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Cross Immunity among the 49.8 KDa Pili Subunit Hemagglutinin Proteins and 7.9 KDa Pili Subunit Anti Hemagglutinin Proteins of *Shigella spp.*

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ABSTRACT

In a recent report, 49.8 kDa pili subunits haemagglutination (HA) protein of *S. dysenteriae* which proved to be an adhesive molecule and allow it to be developed as a vaccine. The purpose of this study was to obtain more information in the immunological response to the 49.8 kDa pili subunit protein *Shigella spp*. Pili subunit proteins *Shigella spp.* isolated according to our procedure manual. The molecular weight (MW) of 49.8HA and anti-HA 7.9kDa were identified using SDS-PAGE. The immunological response was detected using western blotting and dot blotting. Statistical analysis using ANOVA. The results of tests using Western blotting and dot blot method have been found the same immunological reaction between polyclonal antibodies pili 49.8kDa subunits protein of *S. dysenteriae* against 49.8 pili protein subunits of *S. dysenteriae* and 49.8 kDa pili protein subunits of *S. boydii* and polyclonal antibodies pili7.9kDa subunit protein of *S. dysenteriae* against 7.9 kDa pili protein subunits of *S. dysenteriae* and 7.9 kDa pili protein subunits of *S.boydii* 7.9 kDa as well, but not identical immunological reaction between 7.9 kDa pili protein subunits *S. flexneri, S. sonnei* and 49.8kDa pili protein subunits of *S. flexneri, S. sonnei.* There are similarities epitopes 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 epitopes with the 7.9kDa and 49.8 kDa protein pili subunits of *S. flexneri* and *S. sonnei*.

INTRODUCTION

Shigella genus includes four species; *Shigella (S). dysenteriae, S. flexneri, S. sonnei* and *S. boydii*. With the exception of *S. sonnei*, each species can be subdivided into serotypes, namely: *S. dysenteriae* (15 serotypes), *S. flexneri* (6 serotypes and 2 variants), and *S. boydii* (20 serotypes) (Ploeg*et al.*, 2010). *Shigella spp.* causes shigellosis, which is known as bloody diarrhea. The disease causes 120 million clinical cases of severe dysentery every year and is estimated to cause 700,000 deaths. Around 60% mortality is suffered by children under five years old (Ying *et al*., 2005). *V. cholera*e O1 is an adhesive molecule which serves as one of the components of cholera vaccine candidates. It has been tested for its effectiveness on Balb/c mice (Niyogi 2005). A Recent study has apparently found that in the pili subunit of *S. dysentriae* is 49.8 kDaHA protein and anti-HA protein respectively (Agustina *et al.*, 2012). Although the molecular weight of pili subunit HA and anti-HA protein of *S. flexneri, S. sonnei* and *S. boydii* are still unknown. This research was conducted in order to clarify whether there is any cross-immunity among the 49,8 kDa pili subunit HA proteins and the 7.9 kDa subunit pilianti-HA proteins of *Shigella spp*. The result may be very important in designing a vaccine for shigellosis using the components of pili subunit adhesive molecule.

MATERIALS AND METHODS

Culture *Shigella spp.:* Bacteria used in this research were *S. dysentriae, S. flexneri, S. sonnei, S. boydii* with no process of typing. They were derived from the Health Research Laboratory in Surabaya East Java Indonesia. TCG medium was used to enrich the growth of bacterial pili (Sumarno *et al.*, 2011).

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE): To monitor MW of protein HA and anti-HA in *Shigella spp.* pili subunit, SDS-PAGE method was conducted Laemli (Laemli 1970). Protein samples were heated at 100^0 C for 5 minutes in a buffer solution containing 5 mM Tris-HCl pH 6.8, 5% 2-mercapto ethanol, 2.5% w/v sodium dodecyl sulfate, 10% v/v glycerol using color tracker Bromophenol blue. 12.5% mini slab gel with a 4% gel tracking was selected.

Method of pili isolation and protein purification of *Shigella* spp.: The isolation of *Shigella spp.* pili was done using a pili bacterial cutter (PBC) referring to our study (Sumarno *et al.*, 2011). The product of electrophoresis in the form of the gel was cut straight at the desired molecular weight. Then the pieces were cut perpendicularly so each piece would contain

three protein bands. These pieces were then collected and inserted into the tube of dialysis membrane by using electrophoresis running buffer fluid for conducting electro-elution. The dialysis was performed on the product of electro-elution with PBS pH 7.4. Dialysis fluid in the membrane dialysis as a result of electroelution was then ready for hemagglutination test.

