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Characterization Bacterial Biofilm Protein of *Staphylococcus aureus*

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Abstract: Bacterial Biofilm (BF) is a structural form of a group of microorganisms protected by an extracellular matrix which is called Extracellular Polymeric Substance (EPS). Staphylococcus BF EPS consists of Polysaccharide Intercellular Adhesin (PIA), extracellular-DNA, protein and amyloid fibrils. The purpose of this study was to determine the characterization of *S. aureus* bacterial BF protein based on molecular weight, hemagglutinin protein and see the response of BF protein polyclonal antibodies to several antigens from *S. aureus* bacteria. The results of the study of BF protein profiles using SDS-PAGE have a molecular weight of 35, 22.4 and 17 kDa. Protein 22.4 kDa has the highest titer (1/4) in the hemagglutination test using mice erythrocytes, so, it is continued to make polyclonal antibodies. BF protein antibodies of 22.4 kDa *S. aureus* can respond to several antigens from *S. aureus* bacteria which have a molecular weight of 120, 63, 48, 30 and 22.4 kDa. BF protein *S. aureus* compossed polymer protein and as can hemagglutinin protein.

INTRODUCTION

Staphylococcus aureus (*S. aureus*) bacteria is a cause of nosocomial infections that are pathogenic in humans because it has virulence factors such as BFs (BF), Panton-Valentine Leukocidin (PVL) and several enzymes (protease, lipase and elastase) which make it possible to destroy host tissue and metastases to other places (Buyukcangaz *et al*., 2013).

A (BF) is a structural form of a group of microorganisms protected by an extracellular matrix called Extracellular Polymeric Substance (EPS) where EPS is produced by organisms and can defend from adverse environmental influences (Veeregowda and Krishnappa, 2003). BFs can be single or multilayer, containing a population of either homogeneous or heterogeneous bacteria that remain in the matrix and are secreted by BF-forming bacteria (Satpathy *et al*., 2016). Staphylococcus BF EPS consists of Polysaccharide Intercellular Adhesin (PIA), extracellular-DNA, protein and amyloid fibrils. PIA is composed of poly-β (1-6) N-acetylglucosamine (PNAG), partially deacetylated, positively charged whose synthesis is mediated by the ADBC ica locus (Arciola *et al*., 2015).

It has proven that the bacteria *S. aureus* and *S. epidermidis* have Intercellular Adhesion (ica) operons which are responsible for slime production. This operon contains the ADBC ica gene in addition to the ica R gene which functions as a regulatory gene as well. Among the ica genes, the ica A and D genes have been reported to

play an essential role in BF formation. The ica A gene encodes N-acetylglucosaminyltransferase which is the enzyme involved in the synthesis of PIA. The ica D gene has been reported to play an essential role in the maximum expression of N-acetyl-glucosaminyltransferase which leads to the phenotypic expression of polysaccharide capsules (Nasr *et al*., 2012).

The pathogenesis of *S. aureus* bacteria is caused by the combined effects of extracellular and toxin factors along with invasive strains such as attachment, BF formation and resistance to phagocytosis (Chessa *et al*., 2015). The process of *S. aureus* bacterial BF formation occurs through several stages including reversible initial attachment and will become an irreversible attachment, cell aggregation forms a microcolony then followed by cell growth and maturation and BF cell dispersion into planktonic forms for colonization to new surfaces (Gupta *et al*., 2016).

Some strategies and mechanisms for BF inhibition are: bacterial anti BF polysaccharides, anti-BF enzymes, chelating agents, antimicrobial peptides and anti-adhesion agents (Abdel-Aziz and Aeron, 2014).

Hemagglutinin proteins bacteria are identical with moleculeadhesion (Suharsono *et al*., 2014, 2015). BF bacteria are adhesion molecules, therefore, this molecule is a molecule of hemagglutinin.

This research was conducted to determine the characterization of hemagglutinin *S. aureus* BF protein based on the molecular weight of BF protein, molecular weight of BF protein hemagglutinin protein and to examine the polyclonal antibody response of BF proteins to several antigens from *S. aureus*.

