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Reaction Between Shigella dysenteriae Subunit Pili Antigen With 18 KDA Shigella flexneri Subunit Pili Antibody

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Abstract

Shigellosis caused by S. flexneri and S. dysenteriae is the leading cause of death in developing countries. Meanwhile, the development of the Shigella vaccine is still ongoing. This study aims to prove the reaction between the 18 kDa S. flexneri pili sub-unit protein's antibodies and the S. dysenteriae pili sub-unit protein antigen. This study used the Western Blot and Dot Blot tests to determine the antibody-antigen reaction both qualitatively and quantitatively. From the study results, it was found that the 18 kDa S. flexneri 18 kDa pili subunit protein was able to recognize the S. dysenteriae pili sub-unit protein with a molecular weight of 17 kDa and 23 kDa. The protein subunit pili S. dysenteriae with a molecular weight of 17 kDa and 23 kDa probably has an epitope recognized by the antibody to the pili protein subunit S. flexneri 18 kDa. The equivalent zone of the antibody-antigen reaction lies in the concentration of the antibody protein subunit pili 18 kDa S. flexneri titer 1/300 and the concentration of the antigen protein subunit pili 18 kDa S. dysenteriae titer 1/20. So that the higher the dilution of the antibody of the 18 kDa S. flexneri pili subunit, the lower the bond with the S. dysenteriae pili subunit antigen.

1. INTRODUCTION

According to research in 2013, there were 28,000 deaths annually among children under 5 years of age due to shigellosis [1] Lanata et al., 2013). Quantitative molecular analysis of the Global Enteric Multicentre Study (GEMS) identified an increased burden of shigellosis and reported it as a major pathogen among the six main causative pathogens that cause diarrhea in childhood [3]. Shigella infection is a challenge due to antimicrobial resistance. So that WHO Global Antimicrobial Resistance Surveillance identified Shigella as a priority pathogen for the development of new interventions. The Wellcome Trust-BCG

recommended accelerating development of the Shigella vaccine due to difficulties in achieving effective treatment with available antibiotics [4].

Various strategies for making vaccines have been used for decades to develop a safe and effective Shigella vaccine (Camacho et al., 2013; Barry et al., 2013). Although a licensed vaccine is not yet available, these efforts have helped better understand Shigella's immune response, and together with the latest innovative strategies are leading to promising vaccines [6].

Shigella can attach to host cells in 2 ways, namely through pili and Outer Membrane Protein (OMP). Pili can attach to host cells because they contain adhesion molecules (Sumarno et al., 2015). Pili or called fimbriae are involved in a special mechanism (attachment) of bacteria to the host cell. Pili is a major determinant of bacterial virulence because it allows pathogens to attach to host cells [8].

Based on research in 2017, the results showed that the *S. flexneri* pili protein subunit was able to cross-react with Omp *S. flexneri* with Molecular Weight (MW) 23 and 27 kDa, and could cross-react with the pili 18 kDa subunit; 23; 34; and 53 kDa *S. flexneri* [9]. Then from another study in the same year, it was also found that the 18 kDa *S. flexneri* pili subunit protein is an adhesive protein that can help attach the pili bacteria to the enterocyte cells of mice [10]. A 2018 study proved that β defensin in the serum, intestinal mucosa, and lungs of mice was increased by oral vaccination of the 18 kDa protein subunit *S. flexneri* pili for 4 weeks. This study proves that the 18 kDa *S. flexneri* pili subunit protein can be a subunit-based vaccine material [11]. The results of this study could lead to the idea that if the *S. flexneri* pili subunit protein was recognized by pili of the same species, it should also be recognized by *S. dysenteriae* pili. Whereas *S. dysenteriae* is unique among Shigella because it produces Shiga toxin (which exhibits strong neurotoxic, cytotoxic and enterotoxic activity), causes unusually severe clinical disease, and causes widespread pandemic spread [12].

