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The Role of Immunogenic Adhesin *Vibrio alginolyticus* 49 kDa to Molecule Expression of Major Histocompatibility Complex on Receptors of Humpback Grouper *Cromileptes altivelis*

Uun Yanuhar

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***Abstract*—**The purpose of research was to know the role of immunogenic protein of 49 kDa from *V.alginolyticus* which capable to initiate molecule expression of MHC Class II in receptor of *Cromileptes altivelis*. The method used was *in vivo* experimental research through testing of immunogenic protein 49 kDa from *V.alginolyticus* at *Cromileptes altivelis* (size of 250 - 300 grams) using 3 times booster by injecting an immunogenic protein in a intramuscular manner. Response of expressed MHC molecule was shown using immunocytochemistry method and SEM. Results indicated that adhesin *V.alginolyticus* 49 kDa which have immunogenic character could trigger expression of MHC class II on receptor of grouper and has been proven by staining using immunocytochemistry and SEM with labeling using antibody anti MHC (anti mouse). This visible expression based on binding between epitopes antigen and antibody anti MHC in the receptor. Using immunocytochemistry, intracellular response of MHC to in vivo induction of immunogenic adhesin from *V.alginolyticus* was shown.

Keywords*—C.altivelis*, immunogenic, MHC, *V.alginolyticus*.

I. Introduction

HUMPBACK grouper is a teleostei fish with high economical value commodity in Indonesia. Humpback grouper industries often suffer from a massive disease attack caused by vibrosis. Vibrosis triggered by a group of *Vibrio* spp. One of harmful species is *Vibrio alginolyticus (V. alginolyticus)*. This bacterium is among the main disease agent in grouper aquaculture with the mortality rate reached up to 100% [1],[2].

As teleostei, humpback grouper has two body defense systems, i.e. innate and adaptive [3]. Both systems work together to detect any antigen (virus, bacteria, fungi or parasite) which enter host body and subsequently to be destroyed and eliminated from the host. B cell is the main component of adaptive defense system which responsible in synthesizing antibody. Antibody is formed after extracellular antigen entered the host and it will be presented by *antigen presenting cells* (APC) through Major Histocompatibility Complexs (MHC) class II molecule to $CD4^+$ T cell (T helper cell). Afterward, T helper cell synthesis cytokine, i,e. IL-6, to activate B cell and then form antibody [4].

MHC molecule, TCR and Ig are receptors that play a role on fish adaptive defense system, though MHC molecule is the first receptor to bind antigen to be presented to T cell. Previous research have been conducted regarding MHC expression on pathogen infection of parasite *Diplostomum pseudospathacaeum* and *Camallanus lacustris* on three-spined stickleback (*Gasterosteus aculeatus*) [5], *Aeromonas salmonicida*, *infectious salmon anaemia* (ISA) virus and *infectious pancreatic necrosis* (IPN) virus on salmon (*Salmo salar L.*) [6]; and *V. anguillarum* on Japanese flounder (*Paralichthys olivaceus*) [7]. So far, however, only a small number of researches on MHC expression toward the *Vibrio* infection on Humpback grouper have been done, especially in Indonesia. In contrary, detail research about molecule essential for immune system is crucial as valid data on DNA base sequence which code MHC molecule can be used for detecting certain fish disease. The construction of MHC single peptide is also used as a component of vaccine, conjugate of vaccine to kill autoimmune or dysfunction T cells, or for specifically labeled reagent to express specific cloned T cells [8].

MHC class II plays an important role on adaptive immune system. Also, MHC class II is a heterodimer which consist of one alpha and beta chain, both with molecular weight around 30.000 Dalton. Proteins of MHC class II are expressed by *antigen presenting cells* (APC) and play a role in presenting extracellular antigen to T helper cell which triggers humoral immune response. It has been reported that MHC present on all vertebrate except primitive fish with no jawbone [9], [10].

