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Characterization of pili protein with molecular mass of 85 kDa *Escherichia coli* as an adhesin and a hemagglutinin

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ABSTRACT

Background: *Escherichia coli* (*E. coli*) is a rod-shaped gram-negative bacterium included in Multi Drug Resistant (MDR) bacteria. There are several strategies in place to prevent and treat E. coli bacterial infections to reduce some the incidences of MDR. One of them is the development of a protein-based vaccine. Pili is one of virulence factors in E. coli surface proteins that can mediate attachments of bacteria to host cells (adhesin or hemagglutinin) and can be used as protein-based vaccine candidates.
Objective: This study aims to determine characterization of pili protein with molecular mass of 85 kDa *Escherichia coli* bacteria as an adhesin and a hemagglutinin.
Methods: This study is a true experimental laboratories and a descriptive study to determine roles of pili protein with molecular mass of 85 kDa as an adhesin and a hemagglutinin. Samples used in this study were a stock of *E. coli* in the Microbiology Laboratory Faculty of Medicine, Universitas Negeri Jember (UNEJ). A hemagglutination test in this study used mouse erythrocytes while an adhesion test used mouse enterocytes. Isolation and purification of E. coli pili protein, isolation of mouse erythrocytes, isolation of mouse enterocytes, the hemagglutination test and the adhesion test were methods in this study. A data analysis with a correlation-regression was used to determine relationships between the adhesion index and titer pili 85 kDa with a limit of significance, 0.05 ($p < 0.05$).
Results: The hemagglutination test showed no ability of agglutination with the formation of red aggregate points starting from the first dilution. An analysis with Pearson correlation indicated a relationship between titer pili 85 kDa *E. coli* and adhesion index ($p = 0.009$; $R = -0.921$).
Conclusion: Pili protein 85 kDa *E. coli* plays a role as an adhesin protein and do not have a role as a hemagglutinin protein.

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 rod-shaped gram-negative bacterium. *E. coli* can cause diseases of the gastrointestinal tracts, namely strains that cause diarrhoea or infect intestines 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. A 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 membranes. Membrane proteins will affect the pore size, which makes antibiotic resistant to bacteria.1 Proliferation of multiple MDR strains of drugs in recent years has led to an increase of hospitalization incidences, 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 to exotoxin A protein. The ExPEC4V vaccine is designed to prevent urinary tract infections and complications such as bacteraemia.[2]

E. coli also has surface proteins on its outer membranes 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 mass between 10-100 kDa. A previous study has shown that 32.2 kDa *E. coli* adhesin pili have seemed to inhibit an attachment of

E. coli to human spermatozoa.[3] In this study, adhesion and hemagglutination tests were conducted on the pili protein with 85 kDa, which is an immunogenic protein (among 10-100 kDa) and has a thicker band than other molecular masses. This study aimed to find out that *E. coli* 85 kDa can act as an adhesin and a hemagglutinin.

METHODS

A type of this study was a true experimental laboratories and a descriptive study. Its samples were a stock of *E. coli* in the Microbiology Laboratory Faculty of Medicine, Universitas Negeri Jember (UNEJ). The hemagglutination test used mouse erythrocytes while the adhesion test used mouse enterocytes.

The study was conducted through for several stages. It was *E. coli* pili isolation, molecular mass identification (using Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis/ SDS-PAGE), *E. coli* pili purification, mouse erytrocytes isolation, Hemagglutination (HA) assay, mice enterocytes isolation, and adhesion assay.

Bacteria and Mice strain

Samples used in this study was a stock of *E. coli* that isolated from human in the Microbiology Laboratory, Faculty of Medicine, Universitas Negeri Jember (UNEJ). Mouse used to take erythrocytes and enterocytes were female Balb/c mice aged 6-8 weeks.[4]

E. coli pili isolation

E. coli was taken from Eosin Methylen Blue

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

The obtained pili was dialyzed by using a PBS solution pH 7.4 at 4°C for 2x24 hours to remove any remaining Trichloroacetic Acid (TCA). Results of dialysis were precipitated by using 35% of ammonium sulphate and were centrifuged at 4°C and 6000 rpm. The supernatant was then discarded, pellets were mixed with PBS sufficiently and dialysis was conducted again. The results of dialysis were protein pili and were stored at -20°C for a use in the next stage.[3]

