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The 32 kDa Outer Membrane Proteins of *Klebsiella pneumoniae* Acts as A Bacterial Adhesin

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ABSTRACT

Klebsiella pneumoniae is a bacterium that often causes infection in the human body. At present *K. pneumoniae* can resist some of the antibiotics it has associated with modification of one of the virulence factors possessed by *K. pneumoniae*. One virulence factor of *K. pneumoniae* as pathogen bacteria is Outer Membrane Protein (OMP). The study of adhesin factors in *K. pneumoniae* involving hemagglutinin and adhesin proteins that have been found in the OMP 20 kDa and 40 kDa, but there is still no research that discusses the role of 32 kDa OMP as a hemagglutinin protein and adhesin. The purpose of this study is to determine the role of 32 kDa outer membrane of *K. pneumoniae* as hemagglutinin and adhesin proteins. After isolation of Outer Member Protein (OMP) from the *K. pneumoniae*, which then carried out a hemagglutination test using mice erythrocyte cells and adhesion test using mice enterocyte cells. The results of the hemagglutination test using mice erythrocyte cells obtained the highest hemagglutination titer for the molecular weight of 32 kDa in titers 1/4. The adhesion index with dilution titer has a significant relationship, with a conversion coefficient of 0,813 which means the dilution titer with the OMP adhesion index has a strong relationship with the direction of a positive relationship. The regression test results obtained an R-value of 0,813 which shows a strong relationship, while the R2 value is 66.1%. Conclusion in this study is 32 kDa outer membrane proteins of *K. pneumoniae* acts as a bacterial adhesin.

Keywords: *Klebsiella pneumonia*, outer membrane protein, adhesin.

INTRODUCTION

Klebsiella pneumoniae is a basil Gram-negative bacterium. These bacteria is one of Multi-Drug Resistance (MDR) pathogen so treatment for this infection is difficult *(*Brooks *et al.*, 2013*;* Mędrzycka-Dąbrowska *et al.*, 2021*;* Navon-Venezia *et al.*, 2017*;* Vading *et al.*, 2018). *K. pneumoniae* can cause several serious infections such as pneumonia, liver abscess, bacteremia, sepsis, nosocomial infections and urinary tract infections as well as several other infections (Clegg & Murphy, 2016).

K. pneumoniae is considered the most common cause of hospital-acquired pneumonia in the United States, and the organism accounts for 3% to 8% of all nosocomial bacterial infections January 2010 to June 2012, a study, revealed that 2320 of 2488 (93%) adults had radiographic pneumonia, the mean age of the patients was 57 years; 498 patients (21%) required intensive care, and 52 (2%) died (Ashurst & Dawson, 2021*;* Jain *et al.*, 2015; Jain *et al.*, 2021)

In 2019, pneumonia accounted for at least 14% (740,180) of all deaths in children under five years of age. The highest deaths from this disease were recorded in South Asia and sub-Saharan Africa. Data from the 2016 and 2019 Global Burden of Diseases (GBD) Studies show that lower respiratory tract infections (LRTIs) including pneumonia and bronchiolitis affect 489 million people globally. Children under 5 years of age and adults over 70 years are the population most affected by pneumonia(Abbafati *et al.*, 2020; Torres *et al.*, 2021; Troeger *et al.*, 2017; WHO, 2021)

K. pneumoniae is a major cause of multi-drug resistant (MDR) infections. This bacterium has a central role as a repository and disseminator of genetic determinants of MDR to other bacterial species. As many as > 400 AMR-associated *K. pneumoniae* genes and these strains can persist in the environment for many years. The dramatic increase in microbial resistance and the lack of new antimicrobials mean that there are fewer options for therapy, resulting in increased morbidity and mortality. At present *K. pneumoniae* can resist some of the antibiotics it has associated with modification of one of the virulence factors possessed by *K. pneumoniae.* One of the efforts to overcome this is the development of vaccines. Vaccines can reduce the incidence of infection in individuals, health services and communities thereby reducing the need for antimicrobials, inhibiting the development of antimicrobial resistance and

preventing infection in all age groups. Various platforms have been developed for the *K. pneumoniae* vaccine, including whole cells, capsule polysaccharides (CPS), O polysaccharides (OPS), Multiple antigen-presenting system (MAPS), Outer membrane proteins (OMP), fimbriae/pili, toxins, and additional antigens (Choi *et al.*, 2019; Kennedy & Read, 2017; Klugman & Black, 2018; Poolman, 2020; Wyres & Holt, 2018)

