

The 38.8 kDa Pili Subunit Hemagglutinin Protein of *Acinetobacter baumannii* is an Adhesin Protein that can activate s-IgA Production

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Abstract: *Acinetobacter baumannii* (*A. baumannii*) is a pathogenic bacterium that can cause nosocomial infection in hospitalized patients with many kinds of manifestations. All bacterial infection is initiated by adhesion of bacterium to host cells through certain virulence factor which are called adhesin proteins. The purposes of this study were to isolate the pili subunit hemagglutinin (HA) protein of *A. baumannii* and to prove whether it functioned as adhesin protein and could activate the production of s-IgA. Isolation of pili subunit protein, was done using SDS-PAGE to get the molecular weight (MW) of pili subunit protein and then hemagglutinated. Adhesion test was conducted using pili subunit HA protein which had the highest titer of hemagglutination test, then coated on enterocyte cells of mice at the doses of 400 µg, 200 µg, 100 µg, 50 µg, 25 µg, 12.5 µg, 0 µg (control), respectively. ANOVA analysis results of dose response method from pili subunit HA protein of *A. baumannii* showed a significant difference of the index among treatment doses ($p = 0.000$). It can be seen that lower dose of pili subunit HA protein with MW of 38.8 kDa of *A. baumannii* could increase adhesion index. The 38.8 kDa pili subunit HA protein had significant influence on the adhesion index ($r = -0.729$, $p = 0.000$, R square = 0.532, $p = 0.000$). Measurements of s-IgA concentration using ELISA method. There were differences in the number of s-IgA by ELISA test based on the treatment given to the value of $p = 0.027$ ($p < 0.05$; ANOVA), the number of s-IgA in treatment group I differed significantly from the number of s-IgA in group III ($p=0.039$; Tukey test). It can be concluded that the 38.8 kDa pili subunit HA protein of *A. baumannii* is an adhesin protein and can activate the production of s-IgA.

Keywords: *Acinetobacter baumannii*, pili, hemagglutinin, adhesin protein, s-IgA.

I. Introduction

A. baumannii is a gram-negative bacterium, rod-shaped with a size of 2.5 micron during the growth phase and becomes shorter and rounded at the stationary phase. These bacteria live in pairs or groups, aerobics, nonfermenter, positive catalase, negative oxidase that are widespread in soil and water, and can thrive on skin, in mucous membranes, secretions and hospital environment [1,2,3,4]. *A. baumannii* is a pathogenic bacterium that can cause nosocomial infection in hospitalized patients with many kinds of manifestations such as pneumonia, eye, surgical wound or burns, skin, and urinary tract infections, also bacteremia and septicemia [3,4,5].

The process of bacterial is specific, but generally initiated by adhesion of bacterial cell to host cell using certain protein which are called adhesin proteins. The adhesin proteins are differ among each bacterium to others [6,7]. Braun and Vidotto (2004) found that bacteria *A. baumannii* can agglutinate human erythrocytes AB group, and it's capable of adhering on polysteryn suspected mediated by fimbriae (pili) of *A. baumannii* [8]. Gohl *et al.* (2006) found that the pili of *Acinetobacter sp.* BD413 strain with a MW of 16 kDa functions as an adhesin protein for adhesion on biotic and abiotic surfaces [9]. Sumarno (2011) found that bacteria *V. cholera* had adhesin molecules of pili subunit protein with a MW of 37,8 kDa [10].

Choi *et al.* (2008) found that the bacterial molecule of *A. baumannii* directly responsible for the death of host cells. Among the various molecules of the bacteria, outer membrane protein A from *A. baumannii* (AbOmpA) with a MW of 38 kDa was identified as virulence factor (adhesin) that potential to induce host cell death through both mitochondria and nucleus [11,12,13]. The outer membrane protein (OMP) with MW of 38 kDa activated dendritic cells, which cause CD4+ T cell differentiation into Th1 phenotype [14]. Luo *et al.* (2012) also found that recombinant OmpA can activate humoral immunity, namely IgG [15].

Bacteria *A. baumannii* has pili with a size of 1-3 µm, 2-8 nm in diameter and composed of pili subunits protein called fimbrins or pilins. Pili played important roles in adhesion to inert or living surface, and formation of bacterial biofilm that had been recognized as an important cause of human infections [16].

Until now, there is no study proved that the pili of *A. baumannii* has hemagglutinin protein is an adhesin protein and immunogenic. The purpose of this study was to isolate the pili subunit hemagglutinin protein of *A. baumannii*, and to prove that it is an adhesin protein, which can activate s-IgA production.

