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Increased levels of IL-4 in the Spleen of BALB/c Mice after 65.5 kDa Pili Protein *Klebsiella pneumoniae* Immunization

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Abstract: Pneumonia is an infection of the lung parenchyma caused by *Klebsiella pneumoniae*, resulting in a high mortality rate of millions each year. To reduce these deaths, one potential solution is to create a vaccine that utilizes virulence factors of this microorganism, such as pili. During the early phase of infection, these antigens have a crucial role and can stimulate the production of memory b cells. These cells are activated by IL-4 (interleukin-4) in lymphoid organs, such as the spleen. Pathogen exposure such as virulence factors can stimulate the secretion of IL-4 in the spleen organ. This study aims to investigate the role of *Klebsiella pneumoniae* pili in this process. The research conducted from May to December 2023 at the Microbiology Laboratory, Faculty of Medicine, University of Jember was purely experimental, using Balb/c mice with IL-4 levels in the spleen organ as variables. The study used 15 mice, divided into control (K1), adjuvant (K2), and antigen (K3) groups. The research data were analyzed using non-parametric tests, specifically the Kruskal-Wallis and Post Hoc tests. The Kruskal-Wallis test revealed significant differences (p=0.003). In the Post Hoc test, a significant difference was found between the control and antigen groups (p=0.002). The results conclusively demonstrate that induction of *Klebsiella pneumoniae* pili protein 65.5 kDa significantly increases IL-4 levels in the spleen. Future studies should consider adding serum specimens to provide additional information.
Keywords: Interleukin-4; *Klebsiella pneumoniae*; pili; vaccine.

INTRODUCTION

Pneumonia is a contagious infection that affects the lung parenchyma (Chang et al., 2021). It is the leading cause of death in cases of infection in the United States. In 2018, approximately 450 million people in developing countries suffered from pneumonia. Nusa Tenggara Timur is one of the provinces in Indonesia with the highest incidence of pneumonia (Manuaba et al., 2021). One of the agents that cause pneumonia is *Klebsiella pneumonia* (*K. pneumoniae*), a Gram-negative bacterium that can survive inside macrophages and is the most common cause of lower respiratory tract infections (Agustina et al., 2022; Chang et al., 2021). Infections caused by this microorganism are often exacerbated by inadequate treatment, leading to spread to multiple organs, sepsis, and Multidrug-resistant *Klebsiella pneumoniae* (MDR-KP). The mortality rate of MDR-KP reaches 72% in some hospitals due to antibiotic misuse and the bacteria's inherent resistance to antibiotics (Ejikeme et al., 2021; Poerio et al., 2022). Currently, there is no licensed vaccine for the prevention of *K. pneumoniae*. One possible measure to prevent further deaths from *K. pneumoniae* infections is vaccination. This is because previous vaccines had a short protection duration and did

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not cover all *K. pneumoniae* types (Arato et al., 2021; Choi et al., 2020). Therefore, current research is focused on identifying proteins from *K. pneumoniae* to be used in vaccine development.

Vaccines are created using immunogenic bacterial virulence factors.*K. pneumoniae* has several virulence factors, including capsule, siderophore, lipopolysaccharide (LPS), and pili (Chang et al., 2021). Pili play an important role during the initial phase of infection by attaching the bacteria to host cells due to their adhesive proteins. Compared to other virulence factors, pili have much less variability, making them representative of the entire bacterial genetics and triggering the formation of immune cells through biofilm formation mechanisms. Therefore, pili can be used as a candidate antigen for *the K. pneumoniae* vaccine (Arato et al., 2021).

The immunogenic nature of *K. pneumoniae* triggers the body's immune response to release several cytokines, including IL-3, IL-4, IL-5, IL-12p70, and IL-13. IL-4 plays a crucial role in respiratory tract infections caused by *K. pneumoniae* by regulating the number of alveolar macrophages and neutrophils during the early stages of infection (Moser et al., 2018). IL-4 is an anti-inflammatory cytokine that also functions in B lymphocytes to produce IgG as memory B cells, which are the target of vaccine development (Lee et al., 2015; Pollard & Bijker, 2021). IL-4 is primarily produced by T-helper 2 cells, which can be found in lymphoid organs, particularly the spleen, as the largest lymphoid organ (Lewis et al., 2019). Research was conducted using *Klebsiella pneumoniae* pili protein 65.5 kDa, which found that increase in anti-inflammatory cytokines in the antigen group (Suswati et al., 2022). There has been research on *K. pneumoniae* antigens induced intraperitoneally which influence IL-4 levels (Lee et al., 2015), and research by Hu et al., (2022) which uses *K. pneumoniae* antigens in the form of outer membrane proteins (OMP) with a molecular weight of 38 kDa which influences IL-4 levels, but there is still limited research using the *K. pneumoniae* pili protein so this study aims to analyze the effect of exposure to the 65.5 kDa *K. pneumoniae* pili protein on IL-4 levels in the spleen of BALB/C mice.

