

## Cross Reaction among Antibody Pili subunit Hemagglutinin Proteins and Outer Membrane subunit Hemagglutinin Proteins of *Shigella flexneri*

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### ABSTRACT

*Shigella flexneri* is the most common causal agent of shigellosis. Its pili are composed of pili protein subunits. Adhesion molecules can be found on the pili and outer membrane proteins (Omp). A hemagglutination reaction can be used for screening of adhesion molecules. Objectives: The purpose of this study was to determine the molecular weight of the pili protein subunits and outer membrane proteins of *S. flexneri* that act as hemagglutinin proteins, and to prove whether there is a cross-reaction between antibodies against hemagglutinin pili protein subunits and outer membrane proteins of *S. flexneri*. Methods: Pili protein subunits were isolated using pili bacteria cutters, and the outer membrane proteins were solubilized and obtained using sodium dodecyl sulfate 0.05% as detergent for Omp isolation. The hemagglutination reaction used mice erythrocytes. The cross reactions between subunit pili proteins were conducted by Western blot and Dot blot. Results: Antibodies against hemagglutinin sub unit pili protein 18 kDa responded to pili protein subunits 18 kDa; 23 kDa; 34 kDa; and 53 kDa; and Omp 23 kDa and 27 kDa. Omp and subunit pili proteins *S. flexneri* consists of several identical epitopes that were responsible for the similarity of the response profile in the cross-reactions of antibodies.

**Keywords:** Pili, Omp, hemagglutinin, cross reaction, *Shigella flexneri*

### INTRODUCTION

Shigellosis is a type of diarrhea caused by *Shigella* spp. Shigellosis is an endemic disease that occurs mainly in developing countries and is the most common cause of bloody diarrhea. These diseases are endemic and cause 120 million cases each year with severe dysentery. In developing countries endemic shigellosis is a significant cause of morbidity and mortality especially in children under five [1].

*Shigella* is Gram-negative bacteria in the family Enterobacteriaceae, consisting of non-motile bacilli. *Shigella* genus consists of four species: *S. dysenteriae*, *S. flexneri*, *S. sonnei* and *S. boydii* [2]. From the four species of *Shigella*, *S. flexneri* most commonly causes

dysentery and is recorded as causing 60 % of cases of shigellosis in developing countries [3].

The outer membrane protein (Omp), is known as an adhesion molecule in many species of bacteria and as well as *Shigella*, allowing bacteria to attach to cell receptors. [4]. Many pathogenic bacteria use a two-stage process of attachment: the first stage, where the bacteria is loosely attached with pili, and then the second stage, a stronger bond with the surface cell receptors using Omp. Hemagglutination activity is directly correlated with the colonization ability of *Shigella* spp. in a suckling mouse model [5]. Both pili and Omp hemagglutinin are virulence factors for infection-causing bacteria colonization [6].

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Developing vaccines against bacterial adhesion molecules may have an advantage in that the immune response may better eliminate bacteria and will not generate other side effects when compared with a whole cell-based vaccine or a bacterial lipopolysaccharide. Antibodies produced against Omp *S. flexneri* 34 kDa also may react with other *Shigella* species [4]. Recent research has shown that the Omp *S. flexneri* 2a is one of the most immunodominant antigens in the outer membrane of gram-negative bacteria and has many desirable characteristics for a candidate vaccine. Omp *S. flexneri* 2a is cross-reactive to antigens and common among *Shigella* spp. and is a widely recognized epitope on the cell surface and able to generate protective immunity in mice. Protective immunity involves the participation of both humoral and cellular immune responses, as OmpA increased rapidly induced IgG and IgA in both systemic and mucosal compartments and also activated Th1 cells [7, 8].

Recent studies have clarified that the anti-subunit hemagglutinin pili protein 7.9 kDa and subunit hemagglutinin pili protein 49.8 kDa *S. dysenteriae* are adhesion molecules [9, 10]. Subsequent studies have confirmed that there are similar epitopes between subunit pili protein 7.9 kDa *S. dysenteriae* and subunit pili protein 7.9 kDa *S. boydii*, and between subunit pili protein 49.8 kDa *S. dysenteriae* and pili subunit protein 49.8 kDa *S. boydii* as well, but differences in epitopes than with subunit pili 7.9 kDa and 49.8 kDa protein in *S. flexneri* and *S. sonnei* [11].

