The immunogenicity

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The immunogenicity of polyclonal antibody from induced *Vibrio parahaemolyticus* membrane by Elisa method

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Abstract. Polyclonal antibodies are antibody proteins formed from interaction between antigens that enter a body. It results in immunogenicity. The process of generating polyclonal antibodies begins with purifying a membrane protein. A membrane protein that is potential to make an antibody is the outer membrane protein (OMP) of bacterium *V. parahaemolyticus*. These proteins are injects into an animal's body as the target antigen so that an antibody can be produced. The antibody was obtained by taking the animal's blood sample. It is then isolated in a serum until it generates a polyclonal antibody. ELISA (*Enzyme-Linked Immunosorbent Assay)* is a technique normally used to measure the concentration of an antibody or an antigen in blood or to detect their existence in a sample. The aim of this research was to analyse the immunogenicity of polyclonal antibodies produced from *Vibrio Parahaemolyticus* membranes that was induce using ELISA method. Research findings indicate that the highest OD value of ELISA (0, 404) was obtained on the third week of 1/100 dilution. The highest value of antigens and antibodies interaction was found on 1/100 dilution. Immunization before booster generated relatively small antibody titres because the rat's body was not ready to provide an immune response.

1. Introduction

Vibrio parahaemoliticus belongs to pathogenic gram-negative bacteria whose membrane functions to infect the host. These bacteria have reported to vary levels of pathogenicity [1]. The outer membrane of the bacteria is composed of recognizable glycolipids and proteins, which are identified as substances alien to the host immune system. The membrane proteins play a crucial role in cells and physiological processes has found some factors that influence gram-negative bacteria in mechanizing the pathogenic infection. These include capsules, *fimbriae* (pili), serum resistance, resistensi serum, *lipopolysaccharide* (LPS), and siderophores [2,3]. Additionally, the pathogenicity of Vibrio is determined by some outer membrane proteins such as *Thermostable direct hemolysin* (TDH), TDH-related hemolysin (TRH), lethal toxin and urease [4]. However, TDH constitutes the most influential factor in Vibrio virulence. *Thermostable direct hemolysin* is a protein, which performs hemolysis activities in human erythrocyte *V. parahaemolyticus* virulence falls into two groups: positive Kanagawa Phenomena (KP+) bacteria which carry tdh and/or trh gene and negative *Kanagawa*

Phenomena (KP-) which do not inherit both genes [5]. The KP+ group of bacteria exist due to the production of TDH (*Thermostable direct hemolysin*) toxin proteins by tdh gene. Virulent Enterotoxigenic strains which carry tdh genes has been identified by conducting a test of mutant cloning on "TDH-deficient" KP+ strains which do not indicate any hemolysis reaction on non TDH bacterial mutants [6].

The Outer Membrane Protein (OMP) of *V. parahaemolyticus* consists of some virulent and immunogenic proteins [7]. Suggests that in a laboratory scale, OMP constitutes a potential vaccine component that can help control diseases on fish and meningitis on human [8]. Furthermore, have found out some proofs to the immunogenicity of *V. alginolyticus* immunogenic OMP which was isolated from tiger grouper fish (*Epinephelus fuscoguttatus*) [9]. Negative OMP is a good vaccine candidate since it contains lipoproteins, some virulence factors including siderophores, hemolysin, and porins, adhesin which is known as an antigenic and strong immunogenicity [10,11,12]. Based on the information, it can be found out that *V. parahaemolyticus* has some virulence and immunogenic proteins which can be used as a vaccine and diagnose kit [2,12,13,14].

According to *V. parahaemolyticus* OMP contains some antigenic proteins with various sizes and molecular weights. OMP with 36 kDa protein is immuno-dominant [15]. In addition to that, point out that 38 KDa OMP contains high antigenic proteins which serve an important role in a host-pathogen interaction [16]. Some studies involving *V. cholerae* have shown that when a bacterium is transferred into a new environment, its OMPs synthesis will be changed into an osmolarity on the OMP profile[17,18].