Molecular Weight Identification

SDS-PAGE used to identify the molecular mass of *E. coli* pili. Electrophoresis using the gel contained a 12.5% of mini slab and 4% of stacking gel. The protein marker used was a stained broad range. Gel colouring or staining using Commasie Brilliant dyes Blue R-250. When running electrophoresis with SDS-PAGE, the voltage was used at 120 mV with a current of 400 mA and run for 90 minutes. The sample results of the *E. coli* pili 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 was heated in water at temperature 100°C for 5 minutes.[3]

***E. coli* pili purification**

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

Erythrocytes Isolation

Blood was collected from the mouse's heart, collected in a tube containing 3 mL sodium citrate and 3.5% with EDTA (1 mg/mL) and centrifuged for 10 minutes at 4°C. Plasma and leukocytes were removed and erythrocytes were resuspended and washed 3 times in PBS-glucose buffer for five times and more volumes (138 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4 and 5 mM glucose, pH 7.4). Wash conditions of erythrocytes were 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 of the volume was 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 and 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 rotator plate for 1 minute left at a room temperature for 1-minute, and observed. Observation of erythrocyte agglutination from the highest to the lowest dilution was conducted to determine the amount of the titer.[6]

Mouse Enterocytes Isolation

The Weisser method was used to isolate the mouse enterocytes. The female BALB/C mice were the mouse lines used in the method. The mice were anesthetized by using chloroform on beaker glass, and a surgery was conducted to take enterocytes in the intestine smooth. The small intestine of mouse was opened, cleaned of dirt, and cut into small pieces. Then, the mouse's small intestines were washed with sterile PBS pH 7.4, which contained 1 mM DTT at 4°C. After washing, the enterocytes were put into a liquid containing PBS pH 7.4 and were placed in a shaking waterbath for 15 minutes at 37°C. The supernatant was removed and the tissue settles was transferred to PBS liquid pH 7.4, containing 1.5 mM EDTA and 0.5 mM DTT. The network was placed in the shaking waterbath again for 15 minutes at 37°C, and then the resulted supernatant was discarded. Deposit of tissue were rewashed with PBS and then centrifuged for 5 minutes at a speed of 1000 rpm and repeated three times. The deposit of enterocyte tissue was added with the sterile PBS. The enterocyte was analyzed by using spectrophotometry with a wavelength of 560 nm until it reached a concentration of 106/mL for further prepared adhesion testing.[4]

Adhesion Assay

A method modified by Nagayama was used for perform adhesion test by culturing *E. coli* in lactose broth at 37°C for 24 hours. Bacteria from culture were centrifuged with speed of 6000 rpm at 4°C for 10 minutes to get harvest of these bacteria. The PBS added to the centrifuge precipitate and concentration bacteria were made 108/mL by using spectrophotometry with a wavelength 600 nm. The next process was pili dosage preparation by making multiple dilutions ranging from 0 (Control), 0.72, 0.65, 0.61, 0.4, and 0.35 as a control, P1-P5 respectively. Enterocyte suspension was given at each dose of 300 µL and 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, incubated in the incubator at 37°C for 30 minutes, and centrifuged at 1500 rpm at 4°C for 3 minutes. Deposit of sentrifuge product washed twice with PBS. The residue was taken, and preparations were made smear on the object glass and stained with Gram stain. The preparations that had been stained with the gram were then observed by using microscope with a magnification of 1000 times, and a number of bacteria attaching to the enterocytes were counted to determine the adhesion index. The adhesion index was the average number of bacteria attached to the epithelium, and the count of adhesion index performed in 100 enterocytes of epitheliums.[4]

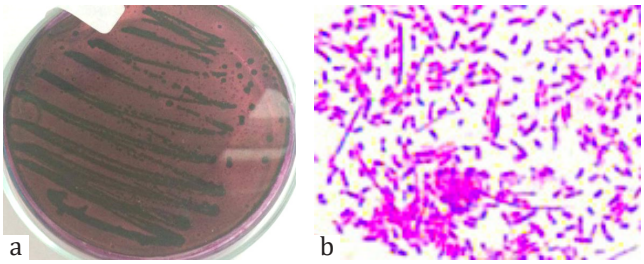
Statistical Analysis

Data of the presence of a hemagglutinin and an adhesin in pili 85 kDa *E. coli* was presented descriptively. Meanwhile, a calculation of the relationship between the pili doses of 85 kDa *E. coli* and the adhesion index was analyzed by using statistical correlation-regression test with a significant limit of 0.05 ($p < 0.05$).