This research was conducted in order to clarify whether there is any cross-reaction among hemagglutinin subunit pili proteins and Omp *S. flexneri*. The result may be very important in designing a vaccine for shigellosis using an adhesion molecule-based vaccine.

## MATERIALS AND METHODS

### *Culture of Shigella flexneri*

Cultures of *S. flexneri* used in this research were derived from the Health Research Laboratory in DI Yogyakarta Indonesia. *S. flexneri* were grown in MacConkey's or salmonella-shigella agars. A Carbonate Thioproline Glutamate (TCG) medium was used to enrich the growth of *S. flexneri* pili. Bacterial culture on TCBS media were harvested and put in a bottle contained 1000 mL of brain-heart infusion broth (BHI). The bottles were then shaken for 30 minutes in a water bath at a temperature of 37°C. Furthermore, from the bottle of 10 mL bacterial suspension included in each bottle that already contained media TCG, then the bacteria in media TCG incubation was conducted at a

temperature of 37°C for 2 × 24 hours.

### *Method of harvesting subunit pili protein and isolating Omp from Shigella flexneri*

In order to harvest the pili, we applied the method using a pili cutter as described in Sumarno [12], which was a slight modification of Evan's method. Modifications that were made included precipitating the free pili and flagella from the bacteria after the last round of cutting and further separation using column chromatography was not used. Pellets were suspended with PBS at pH 7.4 until the volume reached 5 times the volume of the pellet, then SDS was added until the concentration reached 0.05%. Then the mixture was homogenized using a vortex mixer at full speed for 1 minute. The mixture was then centrifuged at 12,000 rpm at 4°C for 15 minutes. The supernatant was collected and stored at 4°C [19].

### *Sodium Dodecyl Sulfate Polyacrylamide Electrophoresis (SDS-PAGE)*

For protein identification and characterization on the electrophoresis gels, the protein stain comassie brilliant blue was used along with standard low-range molecular markers from Sigma Chemical. After calculating the molecular weight of the protein samples, SDS-PAGE was replicated for times to get a sufficient amount of protein for further analyses [13].

### *Subunit pili protein and Omp purification.*

Purification of subunit pili protein and Omp used the method of electroelution [9]. Bands of interest in the SDS-PAGE gels were cut perpendicularly so that each piece contained one protein band. The cut bands were collected and inserted into a piece of membrane tape that was filled with an electrophoresis running buffer. The membrane was put in a horizontal electrophoresis apparatus, and the protein from each eluted for 90 minutes under 120 mV of current. Following this, the membrane tape was dialyzed in a PBS pH 7.4 fluid buffer for 28 h with the replacement of the buffer 4 times in between (replaced 4 times after 7 minute intervals).

### *Hemagglutination test methods*

Hemagglutination assays were performed according to Hanne and Finkelstein [14]. Sample dilutions were made at half-concentration in micro plates with well volumes of 50 µL. In every well, mouse red blood cell suspensions at a concentration of 0.5% was added in the same volume. These were then shaken using a ro-

tator plate for 1 minute. Subsequently plates were incubated at room temperature for 1 hour. The titer was determined by observing the agglutination of red blood cells at the lowest dilution.

### Production of polyclonal antibodies

Mice were acclimatized for 4 days before immunization. The antigen used was subunit pili proteins from *S. flexneri*. Mice were injected with antigen emulsified with Complete Freud's Adjuvant (CFA), intraperitoneal, 100 µg/µL doses. Booster injections were performed in weeks two to four using antigens emulsified with Incomplete Freud's Adjuvant (IFA). A booster dose of 0.1 mL was used in a intraperitoneal injection. Serum was taken 1 week after the last booster [15].

### Western Blotting method

Western blots were performed according to the Bio-rad technical protocol [16]. The SDS-PAGE gels containing protein bands were transferred onto nitrocellulose (NC) membranes using a semi-dry blotter (Bio-rad). After that, the NC membrane was incubated overnight and washed with TBE plus 0.05% tween-20 twice. Next, the primary antibody of mice IgG at concentrations of 1/1000 in TBE pH 7.4 containing 1% solution of BSA was applied to the NC. The NC was washed again using the same solution and then an anti-mouse secondary antibody IgG at a concentration of 1/1000 in TBE pH 7.4 and a 1% solution of BSA were added. Subsequently the NC was washed twice for 5 minutes using TBE pH 7.4 with tween-20 at a concentration of 0.05%. For staining, Cip β tablets dissolved in 10 mL H<sub>2</sub>O were applied after this washing.

