVAPRPVPTPVDNSLVHLDLRF SHPVKALFFAV	300
Query	301	KNVTHRNVQSNYTAASPVYVNNKVNLP LLATNPLSEVSLIYENTPRLHQMGVDYFTSVDP	360
Sbjct	301	KNVTHRNVQSNYTAASPVYVNNKVNLP LLATNPLSEVSLIYENTPRLHQMGVDYFTSVDP	360
Query	361	YYFAPSPPEMDGVMTYCYTLDMGNINFMGSTNYGRLSNVTLSCKVSDNAKTTAAGGGNG	420
Sbjct	361	YYFAPSPPEMDGVMTYCYTLDMGNINFMGSTNYGRLSNVTLSCKVSDNAKTTAAGGGNG	420
Query	421	tgytvaQKFELVVIAVNHNIMKIADGAAGFPIL	453
Sbjct	421	TGYTVAQKFELVVIAVNHNIMKIADGAAGFPIL	453

Figure 5. Amino acid sequence alligment with other highly homolog MCP *Megalocytivirus*

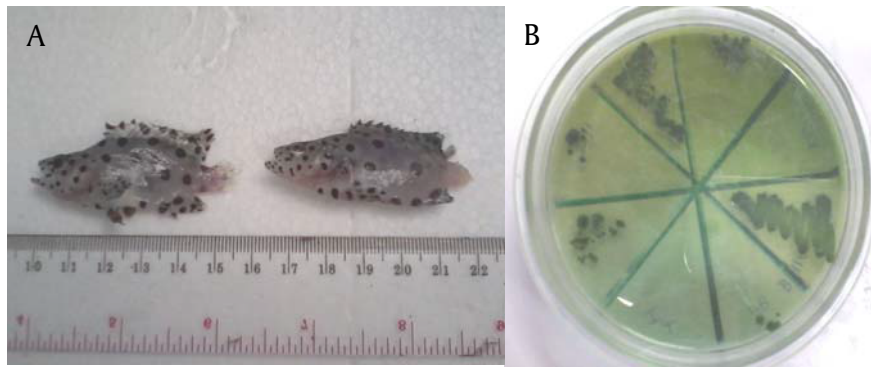


Figure 6. Bacterial infection within challenge test period at 30 day post final vaccination experiment, Bacterial infection caused fin root disease (A); bacteria grew on TCBSA (B)

Table 2. RPS value of the crude subunit vaccine at laboratory experimental scale

Treatments	14 Days post final vaccination		21 Days post final vaccination	
	Mortality (%)	RPS (%)	Mortality (%)	RPS (%)
MCP 1	40.87	54.58	75.66	24.44
CAT (+ control)	90	0	100	0,00
PBS (- control)	90	-	100	-

Table 3. RPS value of the crude subunit vaccine at semi-mass experimental scale

Trial	Treatments	Days after fin root infection		Days post final vaccination					
		Until 22 days		14 days		20 days		30 days	
		Mortality (%)	RPS (%)	Mortality (%)	RPS (%)	Mortality (%)	RPS (%)	Mortality (%)	RPS (%)
1	MCP	-	-	-	-	0	100	66.7	31
	CAT (positive/ protein control)	-	-	-	-	15	0	96.7	0
2	MCP	39.2	15	40	56	75	16.7	80	7,7
	PBS (negative control)	46	0	90	0	90	0	86.7	0

29.0 g at the end of the vaccination period. This value is higher than those of the control group which was injected by the crude protein SUMO CAT vaccine i.e. 26.7%.

Some isolates of *Megalocytivirus* have been known as a complete genome such as ISKNV (AF371960), RSIV-Ehime1 strain (BD143114, AB104413), rock bream iridovirus (RBIV) (AY532606), orange-spotted grouper iridovirus (OSGIV) (AY894343), TRBIV (GQ273492) and large yellow croaker iridovirus (LYCIV)

(AY779031). Based on the complete genome, structure of MCP RSIV-Ehime strain was known to be the genes that are common to *Megalocytivirus* (Kurita & Nakajima, 2012). Some capsid protein encoding ORF on *Megalocytivirus* genome have been developed as a vaccine, such as ORF and ORF 7L 11L which are the gene encoding the structural proteins of the capsid *Megalocytivirus* (Lua *et al.*, 2005). Instead of MCP has been used as a recombinant protein vaccine (Shimmoto *et al.*, 2010).



This study utilised a crude subunit MCP protein vaccine which was expected to modulate specific immune system controlled by recombinant MCP protein, and non-specific immune system of fish conducting by owned bacteria protein, supported by the research result of Shimmoto *et al.* (2010). MCP vaccine has the potential to induce specific immune system that reflected by the value of the RPS. In another study, the increase in specific immunity was also achieved when the recombinant RSIV vaccine was used to alleviate RSIV infection, signified by the increase in serum antibody and expression of MHC class 1 which is display intracellular proteins to cytotoxic T cells (CTLs), and also present peptides generated from exogenous proteins (Caipang *et al.*, 2006).

Results of the vaccination in the laboratory scale and or semi-mass scale showed a good protective effect in 14 days post vaccination. However, this good protective effect started to decline at days of 20-21. This result suggested that the protection period of the vaccination using subunit MCP protein vaccine by single or double (booster) application with an interval of 7 days are still short. Therefore, it is required to apply booster vaccine at 14-20 days after the first vaccination. This booster vaccine is intended to maintain high immunity level after 14 days post vaccination. In addition, Adjuvants could also be added to the vaccine to improve the efficacy and stability of the vaccine formulations to give a longer period of protection. Further research needs to be done to improve the protection period by addition of adjuvants.

## CONCLUSION

GSDIV gene encoding region of the MCP had successfully cloned into the expression plasmid. MCP gene was well expressed and the corresponding protein was obtained. Application of recombinant proteins as a crude subunit vaccine was potential to induce the immune system that reflected by the RPS value, although it provides short protection period (14-20 days).

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