MATERIALS AND METHODS

This research is by doing exploratory laboratory research conducted at the laboratory of microbiology, pharmacology and Biomedicine of the Medical Faculty at Brawijaya University, Malang, Indonesia.

Identification of *S. aureus* bacteria: A total of 7 samples obtained from the laboratory of Saiful Anwar Malang Hospital identified in the microbiology laboratory of the medical faculty of Brawiya University. The initial stage bacteria was inoculated on Blood Agar Plate (BAP) media and incubated at 37°C for 24 h then continued the selection on the Mannitol Salt Agar (MSA) medium, Gram stain, catalase test and coagulase test.

BF formation test with the congo red agar method: The Congo Red Agar (CRA) method is a qualitative screening method for detecting *S. aureus* bacteria in producing BF. The media used consisted of Brain Heart Infusion Broth (BHIB) (37 g L^{-1}) was added with sucrose (50 g L^{-1}) after that No 1 (10 g L^{-1}) and Congo red (0.8 g L^{-1}). Positive results are characterized by the formation of black colony pigments while red colonies are detrimental BF (Freeman *et al*., 1989).

Quantitative test for BF formation with tube method: The tube method was carried out according to what was described by Christensen (Christensen *et al*., 1982). The test organism was inoculated in 10 mL Trypticase Soy Broth (TSB) with 1% glucose in a reaction of the tube. The bacteria incubated at 37°C for 24 h. After incubation, the bacteria were washed and dissolved with Phosphate Buffer Saline (PBS) pH 7.3 and dried. The tube stained with 0.1% crystal violet for 15 min. The remaining dye is removed and washed using distilled water. The tube dried in reverse. BF formation considered positive when the film is seen lined up on the wall and the bottom of the tube. To determine the intensity of the color in the area of the wall and the bottom of the tube than are used the adobe photoshop CS6 application program.

Isolation of *S. aureus* bacterial BF protein: The highest *S. aureus* bacteria that produced BF were inoculated on TSB+glucose 2.5% at 37°C for 48 h. Protein isolation used chloroform methanol precipitation method according to Wessel and Flugge (1984). Protein isolation results from running used SDS-PAGE.

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE): Molecular weight monitoring was carried out with the SDS-PAGE method of Laemmli (1970). Protein samples are heated to 100°C for 5 min in a buffer solution containing 5 m M Tris pH 6.8, 5% 2-mercaptoethanol, 2.5% w/v sodium dodecyl sulfate, 10% v/v glycerol by using bromophenol blue tracking color 12% mini slab gel selected with 4% tracking gel. The electricity voltage used is 120 mV. The color material used is coomassie brilliant blue and the standard molecule GangNam-stain prestained ladder marker protein.

Electroelution and dialysis: Electroelution of protein is done by cutting the gel from running SDS-PAGE according to the desired molecular weight. Gel with the content protein put in a cellophane bag, then added 0.2 M phosphate buffer. The gel in a cellophane bag inserted into the electroelution chamber containing 0.1 M phosphate buffer then electroeluted at 250 V, 20 mA for 60 min and then dialyzed. Dialysis was done by inserting electroeluted cellophane into a glass beaker containing sterile PBS which is then stirred at 4°C for 24 h. The cellophane bag is opened and the protein in it is collected and placed in an eppendorf tube and added with cold

absolute ethanol, stored at 4°C for 24 h. The eppendorf tube was centrifuged 12,000 rpm for 15 min at 4°C. The supernatant is discarded and the sediment is dried then added with a Tris-Cl buffer with a ratio of 1:1 then stored at -20°C. Purification results are used for hemagglutination tests (Agustina *et al*., 2012).