By doing this research, it is expected to find materials for shigellosis vaccine based on protein subunits specifically for *S. flexneri* and *S. dysenteriae.*

2. MATERIALS AND METHODS

Research design

This study is an exploratory study in which proteins from the *S. flexneri* pili subunit protein will be identified, then reacted with the *S. dysenteriae* pili subunit, to determine whether there is a cross-reaction between the *S. flexneri* and *S. dysenteriae*.

Place and time of research

This research was conducted at the Microbiology and Research Laboratory, Faculty of Medicine and Health Science UIN Maulana Malik Ibrahim Malang and Biomedical Laboratory, Faculty of Medicine Brawijaya University. The research was conducted for 3 months, from January 2020 to March 2020.

Tools and Materials

The materials used in this study were: pure culture of *S. flexneri* and *S. dysenteriae*, Salmonella-Shigella Agar (SSA) medium, Bismuth sulfite agar (BSA), Thiaproline Carbonate Glutamate (TCG) agar, Brain Heart Infusion (BHI) broth. and Mac Conkey agar, Balb / C rats, reagents: TCA, tris base, methanol, Tween 20, PBS, glacial acetic acid, glycerol, bromophenol blue, ammonium sulfate, SDS, TEMED, ammonium persulfate, glycine, commassie blue, NaCitrat, tris-HCL, EDTA, EGTA, dithiothreitol, NaCl, ethanol, Na2HPO4, KH2PO4, acrylamide, bis acrylamide.

The tools used in this research are a microtiter plate, microscope, culture bottle, pili cutter, stirrer, incubator, autoclave, micropipette, syringe, water bath, shaker, hot plate, pH meter, centrifuge, horizontal electrophoresis, membrane. dialysis,

electroelution membrane, Eppendorf tube, 15 ml tube.

Research Steps

***Shigella dysenteriae and S. flexneri* cultures**

This study used the bacteria *S. dysenteriae* and *S. flexneri* from the Labkesda Yogyakarta. The bacterial growth media used were Salmonella Shigella Agar selective media and Mac Conkey media. Then to enrich the growth of pili planted in TCG media. Then the culture is harvested by scraping, and the bacterial suspension is put into a bottle containing 1000 ml of cerebral brain infusion (BHI) solution.

Pili method isolation from *S. dysenteriae* and *S. flexneri*

The results of the collection of bacteria from TCG media were collected in one bottle which was then added with trichloracetic acid (TCA) with a concentration of 3%. Then a pili shaver is prepared, which is a pili bacteria cutter. The supernatant resulting from cutting the bacterial pili was then stored at 40°C.

Electrophoresis (SDS-PAGE)

Monitoring the molecular weight of bacterial pili was carried out using the SDS-PAGE electrophoresis method. After obtaining the molecular weight, the desired protein multiplication was carried out by the electroelution method.

Electroelution and Dialysis

The electrophoretic protein is then cut to the desired molecular weight. The sliced protein is placed into a petri dish containing a flowing buffer. The dialysis method was carried out by inserting electroeluted cellophane into a beaker glass containing a sterile phosphate buffer salt solution which was then mixed with a stirrer at 4 oC for 24 hours.

Polyclonal antibody (IgG) production

Before vaccination, the mice were acclimatized for 1 week. The antigen used was 18 kDa *S. flexneri* pili subunit protein obtained from dialysis. Mice were injected intra-peritoneally with the antigen emulsified with Complete Freud's Adjuvant (CFA) at a dose of 250 µg / 0.3 ml PBS. One week later a booster dose of 0.1 ml was given by intraperitoneal injection. This booster dose is given 3 times at an interval of 1 week. One week later the mice were sacrificed and the serum was taken.

Check Board Test

To see the effectiveness of the antigen reaction in the form of *S. dysenteriae* pili protein and antibody in the form of IgG induced by the *S. flexneri* pili protein subunit, a checkboard test was performed. This checkboard test uses the dot blot method by reacting to the *S. dysenteriae* pili subunit antigen and the resulting antibody (IgG). The results from the checkboard will get the best antigen and antibody concentrations that will be used as a concentration reference for western blot and dot blot tests.

