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# The Role and Expression CD-4 and CD-8 Cells on Receptor Protein of Humpback grouper *Cromileptes altivelis* to Defense of Viral Nervous Necrotic Infection

Uun Yanuhar

Laboratory of Waters Sciences and Marine Biotechnology
Faculty of Fisheries and Marine Science, Brawijaya University, Malang, East Java, Indonesia
Email : uunyanuhar@yahoo.com,

**Abstract.** Humpback grouper *Cromileptes altivelis* is one of non oil favorite export of Indonesia until now. The decrease of this cultured is caused by *Viral Nervous Necrotic* (VNN) occasioned grouper mortality till 100%. Mechanism of viral infection is not apart from role and function of grouper receptor, especially brain defence which mediated by nervous cell. Research objective is to identify how a role and expression of cluster differentiation 4 (CD-4) and cluster differentiation 8 (CD-8) cells to defense of VNN infection in *C. altivelis*. Methods are isolate, identify and characterize of receptor protein of brain *C.altivelis* infected by VNN by exploration method, SDS-Page, measure to expression of CD-4 and CD-8 cells on receptor protein grouper using Confocal Laser Scanning Microscope (CLSM), response immune with analyze using immunohistochemistry and dot blott methods. The result indicate that receptor protein of brain *C.altivelis* exposed by VNN protein 32,5 kDa expressed CD-4 and CD-8 cell that the function of defense to VNN after use detected with primary and secondary antibody CD-4 and CD-8 cell from mouse Imunoglobulin G (IgG). Receptor protein of brain of *C. altivelis* has function to response immune system of grouper to defense VNN infection through expression of both CD-4 and CD-8.

**Keywords:** *C. altivelis*, CLSM, CD-4, CD-8, VNN

## 1. Introduction

Humpback grouper is a serranidae family with widespread population in tropical and sub tropical water territory and product for seafood that is very favorites in world [1]. 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, in Indonesia, it explained that one of constraint in Humpback grouper cultured is limitation of supply of seed caused by pathogen infection resulting mortality more than 80%, even till 100% [3]. In East Java, seeding industry also get a loss as 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] [4]. 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) [5]. Therefore, the problem needs an attention seriously and prevention because of loss resulted stress and corrupt in industrial cultured.

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, and kidney. In this research, it will be seen VNN infection in grouper brain as agent that is very endangers. The role of target of receptor protein is important to neutralize VNN [6].

Studies on functional immunology of T cell activities are still at their beginning, and much work is needed to investigate T cell responses in grouper species. In higher vertebrates two major subsets of T lymphocytes exist: CD-4 positive T helper (TH) cells, and CD-8 positive T cytotoxic (TC) Cells [7]. Both

CD-4 and CD-8 positive T cell subsets in a teleost show characteristics similar to those of mammalian CD-4 and CD-8 positive T lymphocytes in terms of the morphology, tissue distribution and gene expression [8]. In Atlantic halibut, CD-4 protein is structurally similar to other CD-4 homologues in fish and mammals. Its function as co-receptors for the T-cell receptor (TCR) on T lymphocytes, and collectively the CD-4 and CD-8 co-receptors are very important for all kinds of T-cell activation [7].

In host, infection of viral was occur through intracellular mechanism so it need to expression of Major Histocompatibility Complex (MHC) molecule for stimulation resulted immune cell as expression of CD-4 and CD-8 cell resulted by T cell [9]. In case viral infection, CD-4 and CD-8 functions are to bind, defense and eliminate antigens or viral [10]. It is required to understand the role of this molecule in immune response. The research objective is to identify how a role and expression CD-4 and CD-8 cells on receptor protein of Humback grouper to defense of VNN.

## 2. Material and Method

**Animals**

Fish samples which infected by VNN (confirmed by PCR methods) are collected from Institute for Brackishwater Aquaculture (BBAP), Situbondo, Indonesia. Research applies *C.altivelis* in stadia larva by length of 1-3 cm, 5-7 cm, 10-15 cm infected by VNN and control. Each treatment use 30 fishes in controlled by salinity of 30-33 ppm

**Isolation of brain organ of Humpback grouper and VNN protein**

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 secting sterile set. Brain is homogenized by sterile mortar and conducted isolation of nervous cell by adding extract buffer with ratio 2 ml : 1 mg organ. Hereinafter, homogenate was centrifuged 50000 rpm by ultrahigh centrifuge during 1 hour to dissociate debris with nervous cells. Nervous cell which have been degradation checked its morphology and then conducted isolation of receptor protein of nervous cell. To obtain VNN protein, sample collection was re-centrifuged by 150000 rpm for 3-5 hours. Supernatant is dissociated with pellet. That supernatant was crude protein VNN, packed into sterile eppendorf and kept in freezer - $80^0$C until next test.

