

The Function of Receptor Protein Humpback Grouper *Cromileptes altivelis* in Expression and Proliferation of CD4 and CD8 cells in Defence Immunity of Viral Nervous Necrotic Infection

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Abstract—Mechanism of viral infection is not apart from role and function of grouper receptor, especially brain defence which mediated by nervous cells, heart and kidney. This research is to identify how a role and expression of CD4 and CD8 cells to defence of VNN infection in *C. altivelis*. Receptor protein in brain, heart and kidney organ from *C. altivelis* infected by VNN were isolate, identify and characterize using SDS-Page. The expression of CD4 and CD8 cells on receptor protein grouper observed by Confocal Laser Scanning Microscope (CLSM) and the response and specificity of receptor protein shown by dot blotting and western blotting respectively. Cross reactivity of specificity of organ showed by immunocytochemistry and immunohistochemistry methods. The result indicate that receptor protein which is in nervous cells on brain organ, heart and kidney organ by molecule weight 32.5 kDa is receptor protein expressed on nervous cells on brain organ, heart and kidney of *C. altivelis*. It give a response to recognize VNN infection evidenced by antibody yield which have cross reaction through dot blotting, western blotting inspection and expression of receptor showed by fluorescence color in CLSM inspection and immunocytochemistry and immunohistochemistry. Protein of nervous cells on organ of brain, heart and kidney of *C. altivelis* by 32.5 kDa is a receptor protein which have function to recognize antigen VNN and the roles for expression of CD4 and CD8 cells.

Index Terms— *C. altivelis*, CD4, CD8, VNN.

I. INTRODUCTION

Humpback grouper is a serranidae family with widespread population in tropical and sub tropical water territory and product for seafood that is very favourite in world [1]. As one of non oil export commodity, this species is the most popular among fish species living in rock and becomes important economic commodity of fishery in Asia Pacific region [2]. Furthermore, it explained that one of constraint in Humpback grouper (*C. altivelis*) cultured in Indonesia is limitation of supply of seed caused by pathogen infection causing mortality more than 80%, even till 100% [3]. Therefore, the problem needs an attention seriously and prevention because of loss resulted stress and corrupt in industrial cultured.

One of cause of industrial loss in grouper cultured is VNN attack. VNN causes retinopathy and encephalopathy which

having wide host range. It is included one of epidemic disease almost in the world and inscribed in OIE (Office International des Epizooties) [4]. VNN is reported to attack some fish species as Japanese parrot fish (*Oplegnathus fasciatus*) [5], redspotted grouper (*Epinephelus akaara*) [6], striped jack (*Pseudocaranx dentex*), Japanese flounder (*Paralichthys olivaceus*), tiger puffer (*Takifugu rubripes*), kelp grouper (*Epinephelus moara*) [7] and barfin flounder (*Verasper moseri*), barramundi (*Lates calcarifer*) [8] [9], turbot (*Scophthalmus maximus*) [10] and sea bass (*Dicentrarchus labrax*) [11].

In Indonesia, it is reported that VNN has attacked most of grouper cultured with mortality of 100%. In East Java, seeding industry also get a loss as result of VNN attack in seed stadium and even in adult (data is not publicized). The symptom is fish whirling, happened sleeping dead or fish resides in base like death and existence of unnatural fish behavior symptom [3].

Important role of defense system of grouper to VNN is receptor protein expressed at part of grouper body. VNN target on grouper are eye, brain, kidney, flesh, liver and also gill. In this research, it will be seen VNN infection in grouper brain as agent that is very endangers because VNN can weaken fish nervous system so that fish will loss nervous control, happened weakness of motion, and finally death. The role of nervous cells to VNN agent is target of protein receptor which is important to neutralize VNN besides other organ as heart and kidney that functions for circulation and osmoregulation of blood in body fish.

Something that causes the mechanism of this viral infection is the bonding between VNN adhesin and its receptor molecule in grouper organs. Viral adhesin can be in the form of viral basic component namely coat protein and nucleic acid. Coat protein of VNN is primary factor in mechanism of virus infect the host (humpback grouper) where the protein have a role in attachment of viral to host receptor. It has been known that one of adhesin of VNN is haemagglutinin [3]. Further development both adhesin and its receptor can be exploited to produce a protein material for a diagnostic tools to virus infection and so its prevention for fish cultured industry. For this purpose, the research objective is to identify how a role and expression of receptor protein from grouper brain, heart and kidney mediated by nervous, heart and nephrose cells in infection mechanism of VNN.