Western blot method

SDS-PAGE gel containing protein bands was transferred to nitrocellulose paper using a semi-dry blotter. Then through several stages, namely the provision of primary, secondary, SAHRP antibodies. The western blot results in the form of a blue line on the cellulose membrane which shows the molecular weight of the detected antigen protein.

The dot blot method

The dot blot test is performed to detect the titer of dilution with the strongest antigen and antibody reaction. The spots show purplish-blue dots which indicate that the pili protein is bound by anti-pili protein antibodies. Purplish blue teat on the NC membrane indicates a specific antigen-antibody response.

Approval of Experimental Animal Research Ethics

The implication of using experimental animals by administering intraperitoneal vaccines in the form of Shigella pili subunit proteins that do not carry virulence factors and

isolating the blood of mice by drawing blood from the mice's hearts. Mice were sacrificed through cervical bone dislocation and performed by trained personnel. After that, the carcasses of mice are buried in a suitable place, so as not to cause the impact disease transmission to other animals or the environment.

3. RESULTS and DISCUSSION

Results of *S. flexneri* and *S. dysenteriae* pili protein isolation

After cutting the pili with pili cutter bacteria, protein isolation was carried out using the SDS-PAGE electrophoresis method. The pili protein profiles of S. *flexneri* and S. *dysenteriae* are shown in Figure 1.

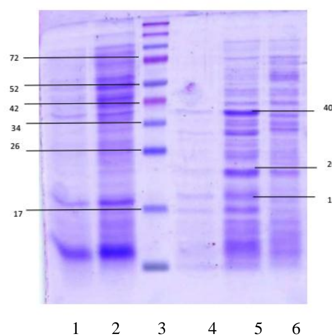

Figure 1. Pili protein profiles of S. *flexneri* and S. *dysenteriae*

Notes:
1.First cut of S. *dysenteriae* 1 pili, 2.Second cut of S. *dysenteriae* pili, 3.Markers, 4.First cut of S. *flexneri* pili, 5.Second cut of S. *flexneri* pili, 6.Third cut of S. *flexneri* pili

The profiles and section molecular weight calculations of S. *flexneri* and S. *dysenteriae* pili show a similar picture. Meanwhile, from the calculation of the molecular weight, the protein in each piece of pili was obtained with the clearest molecular weight of 40 kDa; 20 kDa; and 18 kDa.

Antigen and Antibody check board

An antigen and antibody checkboard is performed to see the effectiveness of the antibody response to the antigen. The antigen used was the 18 kDa *S. dysenteriae* pili subunit protein, while the antibody used was the 18 kDa *S. flexneri* pili subunit protein. The checkboard test will be used as the basis for the antibody-antigen reaction test using the Western Blot and Dot Blot methods. The results of the density measurement showed that the pili protein antigen 18 kDa *S. dysenteriae* had the strongest reaction seen at 1/20 dilution, while antibodies were seen at 1/300 dilution.

Table 1. The results of the 18 kDa S. flexneri protein antibody checkboard against the 18 kDa S. dysenteriae protein antigen

Ag/Ab	**1/100**	**1/200**	**1/300**	**1/400**	**1/500**	**1/600**	**1/700**	**1/800**
1/10	131,12	120.3	94,20	123,81	101,11	127,75	102,74	74.05
1/20	78,52	88,6	**50,72**	113,34	87,057	101,85	88,30	86,53
1/40	68,59	86,9	54,76	123,44	72,74	112,49	59,91	54,72
1/80	88,96	79,4	65,78	143,7	109,73	115,42	55.54	79,57
1/160	65,70	64,2	58,9	102,23	70,85	101,24	54,93	54,21

Antigen-antibody reaction with Western Blot

The Western Blot test was used to see the antigen-antibody reaction using the 18 kDa S. flexneri pili subunit protein antibody with S. dysenteriae pili slices.