MHC class II has been found on APC (dendrite cell, macrophage, or B cell) and act in presenting extracellular antigens to helper 2 T cells (Th2 cell). Th2 cell, subsequently, secretes cytokine in form of interleukin-2 (IL-2), IL-4 and IL-5 that stimulate B cell to proliferate; differentiate to be plasma cell and B cell memory; and to stimulate plasma cell to

Uun Yanuhar is with the Faculty of Fisheries and Marine Sciences, Brawijaya University, Malang, East Java, Indonesia (corresponding author to provide phone: +623417354944,+6281931892262;fax:+62341553512; e-mail: uunyanuhar@ yahoo.com).

produce antibody [11]. Accordingly, study on molecules expression in regard to immune system of humpback grouper toward *V. alginolyticus* infection, especially immunogenic protein of *V alginolyticus* that trigger MHC class II expression, is indispensable. The purpose of this research was to investigate the role of adhesin protein 49 kDa as immunogenic protein of *V alginolyticus* that express MHC class II molecules on Humpback grouper receptors. The observation was conducted qualitatively on blood cell of humpback grouper.

II. Materials and Methods

Place and Time of Research:

Research was conducted on Central Laboratory of Natural Sciences – Brawijaya University, Laboratory of Biomedical and Laboratory of Microbiology – Faculty of Medicine Brawijaya University, Laboratory of Biomolecular – Faculty of Sciences Brawijaya University, and Laboratory of Aquatic Sciences and Marine Biotechnology Brawijaya University. It was carried out from February to December 2009.

Bacteria and humpback grouper blood isolation

Bacterium used was *V.alginolyticus* obtained from Laboratory of Microbiology – Faculty of Medicine Brawijaya University. 250-300 g of Humpback groupers were employed in order to obtain blood cells.

Isolation of V. alginolyticus crude protein

Isolate of *V. alginolyticus* was cultured on liquid BHI (Brain Heart Infusion) media (Difco) and incubated for 48 hours at room temperature. The culture product was then centrifuged for 4500 rpm on room temperature to obtain pellet as whole cells of bacteria. Crude protein of *V. alginolyticus* was isolated by means of homogenizing the whole cells using mortar and adding extract buffer (1:2 w/v). Homogenate was then centrifuged at 10.000 rpm (Hettich, Jerman) for 20 minutes at 4 $^{\circ}$C. Supernatant was collected as crude protein of *V. alginolyticus*. Crude protein of *V. alginolyticus* was employed with Sodium Doudecyl Sulfate polyacrylamide gel (SDS-page), while the rest was kept in freezer (-20 ^{0}C) for test.

Fractionation of crude protein

Crude protein of *V. alginolyticus* was fractionated using SDS-Page (Biorad) with the concentration of 12,5% gel for separation and track gel 4% with voltage 90 V, 400 mA, for 120 minutes using coomassie brilliant blue and standard molecule of Low Range Marker PRO-STAIN^{TM} (Fermentas). The molecule weight of protein band was calculated using linear equation of separating gel distance. Protein band of each crude protein was selected to be used as protein adhesion candidate based on major band took place and not attach with the other protein bands (non-dimer). The cutting of protein band was done with gel cutter. Cut gel was put into cellophane sack filled with running buffer solution for electroelution using horizontal protean mini electrophoresis (Biorad).

Hemaglutination (HA) test of V. alginolyticus protein adhesin

HA test of *V. alginolyticus* protein adhesion candidate was conducted according to Hanne dan Finkelstein [12]. Blood was isolated from normal grouper using 1 ml 26 GX½" spuit (Therumo), damped with EDTA 10%. The blood was then washed twice with PBS, homogenized and then centrifuged with 3500 rpm for 10 minutes. Grouper erythrocyte was then dissolved using PBS (1:200) to be used for HA test.