RESULTS

The results of identification of *E. coli* with a 1000x magnification showed morphology of rod-shaped bacteria and Gram-negative bacteria (Figure 1). The bacteria were cultured in Eosin Methylen Blue Agar (Figure 1), harvested, and isolated. Furthermore, pili electrophoresis used SDS-PAGE to determine the molecular mass of proteins. The SDS PAGE results indicated that the molecular mass 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



a b

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

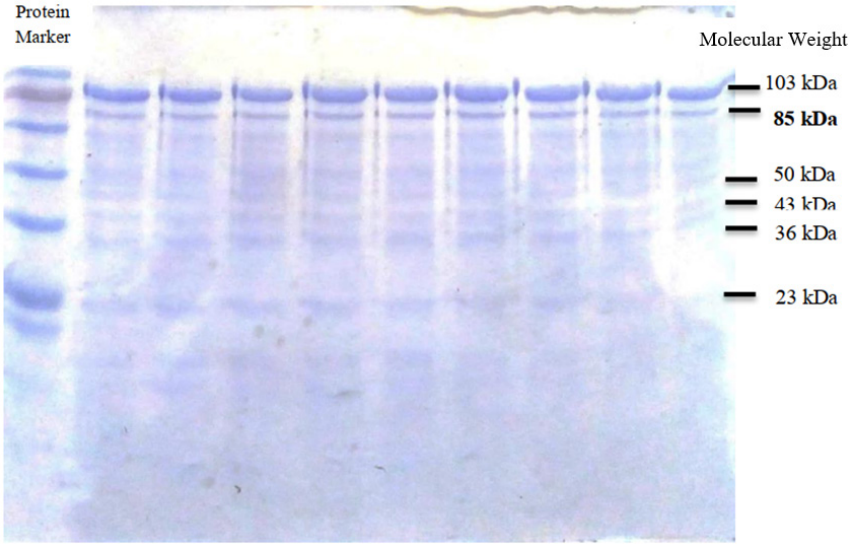


Figure 2. Profiles of pili protein 85 kDa *E. coli* produced by SDS-PAGE electrophoresis with each well filled with the fourth slice of protein the result of the pili protein isolation process.

erythrocyte deposition in each well (Figure 3). Hemagglutination test results conducted in this study showed red aggregate starting points of dilution ½ to $^1/_{1024}$, which marked the pili protein 85 kDa of *E. coli* and did not have ability to agglutinate erythrocytes.

Adhesion test using mouse enterocytes with different concentration of 0.72 ; 0.65; 0.61; 0.4; and 0.35 g/dL (Figure 4) could be seen in the spectrophotometric results. On observation with a microscope with a magnification of 1000x, *E. coli* bacteria appeared darker than

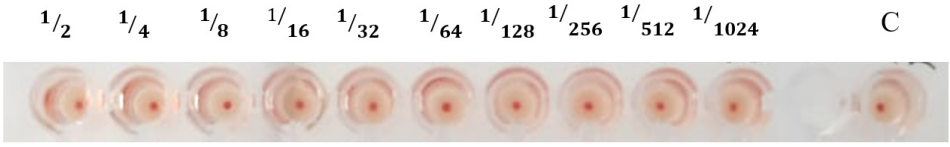


Figure 3. Hemagglutination test results with various dilution concentrations from ½ to $^1/_{1024}$