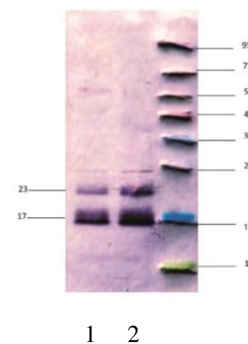

Figure 2. Results of Western blot test on 18 kDa S. flexneri pili subunit protein with S. dysenteriae pili pieces

Notes :
1.The antigen-antibody reaction between second cut of S. dysenteriae pili and the 18 kDa S. flexneri pili subunit protein antibody, 2.The antigen-antibody reaction between first cut of S. dysenteriae pili and the 18 kDa S. flexneri pili subunit subunit antibody

Figure 2 shows the results of the reaction expression between the antibody from the 18 kDa *S. flexneri* pili subunit protein and the antigen from *S. dysenteriae* pili. From the antigen-antibody reaction test using the Western Blot method with one different antibody and antigen, the molecular weight profile of each can be seen. Proteins that respond to antibody from the 18 kDa *S. flexneri* pili subunit protein to *S. dysenteriae* pili protein are proteins with a molecular weight of 17 kDa and 23 kDa.

Antigen-antibody reaction with Dot blot

The dot blot method was used to observe the antigen-antibody reaction using the 18 kDa *S. flexneri* pili protein subunit antibody with pieces of pili and purified *S. dysenteriae* pili protein. The results were then calculated using Corel Photopaint to see the sharpness of the resulting color. Corel Photo paint calculations from the color gradations on the dot blot are shown in Table 3.

Table 3. Dot Blot Results of 18 kDa *S. flexneri* pili protein subunit antibody to *S. flexneri* and *S. dysenteriae* pili subunit antigen

Ag/ Ab	1/3 00	1/40 0	1/50 0	1/6 00	1/70 0	1/80 0	1/90 0	1/10 00
a	184, 94	186	191,5 8	192,9 6	202, 72	204, 53	204 ,55	205,1 4
b	186,1 3	186, 72	187, 47	194,6 3	196, 24	199, 62	202 ,62	206, 06
c	182,5 8	184, 51	186, 06	198,6 3	198, 91	202, 47	203	203,0 1
d	174,2 2	174, 44	190 ,71	193 ,83	194,15	191,1 1	203, 67	203,7 1

Notes :

a.Pieces of *S. flexneri* pili, b.Subunit pili 18 kDa *S. Flexneri,n*c.Pieces of *S. dysenteriae* pili, d.Subunit pili 18 kDa *S. dysenteriae*

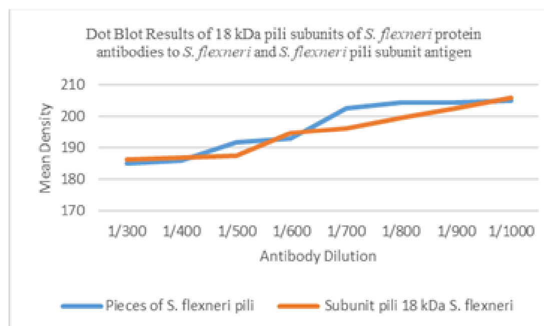

Figure 3. Graph of *S. flexneri* protein dot blot results with *S. flexneri* pili protein antibody.

The results of figure 3 show that there is an increasing trend in the mean density of the results of the reaction of the 18 kDa *S. flexneri* pili protein subunit antibody with pieces of pili and 18 kDa *S. flexneri* pili subunit protein.

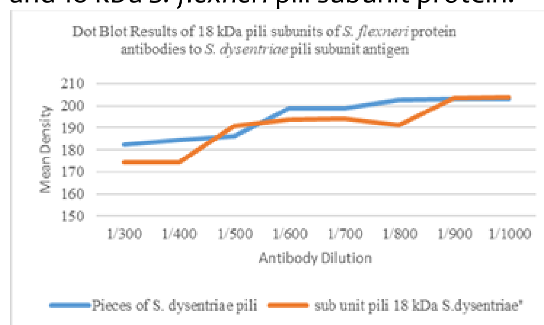

Figure 4. Graph of dot blot results for the S. dysenteriae pili protein subunit with 18 kDa S. flexneri pili protein subunit antibody

The results of figure 4 show that there is an increasing trend in the mean density of the antibody reaction of the 18 kDa *S. flexneri* subunit protein pili with pieces of pili and 18 kDa *S. dysenteriae* subunit protein pili. The higher the mean density of the dot blot results, the lower the density of the antigen-antibody reaction.