HA test was done using 96 wells of V-bottom micro plate. As much as 50 µl of *V. alginolyticus* protein adhesion, which have been determined its molecule weight, was put into wells and a series dilution was made using PBS up to 2^{10} (1/1024). A well contained PBS only was used as negative control. Afterward, dissolved erythrocyte was put into all wells. Microplate was then shaken and HA reaction was observed after 20 minutes. Positive reaction was evident with the absence of sediment (dot) on the bottom of wells.

Electro elution and dialisa

V. alginolyticus protein adhesion candidate on protein band was separated using electroelution method via mini protean horizontal electrophoresis (Biorad). Gel cut from each protein adhesion candidate was put into cellophane membrane and hold with 50 mm cellophane holder, and then electrophoresis 120 V, 400 mA for 60 minutes. Protein was then dialyzed in PBS solution pH 7,4 at 4^{0}C for 48 hours. Liquid on cellophane sacks was then taken and put into microtube and being precipitated by incubating it on acetone (1:1 v/v) for one night at 4^{0}C. Mixture of protein and acetone was then centrifuged at 10.000 rpm for 20 minutes at 4^{0}C. Protein obtained in form of pellet, was air dried and dissolved in 100 µl tris HCl 0,5 M, pH 8,6. Next, the measurement of protein concentration using Nanodrop Spectrophotometry was done using absorbance of 1 on 280 nm

V.alginolyticus infection to produce Serum (IgM) of humpback grouper

Aquariums with the capacity of 18 liters were used as container for bacterial *V. alginolyticus* infection on humpback grouper. Humpback grouper was infected by soaking the fish in infected water with bacteria concentration of 5 x 10^{5} cells/ml media. Each aquarium was filled with four fish. No water exchange was done to maintain bacteria concentration. Five days after infection, blood was obtained from infected fish and normal fish. Blood was taken using 26 G syringe with ½ inch needle through caudalis vena. The needle was soaked with EDTA 10% as anticoagulant. Blood obtained was put into eppendorf and then centrifuged 5000 rpm for 15 minutes. The result was clear solution at upper part (supernatant) as serum and pellet (blood cells) at tube bottom. Supernatant was then separated, put into new eppendorf and kept in freezer (-20°C) for next test.

Clinical test of Specific Protein of V.alginolyticus on humpback grouper

Specific protein of *V.alginolyticus*, which has been dialyzed to be pure protein, employed as injection material or vaccine

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for normal humpback grouper. Grouper were fed as normal condition (by catch fish 2 times/day) and added with vaccine treatment on the first day, booster on day seventh and repeated on week 2 and 3 for 3 times of booster. The expression of MHC class II on fish blood was determined by means of immunocytochemistry examination and SEM.

Specificity test of polyclonal antibody IgM of Humpback grouper - Anti Adhesin of V. alginolyticus with Dot Blot technique

Specificity test of IgM antibody of grouper toward the antigen of *V. alginolyticus* 49 kDa with secondary antibody IgM anti grouper was conducted as follows: Nitrocellulosa (NC) was soaked in PBS for 30 menit; NC was then put on chamber dot blotting. PBS skim 5% (1:10) @50µl was prepared for soaking. It was incubated at room temperature for 1 hour. Afterward, antigen of bakteri *V. alginolyticus* crude protein (protein immunogenic 49 kDa) in PBS skim 5% (1:10) @50µl was incubated for one night at room temperature.

Incubated result was blocked by PBS skim 5% for 1 hour, washed with PBS Tween 0.05% (5x5 minutes), added with primary antibody IgM of grouper fish from infected serum (1 : 50) in PBS skim 5% @50µl and being incubated overnight at room temperature. The result was washed with PBS Tween 0.05% (5x5 minutes); added with secondary antibody (IgM *antigrouper*) (1:50) in PBS skim 5% @50µl; added with chromogen NBT substrate @ 50µl; and incubated at room temperature for 2 hours. The reaction occurred was stopped with dissolving it on aquadest @50µl. Dot blot analysis was conducted by color quantification using *Corel Draw Graphics Suite X4* program. Dot blot test was repeated with the same procedure without *crude protein* adhesin of *V. alginolyticus* 49 kDa addition to observe the level of specificity of antibody produced by infected humpback grouper.