An antibody is a part of body defence used to protect the body from alien substances [19]. A polyclonal antibody is created from an antigen which stimulates the body immunogenicity. A conventional process of generating a polyclonal antibody is by purifying *V. parahaemolyticus* membrane proteins and injecting the antigen target to the rat's body. After that, blood sample is taken and an antibody is isolated in a serum. An immune response will be provided by cells and molecules which construct the immune system to the antigen. A specific response towards intracellular bacteria are contributed by cell mediated immunity (CMI). However, the immunity mechanism will be performed by the T cell lymphocyte. Meanwhile, the effector function will be served by macrophages which are activated by cytokines produced by the T cell (IFN a). This immune response is analogical with slow hypersensitive reaction [20]. According to a polyclonal antibody can only be used as an antigen to detect genus existence since it merely responses to heterogeneous complex antigens which cannot be developed to the species level.

ELISA (Enzyme-Linked Immunosorbent Assay) is a technique normally employed in the immunology laboratory to measure the concentration of blood antigens or antibodies or to recognize the existence of antigens or antibodies in a sample [21]. This method has some advantages such as its simple execution technique which is economical and sensitive. The test can be performed using an available kit or another antigen kit which is developed independently. Treatment serum, positive control serum, and negative control serum are incubated in ELISA tools. To observe the bound antigens or antibodies, secondary antibodies which are found on enzymes such as peroxidase or alkaline phosphatase are added into each well. After the incubation period, the unbound secondary antibodies should be washed off. If added substrates fit, the enzymes will react with them and produce colours. The colours produced will be measured as the function or quantity of the antigens or antibodies. Colour intensity is going to be measured at 405 nm. This colour intensity indicates the number of produced antigens or antibodies. This research, thus, aimed to analyse the immunogenicity of polyclonal antibodies produced from *Vibrio Parahaemolyticus* membranes that were induced using ELISA method.

2. Materials and Methods

Materials used in this research were pure breed of *V. parahaemolyticus* ATCC 17802®, which was obtained from authorised AHM Biotech Indonesia, Bogor, West Java.

2.1 Outer Membrane Protein Isolation

The outer membrane protein was isolated using a technique suggested by which is also a modification of Evan's method [22]. A part of sample used was the sediment part as a result of cutting pili in the last round. The pellet was re-suspended using PBS pH 7,4 until the volume multiplied 10 times. After that, SDS 0,05% was added into 10 ml of PBS 0,005 and it was homogenized until it was dissolved. OMP with 1 SDS was left to be tested on SDS-PAGE.

2.2 Polyclonal Antibodies Production

Four male strains of albino wistar rats (*Ratus Novergitus)* aged three months were immunised subcutaneously with 100 µl OMP in *Freund's Adjuvant* (FA); twice booster with OMP antigens in Freund's complete adjuvant. The first immunization with polyclonal antibodies from the *V. parahaemolyticus* OMP was dissolved in *Complete Freund's Adjuvant* (CFA) by 1:1 [23]. The booster was conducted twice with the Incomplete *Freund's Adjuvant* (IFA) solvent by 1:1 within a 14 days interval. The blood sampled for serum collection was taken four times from lateral veins: before immunization, after the first injection (before the first booster), after the first booster (before the second booster), and after the immunization (after the second booster) [25].

2.3 Animal Experiments Serum Isolation

It was performed by precipitating the animal blood for about 30 minutes. Then, serum was separated by centrifugation and antiserum was separated at 3000 rpm for 10 minutes at a room temperature. The serum was transferred into an eppendorf tube using a pipette and it was then stored in a 4^0C freezer until the purification time.

2.4 Outer Membrane Protein Purification

100-150 µl of serum was taken and put into a microtube. It was then added with SAS 50% by 1:1 and homogenised using vortex. After that, it was incubated overnight at 4^0C. The serum was centrifugated at 6000 Rpm for 15 minutes at 4^oC. Supernatant was thrown out, precipitate was washed using SAS 50% (10 times serum volume), and homogenised using vortex. It was centrifugated at 6000 rpm and 4^0C for 10 minutes. Precipitate was added with 0,05 M buffer phosphate pH 7, and put into a cellophane bag, analysed with buffer phosphate 0,1 pH 7 and kept overnight at a cold temperature. Buffer phosphate in the cellophane bag was added with cold ethanol (the ratio was 1:1), and inverted. It was incubated in a refrigerator for an hour until the sediment appeared and then centrifugated at 6000 Rpm for 15 minutes. The solution was put into a freezer for more or less 15 minutes. Pellet was taken and supernatant was thrown out. The pellet became quite dry, it was added with buffer Tris-C with ratio 1:1 and heated at -20^0C. The process resulted in an antibody protein weighed 38 kDa.

