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Characterization pili protein with molecular weight 85 kDa *Escherichia coli* as protein adhesin and hemagglutinin

dr. Dini Agustina, M.Biomed

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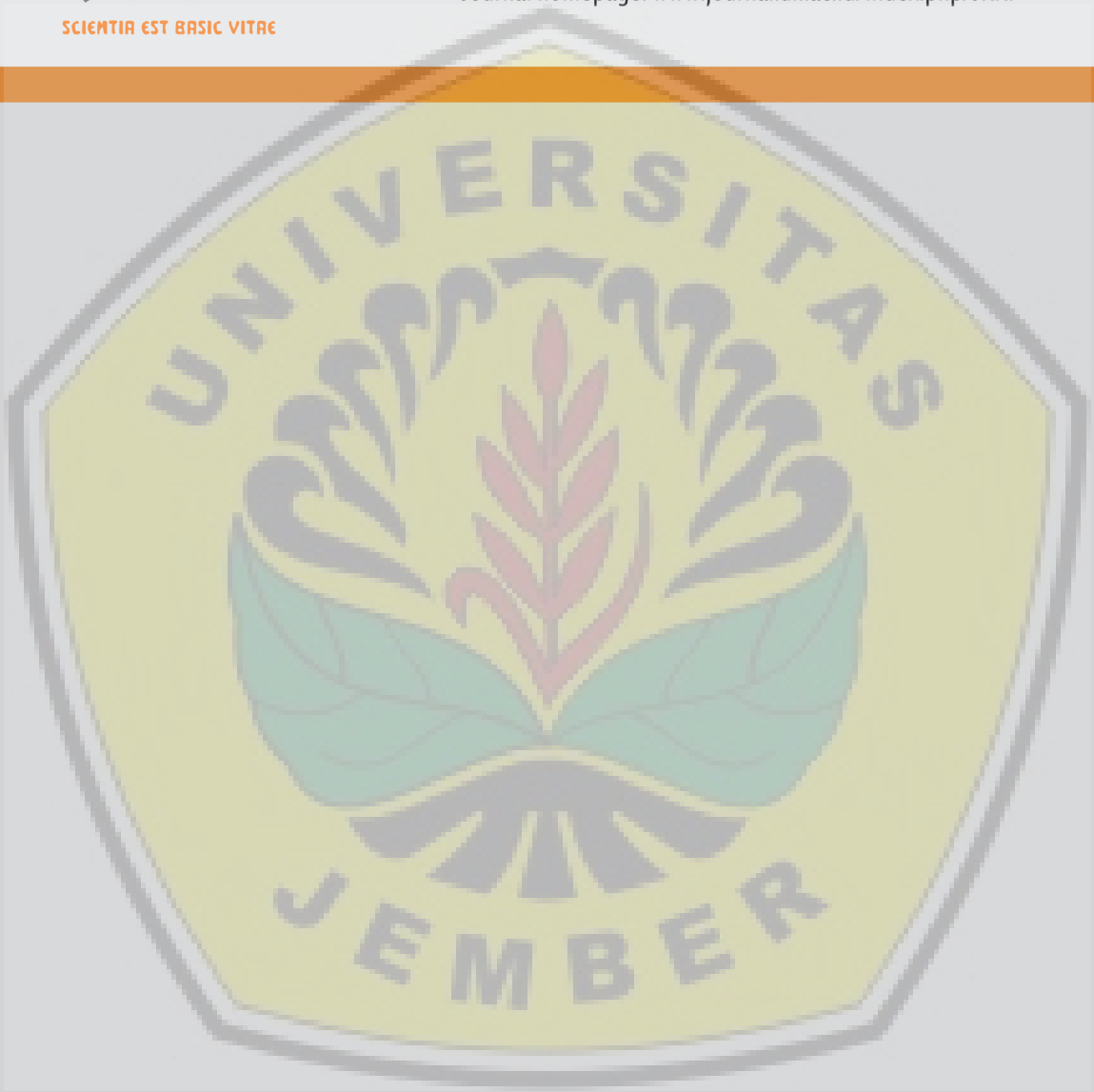