II. MATERIAL AND METHOD

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A. Animals

Fish samples which infected by VNN (confirmed by PCR methods) are collected from The Brackishwater Aquaculture Development Centre Situbondo (BADC/BBAP Situbondo), Indonesia. Research matter applies *C. altivelis* in stadia larva by length of 1-3 cm, 5-7 cm, 10-15 cm infected by VNN and control. Condition is conducted homogeneously between infected fish and control. Each treatment use 30 fishes in controlled sea water salinity of 30-33 ppm.

B. Isolation of brain organ of humpback grouper and VNN protein

A positive sample which infected by VNN was kept in liquid nitrogen. The brain of grouper from positive sample of VNN is isolated in laminar airflow by using a set of sterile scalpel. Brain is homogenized by sterile mortar and conducted isolation of nervous cells by adding extract buffer with ratio 2 ml: 1 mg organ. Hereinafter, homogenate was centrifuged 50000 rpm by ultra high centrifuge during 1 hour to dissociate debris from nervous cells. Nervous cells which have been degradation checked its morphology and then conducted isolation of receptor protein of nervous cells. To obtain VNN protein, collected sample was re-centrifuged by 150000 rpm for 3-5 hours. Supernatant is dissociated with pellet. Such supernatant was crude protein VNN, packed into sterile eppendorf and kept in freezer - 80°C until next test.

C. Isolation of heart and kidney organ of humpback grouper and VNN protein

Heart and kidney of *C. altivelis* are target of VNN. These organs are regulating of spreading VNN in the osmoregulation and circulation process. The heart and kidney from the positive sample which infected by VNN was kept in liquid nitrogen the same with procedure above. Heart and kidney of grouper from positive sample of VNN is isolated in laminar airflow by a set of sterile scalpel and homogenized by sterile mortar and conducted isolation of heart cells and nephrose cells by adding extract buffer with ratio 2 ml : 1 mg organ. After that, homogenate was centrifuged 50000 rpm by ultrahigh centrifuge during 2 hour to dissociate debris from heart and kidney cells. The cells which have been degradation checked its morphology and then conducted isolation of receptor protein. To obtain VNN protein, collected sample was re-centrifuge by 200.000 rpm for 5 hours. Supernatant is dissociated from pellet. The supernatant was crude protein VNN, packed into sterile eppendorf and kept in freezer - 80°C until next test. A different technic of protein isolation of VNN from heart and kidney was in ultracentrifugation and time that used works because in heart and kidney, there are so many tissue that be stretched to release protein receptor.

D. Electrophoreses SDS-Page

Determination of receptor molecule weight and VNN protein is using SDS-Page according to Laemmli methods [11]. Gel is made two layers that is gel as medium separating protein (separating gel) and gel as sample compiler (stacking gel) 12.5%. Separating gel contains acrilamid 30%, TrisHCL 15 M pH 8.8, dH₂O, SDS (*Sodium Dodecil Sulfate*) 10%, APS (*Ammonium Persulfate*) 10%, TEMED (*tetra ethylene diamine*). Stacking gel contains acrilamide 30%, Tris HCL 15 M pH 6.8, dH₂O, SDS 10%, APS 10%, TEMED. Protein

sample SDS-Page is reduced using addition of RSB (1:1) and heated at 100°C for 5'. Sample is packed into gel well by each volume of well 18-20 uL. Protein marker use Low range marker PRO-STAIN™ of 4 µl. Electrophoresis use voltage of 80V, 400 mA for 100'. Gel lifted from chamber and coloration is done by soaking in staining solution (Commassie Brilliant Blue R220) for 30' while be shaken. After that, coloration should be stopped with destaining solution and then counted the protein molecule weight.