### Dot Blot method of examination

Dot blots are used to detect reactions with serological specificity between antigens and antibodies. The NC were trimmed into measuring 7.5 × 11 cm rectangles and inserted between two pieces of metal blotter apparatus. Then they were mounted on the dot blot, incubated overnight at a temperature of 4°C and degassed until the antigen was completely absorbed into the NC membrane. Further TBS blocking was carried out with blocking buffer (containing 50 mM Tris Base, 0.2 M NaCl, 5% skim milk, pH 7.4). Next, 50 mL of primary antibody was added, incubated for 2 h at room temperature and then placed in a shaker. The solution was removed, and then washed 3 times with TBS-0.05% Tween-20. Next, the secondary antibody with 1 : 2500 dilution was added. Again the NC was washed

3 times with TBS 0.05% Tween-20 and chromogenic substrate (BCIP-NBT) was added [17].

### Data analysis

The data obtained from the results of antigen-antibody reaction by Dot Blot method were transformed in *Corel Photo Paint*. The results of the interpretation of data on *Corel Photo Paint* were then analyzed using ANOVA statistical test and correlation test.

## RESULTS AND DISCUSSION

### The isolated sub unit pili protein and Omp of *Shigella flexneri*

The subunit pili protein *S. flexneri* and Omp profiles are shown in Figure 1. Profiles and the calculation of molecular weight (MW) of the subunit pili and Omp of *S. flexneri* showed a similar picture. Our result showed that the isolated protein derived from pili and Omp with a molecular weight of 72 kDa.

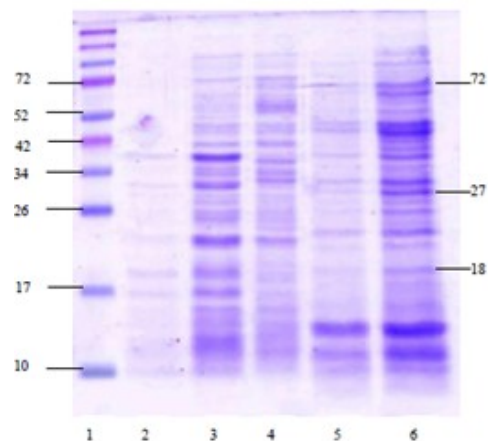


Figure 1. Profiles of pili and Omp of *S. flexneri* (1: marker protein; 2 and 3: pili slices 1 and 2 of *S. flexneri*; 3, 4, and 5 Omp isolation 1 and 2 of *S. flexneri*)

The aim of the erythrocyte agglutination test was to see whether purified subunit pili protein and Omp have the ability to perform mouse erythrocyte agglutination (hemagglutination). The results hem agglutination sub unit pili protein and Omp *S. flexneri* shown in Figure 2. The hemagglutination test results showed that the subunit pili protein 18 kDa *S. flexneri* showed the highest titer (1/128), therefore this protein was selected for further research.

The hemagglutination test carried out on Omp *S. flexneri* from three 3 band purification results are shown in Figure 3. The results showed that the Omp 72 kDa, 27 kDa and 18 kDa proteins can agglutinate erythrocyte at a titer of 1/128.

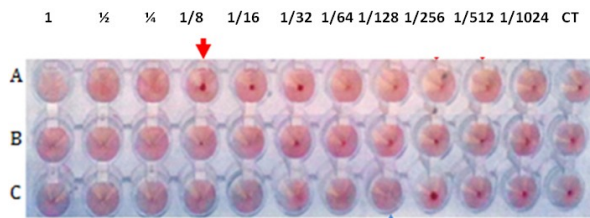


Figure 2. Hemagglutination test of subunit pili protein *S. flexneri* shows negative agglutination (red arrow) and positive agglutination (blue arrow). (A) Hemagglutination result of sub unit pili protein 72 kDa *S. flexneri*. (B) Hemagglutination result of sub unit pili protein 27 kDa *S. flexneri*. (C) Hemagglutination result of sub unit pili protein 18 kDa *S. flexneri*. (CT) Control (PBS + erythrocyte)

