CLARIFICATION OF PROTEIN SUB UNIT PILI AND OUTER MEMBRANE PROTEIN (Omp) *Shigella flexneri* AS ADHESION PROTEIN WITH HEM AGGLUTINATION TEST

Avin Ainur Fitrianingsih[1]

[1] Faculty of Science and Technology, State Islamic of University Maulana Malik Ibrahim, Malang, Indonesia
Email: avinainur@yahoo.com

ABSTRACT

Diarrhea is a serious problem in developing countries. *Shigella flexneri* is the commonest cause bacillary diarrhea in developing countries. Vaccination against bacteria *Shigella spp.* necessary to reduce the incidence of diarrhea bacillary including those caused by *S. flexneri.* This study aims to explore the hemagglutinin protein (a protein that is able to agglutinate erythrocytes) pili and Omp *S. flexneri* alleged role in the adhesion of bacteria to the enterocytes. This study using hemagglutination assay method by reacting antigen derived either from protein sub units pili or subunits Omp *S. flexneri* result of purification, then observed agglutination occurs. At the protein sub units of *S. flexneri* pili showed: the molecular weight (MW) 72 kDa protein occurs in the agglutination titer 1/4, on the MW 27 kDa agglutination titer 1/16, and the MW 18 kDa occurred agglutination in titers of 1/128. While the Omp protein 72 kDa subunit; 27 kDa; and 18 kDa occurred agglutination in titers of 1/128. These results prove that the pili and Omp protein purification results thought to contain adhesion protein that is able to bind erythrocytes or called hemagglutinin protein. The occurrence of agglutination indicated by the absence of erythrocyte sediment at the bottom of the wells. While the deposition that occurs on the bottom of the well indicates a negative result, this is because the erythrocytes are not bound by a protein sub unit of pili and Omp *S. flexneri.*

Keywords

S. flexneri, pili, Omp, hemagglutinin

INTRODUCTION

Shigellosis is 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 . In developing countries endemic *Shigellosis* causes of morbidity and mortality especially in children under five age (Niyogi, 2005). In Indonesia, one type of *shigellosis* is caused by the *S. flexneri* (anonymous, 2005). A study conducted in Jakarta in February 2005 to September 2007 were performed on 612 children aged 0-12 years with diarrhea showed that *Shigella* diarrhea causes 9.3 % of all cases. A total of 63.2 % of *Shigella* isolates are *S. flexneri* (Herwana, 2010).

Transmission of *S.flexneri* is through the fecal-oral route which enters the human body through the consumption of water or food contaminated feces. *S. flexneri* is very easy to infect , where only 10 to 100 microorganisms alone is enough to cause diarrhea . Most of the current knowledge on the mechanisms underlying the pathogenesis of *Shigella* infection comes

from studies of *S. flexneri* (Schroeder & Hilbi, 2008).

Pili and outer membrane protein (Omp) is already known to the adhesin molecule in many species of bacteria and similarly to the family *Enterobacteriacea*. Hem agglutinin protein of bacteria is an indication of the ability of bacterial adhesion and adhesin which are found also possess hemagglutination (Pore*, et al*, 2010). Many pathogenic bacteria use two stage process of this attachment, first with loose ties pili , followed by a stronger bond to the surface of the protein . Both pili and Omp is one of the virulence factors for the occurrence of infection-causing bacteria colonization (Salyer & Whitt, 2002).

The development of molecular adhesin vaccine has advantages on the immune reaction of the body that is formed will form the body's defense system more powerful in eliminating bacteria and does not generate fever like a vaccine of intact bacterial cells or from LPS. While antibodies produced 34 kDa Omp *S. flexneri* also has advantages may react in other *Shigella* species (Pore, *et al,* 2010). 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 of the candidate vaccine (Pore & Chakrabarti, 2013).

This research will be conducted for clarification of protein sub unit pili and sub unit Omp *S. flexneri* alleged hemagglutinin protein so that it can be a vaccine candidate that is expected to stimulate an immune response but has minimal side effects .