**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, $dH_2O$, SDS (*Sodium Dodecil Sulfate*) 10%, APS (*Ammonium Persulfat*) 10%, TEMED (*tetra ethylene diamine*). Stacking gel contains acrilamid 30%, Tris HCL 15 M pH 6.8, $dH_2O$, SDS 10%, APS 10%, TEMED. Protein sample SDS-Page is reduced using addition of RSB (1:1) and heated at $100^o$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$^{TM}$ 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 shake. After that, coloration stopped with de staining solution and counted the protein molecule weight.

**Measurement of protein concentration using nanodrop spectrofotometer**

Receptor protein resulted by dialize 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 dialize 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.

**Observation of Receptor Protein Expression and VNN using CLSM**

Preparation of sample for showing receptor expression from brain organ and nervous cell 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 the n placing on a glass preparation, and depository at – 20°C. Preparation ready to be done for inspection by CLSM.

**Produce of CD-4 andCD-8 cell on receptor grouper**

Produce of CD-4 and CD-8 cell 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 CD-4 and CD-8 cells on receptor is conducted by dot blotting and immunohistochemistry methods.

**Dot Blotting and immunohistochemistry**

Method applied according to Towbin methods [12]. 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) @ 50µl. Incubation at room temperature for 1 hour. Then it is added antigen coat protein VNN in PBS skim 5% (1:10) @ 50µl and incubation over night at room temperature.

Result of incubation is blocked by PBS skim 5% for 1 hour, cleaned by PBS Tween 005% (5x5menit) then added primary antibody from fish serum infected by VNN (1:50), PBS skim 5% @50µl 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% @ 50µl then incubation for 3 hour at ambient temperature and washed by PBS Tween ( 5x5 min.) and added an alkaline phopatase substrate (chromogen NBT) @ 50µl, incubation at room temperature for 2 hour. Reaction which happened is stopped by soaking aquadest @ 50µl then NC taken and cleaned with aquades, and done analysis result of dot blot. For immunohistochemistry, it used brain tissue incubated by H2O2 3% and then procedure sama as dot blot methods but after incubate by primary and secondary antibody mouse IgG anti CD-4 and CD-8 cells, it continuied by SAHRP, Cromogen DAB and Counterstain Meyer Hematoxylin. Response of expression CD-4 and CD-8 observed by inverted microscope.

## 3. Result

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

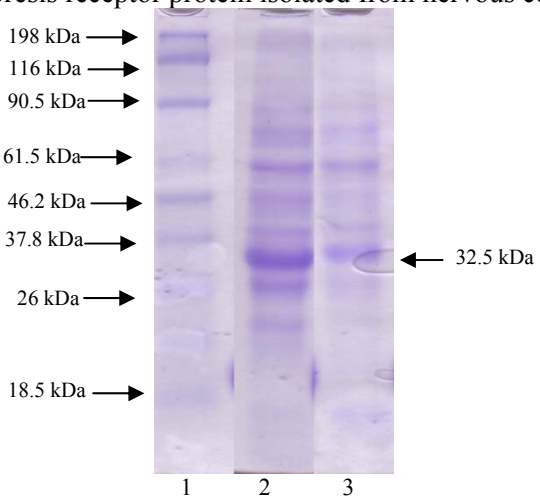


Fig. 1. Electrophoregram receptor protein isolated from nervous cell of *C. altivelis*. Column 1: marker; 2:brain infected VNN; 3: brain receptor of normal grouper;

Protein band of receptor of normal grouper show molecule weight 32.5 kDa, whereas after infected by VNN, protein band of nervous cell 32.5 kDa become thinner than normal protein. Through cytotoxic test, it is known that protein 32,5 kDa is a receptor protein which able to recognize adhesin VNN, and in previous research [6], it is already tested that adhesin VNN is also both adhesin and haemaglutinin.