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Characterization pili protein with molecular weight 85 kDa *Escherichia coli* as protein adhesin and hemagglutinin

Dini Agustina[1], Siti Marissa Aisyah[2], Ika Rahmawati Sutejo[3], Diana Chusna Mufida[1]

[1]Department of Microbiology, Faculty of Medicine, University of Jember, Jember, Indonesia
[2]Medical Student of Medical Faculty, University of Jember, Jember, Indonesia
[3]Departement Biochemistry, Faculty of Medicine, University of Jember, Jember, Indonesia

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***Corresponding author:**
dini_agustina@unej.ac.id

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ABSTRACT

Background: *Escherichia coli* (*E. coli*) is a rod-shaped Gram-negative bacteria that included Multi-Drug Resistant (MDR) bacteria. There are several strategies to prevent and treat E. coli bacterial infections to reduce the incidence of MDR. One of them is the development of a protein-based vaccine. Pili is one of the virulence factors in *E. coli* surface proteins that can mediate the attachment of bacteria to host cells (adhesin or hemagglutinin) and can be used as protein-based vaccine candidates.
Objective: This study aims to determine characterization pili protein with molecular weight 85 kDa *Escherichia coli* bacteria as protein adhesin and hemagglutinin.
Methods: This type of research is a true experimental laboratory and descriptive study to determine the role of 85 kDa molecular weight pili protein *E. coli* bacteria as adhesin and hemagglutinin. The sample used was the stock of *E. coli* in the microbiology laboratory medical faculty of Universitas Jember (UNEJ). The hemagglutination test used mice erythrocytes, while the adhesion test used mice enterocytes. Isolation and purification of *E. coli* pili protein, isolation of mouse erythrocytes, isolation of mouse enterocytes, hemagglutination test and adhesion test were the methods in this study. Data analysis with correlation-regression to determine the relationship between the adhesion index and the titer 85 kDa with a limit of significance. 0.05 ($p < 0.05$).
Results: Hemagglutination test results showed no agglutination ability to form red aggregate points starting from the first dilution. Analysis with Pearson correlation indicates a relationship between 85 kDa *E. coli* titers and adhesion index ($p = 0.009$; $R = -0.921$).
Conclusion: Pili protein 85 kDa *E. coli* has a role as an adhesin protein and does not have a hemagglutinin protein position.

Latar Belakang: Escherichia coli *(*E. coli*) merupakan bakteri Gram negatif yang termasuk dalam bakteri* Multi Drug Resistant *(MDR). Saat ini ada beberapa strategi dilakukan untuk mencegah dan mengobati infeksi bakteri* E. coli *sehingga mengurangi angka terjadinya MDR. Salah satunya dengan pengembangan vaksin berbasis protein. Pili merupakan salah satu faktor virulensi pada protein permukaan* E. coli *yang dapat memperantarai perlekatan bakteri dengan sel inang (adhesin atau hemaglutinin) dan dapat dijadikan kandidat vaksin berbasis protein.*
Tujuan: *Penelitian ini bertujuan untuk mengetahui peran pili 85 kDa* E. coli *sebagai protein adhesin dan hemaglutinin.*

Metode: *Jenis penelitian ini adalah true experimental dan studi deskriptif untuk mengetahui peran protein pili berat molekul 85 kDa bakteri* E. coli *sebagai adhesin dan hemagglutinin. Sampel yang digunakan adalah stok* E. coli *di laboratorium mikrobiologi FK UNEJ. Uji hemaglutinasi menggunakan eritrosit mencit sedangkan uji adhesi menggunakan enterosit mencit. Metode dalam penelitian ini adalah isolasi dan pemurnian protein* E. coli *pili, isolasi eritrosit mencit, isolasi enterosit tikus, uji hemaglutinasi dan uji adhesi. Analisis data dengan korelasi-regresi untuk mengetahui hubungan antara indeks adhesi dan titer 85 kDa dengan batas signifikansi. 0,05 (p <0,05).*

