ORIGINAL ARTICLE

Impact of Inducing 65.5 kDa Pili Protein from *Klebsiella pneumoniae* on IL-5 Concentrations in BALB/C Mice

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ABSTRACT

Introduction: *Klebsiella pneumoniae* is an opportunistic pathogen that cause nosocomial infections in individuals with high risk factors and low immune systems. Treatment of this bacterial infection received special attention because clinical isolates of Multidrug Resistant-*Klebsiella pneumoniae* (MDR-KP) were found. Several studies have shown the potential of the *Klebsiella pneumoniae* pili protein as a vaccine candidate which has the thickest band, namely a molecular weight of 65.5 kDa. Interleukin 5 (IL-5) is a potent pro-inflammatory cytokine that functions in the maturation, proliferation, activation, and migration of eosinophils, which play a role in inflammation during vaccine. This study aims to determine the effect of induction of the 65.5 kDa *Klebsiella pneumoniae* pili protein on the Interleukin 5 immune response. **Methods:** This true experimental study used a randomized posttest-only controlled group design and was conducted from May to September 2023 at the Faculty of Medicine, Jember University. Mice were divided into three groups: K1, induced with phosphate-buffered saline (PBS); K2, induced with Freund's adjuvant and PBS; and K3, induced with 50 µg of 65.5 kDa K. pneumoniae pili protein mixed with Freund's adjuvant. Injections were administered intraperitoneally on days 0, 14, and 28. Blood samples were collected by cardiac puncture, and IL-5 levels were measured using ELISA. **Results:** Mean IL-5 levels were 8.913 ± 1.930 pg/mL, 7.881 ± 1.905 pg/mL, and 8.389 ± 1.289 pg/mL in K1, K2, and K3, respectively. One-way ANOVA showed no significant difference between groups (p = 0.499). **Conclusion:** Induction with the 65.5 kDa K. pneumoniae pili protein did not significantly affect IL-5 immune response.
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INTRODUCTION

Klebsiella pneumoniae is known as one of the opportunistic pathogens that can cause nosocomial infections in individuals with high risk factors and individuals with weakened immune systems (1,2). The treatment of *Klebsiella pneumoniae* bacterial infections is of particular concern because clinical isolates are found in the form of Multidrug Resistant-*Klebsiella pneumoniae* (MDR-KP) which can increase morbidity and mortality from 22% to 72%. In addition, some MDR-KP isolates have evolved to be Extensively Drug-Resistant (XDR), making treatment options in cases of these infections more limited (3–7). Therefore, there is an urgent urgency for alternative treatments that can be used to prevent and treat infections caused by these bacteria.

Along with the development of science, many principles of host immunity have been developed that can be an alternative treatment due to *Klebsiella pneumoniae* infection, one of which is vaccination. One of the vaccine candidates that has received considerable attention is the *Klebsiella pneumoniae* (8). In previous studies, proteins with the thickest band have been isolated, namely with a molecular weight of 65.5 kDa (9–11). The *Klebsiella pneumoniae* antigen protein that enters the body can activate the innate immune system and the adaptive immune system. One of these adaptive immune responses is Interleukin 5 (IL-5) (12).

Interleukin 5 can help in growth factors and B cell differentiation to produce specific antibodies to fight incoming antigens (12–14). In study Herzt (2001),

vaccination against IL-5 resulted in protection against eosinophilic infiltration, the same principal biological effect observed for commercial anti IL-5 antibody therapies. the coupling of IL-5 to VLPs derived from the bacteriophage Qβ readily induced strong and neutralizing antibody responses in mice in the absence of an adjuvant. To determine its immunogenicity, in this study, the 65.5 kDa pili protein *Klebsiella pneumoniae* in BALB/C strain mice was induced to IL-5 levels as one of the cytokines in cases of *Klebsiella pneumoniae* infection. The immunogenic, 65.5 kDa K. pneumoniae pili protein, can be considered as an antigen for vaccine candidates. Previously, it was necessary to conduct research aimed at determining the immune response formed in the form of IL-5 levels in the induction of the 65.5 kDa pili protein *Klebsiella pneumoniae*.