Scanning Electron Microscope Determination (SEM): The instruction method was done according to protocol. The bacterial pellet was isolated from bacteria culture grown in TCG media using centrifugation. The dilution sample had 1:100 ratios with a physiological saline solution in order to separate the bacteria. Preparations were made on the cover glass then fixed by using methanol. Furthermore, the sample was put in the instrument sputter coater for coating with a gold-palladium coating to turn it into a seemingly purple color. Samples were removed from the engine sputter coater and then inserted into the SEM**.**

Hemagglutination test method: Hemagglutination assay was done according to the instructions of Hanne and Finkelstein's (Hanne and Finkelstein 1982). Sample dilutions were made on ½ concentrations 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.

Western Blotting method: Western Blotting method referred to a technical protocol by Biorad (Kyhse-Andersen 1984). The SDS-PAGE gel results containing protein bands were transferred onto nitrocellulose (NC) membrane using a semi-dry blotter (Biorad). The washing was done by using TBE plus 0.05% tween 20 concentrations twice. The primary antibody of mouse IgG concentration 1/1000 in TBE pH 7.4 containing 1% solution of BSA was given to the NC. The anti-mouse secondary antibody IgG concentration 1/1000 in TBE pH 7.4 and 1%BSA were added. The color tablet was used as Cipβ as the material.

Polyclonal antibodies production: Mice were acclimatized for 4 days before immunization referred to ethical clearance. Antigens used were *S. dysenteriae* pili protein. Mice were injected with the antigen that had been emulsified with Complete Freud's Adjuvant (CFA) subcutaneously, 250 µg/0.3 ml PBS doses. Booster injections were performed in week two to four using antigen emulsified with Incomplete Freud's Adjuvant (IFA). 0.1 ml booster dose

was used in a subcutaneous injection. Serum was taken one week after the last booster (Harlow, E.and Lane, and D.1984).

Data analysis: Data obtained from immune response using Dot Blot Method were transformed into Corel Photo-Paint, and then, the data was analyzed using ANOVA statistical test, correlation and regression test.

RESULTS

Morphology of *Shigella* bacteria

Before and after the cutting of pili using PBC (Figure 1A), the whole cell morphology of *Shigella spp.* was observed using a scanning electron microscope as shown in Fig A and B.

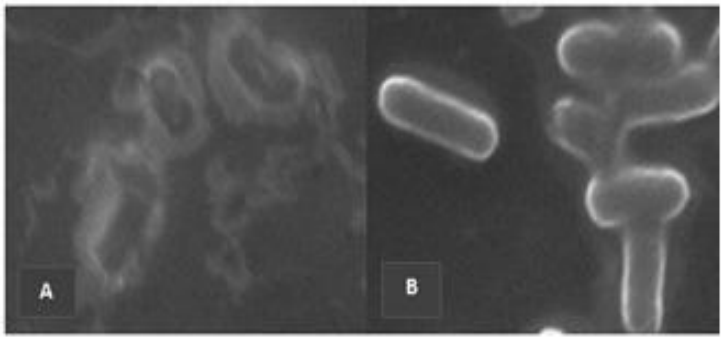

Figure 1A: Morphology of *Shigella dysenteriae* bacteria using a scanning electron microscope/SEM (50.000 x magnifications)

A. Bacterial cells *S. dysenteriae* before pili cutting
B. Bacterial cells *S. dysenteriae* after pili cutting.

The isolated pili proteins of *Shigella spp.*

After cutting the pili, protein isolation was performed using SDA-PAGE method. The *Shigella spp.* sub-unit pili protein profiles are shown in Figure 2A.

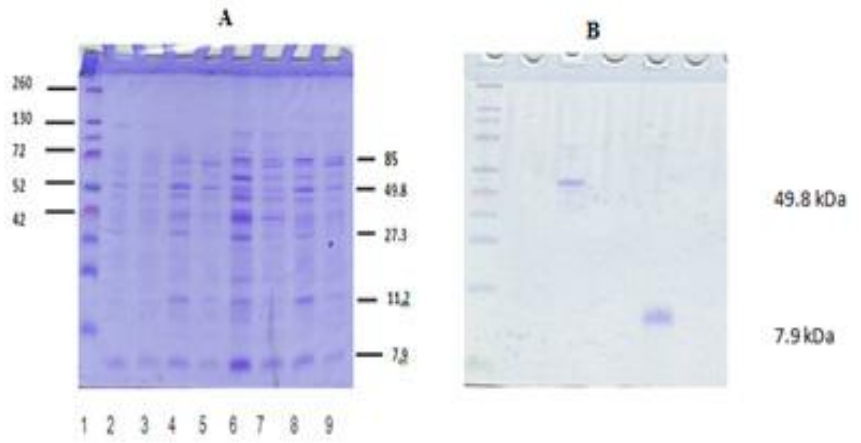

Figure 2A: Profiles of subunit pili proteins of *Shigella spp.* MW result from first and third cutting