Hasil: *Hasil uji hemaglutinasi menunjukkan tidak adanya kemampuan aglutinasi dengan terbentuknya titik agregat merah mulai dari pengenceran pertama. Analisis dengan korelasi Pearson menunjukkan terdapat hubungan antara titer pili 85 kDa* E. coli *dengan indeks adhesi (p= 0,009; R= -0,921).*

Kesimpulan: *Protein pili 85 kDa bakteri* E. coli *memiliki peran sebagai protein adhesin dan tidak memiliki peran sebagai protein hemaglutinin.*

INTRODUCTION

Escherichia coli (*E. coli*) is a Gram-negative rod-shaped bacterium. *E. coli* can cause diseases of the gastrointestinal tract, namely strains that cause diarrhea or which infects the intestine and Extraintestinal Pathogenic *Escherichia coli* (ExPEC). In its treatment, *E. coli* is included in MDR bacteria because it produces extended-spectrum beta-lactamase (ESBL) enzymes. The high level of antibiotic resistance is due to *E. coli* having an outer membrane in its peptidoglycan layer. Beta-lactam antibiotics penetrate *E. coli* through pores in the outer membrane. Membrane proteins will affect pore size, which makes antibiotic resistance to bacteria.[1] The proliferation of multiple MDR strains of drugs in recent years has led to an increase in the incidence of hospitalizations, treatment failures, and mortality. To prevent and treat these problems, protein-based vaccines can be developed. Currently, a candidate for the *E. coli* vaccine, namely ExPEC4V, contains four *E. coli* O-antigens (O1A, O2, O6A, O25B) conjugated protein exotoxin A. The ExPEC4V vaccine is designed to prevent urinary tract infections and complications such as bacteremia.[2]

E. coli also has surface proteins on its outer membrane that play a role in virulence factors. Pili is one of the surface proteins that can mediate bacteria's attachment to host cells (adhesin or hemagglutinin). These can be used as candidates for protein-based vaccines. Immunogenic proteins that can trigger humoral responses and cellular immunity have molecular weights between 10-100 kDa. Previous research has shown that 32.2 kDa *E. coli* adhesin pili have been shown to inhibit the attachment of *E. coli* to human spermatozoa.[3] In this study, adhesion and hemagglutination tests will be carried out on the pili protein 85 kDa, which is an immunogenic protein (among 10-100 kDa) and has a thickness band than other molecular weights. This research aimed to find out that *E. coli* 85 kDa can act as protein adhesin and hemagglutinin.

METHODS

This type of research is a true experimental laboratory and descriptive study. The sample used was the stock of *E. coli* in the microbiology laboratory FK UNEJ. The hemagglutination test used mice erythrocytes, while the adhesion test used mice enterocytes.

The study was carried out through several stages. It is *E. coli* pili isolation, molecular weight identification (using Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis/ SDS-PAGE), *E. coli* pili purification, mice erythrocytes isolation, Hemagglutination (HA) assay, mice enterocytes isolation, and adhesion assay.

Bacteria and Mice strain

The sample used was the stock of *E. coli* isolated from a human in the microbiology laboratory Faculty of Medicine, Universitas Jember. Mice used to take erythrocytes and enterocytes is female Balb/c mice aged 6-8 weeks.[4]

E. coli pili isolation

E. coli was taken from Eosin Methylene Blue agar media for planted on Nutrient Slant and incubated at 37°C for 24 hours to culture the

bacteria. The culture was inoculated onto a 500 mL Erlenmeyer tube containing the Brain Heart Infusion (BHI) solution Broth and incubated for 24 hours. Bacteria 10 mL was poured onto the in 50th 250 mL bottles containing 25 mL of Thiaproline Carbonate Glutamate (TCG) and media incubated at 37°C for 48 hours. TCG media serves as a medium for multiply bacterial pili. Next, the *E. coli* cultures were collected together in a 1000 mL Erlenmeyer tube and ready for pili cutting. *E. coli* pili are prepared to be cut inserted into the pili cutter. Pili is done using a pili cutter at 4°C 3000 rpm for 30 seconds. Sample centrifuged at 4°C, 6000 rpm for 15 minutes. The supernatant was collected in the tube, and the pellets were suspended using PBS pH 7.4 with a ratio of 1:1. The cutting was repeated four times. The fourth slice of pili protein will be used. Furthermore, the supernatant was centrifuged at 4°C 12000 rpm for 15 min to obtain supernatant and pellets.[3]