4. DISCUSSION

This study aims to prove the presence of antigen and antibody reactions between the 18 kDa *S. flexneri* subunit protein pili antibody and *S. dysenteriae* subunit protein pili. The results of the growth of *S. flexneri* and *S. dysenteriae* were then isolated in pills using a bacterial pili cutter, which was cut gradually until the pili on the bacterial cells were clean.

With the isolation of pili, it is expected to obtain adhesion protein from the pili that have completely separated from the membrane. Pili cutting with a pili cutter aims to separate the pili organ from the bacterial cell membrane. These results prove that pili can be isolated from the membrane. Pili found on the surface of bacterial cells is an intermediary tool for bacteria to attach to host cells. Fimbriae of pili and other molecular surfaces are used as a medium for attaching to the surface of host cells through specific receptors. The bond between the adhesive and the receptor will activate signal transduction in the host cell for initial activation and increase bacterial colonization [13].

The results of protein identification showed protein expression bands of *S. flexneri* and *S. dysenteriae* pili. The profile and molecular weight calculations of the *S. flexneri* and *S. dysenteriae* pili slices show the same picture as in Figure 1. The results of the first pili slices appear lighter in color compared to the second and third slices. The difference in the thickness of the ribbon in the first slice and so on is due to the possibility that a small portion of the protein is still cut off in the first slice. Then in the next slice, more protein fractions can be isolated. Besides, the length of time to cut the pili also affects the resulting ribbon expression.

Meanwhile, the molecular weight calculations obtained protein at molecular weights of 18, 20, and 40 kDa in the *S. flexneri* and *S. dysenteriae* pili pieces respectively. From these results, it is molecularly assumed that *S. flexneri* and *S. dysenteriae* pili have the same characteristics. The profiles shown on gel electrophoresis on pili cuttings of *S. flexneri* and *S. dysenteriae* have a fairly large band.

During pili formation, the pili subunits (pilins) are secreted into the periplasmic space via secretory pathways and bind to chaperones (chaperones) which aid in the folding process and prevent premature subunit formation. Then the pili/companion complex is brought to the user of the outer membrane which serves as the pili formation platform. Then, this complex protein forms pores in the outer membrane that allows the bent strands to (Proft & Baker, 2009; Werneburg & Thanassi, 2018).

The western blot method in this study was used to see the reaction between the 18 kDa *S. flexneri* subunit protein antibody and the *S. dysenteriae* subunit pili antigen. Figure 2 shows the expression results of the antibody-antigen reaction of the *S. dysenteriae* pili protein subunit antigen with the 18 kDa *S. flexneri* subunit protein pili antibody. From the antigen-antibody reaction test using the Western blot method with one different antibody and antigen, the molecular weight profile of each can be seen. Proteins that respond to the 18 kDa *S. flexneri* subunit protein pili antibody against *S. dysenteriae* pili protein are proteins with a molecular weight of 17 kDa; and 23 kDa.

The *S. dysenteriae* subunit protein pili with molecular weights of 17 kDa and 23 kDa likely have an epitope that can be recognized by antibodies to the 18 kDa *S. flexneri* subunit protein pili. These results are related to the character of the pili protein in gram-negative bacteria as a medium for adhesion and self-defense so that they can recognize and bind at least one epitope molecule. These results also indicate that there are similarities in the epitopes that make up the protein subunits of *S. flexneri* pili and subunits of *S. dysenteriae* pili so that they can carry out their function as protein adhesins.