1. Marker protein
2. and 3*S. dysentriae*, pilislices 1 and 3
4. And5 *S. flexneri*, pilislices 1 and 3
6. and 7*S. sonnei*, pilislices 1 and 3
8. and 9*S. boydii*, pilislices 1 and 3

Figure 2B: Profiles of pili subunit proteins with MW 49.8 and 7.9 kDa *S. dysenteriae* resulted from electro-elution.

The MW profiles of *Shigella spp.* pili subunit proteins obtained from first and third cuts showed the similarities (Fig 2A). The selected proteins banded on SDS-PAGE which were looked thick were MW: 7, 9, 11.2, 27.3, 49.8 and 85 kDa respectively. Subsequently, the protein selections were performed by hemagglutination assay. Purification of the 49,8kDa pili subunit HA protein and 7, 9 kDa pili subunit anti-HA protein of *S. dysenteriae* resulted from electro-elution can be seen in Fig 2B, whereas the result of purification of *S. dysenteriae* pili subunit protein with MW 11.2, 27.3 and 85 kDa at SDS-PAGE was not shown. Figure 3A and 3B were the results of HA test of purified pili subunit proteins of S. *dysenteriae*, *S. flexneri*, *S. sonnei* and *S. boydii* respectively.

Hemagglutination assay of *Shigella spp.* pili subunit protein

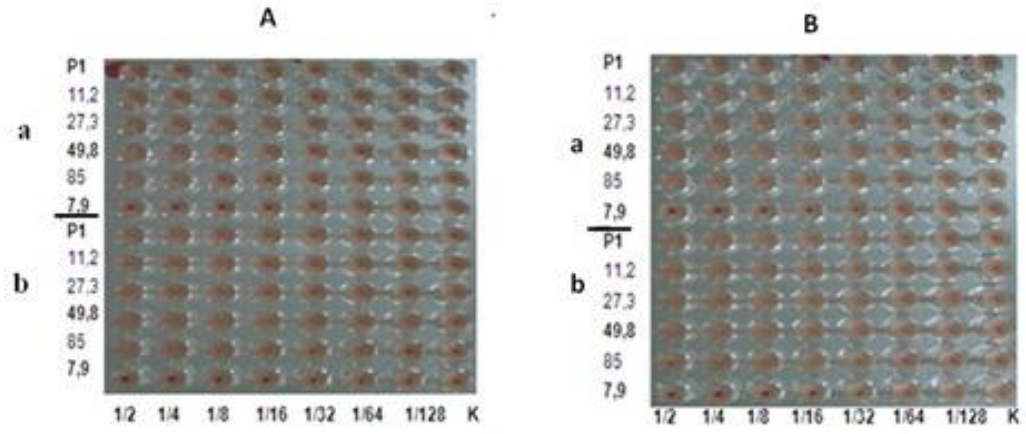

Figure 3A: The result of hemagglutination test for pili subunit proteins of *S. dysenteriae and S. flexneri.*

Figure 3B: The result of hemagglutination purification test of *S. sonnei* and *S. boydii* pili subunit protein.

Description: a. *S. dysenteriae* pili subunit protein with MW; 7.9, 11.2, 27.3, 49.8 and 85 kDa.
b. *S. flexneri* pili subunit protein with MW ;7.9,11.2,27.3, 49.8 and 85 kDa.

K. Negative control

Description: a. *S. sonnei* pili subunit protein with MW; 7.9, 11.2, 27.3, 49.8 and 85kDa
b. *S. boydii* pili subunit protein with MW; 7.9,11.2,27.3, 49.8and 85kDa.

K. Negative control

Figures 3A and 3B that show positive HA protein profiles indicate that there are similarities among the sub-unit pili proteins of *Shigella spp.* The finding of *Shigella spp.* pili subunit HA proteins with MW 85kDa, 49.8kDa, 27.3 kDaand 11.2kDa showed positive results. However, the results of *Shigella spp.* pili subunit HA protein with MW7.9kDa. was negative. From the evaluation of agglutination/sedimentation duration, it is found that its protein is faster than the controls. Highest HA titer was found in the 49.8kDa pili subunit protein.