2.5 Antibody Titres Measurement by ELISA technique

Produced polyclonal antibodies were characterised using ELISA raider method. The ELISA examination referred to a method suggested by Coating buffer was changed into *fresh*. Coating antigen. The concentration of the antigen was 5 μg/ml. It was diluted using coating buffer and put into the microplate, 50 µl into each well. Then, it was incubated at 4°C for 24 hour. The day after, the plate was rinsed three times with PBS Tween 0.2%. Polyclonal antibodies were dissolved in BSA 1% blocking buffer by 1/25, 1/50, 1/100 respectively. Then, they were put into the microplate, 50 µl into each well. It was then incubated at a room temperature for 2 hours. The microplate was rinsed with PBS Tween 0,2 % diluted by 1/1000 and put into the microplate (50 µl). After that, it was incubated at a room temperature for an hour. The microplate was washed with PBS Tween 0,2 % for four times and added with PNPP substrate in 10% ethanolamine. It was then inserted to the microplate, 50 µl into each well and incubated at a dark room temperature for 30 minutes. At this stage, blue colour would indicate the interaction of antigens and antibodies. NaOH 3M was added 50 µl into each well. The blue solution would change its colour into yellow. The titres were measured by ELISA reader at 405 nm. The absorbance result was then converted into a standard curve which presents the OMP concentration.

3. Results And Discussion

3.1 OMP Isolation and Calculation

The results of SDS-PAGE data analysis on the molecular weight of the *V. parahaemolyticus* OMP indicate 31 bands. There are 10 thick bands with molecular weight of 72, 67, 58, 52, 44, 42, 40, 38, 37 and 10 kDa (Table 1).

Table 1. Molecular Weight of the *V. Parahaemolyticus* OMP

Protein Marker	**Molecular weight (OMP)**
260	0
140	0
100	0
70	2
50	2
40	3
35	2
25	0
15	0
10	1

The characterization result of the *V. parahaemolyticus* OMP with SDS PAGE method show that the average molecular weight of the proteins ranged from 35 kDa to 40 kDa. The existence of these two protein groups indicates the difference expression level of each protein, which constructs the OMP. Normally, proteins contained in the OMP are expresses based on the bacterial purpose. These proteins are thus express by cells to maintain cells integrity and serve important roles in cells physiology including in pores making, molecules transport mediation and receptors.

3.2 The Immunogenicity of polyclonal antibodies produced from V. Parahaemolyticus membrane which is induced by ELISA method

Data on the immunogenicity of polyclonal antibodies produced from *V. parahaemolyticus* membrane which is induced by ELISA method was obtained by diluting the antibodies at 1/25, 1/50 and 1/100. The average value of the polyclonal antibodies titres is presented in Table 2.

Table 2. The Average Concentration of Polyclonal Antibodies Titres

	Dilution Level		
Time	0.25	0.50	0.1
Control (pre mium)	0.358		0.37
Before booster	0.286	0.351	0.348
Before booster 1	0.386	0.369	0.389
After booster 2	0.387	0.363	0.404

The data was then analyzing using ELISA Reader (405 nanometer). The result of the titres visualization is described in a graph (Fig. 1).

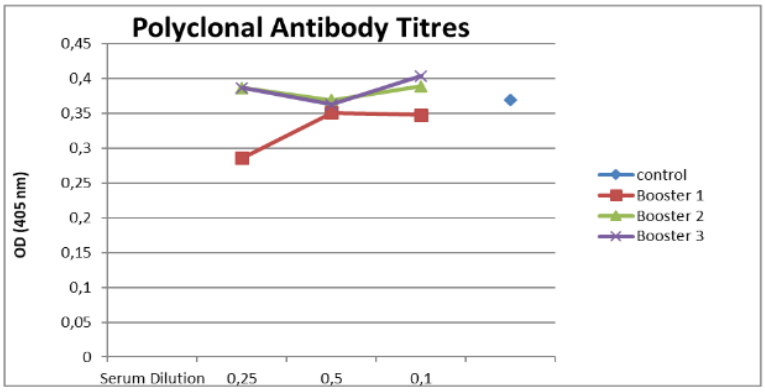

Figure. 1. The Profile of Polyclonal Antibodies Titration from *V. parahaemolyticus* OMP Induction on Albino Rats (*Rattus norvegicus*)