Examination of MHC class II expression with immunocytochemistry

Detection method of MHC with immunocytochemistry was done according to Nanda et al. (2008) where cell was put on cooled chuck (-20 ^{0}C) in CM3050 cryostat (Leica Corp., Deerfield, IL). Tissue (6 µm) was thaw-mounted on Superfrost Plus glass slide (Fisher Scientific, Itasca, IL). Tissue of grouper eye, brain and kidney, exposed with immunogenic protein 49 kDa of *V. alginolyticus* and normal fish, were fixated in 2% paraformaldehyde (pH 7,3) for 10 minutes, washed, incubated with 2% blocking serum (serum from horse or goat), and incubated overnight at 4 ^{0}C with primary antibody mouse monoclonal anti-MHC-II. Afterward, it was countered with secondary antibody of goat anti-mouse conjugated with biotin for 30 minutes at room temperature. Biotin was detected with avidin-biotin peroxides kit (ABC-Elite, Vector Laboratories) using diaminobenzidine tetrachloride as chromogent. Lastly, tissue cut were dehydrated with alcohol and cleaned with xylene. Tissue cut was counterstained with Gill's #3 hematoxylin (1:4), as blue counterstain of nucleus.

Examination of MHC class II expression on grouper cells using SEM

The examination of MHC class II expression was conducted by observing blood cells of grouper treated with immunocytochemistry for 24-48 hours on physiologic condition to maintain cell morphology. After incubation and cold storage at 4^{0}C, sample was ready for SEM (Scanning Electron Microscopy) examination.

III. RESULT

Profile of crude protein of V. alginolyticus

Result of the separation of *V. alginolyticus* crude protein showed 14 protein based on molecule weight (Fig. 1). 49 kDa protein possessed the highest immunogenicity. Proliferation of 49 kDa protein of *V. alginolyticus* was conducted by means of SDS-Page.

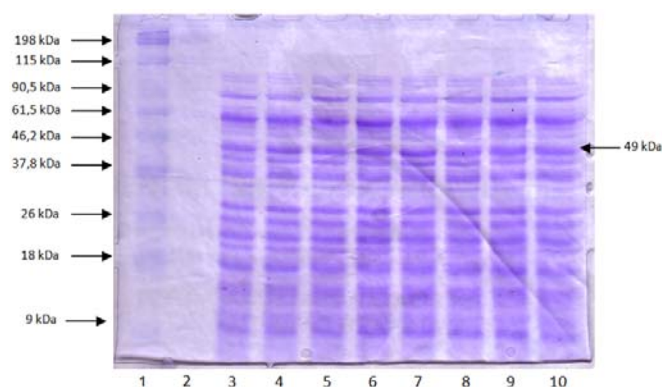

Fig.1 Separation of *V. alginolyticus* crude protein. 1: marker (ProStain), 3-10: crude protein of *V. alginolyticus*

Result of hemaglutination test of immunogenic protein 49 kDa

The result of haemaglutination test showed that protein 49 kDa *V. alginolyticus* demonstrated a high immunogenicity based on its capability to agglutinate grouper erythrocyte up to 1/4times dilution. HA test result of protein 49 kDa is presented in Fig. 2.

Dilution
1/2 1/4 1/8 1/16 1/32 1/64 1/128 1/256 1/512 1/1024 control (-)

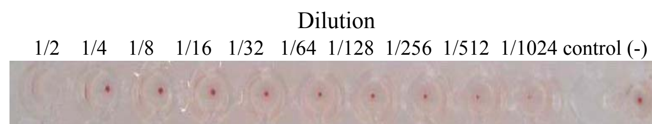

Fig. 2. Result of HA test of immunogenic protein 49 kDa from *V. alginolyticus*

Electroelution and Dialysis of immunogenic protein 49 kDa from V. alginolyticus

Protein suspension as product of electroelution and dialysis was precipitated with acetone to obtain pellet protein. Pellet was then dissolved in Tris HCl pH 6,8. Measurement of protein concentration was done using Nanodrop Spectrophotometer. The result showed that the concentration

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of measured 49 kDa protein was 1,64 mg/ml.