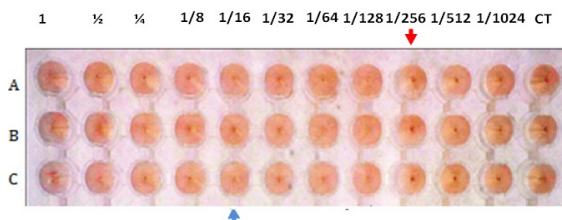


Figure 3. Hemagglutination test of purified Omp *S. flexneri* shows negative agglutination (red arrow) and positive agglutination (blue arrow). (A) Hemagglutination result of Omp 72 kDa *S. flexneri*. (B) Hemagglutination result of Omp 27 kDa *S. flexneri*. (C) Hemagglutination result of Omp 18 kDa *S. flexneri*. (CT) Control (PBS + erythrocyte)

**Antigen-antibody reaction by the method of Western Blotting**

The Western blot test was used to assess the antigen-antibody reaction using antibodies against subunit pili protein 18 kDa *S. flexneri* (the highest titer) to its subunit pili 18 kDa *S. flexneri* antigen, and with others sub unit pili protein and Omp *S. flexneri*.

Figure 4 shows the results of the expression of antigen-antibody reaction of subunit pili protein and Omp *S. flexneri* with antibodies against subunit pili protein 18 kDa *S. flexneri*. Subunits of the pili protein with molecular weights of 18 kDa; 23 kDa; 34 kDa; 49 kDa and 53 kDa *S. flexneri* responded to antibodies against sub unit pili protein 18 kDa *S. flexneri*. Meanwhile antibodies against sub unit pili protein 18 kDa *S. flexneri* responded to OMP *S. flexneri* with a molecular weight of 23 kDa and 27 kDa.

**Antigen-antibody reaction by the method of Dot Blot**

The results of the antigen-antibody reaction using the dot blot method can be seen in Figure 5; the results

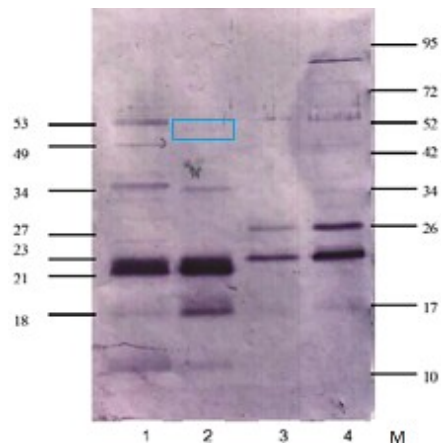


Figure 4. Western blot test results of antibodies against sub unit pili protein 18 kDa among subunits pili protein and Omp *S. flexneri* (1: subunit pili protein *S. flexneri* slice 2; 2: subunit pili protein *S. flexneri* slice 3; 3: Omp *S. flexneri* isolation 1; and 4: Omp *S. flexneri* isolation 2)



Figure 5. Results of Dot Blot among the crude pili, crude Omp and sub unit pili protein of *S. flexneri* with the antibodies against sub unit pili protein 18 kDa *S. flexneri* (1/500, 1/1000, 1/2000.....1/1024000 dilutions). (A) The crude sub unit pili protein, (B) the sub unit pili protein 72 kDa, (C) the sub unit pili protein 27 kDa, (D) the sub unit pili protein 18 kDa, (E) the crude Omp, (E) the Omp 72 kDa (F) the Omp 27 kDa Omp, and (G) the Omp 18 kDa.

of calculation of these antigen and antibody reactions using the semi-quantitative Dot Blot method is depicted in Figure 6.

There were significant differences ( $p = 0.000 < \alpha$ ) on all 8 different types of *S. flexneri* antigens, i.e., pieces of pili 2 *S. flexneri*; subunit pili protein purified 72 kDa, 27 kDa, 18 kDa; crude Omp *S. flexneri*; and subunit Omp 72 kDa, 27 kDa, 18 kDa.

The results of the correlation test between the mean density with 12 concentrations of antibody to subunit pili protein 18 kDa, showed that there was significant



relationship between treatment pili protein 2 *S. flexneri* with antibody concentrations ( $p = 0.000 < \alpha$ ) with a coefficient of correlation  $r = 0.895$ . For subunit pili protein 72 kDa *S. flexneri*, there was a meaningful relationship with pili protein antibody concentrations ( $p = 0.000 < \alpha$ ) with a correlation coefficient  $r = 0.924$ . There was also a significant relationship between subunit pili protein 27 kDa, 18 kDa, Omp *S. flexneri*, subunit Omp 72 kDa, 27 kDa and 18 kDa with pili protein antibody concentrations ( $p = 0.000 < \alpha$ ) with each of the correlation coefficient  $r = 0.932; 0.979; 0.922; 0.913; 0.731; \text{ and } 0.879$ .