E. Measurement of protein concentration using Nanodrop Spectrophotometer

Receptor protein resulted by dialysis uses tubing (SERVA, 5cm dm) is measured its concentration using Nanodrop spectrophotometer at wavelength 280 nm. Absorbance 1 at such wavelength equivalent with concentration of protein 1 mg/ml. Protein sample resulted from dialysis is packed into Nanodrop spectrophotometer for 2 µl. Computer automatically will read concentration of protein in sample. For blanko, it is applied an extract buffer solution.

F. Observation of Receptor Protein Expression and VNN using CLSM

Preparation of sample for showing receptor expression from brain organ and nervous cells, so and heart and kidney organ of humpback grouper is treated using PFA 4% and done a washing sterile PBS sterile for 3 times using sterile DEPC and micro pipette and continued by incubation in DEPC-Sucrose 20% solution in other that protein expression is not contamination. Further, sample is ready to be cut with microtome then placing on a prepare, and depository at - 20°C. Preparation ready to be done for inspection by CLSM.

G. Preparation of tissue processing

Methods of tissue processing includes Cryostat Frozen Sections and paraffin Sections according to Atwood (2001) [12]. Cryostat frozen sections of tissue *C. altivelis* are trimmed to approximately 4 mm³ and snap-freeze in lifuid nitrogen and store at -70°C, if desired the tissue may be embedded in a mixture of polyvinyl alcohol/polyethylen glycol. Using forceps, slowly immerse the base mold in approximately 2 inches of liquid nitrogen, until it is completely frozen, and frozen blocks are wrapped in aluminium foil and store at -70°C. Block to be sectioned are place in the -20°C cryostat for at least one hour prior sectioning. Cut sections 4-10µ thick and thaw mount onto clean glass slides. Use of an adhesive coating or subbing agent, such as poly-L-lysine, charging, or silanization, may improve tissue adherence. Immediately fix in acetone at room temperature for 10 seconds and air dry 12-24 hours.

Methods of paraffin sections includes fix tissue specimens was trimmed to approximately 4 mm³ and dehydrated in graded alcohols, typically, the grading is as follows 60%, 65%, 95%, and absolute. Clear up of alcohol from the tissue may be performed using xylene or a xylene substitute. Infiltrate and embed tissue in liquid paraffin. The temperature should not exceed -58°C. Cut sections 4-6µ thick and mount onto clean glass slides. Use of an adhesive coating or subbing agent, such as poly-L-lisine, chaarging or silanization, may improve tissue adherence. Air-dry for 12-24 hous at room temperature, overnight at 37°C or at 60°C for a hour.

H. Produce of CD4 and CD8 cells on receptor grouper

Produce of CD4 and CD8 cells are done by inducing directly receptor protein material 32.5 kDa through Humpback grouper 300 gram weight. Before, the fish was induced by a complete adjuvant (CFA) and an incomplete (IFA) for three times booster. After third booster, fish serum containing antibody is purified and measurement of response of CD4 and CD8 cells on receptor is done by dot blotting methods, western blotting, Immunocytochemistry and immunohistochemistry.

I. Dot Blotting

Method applied according to Towbin methods [13]. The first procedure is Nitrocellulose (NC) soaked in PBS for 30 min., NC attached in dot blotting chamber, then it is entered healthy fish receptor organ in PBS skim 5% (1:10) @ 50 μ l. Incubation at room temperature for 1 hour. Then it is added antigen coat protein VNN in PBS skim 5% (1:10) @ 50 μ l and incubation over night at room temperature.

Result of incubation is blocked by PBS skim 5% for 1 hour, cleaned by PBS Tween 005% (5x5 minutes) then added primary antibody from fish serum infected by VNN (1:50), PBS skim 5% @ 50 μ l and incubation over at ambient temperature. Result of incubation is cleaned by PBS Tween 005% (5x5min.) then added secondary antibody (AP-Conjugated goat Ig anti-grouper) (1:50) in PBS skim 5% @ 50 μ l then incubation for 3 hour at ambient temperature and washed by PBS Tween (5x5 min.) and added an alkaline phosphatase substrate (chromogen NBT) @ 50 μ l, incubation at room temperature for 2 hour. Reaction which happened is stopped by soaking into aquadest @ 50 μ l then NC taken and cleaned with aquadest, and done analysis result of dot blot.