Result of specification of immunogenic protein 49 kDa from V.alginolyticus toward primary antibody IgM of humpback grouper on fish organ receptor with Dot Blot technique

Serum resulted from clinical test of humpback grouper, injected with immunogenic protein 49 kDa from *V.alginolyticus*, was primary antibody IgM of humpback grouper. This antibody was used in specification test of grouper IgM antibody toward immunogenic protein 49 kDa from *V. alginolyticus* and normal fish using dot blot technique. The result is shown in Fig. 3.

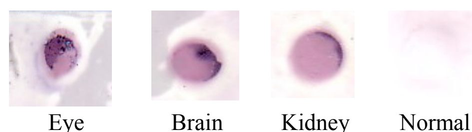


Fig. 3 Result of dot blot test of primary antibody IgM anti- protein 49 kDa from *V. alginolyticus* toward protein receptor on eye, brain and kidney of infected fish and control fish

Result of dot blot test was then analyzed quantitatively using color gradation method Corel Draw Graphics Suite X4. The result is described in Fig. 4.

Based on Fig. 4, color quantification of dot blot test result illustrated that primary antibody IgM anti 49 kDa *V. alginolyticus* showed by purple blue color on formed dot.

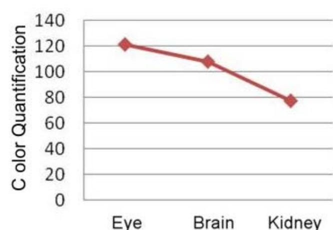
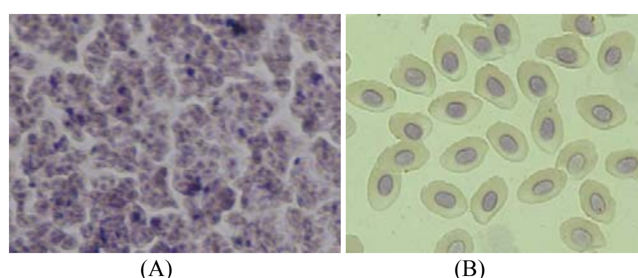


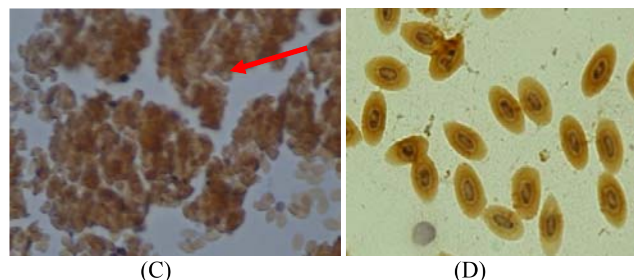
Fig. 4 Color quantification graphic of dot blot test result of primary antibody IgM of immunogenic protein 49 kDa *V. alginolyticus* on infected fish and normal fish; (color 0=black; 255=white)

Result of MHC class II examination of humpback grouper with immunocytochemistry

Result of immunocytochemistry on MHC class II expression of grouper blood cell is shown in Fig. 5.