The attachment of fimbriae or pili and other surface molecules to the surface of a host cell is mediated by specific receptors called adhesion molecules. The bond between the adhesion molecules with the receptor will activate signal transduction in host cells for the initial activation in the pathogenesis process and increased bacterial colonization [18].

The observation and calculation of molecular weight profiles for the pili protein subunits and Omp *S. flexneri* shows the same picture as depicted in Fig 1. The first piece pili subunit band seem thinner than the second and third pieces. Similarly, the same finding was found in the first and second Omp isolation. First isolated produce images that are thinner than in isolation to second. These differences in Omp band thicknesses could be caused by the location of the protein on the cell wall. The thickness differences in the pili protein subunits, however, could be caused by differences in the speeds used in the pili cutter.

During pili formation, subunit pili (pilins) secreted into the periplasmic space through the secretory pathway and binds to chaperon (companion) which assist the folding process and prevent the formation of premature sub-units. Then the complex pili/ chaperones brought into outer membrane usher which serves as a platform for the creation of pili. Then, the complex proteins form pores in the outer membrane that allows impassable helical strands [20, 21].

Three bands of both protein subunits pili and Omp with each molecular weight 72 kDa; 27 kDa; and 18 kDa were taken as the hemagglutinin protein candidate. This selection was based on the relative thickness of the band protein.

The hemagglutination test aims is to find protein hemagglutinin activity of *S. flexneri* pili proteins. The hemagglutinin protein is identical to the adhesion molecule that is responsible for the adhesion of bacteria to the host cell [10]. Therefore, the hemagglutination reaction is a screening tool for bacterial adhesion mole-

cules, which allowed these adhesion molecules to be used as a tool for diagnosis candidates and vaccine component [12, 22].

At times, precipitation of erythrocyte cells occurred in the bottom of the well showing no agglutination. This was caused by the absence of the bond between erythrocyte cells with pili protein subunits *S. flexneri*. Hemagglutinin protein is considered as one of the virulence factors of pathogenic bacteria. Bacteria that are able to perform agglutination and attach to the erythrocyte cell also have the ability to attach to receptors found on host mucosal cells. This is because the receptors that exist in the erythrocyte cell membrane are believed to have similarities with receptors on host cell mucosa [23].

The Western blot was results showed a maximum yield of antigen dilution pili subunits BM 18 kDa protein *S. flexneri* at a titer of 1/20, whereas the maximum results for antibody against pili subunits *S. flexneri* 18 kDa protein was at 1/500.

The Marrack Lattice theory states that if an antigen and antibody molecule react to form a precipitate, it will produce a different concentration ratio depending on the reaction zone in which the precipitin is formed. If the antibody-antigen ratio is above 1, it will settle. Whereas when the ratio is below 1, will remain in complex formed in the supernatant. The results of antigen-antibody reaction can be described in terms of a rainfall curve, which describes a rising curve shape as an excess antibody zone, which means a lot of free antibody molecules are in the supernatant, while a descending curve illustrates an excess antigen zone, which means a lot of free antigen molecules in the supernatant. Maximum precipitation is in the zone where there is no equivalent antigen or antibody detected in the supernatant [24].

From the results of the check board above it can be concluded that the equivalent zone lies in antibody and antigen titers between 1/500 and 1/20. Antigen-antibody bond formation requires the interaction of antibody bivalent and multivalent antigens that produces a complex bond. The more epitopes recognized by antibodies, the more and increasingly complex the bonds formed [25].