Currently vaccine-induced antibodies to KP CPS have been protective in preclinical studies, but the number of CPS strains (> 77) makes vaccines against this virulence factor less viable. So the other platform-based vaccines such as OMP is needed. This protein plays a role in initial attachment to host cells. Some of them were a molecular weight of 20 kDa and OMP 40 kDa (Pertiwi *et al*., 2009; Susilo *et al*., 2013). OMP has a stronger adhesive ability than pili (Agustina, 2017; Li *et al.*, 2014). The ability of OMP to perform adhesion is influenced by its ability to agglutinate erythrocytes (Shareef *et al.*, 2010). Adhesins have good potential as antigen protein in the manufacture of vaccines. Beside that, there are some study proved that OMP was considered a good vaccine candidate, such as, produce cytokines and recruit neutrophils in host defense mechanisms, protection against lung infection and sepsis in murine models and induce innate immune responses and humoral and cellular immunity (Babu *et al.*, 2017; Hussein *et al*., 2018; Lee *et al*., 2015; Pichavant *et al*., 2003). Therefore, vaccines derived from its virulence factors can be an alternative to treat this infection.

Our preliminary study showed OMP of *K. pnemoniae* had molecular weight of 32 kDa because it had a thick and highly concentrated band by electrophoresis. However, this protein has never been studied as an adhesive protein. Therefore, the aim of the study were to determine the role of OMP 32 kDa of bacteria *K. pneumoniae* as hemagglutinin and adhesin proteins.

METHODS

This research was conducted at the Laboratory of Microbiology, Faculty of Medicine and Laboratory of Biology, Faculty of Mathematics and Natural Sciences, University of Jember, which consists of several stages including: identification and culture of *K. pneumoniae* bacteria, isolation of OMP bacteria *K. pneumoniae*, identification of bacterial OMP molecular weight by electrophoresis (SDS-PAGE), purification of *K. pneumoniae* bacterial OMP, hemagglutination test, and adhesion test.

Colonies of *K. pneumoniae* were grown on MacConkey agar and incubated in an incubator for 24 hours at 37 °C in an aerobic atmosphere. The growth of *K. pneumoniae* bacterial colonies on MacConkey media appeared mucoid. After being incubated for 24 hours, biochemical tests were performed, consisting of TSIA, motility, Indole, Methyl Red, Voges-Proskauer, and Citrate (IMViC)

Procedure of hemagglutination test refers to the method used by Li, the protein dilution was made on a U microplate with a volume of 50 l. Mice blood suspension with a concentration of 0.5% was added to each U microplate well and then shaken using a rotator plate for 1 minute. Then let stand at room temperature for 1 hour. The titer was determined by observing the presence of erythrocyte clumping at the lowest dilution (Shareef *et al.*, 2010; Li *et al.*, 1999; Ryu, 2016)

The last procedure was adhesion test, the preeliminary step of this procedure is to prepare epithelial cells and bacterial strains. The epithelial cells used were enterocytes from the small intestine of mice. Enterocytes obtained were counted using a hemocytometer to 10^6/ml. while the concentration of bacteria made 10^8/ml. Furthermore, pili protein was put in a mini tube containing 300 l of PBS and then 300 l of enterocyte and bacterial suspension were added. The mixture was incubated in a shaking incubator for 30 minutes at 37°C. Then it was centrifuged at 1500 rpm, at 4°C for 3 minutes, then the precipitate was washed twice with PBS. The precipitate was taken, blotted on the object glass and painted with Gram stain. The preparations were observed under a microscope with 1000 times magnification, and the average number of bacteria attached to the enterocytes was calculated. Adhesion index is the average number of bacteria attached to enterocytes, calculated for each observation of 100 enterocytes (Di Martino *et al.*, 1995; Letourneau *et al.*, 2011; Nagayama *et al.*, 1995; Noorhamdani, 2013)

The independent variable in this study was the molecular weight of OMP 32 kDa *K. pneumoniae*, while the dependent variables were erythrocyte agglutination and adhesion index. The ethical clearance for this research was issued by the Ethics Commission of the Faculty of Medicine, University of Jember number 1.309/H25.1.11/KE/2019.