Western Blotting pili subunit protein *Shigella spp.*

Western blotting methods were used to analyze the response of antigen-antibody reaction or vice versa. The profile of protein in Figure 4 shows that the subunit pili were composed of

proteins. By using western blotting method it was expected that the 49.8 kDa and 7.9 kDa pili subunit HA protein antibodies of *S .dysenteriae* will cross-react with the 49.8kDa and 7.9 kDa pili subunit proteins of *S. flexneri, S. sonnei* and *S.boydii.*

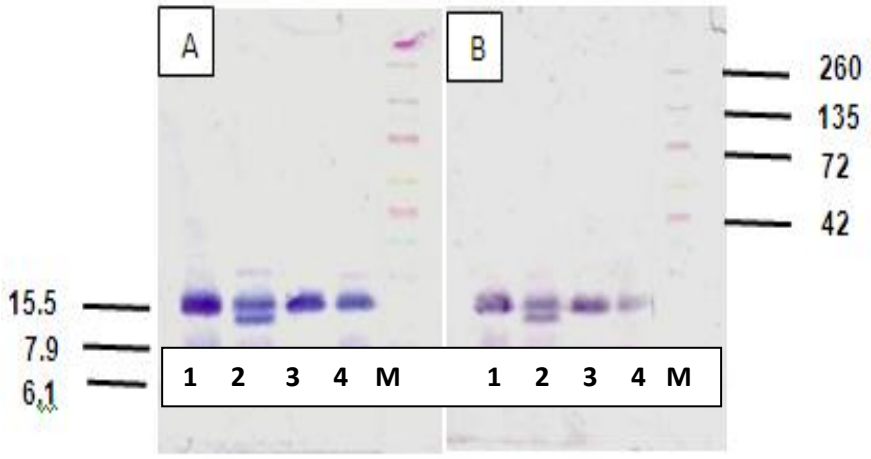

Figure 4: Results of western blotting of the 49.8 kDa and 7.9 kDa pili subunit.

1. *S. dysenteriae*. 3. *S. flexneri* M. Protein marker
2. *S. sonnei* 4. *S. boydii*

HA proteins of *S. dysenteriae* with 49.8 kDa and 7.9 kDa pili subunit proteins of *Shigella spp.*

A. The antigen-antibody reaction between the 49.8kDapili subunit protein of our *S. spp.* and the 49.0 kDa pili subunit antibody protein of *S. dysenteriae.*

B. The antigen-antibody reaction, between the 7.9kDapili subunit protein of *S. dysenteriae*, *S. flexneri, S. sonnei* and *S. boydii* and the 7.9 kDa pili subunit antibody protein of *S. dysenteriae.*

The results of western blotting of both antibodies showed a positive reaction for the pili subunit proteins of MW15.5kDa, 11,2 kDa, 7.9kDa, and 6.1kDa, but negative for the 49.8kDa pili subunit protein.

The results were obtained from the calculation reaction of these antigen and antibody response using semi-quantitative DotBlot method

Antigen-antibody reaction by the method of DotBlot

The results of the antigen-antibody reaction using the DotBlot method can be seen in Fig 5.

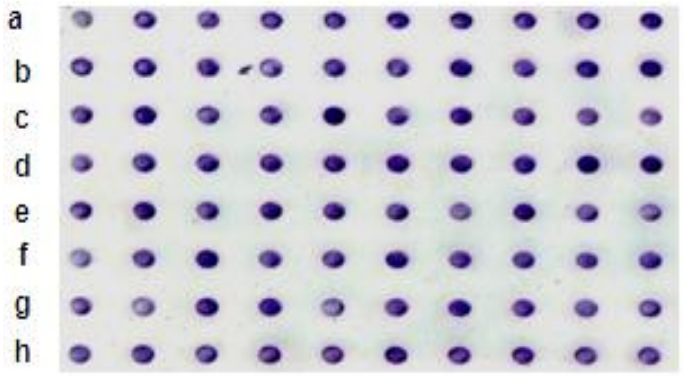

Figure 5: Results of DotBlot among the 49,8kDa and 7.9 kDa pili subunit HA protein of four *Shigella spp.* And the 49.8kDa and 7.9kDa antibody proteins of *S. dysenteriae* (1/1000-1/1953125000 dilution).