II. Materials And Methods

Isolation and identification bacteria *A. baumannii*.

Clinical specimens used to obtain pili protein bacteria *A. baumannii* collected from the urine of urinary tract-infected patients (UTI), sputum, and pus from hospitalized patients which sent to Microbiology Laboratory, General hospital of dr. Saiful Anwar, Malang from January to February 2013. Identification of bacteria *A. baumannii* using microbact system, according to the instructions of Oxoid Microbact Identification Kits. Specimens with single infection of *A. baumannii* were isolated and treated with hemagglutination test, until getting a sample which has the highest HA titer for further steps of research.

Culture *A. baumannii*.

Bacteria *A. baumannii* were culture with method from Ehara [17].

Isolation method of *A. baumannii* pili.

Isolation of *A. baumannii* pili was referred to Sumarno [18].

Sodium dodecyl sulfate - polyacrylamide gel electrophoresis (SDS-PAGE).

Monitoring the molecular weight (MW) by SDS-PAGE was done by applying Laemmli method [19].

Method of Isolation of pili subunit protein of *A. baumannii*.

Research method referred to Sumarno [8].

Hemagglutination test.

Procedure of hemagglutination test used from Hanne and Findkelstain [20].

Isolation of mice enterocytes.

Isolation of mice enterocytes used Weiser method taken from Nagayama [21].

Adhesion test to mice enterocytes.

Procedure of adhesion test bacteria *A. baumannii* to mice enterocytes adapted from Nagayama [21].

Gram staining.

The smear was stained by Gram, to see the big picture and the description of enterocytes morphology and *A. baumannii* adhesion on enterocytes cell [22].

Method of calculating the adhesion index of bacteria to the mice enterocytes.

The adhesion index representing the average number of bacteria per cell was determined by examining 100 cells. The adhesion index was calculated by microscope under oil immersion with 1000 x magnification [23].

Immunization.

In this experimental research using male mice of the species *mus musculus* outbred Balb/C were aged 6-8 weeks, as many as 15 tails mice of the Laboratory of Parasitology, Faculty of Medicine, University of Brawijaya. The mice outbred Balb/C is divided into three treatment groups, each group as much as 5 mices. The experimental research had agreement about ethical clearance from The Ethical Committee Medical Research Faculty of Medicine, University of Brawijaya.

Immunizations were given to Group I: obtained immunization with pili subunit adhesin protein with MW of 38.8 kDa *A. baumannii* 100 µg/100 µl + ISCOM 12 µg/25 µl (under the guidance of ABISCO of ISCONOVA); group II: obtained immunization with pili subunit adhesin protein MW of 38.8 kDa *A. baumannii* 100 µg/100 µl; and group III: as control with PBS 100 µl. Immunizations were given on days 0, 7 and 28 orally. On the day 35, the mice were killed to examined the s-IgA concentrations using ELISA method [24].

Preparation of Mucus.

Preparation of mucus was carried out as follows: intestinal pieces were washed with cold PBS. Then the intestine was opened so that the visible part of the small intestine mucosa exposed. Layer of mucus was collected by scraping longitudinally with spatel and placed in tubes containing sterile PBS and protease

inhibitors. The suspension was shaken, then centrifuged at 12,000 rpm in 4°C for 10 min. The supernatant was taken to perform purification of s-IgA. The supernatant was resuspended with PBS after that dialysis was performed with the use of PBS and used as a sample for examination of s-IgA by ELISA method.

Examination of s-IgA by ELISA method.

s-IgA examination was done by ELISA method using mouse s-IgA ELISA kit from R and D.

Data Analysis.

Statistical analysis used ANOVA. Results of research was significant when $p < 0.05$ was achieved. Post hoc analysis was then performed with the Tukey test if there were significant differences.

III. Results

The *A. baumannii* isolate derived from 521 of urine, 538 of sputum, and 253 of pus samples which had single infection of *A. baumannii* and fulfilling inclusion criteria.

There were eight samples consists of two samples of urine, two samples of pus, and four samples of sputum treated with hemagglutination test on whole cells to determine the samples that had the highest titer, and used for further steps of research. The sixth sample from pus that had the highest titer on dilution 1/16, and the results can be seen in Figure 1.