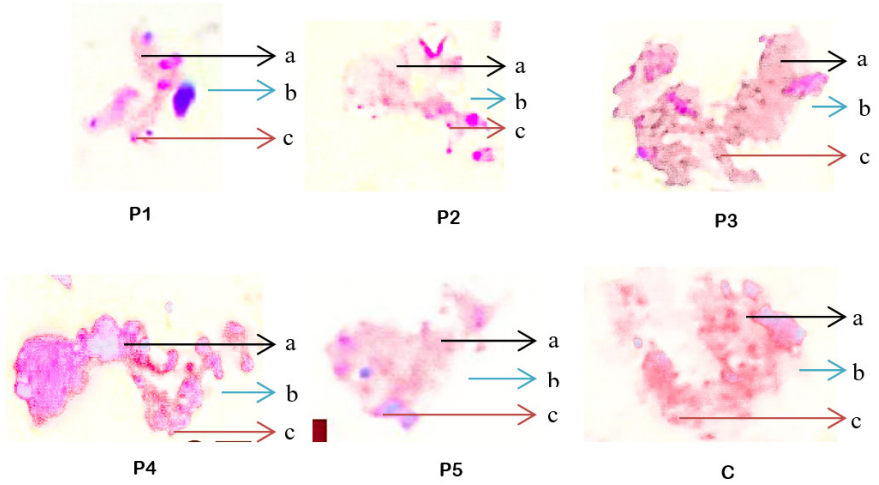


Figure 4. Results of 85 kDa *E. coli* adhesion test showed various adhesion abilities at each dilution.
(P1) First dilution titer at a concentration of 0.72 g/dL;
(P2) Second dilution titer at a concentration of 0.65 g/dL;
(P3) Third dilution titer at a concentration of 0.61 g/dL; (P4) Fourth dilution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

the mouse enterocytes. The number of bacteria attached to enterocytes seemed less at the highest protein concentration (P1= 0.72 g/ dL).

P1 had the highest concentration with the lowest adhesion index, while P5 had the lowest concentration with the highest adhesion index from other dilution titers. Controls had the highest adhesion index (Table 1). Mouse enterocytes in the control were not added to the pili protein, which caused the bacteria not to compete with the pili in terms of adhesion. Mouse enterocytes had been added to several dilutions of pili protein in accordance with 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 meant that the pili titer with the adhesion index had a strong relationship with a direction of the negative relationship. Correlation test followed by a linear regression test with a value of R= 0.921 indicated a strong relationship between variables, whereas R2= 0.848 meant 84.8 % titer pili with 85 kDa affected the 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 a pili protein by observing whether an erythrocyte aggregate of a mouse is formed or not. Hemagglutination test was conducted to determine a protein tested as a hemagglutinin protein, one of the virulence factors that mediate bacterial cell's attachment to a host of erythrocyte.[7] Ability of pili protein to agglutinate erythrocyte is different in each bacterium. A difference can be seen from whether red aggregate point is generated at each microplate v wells. This study shows that pili protein 85 kDa *E. coli* does not cause hemagglutination process with appearance of the red aggregate point starting from the lowest to highest dilution. The red aggregate points produced in this study were mouse 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 the mouse erythrocytes. Factors that influence hemagglutination are different in each bacterium. Factors that can affect hemagglutination are erythrocytes, pH of diluents, and temperature at incubation. Some studies on 100 *E. coli* isolates showed only 9% of P fimbriae 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 types 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 *K. pneumonia* have shown the agglutination of bacterial proteins in the mouse erythrocytes. The agglutination ability is demonstrated by formation of red aggregate points at dilutions of ½ to $^1/_6$.[9] Protein that has proven to play a role as a hemagglutinin can be studied more to obtain a vaccine. Research on the New Castle chicken /Indonesia/GTT/11 genotype VII virus, conducting dilution in the hemagglutination test, showed a vaccination response.[10]