METHODS

Culture *S. flexneri*

Bacteria used in this research is *S. flexneri* that derived from the Health Research Laboratory in DI Yogyakarta Indonesia. *S. flexneri* grown in medium Salmonella and Shigella Agar and Mac Conkey. Carbonate Thiaproline Glutamate (TCG) medium is used to enrich the growth of *S. flexneri* pili. Bacterial culture on TCBS media are harvested using the scrapings then put in a bottle containing 1000 ml of brain heart infusion broth (BHI). Bottle then shaken for 30 minutes in a water bath with a temperature of 37^{o}C. Furthermore, from the bottle of 10 ml bacterial suspension included in each bottle which already contains media TCG, then the bacteria in media TCG incubation was conducted at a temperature of 37^{o}C for 2x24 hours .

Method of pili and Omp isolation of *S. flexneri*

Method of cutting pili using pili cutter referred to Sumarno (Sumarno, *et al*, 2012), with slight a modification from Evan's method. Modifications made in the free pili and flagella precipitated bacteria at the last round cutting and did not do column chromatography separation. Pellets were suspended with PBS pH 7.4 until the volume reaches 5 times, then added SDS until concentration reaches 0.05%. Then performed using a vortex homogenization with full speed for 1 minute.After that centrifugation done with speed 12000 rpm at 4^{o}C for 15 minutes. The supernatant was collected and stored at 4^{o}C.

Sodium Dodecyl Sulfat Polyacrylamide Electrophoresis (SDS-PAGE)

Obtaining the weight of molecules is mostly done by using SDS-PAGE (Laemli, 1970). Protein sample was heated at 100°C for 5 minutes in buffer solution containing 5 mMTris pH 6.8 : 5% 2-mercapto ethanol, 2.5 w/v sodium dodecyl sulfate, 10% v/v glycerol tracking gel 4%. The applied voltage electric current is 120 mV. The colour protein, comassie brilliant blue was used along with sigma standard low range molecular marker. After the calculation of molecular weight of the proteins, multiplication for protein of interest were done in eight gels SDS-PAGE product.

Pili and Omp protein purification

Purification of protein pili and Omp refer to the method of electroelusion (Agustina, et al, 2012). Results of pili and Omp collection were run for electrophoresis by using SDS-PAGE. The results of gel electrophoresis gave us the characterization of pili and Omp proteins. Bands of interest were cut perpendicularly so that each piece contained one protein band. The cut bands were collected and inserted into a piece contained one protein band. The cut bands were collected and inserted into a piece of membrane tape which was filled with an electrophoresis running buffer. The membrane was put in a horizontal electrophoresis apparatus, taking 90 minutes with 120 mV. Following this, the membrane tape was dialyzed with PBS pH 7.4 fluid buffer for 28 h with the replacement of the buffer 4 times in between.

Hemagglutination test methods

Hem agglutination assay was done according to the instructions of Hanne and Findkelstein's (Hanne & Findkelstein, 1982). Sample dilutions were made on ½ concentration in microplate V where each well volume was 50µl. In every well red blood suspension of mice with a concentration of 0.5% in the same volume of 50 µl was added. Then it was shaken using a rotator plate for 1 minute. Subsequently it was placed at room temperature for 1 hour. The titer was determined by observing the agglutination of red blood at the lowest dilution.

RESULTS

The isolated pili and Omp of *S.flexneri*

After cutting pili and Omp isolation was performed using SDS-PAGE electrophoresis method. The *S. flexneri* pili and Omp protein profiles are shown at Fig. 1

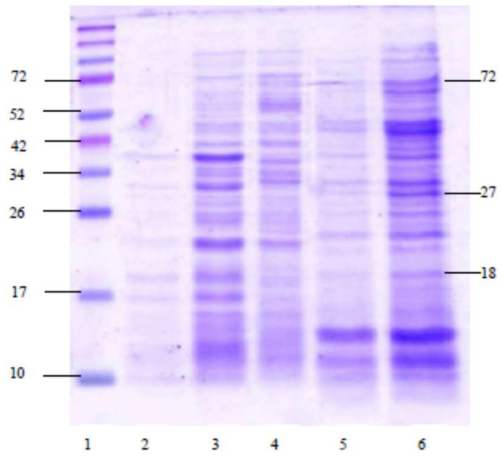

Fig. 1: Profiles of pili and Omp of *S. flexneri*
1 marker protein
2 and 3 *S. flexneri*, pili slices 1; 2; and 3
4 and 5 *S. flexneri*,Omp isolation 1 and 2

Profiles and the calculation of molecular weight (MW) of the pieces pili

and Omp *S. flexneri* showed a similar picture. In this studies taken each piece of pili and Omp with a molecular weight of 72 kDa; 27 kDa; and 18 kDa.