MATERIALS AND METHODS

The following study is a purely experimental study with a randomized posttest only controlled group design through an ELISA test to measure IL-5 levels. This research was carried out with ethical approval by the Ethics Commission of the Faculty of Medicine, University of Jember with number 4962/UN25.1.10.2/KE/2023 which was carried out in the month of May – September 2023. The 65.5 kDa pili protein of *Klebsiella pneumoniae* was obtained by the SDS-PAGE method followed by protein purification.

The sample used was mouse blood serum in the white mouse population of the BALB/C strain. The samples used were 27 male white mice of the BALB/C strain aged 6-8 weeks with a weight of ±25 grams which were randomized into 3 treatment groups, namely the control group induced with PBS, the adjuvant group given Freund's adjuvant and PBS, and the 65.5 kDa pili protein antigen group *Klebsiella pneumoniae* diluted with PBS and then mixed with Freund's adjuvant. After the results of the ELISA reader came out, the serum IL-5 concentration data of the mice blood was carried out by the Shapiro Wilk normality test and the Levene Test homogeneity test which aimed to see whether the distribution of distributed data was normal or not and whether the population variants were homogeneous or not. The IL-5 concentration data was analyzed by parametric test using the One-Way ANOVA test to determine whether the induction of 65.5 kDa pili protein could increase IL-5 levels.

RESULTS

The measurement of IL-5 levels uses the ELISA method with an ELISA reader set with a wavelength of 450 nm. The results of the absorbance obtained are converted in the linear equation y = bx + a so that the equation y = 0.0089x + 0.039 is found (Fig. 1).

Table I is the average of IL-5 level data after the

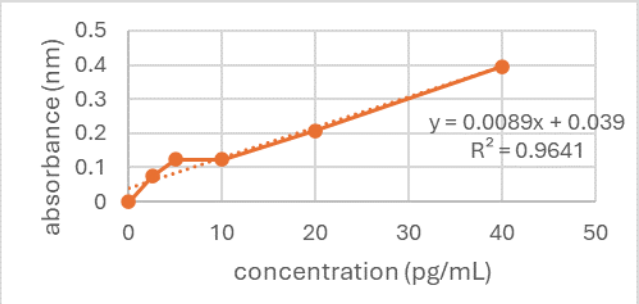

Figure 1: ELISA Standard Curve for Interleukin-5 (IL-5) Measurement

Table 1: IL-5 measurement results from serum blood of mice

Group	Sample (n)	Concentration IL-5 (Mean±SD)
K1	9	8.913±1.930
K2	7	7.881±1.905
K3	9	8.389±1.289
Total	25	8.436±1.697

absorbance value of each treatment group is included in the equation y = 0.0089x + 0.039. Based on the average results of IL-5 concentration measurements, the highest concentration value was obtained in group 1 with a value of 8.913±1.930 pg/mL.

Statistical analysis of IL-5 level results begins with a normality test with the Shapiro-Wilk Test carried out to determine whether the distribution of the data is normal or not. The results of the Shapiro-Wilk Test normality test showed that all data were distributed normally because they had a value of more than 0.05 (p>0.05) (Table II).

Table II: Results of the Normality Test with the Shapiro Wilk Test

Group	Sample (n)	*p-value*
K1	9	0,502
K2	7	0,347
K3	9	0,682

The homogenecity test with the Levene Test was carried out to find out whether the data was homogeneous or not. The results of the Levene Test homogeneity test with a p-value of 1.84 show that all data are homogeneous because it has a p-value value in all categories of more than 0.05 (p>0.05). Based on the normality test and homogeneity test, the data of this study met the requirements of parametric data with normal data distribution and homogeneous data variation. The results of the measurement of IL-5 levels met the requirements of parametric data, so the analysis was continued using the One-Way ANOVA test. The use of the One-Way ANOVA test is used as a parametric test because the variable scale of this study consists of ratio data. The results of the analysis with the One-Way ANOVA test showed that the p value between the treatment groups was 0.499 (p>0.05) which means that the data of this study did not have a significant difference.