J. Western Blotting

This methods are according to Schmidt et al (2001) [14] with modification. Band protein specific with molecular weight that concerned as result of electrophoresis SDS-PAGE was entered to sample buffer containing 1% SDS and 0.1% 2-ME and heated to either 100°C or the indicated temperature for 7 minute. Protein were separated by PAGE (with a 12m5 % acrylamide gel unless otherwise indicated) and then electrophoretically transferred to polyvinylidene difluoride membranes (Biorad). The membrane were blocked with 5% dry milk in PBS containing 1 % Tween 20 (polyxyethylene-sorbitan monolaurate) and then incubated with a 1:25.000 dilution of antiserum anti CD4 and CD8 cells or 1:1.000 dilution of primary antibody anti CD4 and CD8 cells affinity-purified antibody. Membrane were incubated with secondary horseradish peroxidase-labeled anti mouse antibodies at a dilution of 1:5.000 in TBS solution 60 minutes at room temperature and clear in PBST solution. Band weight protein that shows signs be a band protein target in sample that binding by primary antibodies anti CD4 and CD8 cells.

K. Immunocytochemistry and Immunohistochemistry technic

Most of the immunochemical staining were immunocytochemistry and immunohistochemistry using primary and secondary antibody and so streptavidin-avidin to shows cross reactivity between antigen and antibody that performed. In methods of test cross reaction of proliferation

and expression of CD4 and CD8 cells using secondary antibody anti CD4 and anti CD8 cells from mouse IgG with techinc e.i early incubate tissue 30 minutes in normal swine. Tap off serum and wipe away excess, do not rinse. Incubate 30 minute with each of the following 3 reagents; rinse with and place 3-5 minutes in wash buffer after each step. Following gives primary antibody anti CD4 and CD8 cells, and then labeled by biotinylated secondary antibody, and prepared at least 30 minutes for uses streptavidin-avidin complex. Incubate wist substrate until desired staining intensity has developed. Rinse with distilled water, counterstain and cover slip and observation under microscope.

III. RESULT AND DISCUSSION

A. Fractionation of protein

Result of electrophoresis receptor protein isolated from nervous cells of *C.altivelis* shown at Fig. 1.

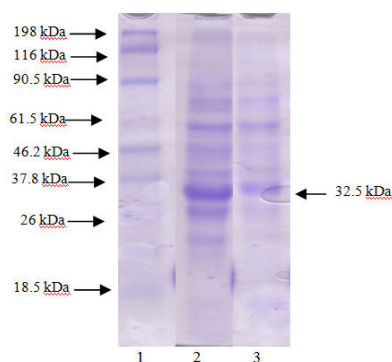


Figure 1. Electrophoregram receptor protein isolated from nervous cells of *C. altivelis*. Column 1: marker; 2: brain infected VNN; 3: brain receptor of normal grouper.

Protein band of receptor of normal grouper show molecule weight 32.5 kDa, whereas after infected by VNN, protein band of nervous cells 32.5 kDa become thinner than normal protein. Through cytotoxic test, it is known that protein with molecule weight 32.5 kDa is a receptor protein which able to recognize adhesin VNN, and in previous research, it is already tested that adhesin VNN is also both adhesin and haemagglutinin.

The results of electrophoresis of protein 32.5 kDa band after immunoblotting with the western blotting test using a PVDF (polyvinylidene difluoride) membrane (Biorad) with incubation of primary and secondary antibodies both anti-CD4 and anti-CD8 with labeler biotin shown in Fig.2.

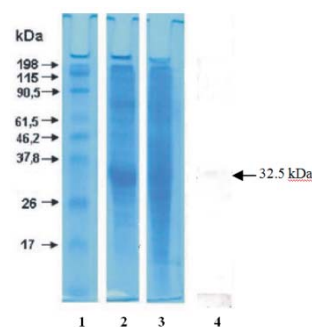


Figure 2. Electrophoregram receptor protein 32.5 kDa of heart and kidney organ *C. altivelis* and western blotting analysis. Column 1. Marker; 2. Expression of Heart organ; and 3. kidney organ; 4. Band weight receptor protein by western blotting analysis.