The pili obtained were dialyzed using a PBS solution pH 7.4 at 4°C for 2 x 24 hours to remove any remaining Tricloroacetic Acid (TCA). Result dialysis was precipitated using 35% ammonium sulfate and centrifuged 6000 rpm 4°C. The supernatant was then discarded, and the pellets were mixed with PBS sufficiently, and dialysis was carried out again. The result of dialysis is protein pili and stored at -20°C for use in the next stage.[3]

Molecular Weight Identification

SDS-PAGE was used to identify the molecular weight of *E. coli* pili. Electrophoresis using the gel was containing a 12.5% mini slab and 4% stacking gel. The protein marker used is a broad stained range: gel coloring or staining using Coomassie Brilliant dyes Blue R-250. When running electrophoresis with SDS-PAGE, the voltage used at 120 mV with a current of 400 mA and run for 90 minutes The results of the *E. coli* pili sample were taken as much as 500 µL after dialysis. Then The sample was added with 500 µL of sample buffer (Bromophenol Blue). After adding the sample buffer, the sample is heated in water at 100 °C for 5 minutes.[3]

***E. coli* pili purification**

The SDS-PAGE gel's molecular weight was calculated and then cut straight according to the desired molecular weight. The resulting cut band on the gel was collected then put into a nitrocellulose sheet to be done electroelution using a horizontal electrophoresis device with a running buffer. The electrophoretic voltage required is 125 mV for 2 hours. Beaker glass containing 1 liter of sterile PBS pH 7.4 was prepared for dialysis electrophoresis and given a magnetic stirrer, then placed in the refrigerator for 2x24 hours. Within 48 hours of dialysis, sterile PBS was changed two times. Fluid from dialysis for 48 hours is ready for the hemagglutination test.[3]

Erythrocytes Isolation

Blood was collected from the mice's heart and then collected in a tube containing 3 mL sodium citrate 3.5% with EDTA (1 mg/mL) and centrifuged for 10 minutes at 4°C. Plasma and leukocytes are removed, and erythrocytes are resuspended and washed three times in PBS-glucose buffer with five times more volume (138 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4 and 5 mM glucose, pH 7.4). The centrifugation wash conditions were the same, and the pellets were buffer rinsed each time to remove the leukocyte lining.[5]

Hemagglutination assay

Serial dilutions on microplates (v shape) were prepared for pili protein dilution, where each well the volume is 50 µL. Dilution was done by adding 50 µL of PBS sequentially or serially to each well, namely 1/2, 1/4, 1/8, 1/16, 1/32, 1/64, 1/128, 1/256, 1/512, 1 / 1.024 and add 50 µL of pili to the first well. The mixture in the first well as much as 50 µL was taken, then diluted to the next well until the last well. The red blood of 0.5% mice as much as 50 µL was added in each well with the same volume. Microplate was placed on a rotator plate for 1 minute, then leave it at room temperature for a 1-minute and observe. Observation of erythrocyte agglutination from dilution the

highest to the lowest is done to determine the amount of titer.[6]

Mice Enterocytes Isolation

The Weisser method was used to isolate mice enterocytes. The female BALB/C mice were the mice lines to be used in the method. The mice were anesthetized using chloroform on beaker glass, then performed surgery to take enterocytes in the intestine smooth. The small intestine of mice is opened and cleaned of dirt and then cut into small pieces. Then, the mice's small intestine was washed with sterile PBS pH 7.4, which contained 1 mM DTT at 4°C. After washing, the enterocytes are put into the liquid containing PBS pH 7.4 then put on a shaking water bath for 15 minutes at 37°C. The supernatant is removed, and the tissue settles transferred to PBS liquid pH 7.4, which contained 1.5 mM EDTA and 0.5 mM DTT. The network is put on the shaking water bath again for 15 minutes at 37°C; then, the resulting supernatant is discarded. Wash it re-settled the tissue with PBS and then centrifuged for 5 minutes at a speed of 1000 rpm and repeated three times. The yield deposits centrifuge (enterocyte tissue) was added with sterile PBS. The enterocyte is analyzed using spectrophotometry with a wavelength of 560 nm until it reaches 106/mL concentration for further adhesion testing is ready.[4]