MATERIALS AND METHODS

The research was conducted using the experimental method of randomized posttest-only controlled group design. Research ethics was approved by The Ethics Committee of the Faculty of Medicine at Jember University with registration number 1807/H25.1.11/KE/2023. The research was conducted from May to December 2023 in the Microbiology and Biochemistry laboratories of the Faculty of Medicine at Jember University.

The mice were divided into three groups: the control group (K1) received only a Phosphate Buffer Saline (PBS) injection, the second group (K2) was treated with Freund's adjuvant, and the last group (K3) was given treatment with protein pili 65.5 kDa of *K. pneumoniae* + Freund's adjuvant and PBS. Each group consisted of five mice, totaling 15 mice. The 'Degree of Freedom Formula' states that the range of the sample in experimental research can be determined: For this research, the minimum sample size was 10/k+1 and the maximum sample size was 20/k+1 (Arifin & Zahiruddin, 2017). Therefore, the appropriate sample size for this study was 5-7 male BALB/C mice, aged 6-8 weeks and weighing 25 grams. This study aimed to measure the concentration of interleukin-4 in the spleen of mice.

Klebsiella pneumoniae bacteria were isolated from the sputum of infected patients and cultured using Mac Conkey (Merck). If the bacteria grew, it was transferred to a bottle of Brain Heart Infusion (BHI) by Merck and incubated at 37 degrees Celsius for thirty minutes. Ten milliliters of the sample were taken and

incubated in Thiaproline Carbonate Glutamate (TCG) media by Merck to observe the pili. The incubation process requires 48 hours at 37 degrees Celsius. After incubation, the suspension was centrifuged at 6000 rpm for 10 minutes with a centrifuge by Hettich Eba 200 to obtain the pili. The pili were then separated from the supernatant using an Omni mixer and centrifuged again.

Experimental animals were acclimated for approximately seven days at a Laboratory animal testing facility and weighed according to established criteria. On day 0, the control group (K1) was induced with 200 µL of PBS, the adjuvant group (K2) was induced with 100 µL of adjuvant and 100 µL of PBS, and the treatment group (K3) was induced with 50 µg of *K. pneumoniae* 65.5 kDa pili dissolved in 100 µL of PBS and 100 µL of adjuvant. The induction continued for 14 days via intraperitoneal injection. The induction was administered three times, on day 0, day 14, and day 28. Complete Freund's Adjuvant (CFA) adjuvant was used for the first induction, while Incomplete Freund's Adjuvant (IFA) was used for the second and third inductions. These adjuvants contain unactivated micro bacteria that are used to boost the priming immunization (Greenfield, 2020).

The experimental animals were terminated 14 days after the third induction using ketamine (90 mg/kgBB) and xylazine (10 mg/kgBB)(IAUCC, 2023). While under anesthesia, the mice's abdomen was opened up to the peritoneal area. The spleen was extracted using a pair of forceps and washed in a pH 7.4 PBS solution before being weighed on an analytical scale. The organ was then pulverized using a mortar and pestle on ice. The resulting homogenate was centrifuged at 3000 RPM for 20 minutes, and the supernatant was transferred to a labeled falcon tube. Following the aforementioned procedure, samples of the spleen organ were tested using an ELISA kit by Eiyue by the assay procedure.

The concentration of IL-4 was analyzed using Statistical Package for Social Science (SPSS) 25.0 with a nonparametric Kruskal-Wallis test. Additionally, a Post Hoc test was conducted to identify any increase in the level of IL-4 (Nahm, 2016).

RESULTS AND DISCUSSION

The results indicate a strong correlation between concentration and absorbance, with a correlation factor close to 1. The linear regression equation used to transform absorbance into IL-4 concentration is y=0.0013x+0.0379 (where x represents concentration and y represents absorbance). The control group (K1) had the lowest average IL-4 level, with a mean value of 61.92 ± 5.95 pg/mL, which serves as a reference for normal IL-4 levels. In diagram 1, the adjuvant group (K2) had a mean IL-4 level of 81.15±7.93 pg/mL, while the antigen group (K3) had the highest average with IL-4 levels of 109.92±26.97 pg/mL.