Hemagglutination test: The hemagglutination test was carried out by first erythrocytes washed with PBS pH 7.4 in a 3500 rpm centrifuge for 10 min (washed 3x) then made a 0.5% suspension in PBS. In the first well, until the twelfth well from the microplate V, 50 µL of PBS was inserted. About 50 µL of sample was added to the well 1 then serial dilution was made into the next well up to well 10. Well 12 was used as a negative control (without being given a sample). Then into each well was added 50 µL of erythrocyte suspension 0.5%, shake using a rotator plate for 1 min then room temperature was left (27°C) until the results were seen. The hemagglutination test results for the sample are read if the negative control well has seen the result, namely the appearance of a red stain (McGAREY and Allred, 1994).

Production of polyclonal antibodies: Mice used in this study were female Balb/c mice aged 6-8 weeks. The isolated 22.4 kDa BF protein antigen was measured for its protein content with nanodrop spectrophotometer and then each of them was extracted to 100 µg/mL in ependorf, then stored at -20°C. Before the injection in mice, BF proteins were emulsified with Complete Freund's Adjuvant (CFA). Mice are injected with antigen at a dose of 10 µg intraperitoneally. The second and third weeks were boosted using the antigen that had been emulsified with Incomplete Freund's Adjuvant (IFA) with the same dose intraperitoneally. Week 4 is carried out a booster with the same Antigen without adjuvants. Serum mice were taken a week after the last mice were immunized by taking the blood of mice as much as 1 mL, then centrifuged at 3,000 rpm for 15 min at room temperature. Serum-containing supernatants are taken while precipitates containing blood cells are removed (Liddell and Cryer, 1991; Swacita *et al*., 2015).

Hemagglutination inhibition test: By using microplate V, into each well a PBS solution of pH 7.4 was inserted as much as 50 µL. Furthermore in the 1st well, serum was added (22.4 kDa BF protein antibody) of 50 µL and serial dilution was carried out until the 10th well. After that, each BF protein was added to each well (according to the highest HA test titre) of 50 µL to the 10th well. Then into each well on microplate v, the erythrocyte suspension of 0.5% mice was added by 50 µL, shaken using a rotator plate for 1 min. Microplate v is left at room temperature until the results are visible. The 11th well was used as a negative hemagglutination control containing serum and erythrocytes without BF protein while the 12th well was used as a positive hemagglutination control containing BF and erythrocyte proteins without the addition of serum (Sumarno *et al*., 2015).

Check board test (dot blot method): The dot blot method is carried out through a nitrocellulose membrane soaked in sterile H_2O for 30 min. Then it is placed in a dot blotter apparatus (Biorad). Through the tool hole, the membrane that has been moistened with TBS is dripped with 50 µL antigen, incubated overnight at 4°C. Then blocking with TBS buffer blocking was incubated overnight at 4°C, the blocking solution was discarded. The primary antibody dripping membrane is 50 µL, incubated for 2 h at room temperature and placed on top of the shaker. The blocking solution was discarded then washed 3 times with 0.05% TBS-20. Secondary, antibodies were added with a 1: 1000 dilution in TBS solution, incubated at room temperature for 1 h above the shaker. Then washed again 3 times with 0.05% 20-TBS. Then the chromogen substrate was added and incubated at room temperature for 30 min. The reaction is stopped by adding H_2O. Then a positive result when dot-dot is formed on nitrocellulose membranes. The quality of the results is seen based on the color gradation. At dilution that produces thick color gradation is the most effective dilution of the reaction. The dilution is then used as the basis for the western blot test (Harlow and Lane, 1988).

Western blot method: Western blot examination is carried out by removing SDS-PAGE resulting gel sheets containing protein bands transferred on nitrocellulose paper using semi-dry trans blot equipment made by Biorad. The way to move protein bands to nitrocellulose study is to use an electric current of 300 mA, 20 V for 120 min (Towbin *et al*., 1979).

Ethical statement: All procedures involving experimental animals were in accordance with ethical standards of Faculty of Medicine, Brawijaya University (No.95/EC/KEPK/03/2017).