Adhesion is an attachment of bacterial cells to the host tissue's cell surface as an initial step of the bacterial infection process. The adhesion test is the test to prove whether the tested protein is an adhesin that plays a role in bacterial adhesion.[11] The adhesion test in this study used healthy mouse enterocytes as a target for adhesion of the pili protein. Previous studies have shown that mouse enterocytes were used to prove roles of adhesion of a bacterium.[12] Results of three repetitions of calculations found that the greater the 85 kDa titer given to mouse's enterocytes, the fewer bacteria that attach to the mouse's enterocytes. Adhesion ability in each bacterium will be different. This is influenced by the molecular mass and receptors that play roles in the adhesion. Receptors on mouse's 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 can be seen by studying of the outer membrane protein of 20 kDa *K. pneumoniae* bacteria, showing that the greater the dilution titer ($^1/_{10}$) was given to the mouse's enterocytes, the less number of bacteria was attached. The lower the dilution titer ($^1/_{10000}$) is assigned to the mouse's 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 a higher adhesion index. This is consistent with Santoso's study showing, that the adhesion index of HA-F36 *Salmonella typhi* protein was lower than HA-036, meaning HA-F36 had a higher adhesion ability.[14] Pili protein, proving to act as an adhesion, can be studied more by focusing on protein-based vaccines. This study shows that lowest dilution titers with the lowest adhesion index are more likely to be vaccine candidates than other dilution titers. A study on the 38 kDa *M. tuberculosis* adhesin induction supplemented with glutamine showed cellular immune responses in kwashiorkor rat model mainly by intestinal CD8 lymphocytes and pulmonary CD8.[15]

Correlation test showed a significant relationship (p= 0.009) with adhesion index, with a correlation coefficient R= -0.921. The pili protein 85 kDa *E. coli* with greater dilution titer can affect the lower adhesion index, so the relationship's direction is reversed or negative.

Every increase of 85 kDa *E. coli* titer is followed by a decrease in the adhesion index to affect the strength of the relationship. These results are similar to a study of Agustina *et al*.who stated that there was 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 32.2 kDa *E. coli* in human spermatozoa have also shown to act as an adhesin.[3]

Regression test was results regression coefficient R= 0.921 that showing both variables have a strong relationship with a determination coefficient R^2= 0.848 or 84.8 %. It is indicates a pili protein dilution titer affecting the amount of 84.8%. In comparison, the adhesion index of 15.2 % influenced by other variables is not examined. Data analysis using correlation and regression tests showed that the results of *E. coli* pili protein with a molecular mass of 85 kDa acted as an adhesin. These results are in accordance with a study by Agustina *et al*. on the 20 kDa outer membrane protein of the *K. pneumonia*, stating that the outer membrane protein acts as an adhesin.[9] This study indicates that pili protein 85 kDa *E. coli* acts as an adhesin although it does not work as a hemagglutinin. The results of pili protein 85 kDa *E. coli* as a protein adhesin can serve as a basis for further study of a *E. coli* vaccine protein-based. A study on the *Enterotoxigenic Escherichia coli* (ETEC) protein that does not prove to be a hemagglutinin shows antigen's effectiveness to inducing anti-adhesin antibodies. The MEFA CFA/I/II/IV ETEC adhesin strain is the strain most likely to be a vaccine candidate.[16] There is a strong correlation between 85 kDa *E. coli* dilution titers and the adhesion index, where the higher the concentration the lower the adhesion index. This is because the pili protein binds to the receptors in the mouse's enterocytes, so the more receptors that have been bound by the pili, the fewer bacteria that attach to the mouse's enterocytes.[10] The pili protein 85 kDa *E. coli* is an immunogenic molecular mass of adhesin bound to mouse's enterocyte receptors. *E. coli* can induce Immunoglobulin G (IgG) as in a study of Sukarjati, stating that the pili adhesin 32.2 kDa *E. coli* in semen of infertile men could cause production of Immunoglogublin G (IgG).[3] However, the 85 kDa *E. coli* pili protein is not a hemagglutinin because it cannot attach erythrocytes. This is likely to occur due to mutation.[4] There is still a shortage of studies that is expected to be developed regarding bacterial pili protein's role as an adhesin and/or a hemagglutinin to produce perfect data that can be used as a protein-based vaccine candidate.

CONCLUSION

Pili protein 85 kDa *E. coli* bacteria has role as an adhesin and do not has a role as a hemagglutinin.

CONFLICT OF INTEREST

The authors don't have any conflict of interest.

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