RESULTS AND DISCUSSION

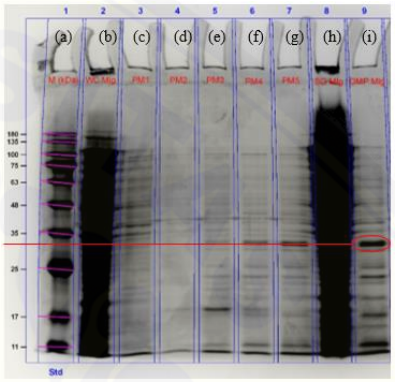
The isolates of *K. pneumoniae* bacteria obtained were examined by Gram staining and observed under a microscope with 1000x magnification. On examination, it was found that the bacteria were rod-shaped and red in color. The red color of the bacteria indicates that the bacteria are Gram-negative. Colonies of *K. pneumoniae* were grown on MacConkey agar and performed of biochemical tests). The results of the

biochemical test (Table 1.) were positive for Voges-Proskauer (VP), indicated by a change in the color of the broth to red after the addition of Barritt A and Barritt B reagents, meaning that the bacteria produced butylene glycol. The methyl red test is carried out to detect the ability of an organism to produce and retain acid which is the result of glucose fermentation. A negative result was indicated by the absence of a color change after the addition of methyl red reagent. In the indole test, it did not appear that the formation of a red ring on the top of the broth after the addition of Kovács reagent proved that the bacteria did not produce indole, meaning that the indole test result was negative. Then, to detect the ability of an organism to utilize citrate as the only source of carbon and energy, a citrate utilization test is carried out. Positive citrate results indicated by growth and color change to blue (Saimin *et al*., 2020; Tankeshawr, 2021).

Table 1. Results of biochemical tests on *K. pneumoniae*

Biochemical test	*K. Pneumoniae*	The studied bacteria
Voges-Proskauer	(+)	(+)
Methyl-Red	(-)	(-)
Indole	(-)	(-)
Citrat	(+)	(+)
TSIA	As/As, gas (+), H2S (-)	As/As, gas (+), H2S (-)

K. pneumoniae isolates were propagated on TCG media. Bacteria were harvested for further isolation of OMP (Figure 2). The results of the hemagglutination test using mouse erythrocyte cells showed the highest hemagglutination titer was at 1/4 (Figure 3). Hemagglutination titer is the occurrence of erythrocyte agglutination at the lowest dilution. This result showed that the higher the dilution, the smaller the hemagglutination titer. The ring-like formation indicates the absence of hemagglutination played by OMP in binding erythrocytes.



(a) Protein Markers; (b) Whole Cells; (c) Pili 1; (d) Pili 2; (e) Pili 3; (f) Pili 4; (g) Pili 5; (h) Bacteria that have been cut pili and their OMP; (i) OMP deductions. The red circle indicates the molecular weight of the OMP is 32 kDa

Figure 2. Protein profile of *K. Pneumonia* using electrophoresis (SDS-PAGE)