(A) (B)

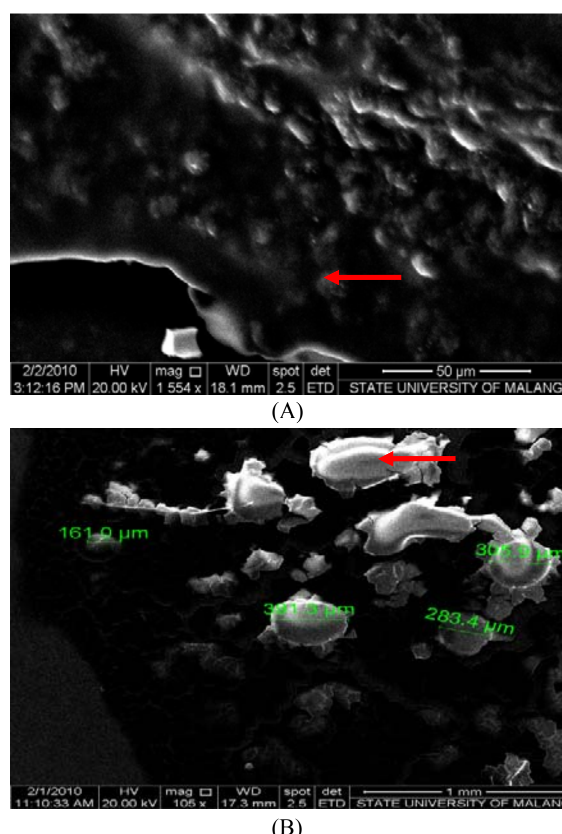


(C) (D)

Fig. 5 Photo of MHC class expression on blood cells (A: colony of normal blood cells, B: normal blood cells, C: colony of blood cells expressing MHC class II, D: blood cells expressing MHC class II) Expression of MHC II was showed by brown color as product of peroxide enzyme with chromogen (marked with arrow)

Result of examination of MHC class II expression using SEM

SEM photo illustrated MHC class II expression as shown with arrow (Fig. 6)



(A)

(B)

Fig. 6 SEM photo of blood cell of humpback grouper resulted from clinical test by injecting immunogenic protein 49 kDa from *V.alginolyticus* intramuscularly and treatment with antibody labeling anti mouse MHC-class II. A. Blood of normal fish without exposing immunogenic protein 49 kDa from *V. alginolyticus* (as shown with arrow). B. Blood of grouper exposed with immunogenic protein 49 kDa from *V. alginolyticus* expressing MHC class II (as shown with arrow)

Based on MHC class II expression of grouper tested with immunogenic protein 49 kDa from *V. alginolyticus*, it showed

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that immunogenic protein 49 kDa was able to trigger MHC expression as examined using immunocytochemistry and structure labeling of antibody using SEM. Qualitatively, MHC class II on grouper blood showed positive expression with brown color and negative expression with purple blue color. Fish blood cell labeled with antibody anti MHC class II showed thicker surface compared to normal fish blood without treatment.

IV. DISCUSSION

In normal condition, fish as well as infected fish have an adaptive body defense. Fish infected with an antigen or bacterium will give adaptive immune response by expressing MHC molecule class II and further by forming antibody produced by B cell. MHC class I or II expression are target organ dependent. MHC expression occurs on brain, muscle cell, upper and lower kidney, colon and small intestine and also gills. The lowest expression on salmon occurs on brain and muscle. Each fish has different infection level depending on fish species and infecting bacteria [14]. MHC class II molecule was also found on leukocyte cell and tumor cell and it was proven by means of RT-PCR on teleostei fish *Sparus aurata* [15].

Experiment to examine molecule MHC expression on humpback grouper has been done [2]. In order to observe MHC class I and II expression, a series of research was conducted on humpback grouper by infecting fish with Vibrio and Viral Nervous Necrotic (VNN) virus. In addition of using Vibrio (*V. alginolyticus, V. parahemolyticus, V. anguilarum, V. harveyi*) and VNN virus, adhesin protein of *V. alginolyticus* 49 kDa was employed. Adhesin protein of *V. alginolyticus* 49 kDa, an immunogenic protein with characteristic of haemaglutinin and adhesin, is able to bind erythrocyte of fish blood so that it can be agglutinated. Further examination showed that adhesin protein of *V. alginolyticus* 49 kDa was capable of expressing CD4 and CD8 cells with the highest response on brain, eye and kidney [2].