DISCUSSION

In this study, the concentration of 65.5 kDa *Klebsiella pneumoniae* pili protein used was 0.64 gr/dl which was measured using the Kingsley method. The concentration of the pili protein was injected into mice as much as 50 µg per mouse to determine the immune response to Interleukin 5 (IL-5) levels. Interleukin 5 is one of the cytokines synthesized and secreted by Th2 cells that functions to help differentiate B cells to form antibodies (15). The results of the One-Way ANOVA test showed that there was no significant difference in IL-5 levels between the treatment groups (p-value 0.499). This shows that the injection of 65.5 kDa pili protein *Klebsiella pneumoniae* cannot induce IL-5 levels in the serum blood of mice. Based on the average results of IL-5 concentration measurement, the highest concentration value was obtained in group 1 with a value of 8.913±1.930 pg/mL, then the concentration value in group 3 was 8.389±1.289 pg/mL, and group 2 was 7.881±1,905 pg/mL was decreasing. This study shows that group 1 as the control group (K1) is higher than the adjuvant group (K2) and antigen group (K3). This is contrary to the research hypothesis that expects IL-5 levels in the antigen group (K3) to be higher than in the control group (K1) and adjuvant group (K2).

The results in this study showed insignificant differences that could be caused by several factors such as the speed of the host's immune response in killing pathogens, other immune responses, injection doses, adjuvant use and molecular weight. A good immune response will be faster and more responsive in radiating bacteria (16–18). Termination of experimental animals carried out 2 weeks after the third injection, when the immune response of mice is good, can cause IL-5 levels to be low before entering the termination time because the cytokines produced by the body are enough to fight antigens, eradicate bacteria and to prevent damage to host tissues (19). This study is in line with a study conducted by Widiatmaja in 2021, the average results of the control group were higher than other treatment groups because they were influenced by the speed of the host's immune response (15). In a 2025 study by Harry et al., increasing plasma levels of IFN-γ, IL-17A, and IL-15 were measured from Day 0 to Day 7 post-vaccination. The results showed that increases in IFN-γ, IL-17A, and IL-15 starting on Day 7 were associated with an effective serological response to vaccination. Meanwhile, another study by Jeffery et al. in 2010 measured serum cytokine levels before vaccination and every other day afterward for two weeks. They found that serum levels of several cytokines, particularly those involving the TNF-α, IL-5, IL-6, G-CSF, and IFN-γ signaling pathways, were increased after primary vaccination. This is indicative of the activation of cell-mediated immunity. NK and T cell-associated cytokine levels post-vaccination are associated with a serological response to vaccination. Given that these findings were based on changes in plasma cytokines at Day 7 post-vaccination, this suggests that IL-5 can begin to be measured as early as the second week post-vaccination (20,21).

Th1 cell host immune response factors can be one of the factors that cause IL-5 levels to be insignificant in each treatment group. Pneumonia caused by bacteria and viruses is more affected by Th1 cells by producing IFN-γ, while Th2 cells are more specific towards the etiology of parasites and chronic bacterial infections (16,18,22). Based on research related to *Klebsiella pneumoniae* derived extracellular vesicles vaccination, it has been shown that the immune response of Th1 cells is higher to produce IFN-γ which aims to prevent tissue damage by suppressing the Th2 response in Peripheral Blood Mononuclear Cells (PBMCs) (17,23). In addition, IFN-γ can selectively suppress GATA3 by targeting the expression of the GATA3 alternative upstream exon (1A) gene which can destabilize Th2 cells by suppressing IL-4 secretion, IL-5 and IL-13 (23). With some of these host immune response mechanisms, it can affect the total IL-5 levels in this study.