Statistical analysis using the comparative Kruskal-Wallis test reveals differences between two or more groups. Additionally, post-hoc analysis is employed to identify where these differences occur. The Kruskal-Wallis test yielded a significant p-value (p=0.003). However, the post-hoc test only showed a significant p-value (p=0.002) for the comparison between the control and antigen groups. The results of all statistical analyses are presented in Table 1 for the Kruskal-Wallis test and Table 2 for the Post Hoc Test.

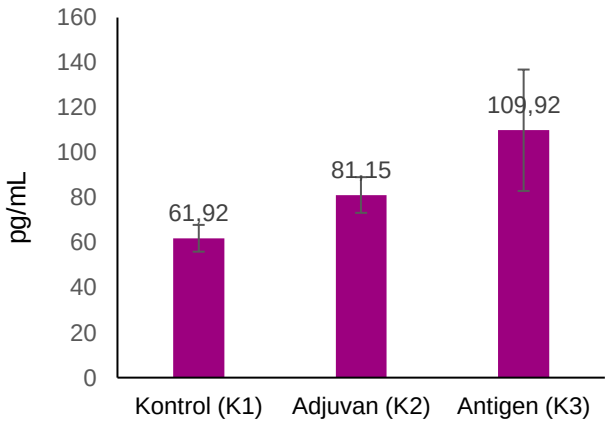

Figure 1. Average IL-4 Levels

Table 1. Result of the Kruskal-Wallis Test for IL-4 Levels in the Entire Sample

Difference between group	*p value*
Interleukin 4 concentration	0,003*

Note: *data was significance p<0,05

Table 2. Result of Post Hoc Test for IL-4 Levels in Each Treatment Group

Variabel	*p value*
Kontrol><adjuvan	0,169
Kontrol><antigen	0,002*
Adjuvan><antigen	0,413

Note: *data was significance p<0,05

The research data shows an increase in IL-4 levels in the adjuvant and antigen groups compared to the control group as the normal value. The Kruskal-Wallis test results showed a significant difference with a p-value of 0.003. The highest increase occurred in the antigen group, followed by the adjuvant group, and the control group in third place. The Post Hoc test showed a significant difference with a p-value of 0.002 in the comparison between the control and antigen groups. The significant difference is caused by the antigen group treatment involving *K. pneumoniae* antigen, indicating the activation of the innate and adaptive immune systems by involving IL-4. During the initial phase of infection, pili are responsible for attaching the antigen to the host cells. This is in line with the theory that this phase leads to the production of several cytokines, mainly IL-4, to regulate the activity of alveolar macrophages and neutrophils during the natural immune system response (Moser et al., 2018). These cytokines are also produced during the adaptive immune response because the presence of *K. pneumoniae* antigen in the host cells causes the differentiation of T lymphocytes into Th2 cells that produce anti-inflammatory cytokines, especially IL-4 (Darwin et al., 2021). IL-4 is an anti-inflammatory cytokine that stimulates B lymphocytes to produce IgG as memory B cells and stimulates T cell differentiation into cytotoxic T cells to lyse antigen *K. pneumoniae* infected cells during adaptive immune response (Widiatmaja et al., 2021). This finding is consistent with Lee et al. (2015) study, which used *K. pneumoniae* antigen-induced intraperitoneally to search for the best vaccine candidate. The research findings showed a significant increase in IL-4 levels (Lee et al., 2015). Additionally, this study's results are consistent with those of Hu et al., (2022) who used *K. pneumoniae* antigen in the form of outer membrane proteins (OMP) with a molecular weight of 38 kDa and found a significant increase in IL-4 compared to the normal value.

The first *K. pneumoniae* vaccine was developed in 1970 and was of the inactivated type. However, this vaccine was only effective in preventing UTIs. Subunit *vaccines* with capsular polysaccharides (CPS) and outer membrane proteins (OMP) have also been developed. However, both parts are ineffective due to the wide variability level, which cannot represent the entire *K. pneumoniae* genetic makeup (Choi et al., 2020). The research conducted by Choi et al. in 2020 demonstrated that a *K. pneumoniae* vaccine with pilus antigens protected test animals from lung infections and sepsis. The use of pili as a vaccine antigen has the advantage of lower variability, allowing it to represent all *K. pneumoniae* gene variants. Pili plays a greater role during the early stages of infection, minimizing potential side effects. Another advantage of using pili as a vaccine antigen is that it has been shown to increase IgG levels in the target animal samples (Arato et al., 2021; Choi et al., 2020; Martin & Bachman, 2018). Based on this background, pili were chosen as the antigen for this study.