RESULTS AND DISCUSSION

Identification of bacteria *S. aureus*: The results of the identification test of *S. aureus* bacteria Table 1. The sample S1-S7 forms β hemolysis in BAP media in the form of gram-positive coccus, positive catalase test, positive coagulase test this shown that the S1-S7 sample is *S. aureus* bacteria.

Table 1: Results of *S. aureus* bacterial identification test

Sample code	Test results: BAP	Gram	Catalase	Coagulase	MSA	Description
S1	Hemolisa β	+	+	+	Yellow colonies	*S. aureus*
S2	Hemolisa β	+	+	+	Yellow colonies	*S. aureus*
S3	Hemolisa β	+	+	+	Yellow colonies	*S. aureus*
S4	Hemolisa β	+	+	+	Yellow colonies	*S. aureus*
S5	Hemolisa β	+	+	+	Yellow colonies	*S. aureus*
S6	Hemolisa β	+	+	+	Yellow colonies	*S. aureus*
S7	Hemolisa β	+	+	+	Yellow colonies	*S. aureus*

Table 2: Test results for the BF formation of the CRA method and the tube method

Sample code	CRA media	Test results: CRA method	Tube method
S1	Black colony	Positive (+)	82.80
S2	Black colony	Positive (+)	122.15
S3	Black colony	Positive (+)	93.09
S4	Black colony	Positive (+)	87.58
S5	Black colony	Positive (+)	86.38
S6	Black colony	Positive (+)	72.49
S7	Black colony	Positive (+)	83.95

BF formation test with CRA method and tube method: The results of the CRA method test Table 2. The positive S1-S7 sample formed a BF with the characteristics of a black colony on CRA media and continued with the tube method to determine the highest BF-producing bacteria. In the tube method if the more intense the color of the BF is formed in the research tube, the smaller the value of mean gray value is. Conversely, if the color of the BF gets brighter and thinner, the greater the value of mean gray value is. Test results of the tube method is obtained the lowest value in the S6 sample with a value of 72.49. This means that the S6 sample is the highest BF producing bacteria. So, this S6 sample is used for the isolation of BF proteins.

Sodium Dodecyl Sulfat Polyacrilamide Gel Electroforesis (SDS-PAGE): To separate protein based on molecular weight, the SDS PAGE method is used. The results of SDS PAGE, obtained protein with various molecular weights as shown in Fig. 1.

The protein profile of *S. aureus* bacterial BF is seen as in Fig. 1 in lanes 2 and 3 containing crude *S. aureus* BF protein obtained 3 protein bands with molecular weight of 35, 22.4 and 17.2 kDa which are not possessed by whole cel *S. aureus*.

Hemagglutination test: Purification BF proteins (BF proteins 35, 22.4 and 17.2 kDa) were tested for hemagglutination to see the ability of the BF protein to agglutinate mouse erythrocytes.

From the results of the hemagglutination test in Fig. 2 it turns out that there is a difference in the mice

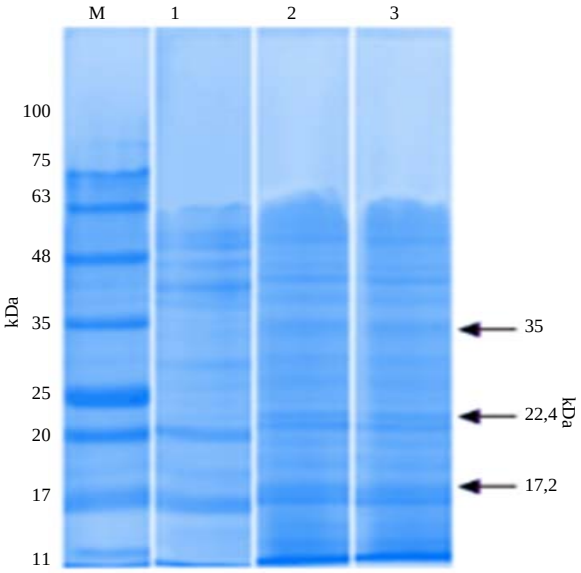

Fig. 1: SDS-PAGE test results. M: Marker, 1: Whole cell *S. aureus*, 2, 3: Crude *S. aureus* BF protein