Adhesion Assay

The method modified by Nagayama is used to perform an adhesion test by culturing *E. coli* in lactose broth at 37°C for 24 hours. Bacteria from the culture were centrifuged with a speed of 6000 rpm at 4°C for 10 minutes to get a harvest of these bacteria. PBS was added to the centrifuge precipitate, and concentration bacteria were made 108/mL using spectrophotometry with 600 nm. The next process is pili dosage preparation by making multiple dilutions ranging from 0, 0,72; 0,65; 0,61; 0,4; and 0,35 g/dL, as a control, P1-P5 respectively. Enterocyte suspension was given at each dose of 300 µL and then shaken gently, shaking the incubator at 37°C for 30 minutes. *E. coli* suspension was added to each pili mixture and the enterocyte suspension as much as 300 µL and then incubated in the incubator at 37°C for 30 minutes. Then centrifuged at 1500 rpm at 4°C for 3 minutes. Wash twice as much as the resulting sediment centrifuge using PBS. The residue is taken, and preparations are made smear on the object-glass, then stained with Gram stain. The preparations that had been stained with gram were then observed using a microscope with a magnification of 1000 times and counted the number of bacteria attaches to the enterocytes to find out the adhesion index. The adhesion index is the average number of bacteria attached to the epithelium and the count performed on 100 enterocyte epithelium.[4]

Statistical Analysis

Research data on the presence of hemagglutinin and adhesin proteins in pili 85 kDa *E. coli* was done descriptively. Meanwhile, the calculation of the relationship between the pili doses of 85 kDa *E. coli* with the adhesion index was analyzed using a statistical correlation-regression test with a significant limit of 0.05 ($p < 0.05$) previously tested for normality.

RESULTS

The identification of E. coli with a 1000x magnification microscope showed the morphology of rod-shaped bacteria and were Gram-negative bacteria (Figure 1). The bacteria were cultured in Eosin Methylene Blue Agar (Figure 1) and then harvested and isolated pili. Furthermore, pili electrophoresis uses SDS-PAGE to determine the molecular weight of proteins. The SDS PAGE results showed that the pili protein's molecular weight was 103 kDa, 85 kDa, 50 kDa, 43 kDa, 36 kDa, and 23 kDa (Figure 2).

Hemagglutination test results were read out at the 60th minute by observing erythrocyte deposition in each well (Figure 3). Hemagglutination test results conducted in this study showed a red aggregate starting

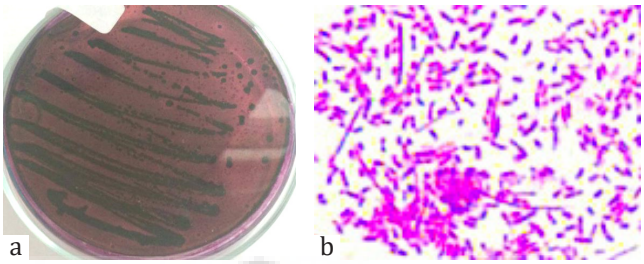

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Figure 1. Results of identification of E. coli bacteria.
(a) Colonies of bacteria growing on EMB media and shown green metallic sheen formation;
(b) Rod-shaped and Gram-negative bacteria