a. the 49.8 kDa pili subunit protein of *S. dysenteriae* was blotted with the49.8kDapili sub-unit protein antibody protein of *S. dysenteriae* with serial dilution.

b. the 49.8 kDa pili subunit protein of *S. flexnerii* was blotted with the 49.8kDapili sub-unit protein antibody protein of *S. flexnerii* with serial dilution.

c. the 49.8 kDa pili subunit protein of *S. sonnei* was blotted with the 49.8kDapili sub-unit protein antibody protein of *S. sonnei* with serial dilution

d. the 49.8 kDa pili subunit protein of *S. boydii* was blotted with the 49.8kDapili sub-unit protein antibody protein of *S. boydii* with serial dilution.

e. the 7.9 kDa pili subunit protein of *S. dysenteriae* was blotted with the 7.9 kDapili sub-unit protein antibody protein of *S. dysenteriae* with serial dilution.

f. the 7.9 kDa pili subunit protein of *S. flexnerii* was blotted with the 7.9kDapili subunit antibody protein of *S. flenerii* with serial dilution

g. the 7.9 kDa pili subunit protein of *S. sonnei* was blotted with the 7.9kDapili subunit antibody protein of *S. sonnei* with serial dilution.

h. the 7.9 kDa pili subunit protein of *S. boydii* was blotted with the 7.9 kDapili subunit antibody protein of *S. boydii* with serial dilution.

Data obtained from immune response using Dot Blot Method was transformed into Corel Photo-Paint

The proteins that function as an antigen were *S. dysenteriae*, *S. sonnei*, *S. flexneri* and *S. boydii* sub-unit pili proteins with MW 49.8kDa and the one that functions as an antibody was *S. dysenteriae* pili subunit with MW 49.8and 7.9 kDa. The results of the reaction calculation of these antigen and antibody response using semi-quantitative DotBlot method are depicted in Fig 6.

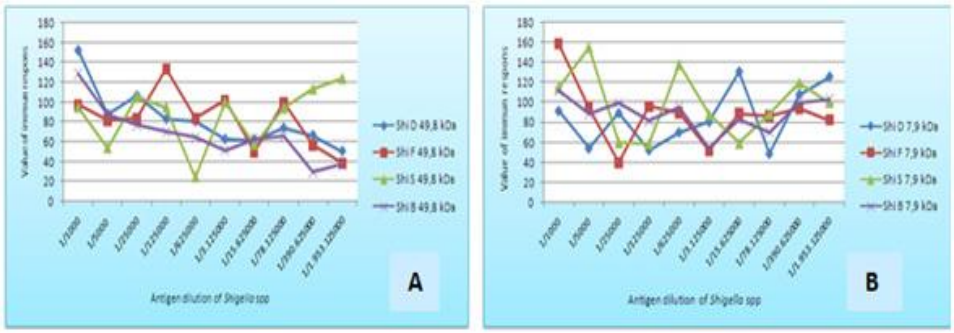

Figure 6A: Graph of the results of DotBlot method of *S. dysenteriae, S. sonnei, S. flexneri* and *S. boydii* sub unit-pili proteins having MW 49.8kDawith *S. dysenteriae* subunit pili antibody protein with MW 49.8kDa.

Figure 6B: Graph of the results of DotBlot method of *S. dysenteriae, S. sonnei, S. flexneri,* and *S. boydii* sub-unit pili protein with MW 7.9k Da using *S. dysenteriae* sub-unit pili antibody protein with MW 7.9kDa.

There was a significant correlation of an immune response between the *S. dysenteriae* sub-pili antibody and protein MW 49.8 and 7.9 kDa and *S. boydii* sub-unit pili proteins MW 49.8 and 7.9 kDa ($p = 0.013 < \alpha$). Whereas, there was no significant correlation of an immune response between the *S. dysenteriae* sub-pili antibody and protein MW 49.8 and 7.9 kDa and *S. flexneri* and *S. sonnei* pili protein MW 49.8 and 7.9 kDa ($p = 0743 > \alpha$)

DISCUSSION

Shigella dysenteriae are rod shaped bacteria as shown in Fig 1A and 1B. Those figures show that after cutting the pili using PBC designed by Sumarno (Fig 1), the pili of *S. dysentriae* were totally cut and the bacteria were bare, but the cell wall was still intact. This result was not different from another study for pili isolation of *V. cholerae O1* although the examination used electron transmission microscope (TEM) (Sumarno*et al.,* 2011).