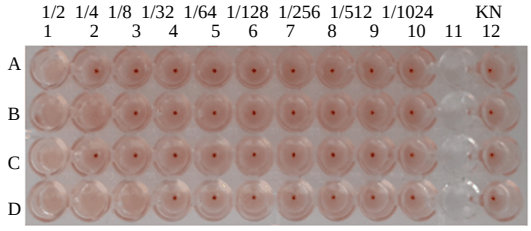

Fig. 2: Results of the hemagglutination test. A: 29.8 kDa BF protein, B: 22.4 kDa Bfprotein, C: 17.2 kDa BF protein, D: crude BF protein, KN: negative control

agglutination of erythrocytes. In the molecular weight BF protein 35, 22.4, 17.2 kDa and the crude, theagglutination titer are 1/2, 1/4, 1/2 and 1/8, respectivelly. The 22.4 kDa protein was chosen to produce polyclonal antibodies in mice because it has the highest titer compared to purified BF proteins (35 and 17.2 kDa).

Hemagglutination inhibition test results: Hemagglutination inhibition test was conducted to

Table 3: The results of the check board test between BF protein antigen 22.4 kDa and *S. aureus* 22.4 kDa BF protein antibody

Variables	1/100 (1)	1/200 (2)	1/300 (3)	1/400 (4)	1/500 (5)	1/600 (6)	1/700 (7)	1/800 (8)	1/900 (9)	1/1000 (10)	Kag (11)	Kab (12)
1/10 (A)	129.25	126.01	110.66	117.62	112.58	119.44	123.21	121.63	121.32	122.50	137.80	128.53
1/20 (B)	117.35	122.13	106.29	120.93	120.18	120.26	121.46	118.89	122.62	127.15	139.72	128.77
1/40 (C)	107.16	109.54	104.45	119.08	112.92	118.98	121.78	119.49	122.85	122.34	143.59	129.02
1/80 (D)	108.30	107.20	108.25	111.88	119.72	128.65	130.01	121.96	130.79	135.56	142.34	138.63
1/160 (E)	116.75	108.53	103.74	119.30	119.74	124.31	133.67	126.58	128.63	136.67	138.11	128.96
1/320 (F)	112.47	104.71	106.11	114.96	120.05	126.63	131.68	131.92	130.11	138.32	145.32	138.47
1/640 (G)	102.15	102.02	**101.48**	121.84	125.97	132.88	135.83	129.88	120.19	137.15	143.41	126.75
1/1280 (H)	109.11	102.42	107.27	123.88	125.60	134.35	134.97	132.99	134.72	137.34	146.93	133.44

Bold value is significant

determine the ability of polyclonal antibodies 22.4 kDa in inhibiting the hemagglutination process mouse erythrocytes. The result of the hemagglutination inhibition test was positive if there were deposits of erythrocyte mention as found in negative control wells whereas in wells that did not show sediment but agglutination occurred it would be considered harmful (Fig. 3 and 4).

Check board test (dot blot method): Check board test is used to see the effectiveness of the reaction between antigens and antibodies to be tested. Check board tests are carried out using the dot blot method by reacting antigens and antibodies that have been produced. Antigen is diluted with a 1/10-1/1280 dilution while BF antibody is 22.4 kDA with a dilution of 1/100-1/1000. The results of the check board test (dot blot method) qualitatively can be seen in Fig. 5.

The results of the checkboard test in Fig. 5, the density measurements using Adobe Photoshop CS6 are shown with the mean value, the results are in Table 3. Where the smaller the mean value means the greater density. The results of the density measurements show that the strongest reaction is seen at 1/640 dilution for the antigen while the antibody is seen at 1/300 dilution (the mean value is 101.48). The antibody dilution titers are used as a basis for conducting western blot tests.