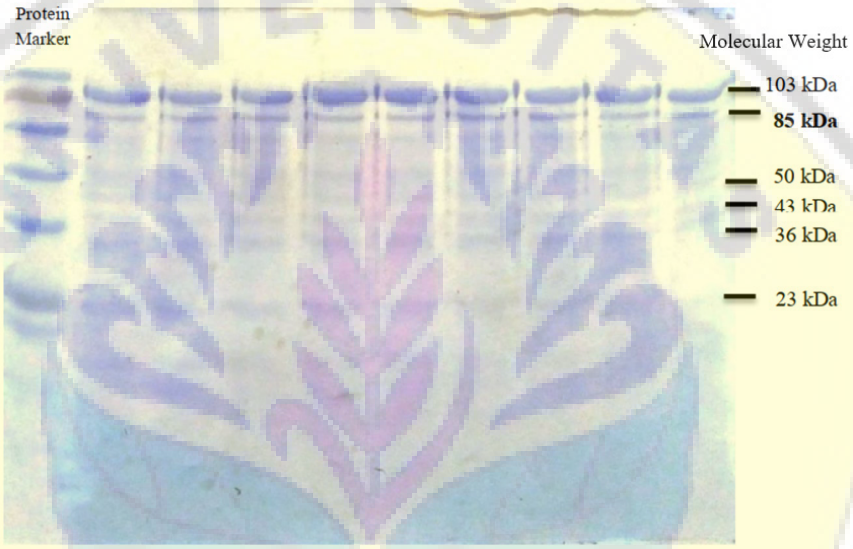

Figure 2. Profiles of pili protein 85 kDa *E. coli* produced by SDS-PAGE electrophoresis with each well filled with the fourth slice of protein resulted from the pili protein isolation process.

point of dilution 1/2 to 1/1024, which marks the pili protein 85 kDa of E. coli do not have the ability to agglutinate erythrocytes.

Adhesion test using mice enterocytes with different concentration of 0,72; 0,65; 0,61; 0,4; and 0,35 g/dL (Figure 4) which seen in the spectrophotometric results. On observation with a microscope with a magnification of 1000x, E. coli bacteria appear darker than mice enterocytes. The number of bacteria attached to enterocytes looks less at the highest protein concentration (P1= 0.72 g/ dL).

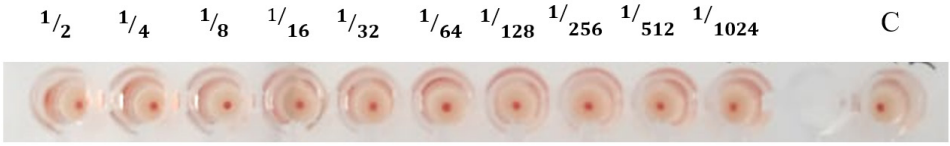

Figure 3. Hemagglutination test results with various dilution concentrations from ½ to $^1/_{1024}$

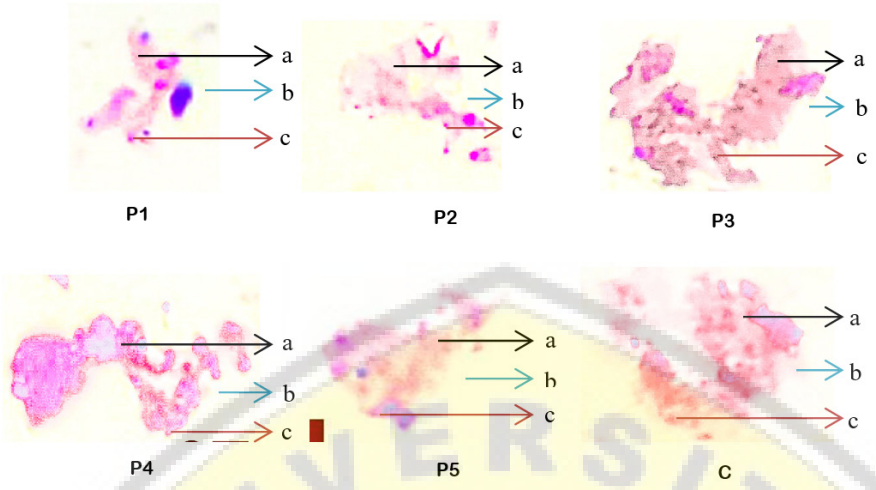

Figure 4. Results of 85 kDa *E. coli* adhesion test showed the results of various adhesion abilities at each dilution.
(P1) First dilution titer at a concentration of 0.72 g/dL;
(P2) Second dilution titer at a concentration of 0.65 g/dL;
(P3) Third dilution titer at a concentration of 0.61 g/dL; (P4) Fourth dilution titer at a concentration of 0.4 g/dL;
(P5) Fifth dilution titer at a concentration of 0.35 g/dL;
(C) Control;
(a) mice enterocytes; (b) Extra Cellular Matrix (ECM); (c) *E. coli*