The result of response of CD4 and CD8 cells on protein receptor grouper that induce by 32.5 kDa of VNN after tested by reacting between antibody of anti CD4 and CD8 cells on receptor grouper on organ brain, heart and kidney show a cross reaction which is specific between antigen VNN infection and receptor protein of organ *C. altivelis*.

B. CLSM observation

The observation to expression and proliferation of receptor protein of nervous cells on brain organ, heart and kidney of *C. altivelis* respectively using CLSM show that receptor expression measured give auto fluorescent intensity as shown in Fig. 3a – 3d.

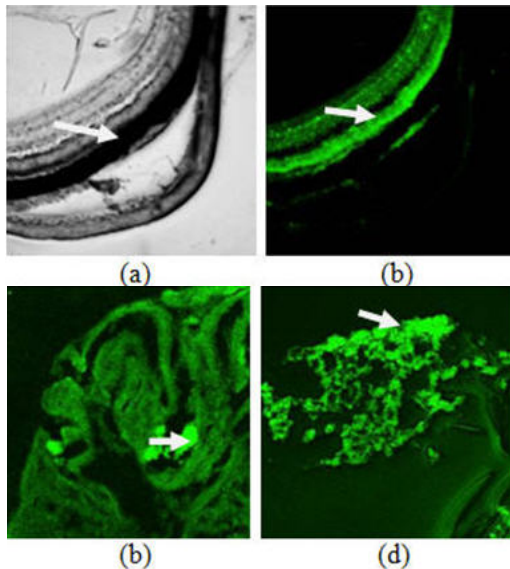


Figure 3. Expression of receptor protein of nervous cells on brain organ, heart and kidney organ of *C. altivelis* that infected by VNN and treatment of receptor protein of *C. altivelis* done by infected of VNN and protein immunogenic VNN with molecule weight 32.5 kDa Degree of the autofluorescence receptor is measurement using CLSM and results confirmed by line shape white. A. Control; B. Nervous cells on brain organ, C. Heart organ and D. Kidney organ

VNN is a class of viruses of single strand positive sense, ss (+) Ribonucleic acid (RNA) that causes the disease retinopathy and encephalopathy in grouper *C. altivelis*. This virus has a single strand of genetic material in the form of RNA that can directly act as a template in the synthesis of amino acids [10]. VNN does not have the envelope so that in inducing infection process of its host, VNN infect its host through an attachment immediately to the host receptor organs such as brain, heart and kidney (Fig. 3). The results of this study is reinforced by the opinion of Murphy et al (2008) [15] that the VNN immediately held an attachment to receptors which then held uncoating host the virus to insert genetic material in host cells or intracellular infection by leaving coat protein outside the cell. The coat protein is a protein constituent VNN virion structures, therefore the coat protein is crucial structure. Coat protein is not only a role in viral nucleic acid packaging VNN but at the same coat protein has a primary status in the process of infection in target cell (*C. altivelis*). This study result the immunogenic protein of VNN that is VNN coat protein with molecular weight of 32,5 kDa. This protein have a nature to evoke cellular immune system that is expressed and proliferated both CD4 and CD8 cells in the brain, heart and kidney organs of the grouper as result of exposure 32.5 kDa immunogenic

protein in vivo.

C. Response of CD4 and CD8 cells

Response of CD4 and CD8 cells on receptor protein of grouper that induce by 32.5 kDa of VNN after tested by reacting between antibody of anti CD4 and CD8 cells on receptor grouper show a cross reaction between antigen VNN and receptor protein with dot blotting analysis on brain, heart and kidney organ as shown in Fig. 4

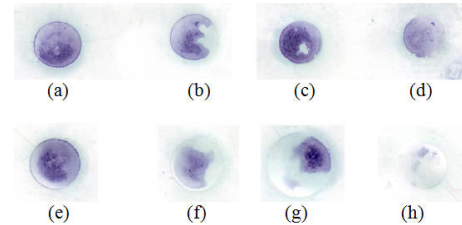


Figure 4. Response of CD4 and CD8 cells on receptor with inducer of 32.5 kDa of protein of nervous cells and antigen VNN. Positive control for CD4 and CD8 cells on brain (a); Positive cross reaction protein VNN 32.5 kDa to following: CD4 cells on brain (b); CD8 cells on brain (c); CD4 cells on heart (d); CD8 cells on heart (e); CD4 cells on kidney (f); CD8 cells on kidney (g); Negative control (h)