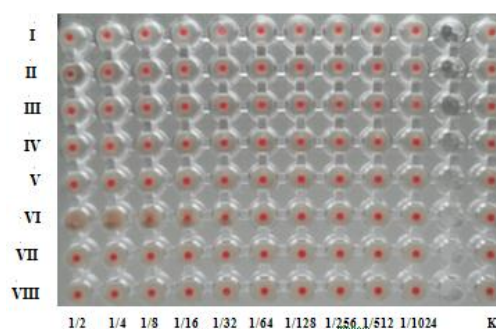


Figure 1. Results of hemagglutination test to whole cells of Acb.

Description: sample from urine (I,IV), sample from sputum (II,V,VII,VIII), and sample from pus (III,VI)

K: erythrocytes and PBS 7.4

Sixth samples that had the highest titers in hemagglutination test were planted on TCG-BHI medium for 24 hours, then harvested for cutting of pili using bacterial pili cutter. Pieces of pili were composed from pieces of the first to fourth, and the residual of bacteria of the fourth pieces were performed with SDS-PAGE. Results of electrophoresis as in Figure 2.

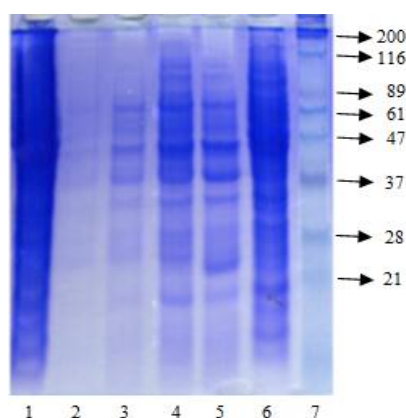


Figure 2. Results of electrophoresis from pieces of pili with SDS-PAGE (12.5%) method.

Description: well 1: The residual of bacteria of the fourth pieces, well 2: The fourth piece, well 3: The third piece, well 4: The second piece, well 5: The first piece, well 6: The whole cells of bacteria, well 7: Marker (Nacalai tesque, product no.02525)

Pieces of *A. baumannii* pili were treated with the hemagglutination test, whole cell, first, second, third, fourth pili pieces, and the residual of the pili fourth piece. Hemagglutination test using erythrocytes from human with O blood group and mice. The results are given in Figure 3, and these showed that the second piece of the pili had the highest titer 1/16.

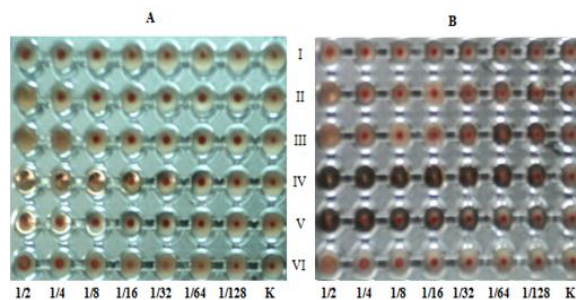


Figure 3. Result of the hemagglutination test from pili pieces.

Description: A: Human erythrocytes of O bloodgroup, B: Erythrocytes of mice.

I: The whole cells of bacteria, II: The first pili piece, III: The second pili piece, IV: The third pili piece, V: The fourth pili piece, VI: The residual of bacteria of the fourth pieces, K: erythrocytes and PBS 7.4.

Figure 2. showed that a good expression of the protein band is the second piece, and the Figure 3 showed that the second piece of the pili had the highest titer 1/16 both on erythrocytes from human with O blood group and mice, hence the second piece of pili was performed the SDS-PAGE showing the presence of several prominent proteins, a protein with a MW of 68 kDa, 44.4 kDa, 38.8 kDa, and 36.7 kDa, respectively, as shown in Figure 4.

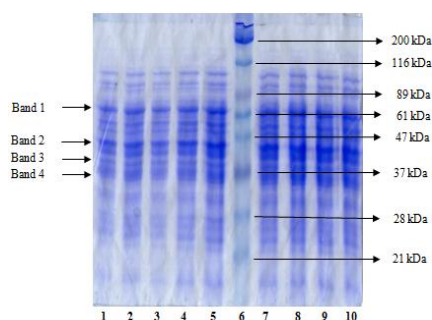


Figure 4. Profil of pili subunit protein from the second piece of bacterial pili of *A. baumannii*.

Description: 1-5, 7-10: The second piece of pili, 6: Marker (Nacalai tesque, product no.02525)

The choiced of four bands were electroeluted, dialysed, and hemagglutinated. The results of hemagglutination test for fourth of pieces bands can agglutination erythrocytes, but it was found that the third piece band, 38.8 kDa, had the highest titer 1/128 (line A-III) to human erythrocytes of O blood group and the titer 1/8 to erythrocytes of mice (line B-III). The result could be seen in Figure 5.