P1 has the highest concentration with the lowest adhesion index, while P5 has the lowest concentration with the highest adhesion index from other dilution titers. Controls had the highest adhesion index (Table 1). Mice enterocytes in control were not added to the pili protein, which caused the bacteria not to compete with the pili in terms of adhesion. Mice enterocytes have been added to several dilutions of pili protein by the specified titer, causing bacteria to compete with each other in terms of adhesion.

Pearson correlation test obtained p= 0.009 and R= -0.921. The correlation coefficient of -0.921 means that the pili titer with the adhesion index has a strong relationship with the negative relationship's direction. A correlation test followed by a linear regression test with a value of R= 0.921 indicates a strong relationship between variables, whereas R2= 0.848 means 84,8 % titer pili 85 kDa affect adhesion index.

Table 1. Adhesion index of E. coli in enterocyte cells of mice

Repetition	Dilution					
	P1	P2	P3	P4	P5	C
I	315	676	815	861	997	1139
II	377	636	703	847	1024	1051
III	414	728	822	952	989	1248
Average	368,67	680	780	886,67	1003,33	1146

DISCUSSION

Hemagglutination test is a test on pili protein by observing whether the erythrocyte aggregate of mice is formed. A hemagglutination test was carried out to determine the protein tested as a hemagglutinin protein, one of the virulence factors that mediate bacterial cells' attachment to the host erythrocyte.[7] The ability of pili protein to agglutinate erythrocyte is different in each bacterium. The difference can be seen from whether a red aggregate point is generated at each microplate v wells. This research showed that pili protein 85 kDa E. coli does not cause hemagglutination process with the appearance of red aggregate point starting from the lowest to highest dilution. The red aggregate points produced in this study were mice erythrocytes, which were not agglutinated and settled on the bottom of the well. This happens because the pili protein is unable to attach itself to mice erythrocytes. The factors that influence hemagglutination are different in each bacterium. Factors that can affect hemagglutination are erythrocytes used, pH of diluents, and temperature at incubation. Research on 100 *E. coli* isolates showed only 9% of P fimbrae or P pili and 14% of pili type 1 had hemagglutination ability, while 73 other isolates were not. This happens probably because of several mutations. Other possible causes include host character factors such as the type of infection and predisposing factors that determine the host and pathogen's interaction in vivo.[8] Other studies on the outer membrane protein of 20 kDa bacterium *K. pneumonia* have shown the agglutination of bacterial proteins in erythrocytes in mice. The agglutination ability is demonstrated by the formation of red aggregate points at dilutions of 1/2 to 1/6.9 Protein that has been proven to play a role as a hemagglutinin protein can be continued with research to obtain a vaccine. A study on the New Castle chicken / Indonesia/GTT/11 genotype VII virus, carried out dilution in the hemagglutination test, showed a vaccination response.[10]