The Western blot results indicate the ability of the antibodies to respond to the pili protein antigen from *S. dysenteriae*. Besides recognizing the protein epitopes of *S. flexneri* pili, the antibody to the 18 kDa pili protein can also recognize the protein epitopes of *S. dysenteriae* pili. So the *S. dysenteriae* pili protein may have identical properties because it can be recognized by the antibody to the *S. flexneri* pili protein.

Before doing the Western blot in observing the antigen-antibody reaction, in this study a checkboard was carried out to see the

effectiveness of the antigen-antibody reaction that occurred. The results of the checkboard reaction showed that the 18 kDa subunit protein pili antigen was seen at 1/20 dilution, while the antibody was seen at 1/300 dilution. Antibody titer is the antibody content measured by titration. Antibody titer is one indicator of antibody-antigen reaction. Each antibody has two arms, each of which can bind to an epitope. When an antibody binds to two antigens, the two antigens bind together by the antibody. Lattices can form because antibodies bind more antigens together, producing precipitins. Most precipitin tests use a polyclonal antiserum rather than a monoclonal antibody because polyclonal antibodies can bind to multiple epitopes, making lattice formation more likely. Although monoclonal antibodies, can bind to multiple antigens, the binding will occur less frequently, so there is much less chance of visible deposits forming.

Based on the lattice theory (The Lattice theory) put forward by Marrack, if the antigen and antibody molecules are reacted it will produce precipitation reactions with different ratios for different concentrations, depending on the precipitin reaction zone where they are formed. If the antibody-antigen ratio is above 1, precipitates will be formed. Meanwhile, if the ratio is below 1, the dissolved complex will still form and remain in the supernatant. The result of this antibody-antigen reaction can be described in the form of a precipitation curve, where the shape of the rising curve represents the antibody excess zone, which means that many free antibody molecules are present in the supernatant. Meanwhile, the downward curve illustrates the excess antigen zone, which means that many free antigen molecules are present in the supernatant. Meanwhile, the maximum precipitation is in the equivalent zone where neither antigen nor antibody is detected in the supernatant [16].

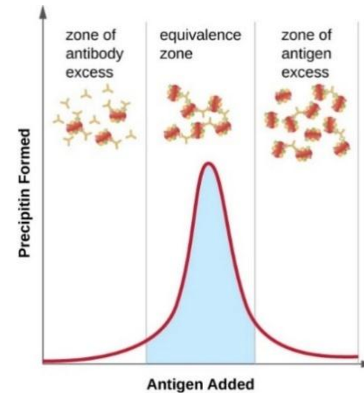

Figure 5. Presipitation curve [16]

Based on the results of the checkboard above, it can be concluded that the equivalence zone lies in the antibody titer 1/300 and antigen titer 1/20. The formation of this antibody-antigen bond requires the interaction of a bivalent antibody and a multivalent antigen that produces a complex bond. The more epitopes recognized by the antibody, the more complex the bonds will be.

Based on the results of the next dot blot, namely by reacting to various antigens, there was an increase in the average reaction density trend, but the results of this study could not be performed in statistical analysis because there were no repetitions for each treatment. The result of the mean density increase means that the antigen-antibody bond has not reached the equivalence zone, because the excess antigen is already large so there are no more bonds with antibodies from the 18 kDa pili subunit *S. Flexneri.*

5. CONCLUSION AND RECOMMENDATIONS

Conclusion

1. The 18 kDa *S. flexneri* subunit protein pili antibody can be responded to the 17 kDa and 23 kDa subunit protein pili *S. dysenteriae.*
2. The equivalent zone of antibody-antigen reaction was located in the antibody concentration of 18 kDa *S. flexneri* subunit protein pili titer 1/300 and antigen concentration of 18 kDa *S. dysenteriae* subunit protein pili titer 1/20.

Recommendations

1. It is necessary to prove the presence of an antibody-antigen reaction against the *S. flexneri* pili subunit protein using the *S. dysenteriae* pili subunit antibody.
2. Further research is needed to develop vaccines based on adhesin molecules

6. ACKNOWLEDGMENT

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