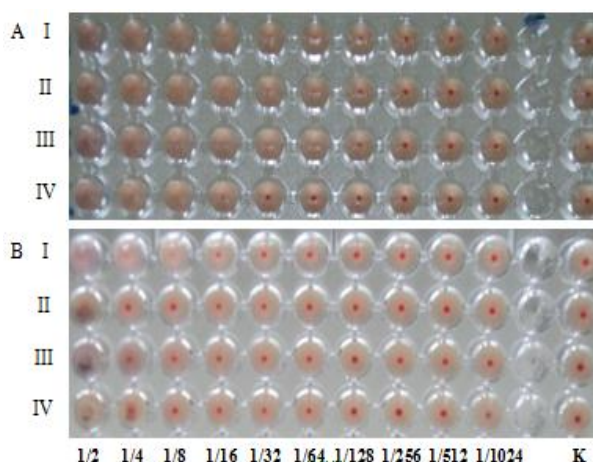


Figure 5. Result of hemagglutination test from the four bands of pili subunit HA protein.

Description: A: Human erythrocytes of O blood group, B: Erythrocytes of mice.

I: The first band with MW of 68 kDa, II: The second band with MW of 44.4 kDa, III: The third band with MW of 38.8 kDa, IV: The fourth band with MW of 36.7 kDa, K: erythrocytes and PBS 7.4.

The third piece band of pili subunit protein, MW 38.8 kDa had the highest titer was continued with adhesion test. The results were given in Figure 6 A to G.

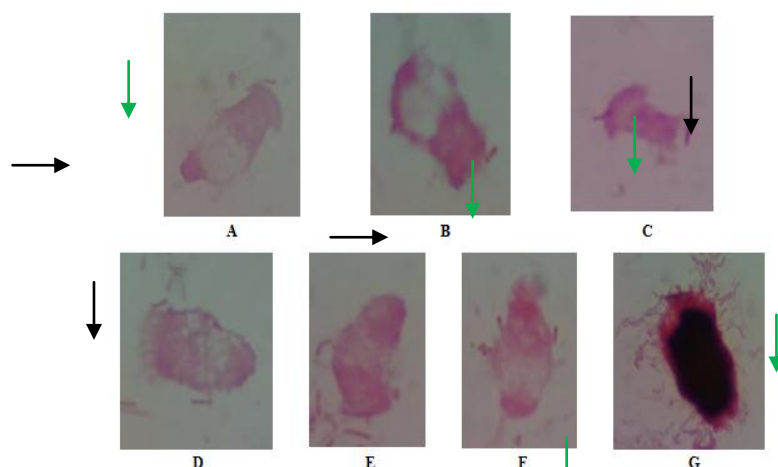


Figure 6. The result of attachment of *A. baumannii* to enterocytes after coated with pili subunit protein of 38.8 kDa at dose of 400 µg (A), 200 µg (B), 100 µg (C), 50 µg (D), 25 µg (E), 12.5 µg (F), and 0 µg (control) (G).

Description: Photomicroscope Olympus with 1000 X magnification. There were differences number of *A.baumannii* attached to enterocyte after coated with pili subunit protein Of 38.8 kDa (A-F) compared with control (G). The green arrow: bacteria of *A. baumannii*, and the black arrow: enterocyte of mice.

The adhesion index (AI) is the numbers of bacteria which attach to enterocytes. The AI was calculated by counting the numbers of bacteria attach to 100 enterocytes, and as shown in Table 1.

Table 1. The result of statistical analysis of the effect various dose of pili subunit protein with MW of 38.8 kDa to the AI

Code	Dose of protein	n	Mean±SD	p-value
A	400 µg	3	78.33±1.53 ^a	0.000
B	200 µg	3	102.00±1.00 ^b	
C	100 µg	3	143.67±1.53 ^c	
D	50 µg	3	193.33±3.51 ^d	
E	25 µg	3	293.33±2.08 ^e	
F	12,5 µg	3	405.00±1.00 ^f	
G	0 µg (Kontrol)	3	560.33±2.08 ^g	

Note: different notations at each Mean±SD value meant that there were significantly different ($p < 0.05$)

The results above showed that pili subunit protein with MW of 38.8 kDa were the hemagglutinin protein and adhesin protein. It was used as antigens to the experimental study to prove that it can activate s-IgA production. The results of the s-IgA concentrations showed that group I had amount of 4.77 µg/ml, group II had amount of 4.53 µg/ml, and group III had amount of 4.51 µg/ml, respectively (figure 7).