The OD values on control (Pre-mium) were 0,358 and 0,37. Before booster, the titres values were 0.286, 0.351 and 0.348. After booster 1, the values increased to 0.386, 0.369 and 0.389, and after booster 2, the values changed into 0.387, 0.363 and 0.404. These research findings suggest that there was an increase in 1/25 OD control concentration but the values decreased along with the immunization process. Meanwhile, before immunization and after booster 1, the OD values rose not higher than the OD values after booster 2. It indicates that before booster and after booster 1, the polyclonal antibodies were not yet formed on the *V. parahaemolyticus* outer membrane. The highest OD value (around 0, 404) ELISA was found at 1/100 dilution in the third week. The results of ELISA examination on the next polyclonal antibody titres were used as the basis of serum dilution used in the dot blot and western blot analyses.

The results of antibody titres analysis show that there was a difference between control rat and experiment rat after immunization. The antibody titres were taken from the highest dilution level of 1/100 during control period, first immunization before booster, second immunization booster 1, and third immunization booster 2. It was found out that the antibody titre before immunization (0,358) was different from immunization I (0, 348), II (0, 389), and III (0,404). The antibody titre before booster was relatively smaller than of booster 1 and booster 2. It happened because adjuvants were added into the antigens during booster 1 before it was injected to the rat's body. *Complette Freund's Adjuvant* (CFA), which contains Mycobacterium tubercolosum, is an adjuvant, which used in the first immunization. *Mycobacterium tubercolosum* would stimulate and increase specific immune responses towards the antigens. However, second booster used *Incomplete Freund's Adjuvant* (IFA), which contains no *Mycobacterium tubercolosum* to avoid hypersensitive responses.

The interaction between the antigens and antibodies is at the highest when diluted at 1/100. The antibody titre value before booster was smaller because the rat's body was not prepared to form an immune response. This research finding is in line with which says that one of the functions of the immune system is the memory function [20]. This function refers to the ability to respond quickly to pathogenic alien substances. The second response is much faster and stronger than of the first contact. According to, the titres values after second booster increased because after the first immunization the body had recognized the OMP 38 kDa antigens. However, according to, this value increase will only last for 3 weeks during the treatment; no treatment will result in the decrease of the antibody value [25].

The capability of the immune system to provide responses, recognize, and distinguish numerous target molecules at the first time creates a phagocytosis antigen from polymorphonuclear phagocyte cells or macrophages. These immune responses initiated when B or T cells bound like a lock and the padlock. When a protein enters the boundary, it will identified by the cells as an alien substance. This, therefore, stimulates the immune responses as macrophages act as the Antigen Presenting Cell (APC) react towards it. These cells will capture a small number of antigens and expose them to the cell surface to recognise by the T cell lymphocyte helper (Th or T helper). The Th cell will activated and the cell activation will result in the activation of other lymphocyte cells such as B cell lymphocyte or cytotoxic T cell lymphocyte. The cytotoxic T lymphocyte will proliferate and perform an effector function to eliminate antigens [20].

4. Conclusion

The results of analysis on the immunogenicity of polyclonal antibodies produced from the outer membrane protein of *V. parahaemolyticus* which was induced with ELISA method show that there was an increase in OD control value at 1/25 dilution level. The value, however, decreased as the immunization process occurred. The OD values were founding that after first booster was smaller than after booster 2. It indicates that before booster and after booster 1, the body had not generated polyclonal antibodies to respond to the *V. parahaemolyticus* OMP. The interaction between antigens and antibodies was at the highest at the 1/100 dilution level. The antibody titre value during the immunization before booster was relatively small because the rat's body was not prepared to form any immune responses.

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