As seen in Figure 4, a cross-reaction between antibodies pili subunit 18 kDa protein in Omp *S. flexneri* was found. The results show that the protein responding to the antibody against pili subunits *S. flexneri* 18 kDa protein was a subunit of pili protein *S. flexneri* with a molecular weight of 18 kDa, 21 kDa, 34 kDa, 49 kDa and 53 kDa. While the protein responding to the

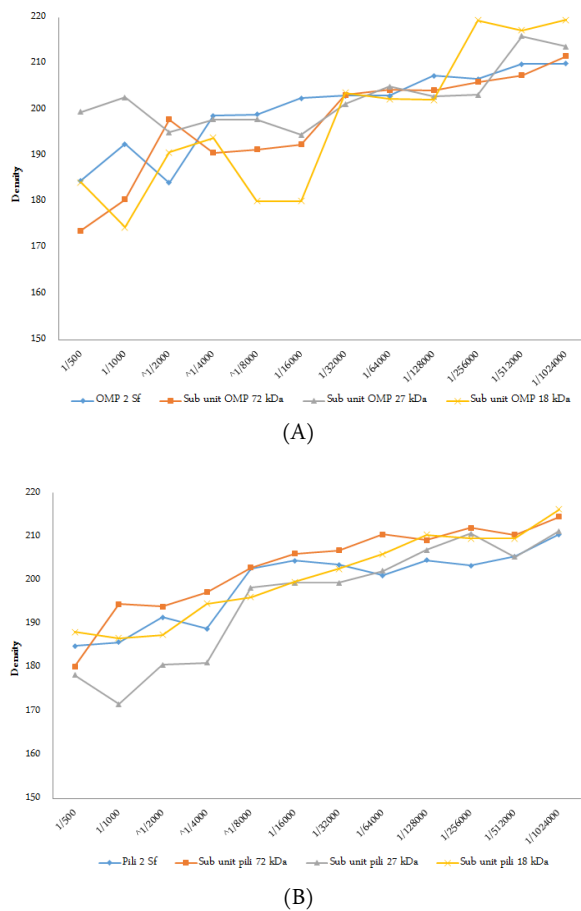


Figure 6. (A) The result of dot blot protein crude pili and sub unit pili protein purified with antibodies against sub unit pili protein 18 kDa *S. flexneri*. (B) The result of dot blot protein crude Omp and Omp purified with antibodies against sub unit pili protein 18 kDa *S. flexneri*

antibody against Omp *S. flexneri* was a protein with a molecular weight of 23 kDa and 27 kDa. This result seemed to be caused by a common epitopes which found on the sub unit pili protein and Omp *S. flexneri*. This study results are related to the character of pili proteins in gram-negative bacteria as a group of media adhesion and self-defense, in order to recognize and bind to at least one epitope molecule. The results also showed that there are similarities epitopes of the protein constituent subunits pili and Omp *S. flexneri*, so both can function as adhesin proteins.

Based on Anam (2015), it was found that there are predicted epitope similarities between the 7.9 kDa protein pili subunits of *S. dysenteriae* with 7.9 kDa protein pili subunits of *S. boydii* and the 49.8 kDa protein pili subunits of *S. dysenteriae* with 49.8 protein pili subunits of *S. boydii* as well, but differences in the epi-

topes between the 7.9 kDa and 49.8 kDa protein pili subunits of *S. flexneri* and *S. sonnei*. [11].

The statistical analysis found that there were significant differences in treatment between antibody reaction with the antigen pili proteins ( $p = 0.000 < \alpha$ ). The value of the lowest mean reaction between antibodies contained in the pili with an antigen protein is 27 kDa subunit. This means that the antigen protein 27 kDa subunit of pili reacted most stably/strongly with the pili protein antibodies. In the treatments with Omp antigens and antibodies to the subunit pili protein 18 kDa there were also significant differences ( $p = 0.000 < \alpha$ ). This seems to mean that the antibody sub unit pili protein when reacted with Omp antigen with different molecular weights will produce different antibody-antigen bond strengths.

Furthermore, the correlation of test results also showed significant correlation ( $p = 0.000 < \alpha$ ) between the treatment of pili proteins and antibody concentration Omp against pili proteins 18 kDa *S. flexneri*. It can be concluded that the smaller concentration of antibody gave a smaller density value of the antigen antibody reaction. This may indicate an antigen excess.

## CONCLUSION

The results of this study can be summarized as follows: hemagglutinin subunit pili protein and Omp 72 kDa, 27 kDa and 18 kDa is present in *S. flexneri*. Antibodies against hemagglutinin subunit pili protein 18 kDa can responded to the sub unit pili proteins 18 kDa, 23 kDa, 34 kDa, and 53 kDa, as well as to Omp 23 kDa and 27 kDa. This likely indicates that the sub unit of pili and Omp *S. flexneri* consist of several identical epitopes and is thus responsible for the similarity of the response profile in the cross-reactions of antibodies.

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