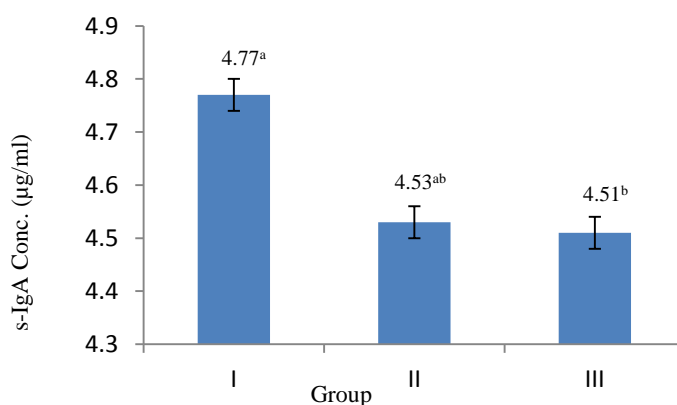


Figure 7. The results of s-IgA concentrations.

Note: different notations meant that there were significantly different ($p < 0.05$), and the same notations meant were no significantly different ($p > 0.05$).

IV. Discussion

Selected samples of *A. baumannii* collected from urine, pus and sputum were treated with hemagglutination test using human erythrocytes of O, A, B, and AB blood groups and mice on the whole cells. Hemagglutination test is a method to determine the titer of hemagglutinin protein of bacteria *A. baumannii* which could agglutinate erythrocytes [21]. The results showed that the sixth samples could agglutinate human erythrocytes of O blood group and mice erythrocytes. The highest titer of hemagglutination test was selected for bacterial samples and the results can see in Figure 1. From this result, it can be assumed that the sixth sample of bacteria *A. baumannii* is pathogen nosocomial bacteria, which can colonize and infect to the host.

The results above, showed that the human erythrocyte of O blood group has a suitable and complementary receptors with bacterial adhesin protein *A. baumannii*, compared with the human erythrocytes of blood group of A, B, and AB. It can be concluded that human erythrocytes of O blood group are more susceptible to bacterial infections of *A. baumannii* than blood group of A, B, and AB. Noorhamdani (2005) found the bacteria of *A. baumannii* has the F16 fimbria proteins that could agglutinate human erythrocytes of O blood group and mice erythrocytes, but did not agglutinate erythrocytes rat, guinea pig, sheep, human of A, B, and AB blood group [25]. Sumarno (2011) found the bacteria of *V. cholerae* 01M094V strain that could agglutinate human erythrocytes of O blood group and mice erythrocytes, but did not agglutinate erythrocytes guinea pig, human of A, B, and AB blood group [8].

Samples of the highest titer had been used for further study, in which the bacteria of *A. baumannii* were cultured in biphasic media TCG-BHI for 48 hours in order to grow the bacterial, and then harvested. The bacteria were cut with pili bacterial cutter until the supernatant of the bacterial pili pieces was already clear.

The results of cutting bacterial pili of *A. baumannii* consisted from pili of the first, second, third, fourth pieces, and the residual of the pellets of the fourth pieces treated with SDS-PAGE. Hemagglutination test showed that the second piece of the pili had the consistent bands expression and highest titer (Figure 2 and Figure 3). SDS-PAGE is method to gained the molecular weight and consistent bands expression of pili subunit protein. Bacteria having a structure that functions to attach to hosts are pili or fimbria composed of a protein called pilin and afimbria, a protein found on the surface of bacterial cells (OMP) [6]. Moreover, the second piece of pili which was performed the SDS-PAGE showing the presence of several prominent proteins, such as proteins with MW of 68 kDa, 44.4 kDa, 38.8 kDa, and 36.7 kDa, respectively, as shown in Figure 4. The fourth of protein bands piece showed a dominant and consistent, so can be assumed that it has more and better than the other pieces of bands. Research conducted by Sumarno found the molecular weight profile of the protein pili subunits from both *Salmonella thypi* and *Vibrio cholerae* [8,18].

The fourth of bands were electroeluted, dialysed, and then hemagglutinated. The results of hemagglutination test for fourth of pieces bands could agglutinate erythrocytes, but it was found that the third piece band, 38.8 kDa, had the highest titer to human erythrocytes of O blood group and could be seen in Figure 5. It's mean that pili subunit protein with MW of 38.8 kDa is a hemagglutinin (HA) protein. Adhesin protein in some bacterial form of protein that can agglutinate erythrocytes known as hemagglutinin protein [21].