Western blot method: Western blotting method is a method that reacts specific antibodies with antigens and this method has been routinely used for protein analysis. Blotting means transferring samples from gel electrophoresis to nitrocellulose membranes and carried out with antibodies on the membrane surface.

The results of the western blot test in Fig. 6 shown a positive reaction of the BF protein subunit with a molecular weight of 120, 63, 48, 30 and 22.4 kDa which are indicated by the appearance of protein bands in lanes 1-6.

Identification of *S. aureus* bacteria was carried out using BAP media, MSA media, gram staining, catalase test and coagulase test. BAP media is used to determine the ability of bacteria to halt erythrocytes and

Fig. 3: Test results of *S. aureus* BF protein hemagglutination test. Number 1/2-1/2048: shown the sample titer (dilution) in the hemagglutination test KN = Negative Control, Samples are crude *S. aureus* BF protein and the results were positive on titers 1/4. The antigen titers were used for hemagglutination inhibitory tests

Fig. 4: Test results of hemagglutination inhibition. Number 1/2-1/1024: shows the sample titer (dilution). KN = Negative Control, KP = Positive Control. Samples were serum (*S. aureus* 22.4 kDa BF protein polyclonal antibody) positive on titers 1/4

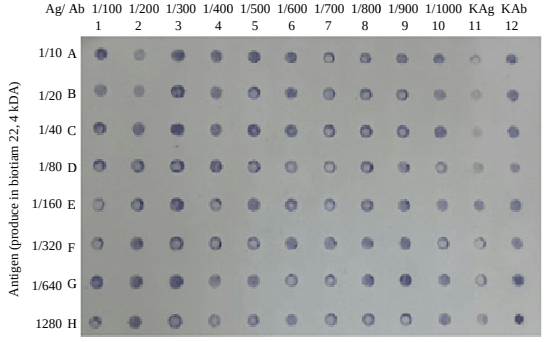

Fig. 5: Check board test results between 22.4 kDa BF protein antigen and 22.4 kDa *S. aureus* BF protein antibody. Columns 1-10 are antibody dilution 1/100-1/1000, column 11: KA (Antigen control) column 12: KAb (Antibody Control), Line A-H is antigen dilution (1/10-1/1280)

hemoglobin. There are 3 types of hemolysis, namely hemolysis a (alpha), hemolysis β (beta) and hemolysis?

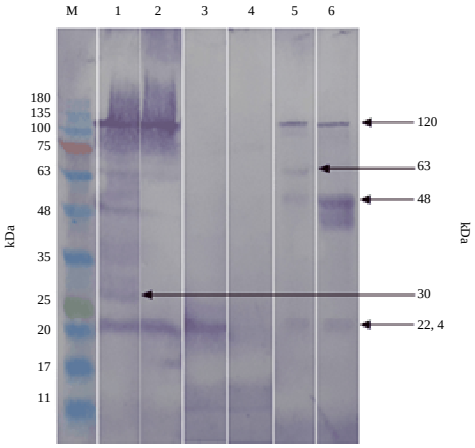

Fig. 6: Western blot test results. M: Marker, 1: Whole cel *S. aureus* strain 1; 2: Whole cel *S. aureus* strain 2; 3: Protein 22,4 kDa; 4: Protein 35kDa; 5: Crude protein BF*S. aureus* 1; 6: Crude protein BF*S. aureus* 2

(gamma). Gram staining is used to observe the morphology, size and composition of bacterial cells. *S. aureus* is a Gram-positive bacteria in the form of coccus which produces purple in Gram staining. The color purple caused by the bacteria maintaining the first color, namely gentian violet. The content of the cell wall influences the difference in Gram's character, i.e., Gram-positive bacteria contain which is thicker peptidoglycan compared to Gram-negative (Leboffe and Pierce, 2011).