After the pili and Omp proteins purified then hem agglutination test to look at the ability of these proteins to agglutinate erythrocytes of mice. Hem agglutination test results that have been purified pili and Omp protein shown in Fig.2.

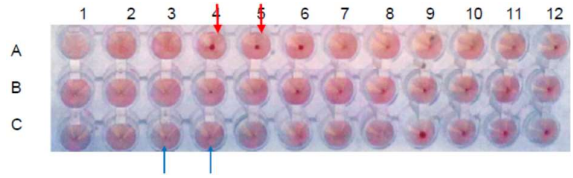

Fig.2 : The result of hemagglutination test of purified protein subunit pili*S. flexneri*
(→) negative agglutination, (→)positive agglutination.

A. Hem agglutination result of sub unit pili 72 kDa *S. flexneri*
B. Hem agglutination result of sub unit pili 27 kDa *S. flexneri*
C. Hem agglutination result of sub unit pili 18 kDa *S. flexneri*

1 – 11 : Dilution antigen : 1, ½, ¼ 1/8, 1/16, 1/32, 1/64, 1/128, 1/256, 1/512, 1/1.024

12 : control (PBS + erythrocyte)

From the results of the hem agglutination test showed that the pili with MW 18 kDa showed the highest titer of 1/128 and can be used as a protein hem agglutinin.

Agglutination test carried out on the Omp *S. flexneri* which consists of 3 bands . Hem agglutination test results that have been purified Omp shown in Fig. 3.

From the results of the hem agglutination test showed that the Omp with a molecular weight of 72 kDa;27 kDa and 18 kDa can agglutinate erythrocyte from titer 1/128 and can be used as a protein hem agglutinin.

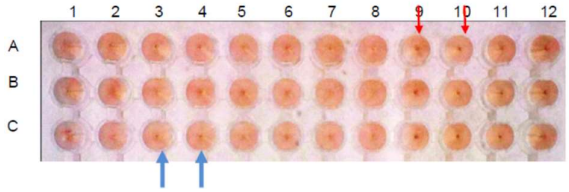

Figure 3. The result of hem agglutination test of purified protein sub unit Omp *S. flexneri*
(→)negative agglutination, (→)positive agglutination.

A. Hemagglutination test result of sub unit Omp 72 kDa *S. flexneri*
B. Hemagglutination test result of sub unit Omp 27 kDa *S. flexneri*
C. Hemagglutination test result of sub unit Omp18 kDa *S. flexneri*

1 – 11 : Dilution antigen : 1, ½, ¼ 1/8, 1/16, 1/32, 1/64, 1/128, 1/256, 1/512, 1/1.024

12 : control (PBS + erythrocyte)

Isolation and Characterization of *S. flexneri*

Based on research Anam in 2012, there are differences in morphological picture of bacteria before and after cutting pili with pili cutter. Observation using electron microscopy aims to look at the morphology of the bacteria prior to cutting and after cutting. From the observation of bacteria using an electron microscope to prove that the tool is effective in cutting pili of *Shigella* bacteria (Anam, *et al*, 2015). Pili found on the surface of the bacterial cell is a tool intermediary of bacteria in conducting attachment on the host cell. Fimbriae or pili and other surface molecules are used as media to stick to the surface of the host cell via a specific receptor. The bond between the adhesin with the receptor will activate signal transduction in host cells for the initial activation as well as an increase in bacterial colonization (Forest, 2007).

In this study, Omp isolation of *S.flexneri* by using a solution of Sodium Dodecyl Sulfate (SDS) 0.05%. Sodium Dodecyl Sulfate is an anionic detergent capable of disrupting the cellular structure and cause the denaturation of cells. This detergent break the membrane proteins by mimicking the mechanism of lipid bilayer environment. Detergent containing compounds that can lower the surface tension through hydrophobic interactions (G-Biosciences, 2010).