Adhesion is the attachment of bacterial cells to the host tissue's cell surface as the initial step of the bacterial infection process. An adhesion test is a test to prove whether the protein tested is an adhesin protein that plays a role in bacterial adhesion.[11] Adhesion test in this study used healthy mice enterocytes as a target for adhesion of pili protein. Previous studies have shown that mice enterocytes are used to prove the role of adhesion of a bacterium.[12] The results of three repetitions of calculations found that the greater the 85 kDa titer given to mice's enterocytes, the fewer bacteria that attach to the enterocytes of mice. Adhesion ability in each bacterium will be different. This is influenced by the molecular weight and receptors that play a role in the adhesion. Receptors on mice enterocytes bind to pili in various titers so that the more receptors have been secured, the fewer bacteria are attached to the enterocytes.[13] This is by studying the outer membrane protein of 20kDa *K.pneumoniae* bacteria, which showed that the greater the dilution titer (1/10) was given to the enterocytes of mice, the less amount of bacteria was attached. The lower the dilution titer (1/10000) assigned to the mice enterocytes, the more the number of bacteria is attached.[9] The lowest dilution titer with the lowest adhesion index can inhibit bacterial adhesion compared to the higher adhesion index. This is consistent with Santoso's research, which showed that the adhesion index of HA-F36 Salmonella typhi protein was lower than HA-036, which means HA-F36 had a higher adhesion ability.[14] Pili protein, which is proven to act as an adhesin protein, can be continued with research related to protein-based vaccines. This study shows that the lowest dilution titers with the lowest adhesion index are more likely to be vaccine candidates than other dilution titers. Research on the 38 kDa M. tuberculosis adhesin protein induction supplemented with glutamine supplementation showed cellular immune responses in kwashiorkor model mice mainly by intestinal CD8 lymphocytes and pulmonary CD8.[15]

The correlation test showed a significant relationship (p= 0.009) with the adhesion index, with the correlation coefficient R= -0.921. The

pili protein 85 kDa E. coli with greater dilution titer affects the lower adhesion index, so the relationship's direction is reversed or negative. Every increase of 85 kDa E. coli titers is followed by a decrease in the adhesion index to affect the strength of the relationship. These results are similar to the study of Agustina et al. which states that there is a significant relationship (R= -0.562) between the 20 kDa *K. pneumoniae* Outer Membrane Protein (OMP) titer and the adhesion index.[9] Other studies of pili protein 32,2kDa *E. coli* in human spermatozoa have also been shown to act as adhesin proteins.[3] Generates regression test regression coefficient R= 0.921, which shows both variables have a strong relationship with the determination coefficient R2 = 0.848 or 84,8 %, this coefficient indicating a dilution titer pili proteins affect the amount of 84.8%. In comparison, the adhesion index of 15.2 % is influenced by other variables not examined. Data analysis using correlation and regression tests showed that the results of *E. coli* pili protein with a molecular weight of 85 kDa acted as adhesin protein. These results are in accordance with a research by Agustina et al. on the 20 kDa outer membrane protein of the *K. pneumoniae*, which states that the outer membrane protein acts as an adhesin protein.[9]

This study indicates that pili protein 85 kDa *E. coli* acts as an adhesin protein, although it does not work as a hemagglutinin protein. The results of research pili protein 85 kDa *E. coli* as a protein adhesin can serve as the basis for further research on efforts development of an *E. coli* vaccine protein-based. Research on the Enterotoxigenic *Escherichia coli* (ETEC) protein that is not proven to be a hemagglutinin protein shows antigens' effectiveness in inducing anti-adhesion antibodies. The MEFA CFA/I/II/IV ETEC adhesin protein strain is most likely a vaccine candidate.[16] There is a strong correlation between 85 kDa *E. coli* dilution titers and adhesion index, where the higher the concentration, the lower the adhesion index. That is because the pili protein binds to the receptors in the mice's enterocytes, so the more receptors that have been bound by the pili, the fewer bacteria that attach to the mice enterocytes.[10] The pili protein 85 kDa E. coli is an immunogenic molecular weight adhesin protein bound to mice enterocyte receptors. *E. coli* can induce IgG as in research Sukarjati, which states that the pili adhesin 32,2 kDa *E. coli* in the semen of infertile men can cause the production of Immunoglobulin (IgG).[3] However, the pili protein 85kDa *E. coli* is not a hemagglutinin protein because it cannot attach erythrocytes. This is likely to occur due to mutation.[4] There is still a shortage of research that is expected to continue to be developed regarding bacterial pili protein's role as an adhesin protein and/or hemagglutinin protein to produce perfect data and can be used as a protein-based vaccine candidate.

CONCLUSION

Pili protein 85 kDa E. coli has a role as adhesin protein and does not has a role as hemagglutinin protein.

CONFLICT OF INTEREST

The authors don't have any conflict of interest.

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