Protein of *V. alginolyticus* 49 kDa is called as immunogenic protein for it is able to stimulate immune system on grouper. Vibrio bacteria, including Enterobacteraceae, generally have 3 antigen structures, i.e.: somatic antigen (Ag O) associated with lipopolysaccharide (LPS) bacteria, capsular antigen (Ag K) associated with extracellular layer of capsule, and flagella antigen (Ag H) associated with flagellum [16]. Further explained that antigen as immunogenic (able to respond immune system) has molecule weight for more than 4000 Dalton (4 kDa). If molecule weight of antigen is less than 4 kDa, it has to be conjugated with other protein known as hapten. Thus, immunogenic protein of 49 kDa from *V.alginolyticus* is called as immunogenic protein [17].

From the expression of adaptive immune system on humpback grouper, measured qualitatively based on MHC class II expression of tested fish, it was expected that fish with size of 250 -300g were already have perfect immune system so that cells and immune molecules produced could be expressed optimally. Results of specificity test of adhesin protein of *V.alginolyticus* 49 kDa showed that the protein was capable of expressing strong antibody on eye, brain, and kidney. It was shown on dot blot test with antibody IgM anti grouper. Expression of MHC II molecule on humpback grouper, which has been clinically tested with immunogenic protein of 49 kDa, showed that MHC II was expressed on blood cell of humpback grouper through immunocytochemistry test (Fig. 5). It was convinced with the result of the examination of antibody binding anti MHC class II with SEM (Fig. 6). MHC expression caused by antigen infection was also found on salmon, where MHC class II expression occurred on gills [3]. It is evident that immunogenic protein of *V. alginolyticus* 49 kDa was able to trigger adaptive immune response of humpback grouper by expressing MHC class II on fish blood cell.

Basic theory of MHC molecule expression can be explained as follows. Each extracellular antigen which enters fish body, including *V.alginolyticus*, will be captured by APC and split into smaller molecules. The molecules are then expressed by MHC class II on APC cell surface to be presented to Th2 cell. Activated Th2 cell will produce cytokines i.e. IL2, IL4, IL6, and IL10 as signal for B cell. Activated B cell will proliferate and differentiate become plasma B cell and memory B cell. Plasma B cell play a role in secreting specific antibody for antigen, while memory B cell will express specific receptors for antigen on the surface and recognized antigen when further infection occurs [19].

Peptides bind by MHC class II are originated from two sources i.e., exogenous antigen swallowed in endocyte and endogenous self-antigen. Immature dendrite cells are able to expressed 10^6 MHC II molecules on the surface, while mature cells expressed lower than immature condition. The reasons for this phenomenon is low efficiency of most protease in degrading antigen on immature cells, and also caused by the presence of protease inhibitor such as cystatin C. The other reason is not all endosome or phagosome own access to MIICs (MHC class II compartment) as MHC class II contain peptide. Immature dendrite cell have shelf life for ≤10 hours as the cell continually consume and deliver peptide into endosome where later peptide change or degradation occur in lysosome [17].

MHC molecule is polymorphic protein, which also coded by gen located on chromosome short arm no 9. MHC is divided into two classes i.e. MHC class I which represent intracellular antigen and MHC class II which represent extracellular antigen. Most MHC class I are expressed by each nucleic cell while MHC class II is presented on APC (antigen presenting cell), mature B cell and activated T cell [19]. MHC molecule will reside on peptide expressed allele and can be detected by staining or up to gen level with T-PCR. MHC class II molecule expression on humpback grouper, triggered with induction of immunogenic protein of *V. alginolyticus* 49 kDa, is a new novelty which possessed the highest expression. Further study on gen construction of MHC class II on humpback grouper is recommended to develop medicine

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material and diagnostic for Vibrio attack, especially in Indonesia

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