The selection of *K. pneumoniae* with a molecular weight of 65.5 kDa as the antigen in this study is based on several reasons. The 65.5 kDa protein pili are the thickest and therefore have the highest concentration compared to others (Agustina & Mufida, 2021; Suardana, 2017). According to Sirat, (2021), antigens with a molecular weight >10 kDa are immunogenic. The theory is supported by Hu et al., (2022) study, which used outer membrane proteins (OMPs) as a candidate vaccine for *K. pneumoniae*. The OMP with a molecular weight of 38 kDa was found to trigger an immune response in animal samples. Additionally, the hemagglutination test of the 65.5 kDa pilus also yielded positive results with a titer of 1/8. This indicates that the pilus with that molecular weight can adhere to target cells (Agustina et al., 2022).

The research findings indicate an increase in IL-4 levels, particularly in the antigen group. This increase in IL-4 serves as an indicator for the formation of memory B cells, which are the primary target for vaccine development (Darwin et al., 2021; Pollard & Bijker, 2021). The cytokine also stimulates the differentiation of CD4+ T cells, which play a role in the maturation and proliferation of B cells, resulting in the production of specific antibodies, including B memory cells. Therefore, pili are strong candidates for the production of a *K. pneumoniae* vaccine (Pollard & Bijker, 2021).

The comparison of IL-4 levels between the control and adjuvant groups through Post Hoc analysis showed no significant difference with a p-value of 0.169. However, the average IL-4 level in the adjuvant group was higher than that in the control group. This is because the administration of Freund's Adjuvant in the adjuvant group can optimize the body's immune response, particularly by stimulating Th2 cells to produce IL-4 (Fan et al., 2022; Greenfield, 2019). The increase in IL-4 levels in the adjuvant group is also due to Freund's Adjuvant, which causes inflammation at the induction site. This was demonstrated in a study by Noh et al. in 2021, which induced CFA on the paw of a test animal, resulting in a series of inflammatory reactions that also involved IL-4 (Darwin et al., 2021; Noh et al., 2021). However, the IL-4 level in the adjuvant group was not as high as in the antigen group because the antigen was not given as the main trigger for the inflammatory response. The adjuvant group also involves administering IFA during the second and third inductions, which serves not only as a booster but also to minimize the inflammatory effects produced by CFA (Fan et al., 2022).

The Post Hoc test results also indicate that there is no significant difference in the IL-4 levels between the antigen and adjuvant groups with a p-value of 0.413. The insignificant difference may be due to the standard deviation value of the antigen group, which is 26.97 pg/mL, and one individual in the antigen group having an IL-4

level of 151.62 pg/mL. This could be attributed to the varying immune responses of each mouse. Each animal individual tries to produce a variety of antibodies that generate different immune responses, which can obscure research results (Darwin et al., 2021; Suswati et al., 2022). The average values of both groups' observations indicate a higher IL-4 level in the antigen group. This is because the antigen group involves the administration of *K. pneumoniae* 65.5 kDa pilus antigen and adjuvant. Adjuvants are additional substances in vaccines that maximize the antibody response to the given antigen (Fan et al., 2022). According to Suswati et al., (2022), a group receiving only adjuvants is necessary as a control to assess the effectiveness of the pilus protein in triggering an immune response. The results of this study are consistent with those of Suswati et al., (2022), who found a higher level of IL-10 as an anti-inflammatory cytokine in the antigen group that received the 65.5 kDa *K. pneumoniae* pilus protein compared to the adjuvant group.

The analysis of the study found no significant difference, leading to the conclusion that the 65.5 kDa *K. pneumoniae* pilus protein is a weak immunogen, particularly in triggering the production of IL-10 by Th2 cells. The comparison of antigen and adjuvant groups in this study yielded similar results to those of Suswati et al., (2022). Therefore, it can be concluded that the 65.5 kDa pilus protein will produce a more maximal immune response, particularly in the production of IL-4 when Freund's Adjuvant is added. This is supported by the increased IL-4 levels in the adjuvant group, even without antigen administration during treatment (Suswati et al., 2022).

This study also has limitations, specifically the inability to measure IL-4 levels before treatment. As a result, researchers are unable to determine the condition of the mice other than through physical examination.

CONCLUSION

Based on the results of this study, it can be concluded that there is a significant increase in IL-4 levels in the spleen of BALB/c mice after immunization with the 65.5 kDa pili protein of *Klebsiella pneumoniae*. These results represent the initial stage in the development of a *Klebsiella pneumoniae* vaccine, and further research is required. To enhance future research, it is recommended to include serum specimens to provide additional information regarding the impact of *Klebsiella pneumoniae* pili protein 65.5 kDa on stimulating IL-4 production.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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