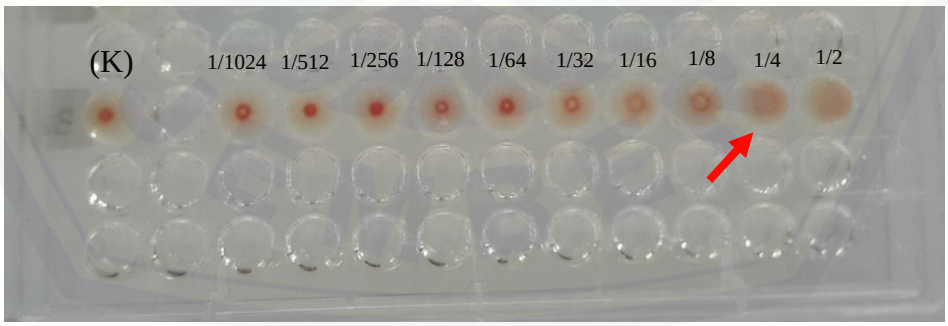


Figure 3. Result of Hemagglutination test OMP *K. Pneumoniae* with various dilution concentrations from ½ to 1/1024. (K) negative control (red arrow) highest titer

The protein OMP 32 kDa *K. Pneumoniae* can hemagglutinate at a dilution titer of 1/4, which means the protein is a hemagglutinin protein.. Hemagglutinin protein is a protein that can coagulate erythrocytes in mammals. The hemagglutinin protein possessed by a bacterium indicates that the bacterium has the ability to adhesion to host cells, which is an important process for the onset of clinical symptoms of a disease. The ability of hemagglutinin protein to coagulate red blood cells in animals and humans is not the same (Agustina *et al*., 2014; Suswati *et al*., 2020). There have been many studies on adhesin proteins from various bacteria, each

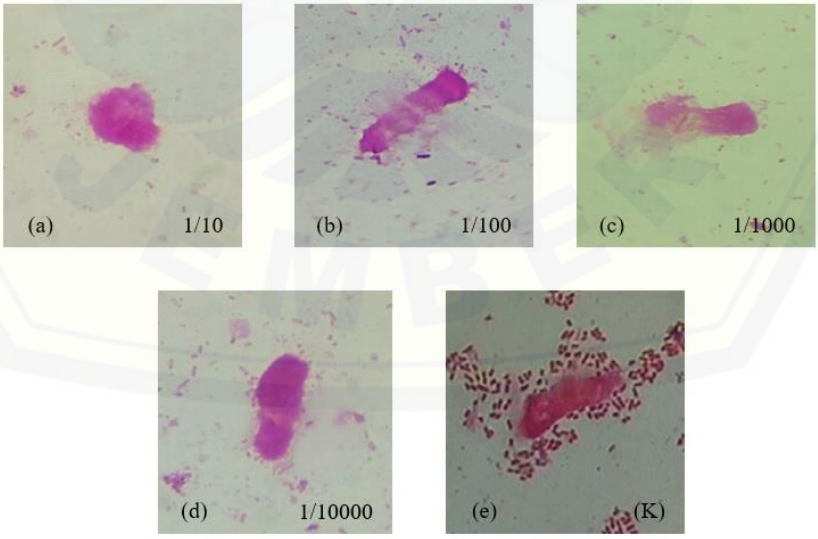
bacteria has a unique characteristic in its ability to agglomerate erythrocytes in mammals (Otto, 2014). In the hemagglutination test using 35 kDa OMP on P. mirabilis bacteria, it was found that the molecular weight OMP was able to agglutinate mouse erythrocyte cells up to a dilution of 1/512. In another study using 55 kDa OMP of S. typhi bacteria, it showed that OMP showed the ability to agglutinate mouse erythrocyte cells up to 1/128 dilution (D. Mufida *et al*., 2012; D. C. Mufida *et al*., 2009; Saptaningtyas *et al*., 2015). Hemagglutination activity of Campylobacter pylori bacteria on erythrocytes of mice, rabbits, guinea pigs, sheep, horses, and humans resulted in a positive reaction (Chmiela & Kupcinskas, 2019; Kao *et al*., 2016; Nakazawa *et al*., 1989). Acinetobacter has a 16 kDa protein fimbriae which is able to agglutinate rat erythrocytes and blood type O in human erythrocytes, but cannot hemagglutinate erythrocytes in sheep, guinea pigs, mice and human blood groups A, B (Noorhamdani, 2005). In this study, OMP K. pneumoniae 32 kDa was able to agglutinate mice erythrocytes. This shows that the hemagglutination activity of *K. pneumoniae* bacteria is different from other bacteria.