Result of observation to expression of receptor protein of nervous cell *C. altivelis* using CLSM show that receptor expression measured give auto fluorescent intensity as shown in Figure 2.

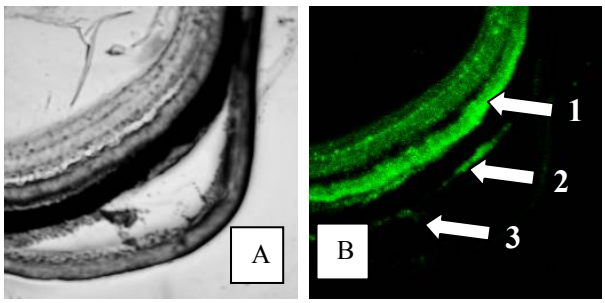


Fig. 2. Expression of receptor protein of brain tissue 32,5 kDa *C. altivelis* infected VNN using CLSM. A. Control; B: Nervous cell infected by VNN with auto fluorescence (arrow: 1: strong, 2. Intermediate; 3. Weak).

Measurement result of response of CD-4 and CD-8 cell on receptor grouper that induce by 32,5 kDa of VNN after tested by react between antibody of both anti CD-4 and CD-8 cell and grouper receptor show a cross reaction between antigen VNN and receptor protein as shown in Fig. 3.

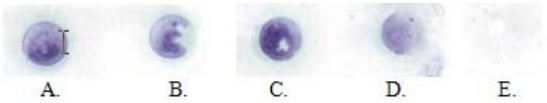


Fig. 3. Response of CD-4 and CD-8 cells on receptor with inducer protein 32,5 kDa and antigen VNN. A. Positive control for CD-4 cell; B. positive cross reaction to CD-4 cell; C. Positive control for CD-8 cell. D. Positive cross reaction to CD-8; E. Negative control.

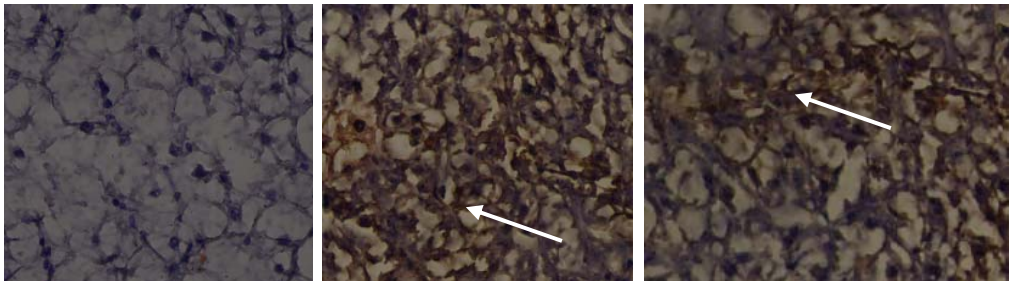


Fig. 4. Immunohistochemisty using mouse conjugate secondary antibody IgG anti CD-4 and CD-8 on brain tissue. A: Normal cell; B. expression CD-4, and C. CD-8

This result show that receptor 32,5 kDa is estimated a receptor protein expressed on brain tissue of grouper and have a role to expression on CD 4 and CD-8 cell 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 tissue indicated by brown color (Fig 4). It is expression of CD-4 cells and CD-8 in the receptor protein in brain tissue of grouper after incubation with mouse IgG conjugate secondary antibody anti CD-4 and CD-8.

## 4. Discussion

Immune system of fish between is MHC class II (*major histocompatibility complex 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 cell, monocyte and B cell. Its expression is able to be induced on other cell 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*) [13] [14], proliferation and differentiation of immune cell as CD-4 and CD-8 that function for protection of viral. Tcell receptor was able to recognize only an antigen represented by molecule of MHC class II [15]. Syntheses chain of α and β MHC class II is occurred in rough reticulum endoplasm together a secial chaperon namely invariant chain (II) 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 [16]. Released of calnexin in parallel with complex formation nonamer $(\alpha\beta)_3Ii_3$ 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 [13] [16]. 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 [13]. 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 [13] [17]. 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 [18]. 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 cell 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.

## 5. Conclusion

The research conclusion is protein from nervous cell of grouper brain with molecule weigh of 32,5 kDa which constitute grouper receptor protein able to recognize VNN infecting grouper can induce expression of CD-4 and CD-8 cell that function for immune system for grouper.

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