Adhesion is the attachment process of bacteria on the surface of host cells. Bacterial cells that enter to the host body should conduct initial adhesion as the mechanism of infection, without adhesion the bacteria will be removed from the body through mucous fluids or body fluids that pass through the surface of the host cells. Hemagglutinin protein is identical with the adhesin protein as cell surface receptors that are responsible for cell interactions or interactions between cells and the extracellular matrix. The process of bacterial infection begins with adhesion of a bacterial cell to host cells through either adhesin virulence factors derived from pili and non-pili, for example, outer membrane proteins. The adhesion of bacterial cell to the host cells is specific [6,7]. In this study, the third band of pili subunit protein of *A. baumannii* was continued with adhesion test obtained the highest titer, it was 38.8 kDa. The results given in Figure 6 and Table 1 showed that the larger the dose of protein coated, the less bacteria attached to the enterocytes of mice. It meant that the more receptors are saturated by the hemagglutinin protein the less bacteria that attach to the receptors. Adhesion of bacteria on surface of eucariota cells could inhibit with adhesin protein had isolated [26].

ANOVA analysis results of dose response method from pili subunit HA protein with MW of 38.8 kDa of *A. baumannii* showed significant differences of the index among treatment doses ($p = 0.000$). It's means the larger dose of pili subunit HA protein with MW of 38.8 kDa of *A. baumannii* could reduce adhesion index. Pili subunit HA protein with MW of 38.8 kDa had a close relationship and a significant influence on the adhesion index ($r = -0,729$, $p = 0,000$, $R \text{ square} = 0532$, $p = 0.000$). Regression test showed that a significant correlation between pili proteins MW of 38.8 kDa and an adhesion index of bacteria *A. baumannii* in the mice enterocytes. The adhesion and adhesion inhibition tests empirically exhibited that the pili subunit HA protein with MW of

38.8 kDa as an adhesin protein. These results are consistent with Sumarno found pili subunit HA protein with MW of 37.8 kDa bacteria of *Vibrio cholerae* O1M09V as an adhesion protein [8].

B cells that changed to be IgA plasma cells that had been induced and stimulated by antigen in the germinal center will cause an interaction between B cells, follicular dendritic cells that present antigen to local CD4 T cells and facilitates B cell proliferation, class switch recombinant (CSR), somatic hypermutation and affinity maturation required for efficient humoral immune response [27]. In the plasma cells, IgM changed to be IgA that induced by cytokines, transforming growth factor- β (TGF- β) and interleukin-5 (IL-5). The s-IgA in lamina propria that transported across through epithelial cells to luminal surface by an IgA-specific factor receptor. On the luminal surface, the IgA will released from the bound receptor, then it can recognizes to ingested of the microbes and their toxin. The repeated exposure of protein antigen will produce antibodies with increased affinity toward the antigen. This process is called affinity maturation, it can cause the production of antibodies with increase in capacity of binding and neutralizing the microbes and toxins [28].

The experimental study conducted the Elisa test to measure levels of s-IgA. In this study, protein that act as antigen was a pili subunit protein *A. baumannii* with MW of 38.8 kDa, and could be seen in Figure 7. Immunizations were repeated on days 0, 7, 28 to stimulate the production of IgM antibodies to IgA antibodies in plasma cells.

ANOVA showed that the significance value of 0.027 ($p < 0.05$), it found that there are differences in the number of s-IgA concentration based on the treatment given. Then, Tukey test showed that the s-IgA concentration in group I treatment (antigen + iscom) differ significantly from that of the s-IgA concentration in group III treatment (control) ($p = 0.039$). Results of this experimental study showed that the pili subunit HA protein *A. baumannii* with MW of 38.8 kDa could activated s-IgA in the treatment to group I and II was higher than the treatment to group III (control). These results are consistent with Czinn which found that there was an increase of s-IgA concentration in gastric of mice after intragastric immunization with killed *H. pylori* combined with cholerae toxin (CT) [29]. Setyorini *et al.* (2013) also found an increased in levels of s-IgA in the intestine of mice after oral immunization pili subunit protein 49.8 kDa of bacteria *S. dysenteriae* combined with iscom that compared with the control group [24].

This study concluded that the pili subunit HA protein of *A. baumannii* with a MW of 38.8 kDa is an adhesin protein and can activate the production of s-IgA.

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