The salt agar mannitol medium (MSA) is used for isolation and distinguishes the pathogenic Staphylococcus bacteria, especially, *S. aureus*. *S. aureus* bacteria in MSA media showed yellowish white colony growth surrounded by yellow zone because of the ability to mannitol ferment. Bacteria that ferment mannitol will produce acid, causing changes in the red phenol indicator to change from red to yellow (Prescott and Harley, 2002). The results of SDS PAGE in Fig. 1 show that the *S. aureus* BF protein has a molecular weight of 35, 22.4 and 17.2 kDa.

Hemagglutinin is one of the bacterial components that mediate the attachment of bacterial cells to red blood cells (Rupp *et al*., 1955). *S. aureus* bacteria which have hemagglutinin protein have a higher adhesion ability in epithelial cells compared to *S. aureus* which does not have hemagglutinin protein (Abrar *et al*., 2012). The role of hemagglutinin in adhesion is predicted as an essential factor for virulence of *S. aureus* (Wahyuni *et al*., 2005).

Hemagglutination test was carried out on *S. aureus* BF protein consisting of 35 kDa, 22.4 kDa, 17.2 kDa and crude protein BF. The titer hemagglutination test results shown: 1/2, 1/4, 1/2 and 1/8, respectively. The hemagglutination test results in each molecular weight are not the same. The result due to the ability of BF proteins that can bind erythrocyte cells. If the protein can bind erythrocyte cells at a higher dilution, the protein can adhere more strongly because at the highest dilution it can bind erythrocyte cells. In this study, hemagglutinin protein obtained with a molecular weight of 22.4 kDa. Rares found the possibility of hemagglutinin protein 22.4 kDa is the same as the *S. aureus* hemagglutinin protein with a molecular weight of 22.38 kDa (Rares, 2011). Several studies on cellular hemagglutinin protein have widely studied including 49.8 kDa pili protein Shigella dysenteriae, Vibrio cholerae 38 kDa pili protein and 76 kDa in OMP10 while Proteus mirabilis P355 has pili hemagglutinin protein with a molecular weight of 35.2, 27.9, 24 and 22 kDa (Mufida *et al*., 2012).

The results of the hemagglutination inhibitory test showed that the polyclonal antibody of *S. aureus* BF protein 22.4 kDa formed was able to inhibit hemagglutination until 1/4 dilution while the subsequent dilution in which the polyclonal antibody concentration was smaller showed a negative result which mean that hemagglutination occurred in mice erythrocytes. In the hemagglutination inhibition test, the process of inhibiting hemagglutination is mediated by polyclonal antibodies which play a role in binding hemagglutinin proteins so that the hemagglutination process in mouse erythrocytes does not occur.

Antigen check board and antibody tests are conducted to see the effectiveness of antibody responses to antigens. The antigen used was *S. aureus* BF protein 22.4 kDa, while the antibody used was 22.4 kDa BF protein antibody. The checkboard test used as a basis for the antibody-antigen reaction test using the Western Blot method.

BF protein antibodies 22.4 kDa *S. aureus* can respond to several antigens from *S. aureus* which have a molecular weight of 120, 63, 48, 30 and 22,4 kDa. Several things can cause the appearance of many protein bands on western blot results. A protein hemagglutinin with a high molecular weight, the cause of which is likely to be a process of glycosylation and multimer formation whereas if its appearance is a protein with a lower molecular weight protein degradation may occur, the cleavage process or because the protein has the same epitope. Another most likely cause is the use of polyclonal antibodies as primary antibodies (Moore, 2009; Kalanjati, 2011; Mahmood and Yang, 2012).

From the results the question arises is protein BFP as molecule adhesion and how is the relationship with cellular adhesion molecule *S. aureus*?. The result is essential in designing the vaccine of *S. aureus* base on the molecular adhesion as like pertussis acellular vaccine (Inatsuka *et al*., 2005).

CONCLUSION

Protein 22.4 kDa BFs antibodies can respond to several antigens from *S. aureus* bacteria which have a molecular weight of 120, 63, 48, 30 and 22.4 kDa.

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