Profile of *Shigella spp.* bacterium pili subunit protein molecule weighs in SDS-PAGE are shown in Fig 2. There were similarities of pili subunit proteins of the four *Shigella spp.* bacteria. The result is not different compared to previous results in an effort to propose the isolation of *V. cholerae* O1 (Sumarno *et al.*,2011). The results of these studies suggest that isolation of protein sub-unit pili using PBC is consistent.

Figure 2 also showed that the pili subunit proteins with MW 85 kDa, 49.8kDa, 27.3 kDa, 11.2 kDa and 7.9 kDa were likely to belong to *Shigella spp*. All of the bacterial pili are actually composed of protein subunits (Soto and Hultgren 1999). Almost all protein sub-units pili are generally HA molecules such as those found in *V. cholerae O1*. Doing an HA test requires a pure protein that could be obtained using electroelution methods (Agustine *et al.,* 2012, Sumarno *et al.*, 2011)

In relation to the results of electro-elution of the 85 kDa, 49.8 kDa, 27.3 kDa, 11.2 kDa and 7.9 kDa pili subunit proteins, subsequently, HA tests showed positive results as shown in Fig 3A and 3B. Interestingly, there was no agglutination of 7.9 kDa pili subunit protein as in the negative control, but the sedimentation rate was faster. Therefore, it was assumed that the 7.9 kDa pili subunit protein is anti-HA protein. The result was not different to those found in previous studies on *S. dysenteriae* and *V. cholerae O1* bacteria. The result also suggested that sub-unit anti-HA protein is proposed as a molecule adhesion.

Figure 4 showed that there were similarities among the results of the 49.8 kDa and 7.9 kDa pili subunit protein antibody using Western Blotting method. The two types of antibodies showed identical positive reactions to the 15.5 kDa, 7.9 kDa of pili subunit protein of *S. dysenteria* and *S. boydii*. Positive results of antigen-antibody reactions found in the two types of proteins showed the presence of two bands. Both of these pili subunit proteins, when compared with pili subunit protein of S. flexneri (4 bands) and S. sonnei (1 band), were not identical. Therefore, the response results of antigen-antibody reactions among *S. flexneri* and

S. sonnei pili subunit proteins with MW 49.8 kDa and 7.9 kDa were different. The positive response of the pili subunit protein with MW 15.5 kDa was consistent in *Shigella spp*.

The results of western blotting of the 49.8 and 7.9 kDa pili subunit antibody proteins of *S. dysenteria* and *S. boydii* could predict the protein composition of pili subunit HA. Both of these pili were composed of HA polymer molecules. The smallest unit of HA protein might be a monomer of pili subunit protein with MW of 7.9 kDa and it could act as an anti-HA (Fig 4A and 4B). Pili sub-unit protein with MW 15.5 kDa acted as a dimer HA (responding) and pili subunit protein with MW 49.8 kDa acted as a hexamer (non-responding). The epitope, which can stimulate pili subunit antibody protein with MW of 7.9 and 49.8 kDa in NC, was not located on the surface (hidden epitope). Therefore, the results of western blotting were negative (Huang and Honda *et al.*, 2006).

From the data in the Fig 6A and 6B, the treatment of dilution varying pili subunit proteins with MW 49.8kDa and7.9kDafrom four different types of *Shigella spp.* with both antibodies were followed up by the regression analysis. The dosage of an antibody selected from the results of the check board using the dot blot was 1/1280.

The result showed that the 7.9kDapili subunit protein of *S. dysenteriae* has a correlation with the 7.9kDapili subunit protein of *S. boydii*, and the 49.8 kDa pili subunit protein of *S. dysenteriae* with the 49.8kDa pili subunit protein of *S. boydii*as well, but not with the 7.9kDa protein of *S. flexneri and S. sonnei*. The antibodies used were polyclonal thus is assumed that there are similarities between epitopes found in7.9 kDa pili subunit protein of *S. dysenteriae* with 7.9 kDa pili subunit protein *S. boydii* and in 49.8kDa pili subunit protein of *S. dysenteriae* between 49.8kDa pili subunit protein *S. boydii* as well. While there are differences when compared with the7.9kDa and 49.8kDa protein pili subunits of *S.flexneri/sonnei*. Additionally, the result may be used as a consideration in developing shigellosis vaccine containing the bacterial adhesive molecular component.

CONFLICTS OF INTEREST

All contributing authors declare no conflict interest

ACKNOWLEDGMENTS

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