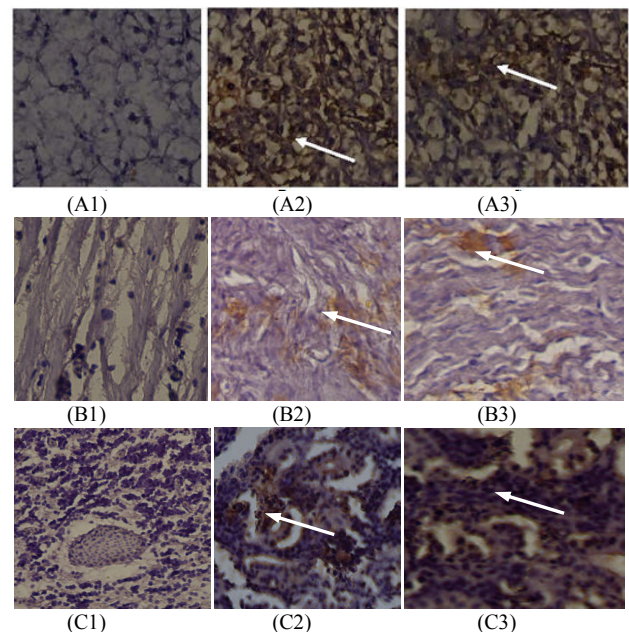


Figure 5. Immunohistochemistry using mouse conjugate secondary antibody IgG anti CD4 and CD8 cells. On brain tissue: Normal cells (A1); expression of CD4 cells (A2), and CD8 cells (A3); On heart tissue: Normal cells (B1), Expression of CD4 cells (B2) and CD8 (B3). On kidney tissue: Normal cells (C), Expression of CD4 (C2) and CD8 cells (C3).

This result show that receptor 32.5 kDa is estimated a receptor protein expressed on brain heart and kidney tissue of grouper and have a role to expression on CD4 and CD8 cells which shows expressing defense immune system on grouper. It is characterized by the presence of cross reaction between immunogenic protein receptor VNN 32.5 kDa and brain, heart and kidney tissue indicated by brown color (Fig 4). It is expression of CD-4 cells and CD-8 in the receptor protein in brain, heart and kidney tissue of grouper after incubation with mouse IgG conjugate secondary antibody anti CD4 and CD8.

CD4 and CD8 cells in grouper cells generated in this study is the expression of immune cells that are formed due to exposure to an antigen. Exposure to foreign materials or antigens can evoke cellular immune responses through the

proliferation and differentiation of dendrite cells into Th1 cells and Th2 cells, where molecules CD4 and CD8 cells produced by differentiation of cytotoxic T cells (Tc) [16]. This shows that the result of expression on CD4 cells and CD8 cells in tissues of grouper *C. altivelis* exposed to an immunogenic protein 32.5 kDa VNN provide the level of cross reaction of antigen and antibody formation of anti-CD4 and CD8 cells as a cellular defense system of grouper *C. altivelis*.

Preparation of tissues from organs of grouper *C. altivelis* (brain, heart and kidney) as results of in vivo test for detecting the expression and proliferation of CD4 and CD8 cells through immunohistochemistry techniques showed that in cells located in tissues and organs grouper *C. altivelis* (brain, heart, and kidney) in Fig. 5 provide the level of specificity of cross-reaction between an antigen that is VNN immunogenic protein of 32.5 kDa and formed antibody or immune cells (CD4 and CD8 cells) as a defense of the fish cellular immune system. This proves that the VNN immunogenic protein 32.5 kDa as an adhesin protein is pathway portal of entry of VNN to host cells such as reinforced by Madigan et al (2003) [18].