Profiles and the calculation of molecular weight of the pieces pili and Omp *S. flexneri* shows a similar picture as shown in Fig. 1. Results of the first pieces of pili seem thinner band than the second and third pieces. Similarly, the isolation of Omp result in the isolation of the first and second, where the results of the first isolation produces a picture that is thinner than the second piece. Differences in the thickness of the ribbon on the first piece and so on because the first cut is likely to remain slightly truncated protein fraction. Then on the next piece of a growing number of increasingly protein fraction can be isolated.

The third band from both pili and Omp protein consisting of a protein with a molecular weight of 72 kDa; 27 kDa; and 18 kDa proteinadhesion is taken as a candidate. Making the protein band because of the many proteins in SDS-PAGE profile has a thicker tape from another tape.

During the formation of pili, pili subunits (pilins) secreted into the periplasmic space through these cretory pathway and bind to the chaperon (companion) who assist the folding process and prevent the formation of sub-units are premature. Then the complex pili/chaperones were taken to usher outer membrane that serves as a platform for the formation of pili. Then, the complex proteins form pores in the outer membrane that allows thehelical strands can be skipped (Soto & Hultgren, 1999) (Proft & Baker, 2009).

Hemagglutination test results aimed at finding protein hemagglutinin protein pili owned by *S. flexneri.* Hemagglutinin protein is a nadhesin protein that mediates attachment of the bacteria to the host cell. This is done to confirm and prove that the pili proteins of *S. flexneri* as adhesion protein.

The occurrence of agglutination indicated by the absence of erythrocyte sediment at the bottom of the wells.While the deposition that occurson the bottom of the well indicates a negative result. This is because the erythrocytes are not bound by a protein sub unit of pili *S. flexneri.*

These result prove that the pili proteins of *S. flexneri* adhesion is thought to contain protein which is a protein that is able tobind erythrocytes or called hemagglutinin protein. Pili adhesion proteins on the bacteria *S. flexneri* is the intermediary in conducting attachment to the host cell enterocytes. Pili which is also known as fimbriae, is one of the adhesion factor is expressed by most gram-negative bacteria. Fimbriae is a protein polymer bacterial cell surfaces as an important mediator of the interaction of bacteria to the host and persist in the environment, the development of biofilms, motility, colonization and invasion of thecells (Burrows, 2005).

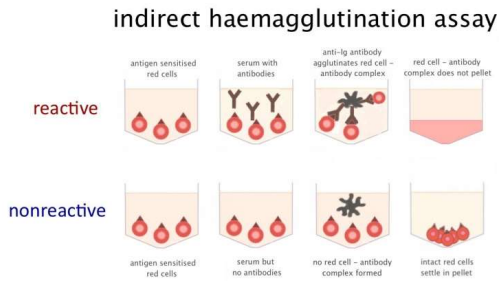

Figure 4. The principle of indirect hemagglutination test (Hay, 2002)

Hemagglutination test by bacterial pili serve as a useful model system for initial determination mechanism *S. flexneri* pili attachment to host cells enterocytes .Pili is a filamentary structure on the surface of bacterial cells composed of mostly formed from the repetition of a single protein subunit . Fimbriae binding of the adhesion (tip) that serves to bind to receptors on the host cell , and generally found at the ends of the structure of pili (Starks*, et al*, 2006).

Hemagglutinin protein is considered as one of the virulence factors of pathogenic bacteria. Based on the assumption that the bacteria are able to agglutinate erythrocytes have the ability to do a mucosal cell adhesion receptors on the host because the erythrocyte membrane is believed to have similarities with receptors on host mucosal cells (Chmiela, et al, 1997), the pili *S. flexneri* that have haemagglutinin protein is believed to be able to perform on cell adhesion host. Erythrocytes is required by all cells of the body in general, therefore, have receptors on each cell of the body. Protein sub unit pili and Omp with MW 72 kDa; 27 kDa; and 18 kDa *S.flexneri* can agglutinate erythrocytes of mice, then in theory this should pili hemagglutinin protein also mediates the process of attachment (adhesion) *S. flexneri* into enterocytes, here in after referred to as the adhesin molecule.

CONCLUSION

Based on the results and discussion of this study concluded that the protein sub unit pili and Omp *S. flexneri* with MW 72 kDa; 27 kDa; and 18 kDa can agglutinate erythrocytes of mice as evidenced by the hemagglutination test .

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