Based on adhesion test (Figure 4) of *K. pneumoniae* in mouse enterocytes, it showed that the lower the OMP concentration given, the greater the attachment index of *K. pneumoniae* to enterocyte cells (Table 2).

Table 2. Results of *K. pneumoniae* Adhesion Index on Enterocyte Cells Mice

Dilution Titer	Adhesion Index
1/10	765
1/100	793.33
1/1000	806.33
1/10000	981.67
0	1788

Adhesion is the initial stage of the colonization process of bacteria in the human body. Pathogenic bacteria must attach to host cells to initiate infection. This process is required for colonization in host tissues and is mediated by bacterial surfaces that have adhesive properties, such as lectins capable of recognizing glycoprotein residue oligosaccharides or glycolipid receptors on host cells (Anderson *et al*., 2007). To prove the role of OMP 32 kDa *K. pneumoniae* as an adhesive molecule, it was tested to determine the effect of protein concentration on the attachment of bacteria to mouse enterocytes which have receptors for molecular ligands to form adhesins, especially those owned by OMP (Agustina *et al.*, 2020; Finka *et al.*, 2019). The concentrations of OMP dilution titers that were made were 1/10, 1/100, 1/1000, 1/10000, and 0 as controls. The results showed that the number of bacteria attached to enterocyte cells increased with decreasing the concentration of OMP given.



(a) First dilution titer at a concentration of 1/10; (b) Second dilution titer at a concentration of 1/100; (c) Third dilution titer at a concentration of 1/100; (d) Fourth dilution titer at a concentration of 1/1000; (e) Control

Figure 4. Results of 32 kDa *K. pneumoniae* OMP adhesion test with graded dilution. Observation using microscope with a 1.000x magnification

The ability of bacteria to adhere to the surface of host cells has a relationship with the role of antigens on the surface to attach to surface receptors, both specific and non-specific. In specific adhesion, bacterial attachment is mediated by host cell receptors capable of binding to bacterial surface antigens. These surface antigens are generally referred to as adhesins and can be pili, fimbriae, capsules, or other structural components (Abrar *et al*., 2013). There are three types of adhesion-receptor according to Pardi (2010). The first type is based on the introduction of lectins-carbohydrates. The second type involves the interaction of proteins present in bacteria with proteins on the mucosal surface of the host cell. The third type involves interactions between proteins and lipids, where lipids can be present on the surface of bacterial cells or host cells. Most of the adhesins are specific to carbohydrates and are known as lectins (Pardi, 2010). Lectins will bind bacteria with carbohydrates from glycoproteins or glycolipids found in epithelial cells or other cells of the host. An example of a lectin-carbohydrate bond is a lectin with a glycoprotein receptor. These lectins can be in the form of fimbriae structures. Lectins can be in the form of fimbriae structures, capsules or OMP components of gram-negative bacteria (Sahly *et al.*, 2008).

Several studies have shown that the receptor and the adhesive molecule that act as a specific ligand that mediates bacterial adhesion will attach to the isolated receptor or analog receptor, and the isolated or analog adhesive molecule will bind to the surface of the host cell. This is consistent with the explanation that bacterial adhesion itself can be inhibited by adhesion molecules; or isolated receptor molecules, or molecules analogous to adhesive molecules and receptors; enzymes or chemical components damage adhesin molecules or receptors, as well as specific antibodies caused by molecular adhesions (Fitrianingsih, 2017; Murray *et al*., 2013; Paczosa & Mecsas, 2016). This study has two limitations, the first limitation is that the measurement of protein level by spectrophotometry is not carried out, due to the limited amount of protein available and the second is there is no in-depth analysis of the correlation between variables. With the results of this study, it is hoped that it can contribute to further research to develop vaccine-based adhesin proteins in overcoming various diseases caused by *K. pneumoniae*

CONCLUSION

Based on the results of the research conducted, this study concludes that The 32 kDa Outer Membrane Proteins of *Klebsiella Pneumoniae* can act as adhesin proteins.

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