This study has shown that the immune system of grouper *C. altivelis* have defense mechanism in the form of CD4 and CD8 cells. The process of formation of CD4 and CD8 cell is the result of proliferation and differentiation starting with the entry process of antigen into the cells and tissues of grouper *C. altivelis*. VNN as intracellular antigens is become smaller molecules in time of entry into the host. Further, pieces of peptide be sent into the reticulum endoplasmic by transporters associated with antigen processing (TAP). Such peptide is bound by MHC class I molecules, to be brought to the surface and subsequently presented by T cells CD8 + (cytotoxic T cells (Tc)). Tc cells have two separate systems in helping cytotoxic function as exocytosis pathways and interactions between Fas ligand (FasL) expressed on the surface of T cells and Fas expressed on target cells. Tc cells which already bind to target cells insert perforin and granzyme in the cytosol of host cells. Perforin will spread and form pores in the cell and cause granzyme entry into the host cell. Granzyme will induce the process of apoptosis leading to cell death.

Other fish immune system is the major histocompatibility complex class II (MHC II). Any antigens entering into the host's body will be captured by Antigen Presenting Cell (APC). APC will split antigen molecules into smaller molecules. Then, these molecules will be expressed by MHC molecules. MHC is grouped into two namely MHC class I and II with function presenting antigens intracellular and extracellular, respectively [9].

Immune system of fish MHC class II have important role in response of vertebrate immunity including fish to viral molecule. MHC class II is expressed definitely on cells represent antigen (*antigen presenting cells*) like macrophage, dendritic cells, monocyte and B cells. Its expression is able to be induced on other cells type after stimulated by cytokine, for example interferon γ . MHC class II have function to represent antigenic peptide from extracellular pathogen to T cell receptor (CTL = *cytotoxic T lymphocyte*) [19] [20], proliferation and differentiation of immune cells as CD-4 and CD-8 that function for protection of viral. T cell receptor was

able to recognize only an antigen represented by molecule of MHC class II [21]. Syntheses chain of α and β MHC class II is occurred in rough reticulum endoplasm together a special chaperon namely invariant chain (I_I) which known having molecule weight 31-33 kDa. Early, chain of α and β MHC class II appear temporary joint in high molecule weight aggregates containing protein binding heavy chain immunoglobulin (BiP) and so assemble in to oligomers containing invariant chain together with calnexin [22]. Released of calnexin in parallel with complex formation nonamer ($\alpha\beta$)₃I_I cannot bind peptides. Nonamer complex released from endoplasmic reticulum to Golgi body and *trans-Golgi network* (TGN) and further in endocytic compartment (endosome, pre-lisosome, *lysosomal-like vesicle*, MIIC, CIIV or CPL) which is place of antigenic peptide degradation [19] [22]. MIIC (MHC II compartment) is place of heaping (storage) and preparation of molecules MHC class II for binding with antigenic peptide coming from degradation of exogenous protein [19]. MHC class II molecule also binds a heterogeneous peptide coming from antigenic pathogen and presents it to surface of cells presenting antigen. Molecules MHC class II in the form of heterodimer is consisted of polypeptide chain known to have weights molecule α (32 kDa) and β (29 kDa) which the two of them binding integral membrane protein with molecular weight around 26 kDa [19] [23]. VNN at grouper are most happened at brain organ, kidney, lymph and lien, and eye, although at other tissue area also this virus is detected. It has been known that VNN is RNA virus having genetic matter in the form of single chain RNA (+) strand and has coat protein with molecule weigh 40-45 kD [24]. In this research, it has been found at stadia larva 1-3 cm that attack mechanism of this VNN has been investigated that receptor grouper have a part in binding VNN which showed by level of auto fluorescent receptor to VNN attack, which will be checked furthermore at expression level molecular relates to function of CD-4 and CD-8 cells in presenting of grouper. It has been proved by that VNN attacking this grouper can be exploited based on presentation of antigen and receptor molecule for developing diagnostic material of VNN and further development to find drug material for ongoing control of VNN attack

IV. CONCLUSIONS

The results showed that immunogenic protein 32.5 kDa VNN in groupers *C. altivelis* has a function to evoke immune system. Proliferation of CD4 and CD8 immune cells in the organ receptors indicate that protein 32.5 kDa immunogenic VNN in groupers could suppress the proliferation of VNN in vivo so the spreading pathways from the brain to the heart and kidney also be inhibited because of the fish immune system is formed that is able to suppress proliferation VNN throughout the body. VNN immunogenic proteins 32.5 kDa as the coating protein can be used to suppress the proliferation of viruses and raise proliferation and expression of the immune system of fish.

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