The injection dosage factor can be one of the factors that cause the incompatibility between the theory and the results of this study. The use of the 65.5 kDa pili protein antigen dose was 50 µg and Freund's Adjuvant used was 100 µl. Based on the standard of intraperitoneal immunization in mice, the usable antigen dose is 50-100 µg in 200 µl of PBS, the usable volume of CFA is <200 µl and the usable volume of IFA is a maximum of 300 µl (24–28) The antigen and adjuvant doses used in this study could not significantly induce IL-5 levels. This can be due to the influence of the molecular weight of IL-5 tends to be higher, requiring higher stimulation as well (10). The dose of pili protein used was 50 g in each mouse, this is in accordance with a similar study in the form of measuring IL-4 levels in the Spleen of BALB/c Mice after 65.5 kDa Pili Protein *Klebsiella pneumoniae* Immunization. In the study used a dose of 50 g pili with the same treatment of the three groups as the following study. Where the results of a similar study showed significant results in increasing IL-4 in the Spleen of BALB/c Mice after 65.5 kDa Pili Protein *Klebsiella pneumoniae* Immunization (29).

The adjuvant selection factor is also one of the factors that cause the inconsistency between the theory and the results of this study. Adjuvant is a substance that is injected together with antigens that are intended to increase the humoral and/or cellular immune response to antigens so that the formation of a symptomatic immune response is intense, long-lasting, and rapid (30). The adjuvant used in this study is Freund's Adjuvant which consists of Complete Freund's Adjuvant (CFA) and Incomplete Freund's Adjuvant (IFA). Freund's Adjuvant is an adjuvant with a mineral oil emulsion that functions to induce a longer antibody response by forming oily

antigen deposits so that there is a possibility of antigen release occurring little by little (28,30) This mechanism can be a factor in the low IL-5 in group 3 when compared to group 1 because there is not enough concentration of antigens to form an immune response that is released so that IL-5 is also formed to be longer. This research aligns with research by Widiatmaja et al., which showed that the average results of the control group were higher than those of the other groups. This could be due to several factors, such as the different immune responses of each experimental animal. Each individual's immune response to a vaccine can be influenced by several factors, including the immunization route (oral or injection), antigen form (live, killed, soluble, peptide subunit, and specific), dose, host, pathogen genetics, and pre-existing immunity (11).

There have been no studies that specifically address the effect of sensitivity of IL-5 cytokine levels that are practically like IL-4. However, some studies have linked IL-4 to other cytokines in the same region, namely region 31. In the study conducted by Junttila by observing the sensitivity of IL-4 and IL-13 where IL-13 is like IL-4 but has a higher molecular weight. Juntilla's research formed five treatment groups, namely concentrations of 0.1 ng/ml, 1 ng/ml, 10 ng/ml, 100 ng/ml, and 1000 ng/ml, the results showed that macrophages were able to detect IL-4 at a concentration of 0.1 ng/ml and to activate STAT6 phosphorylation required a concentration of IL-4 of 1 ng/ml. Meanwhile, IL-13 requires a concentration of 10 ng/ml to be detected and requires a concentration of 100 ng/ml to activate the phosphorylation of STAT6 (12,29,31). This shows that a higher molecular weight can cause the required sensitivity to be higher. In this study, it is likely to have almost the same mechanism as the study conducted by Junttila where IL-5 has a higher molecular weight.

Based on research on Eosinophil Differentiation Factor (EDF) conducted by Mecalf in 1983, this interleukin is similar to IL-4 with a higher molecular weight. These molecules were named T Cell-Replacing Factor (TRF), B Cell Growth Factor II (BCFGII), and B Cell Differentiation Factor μ (BCDFμ), as these molecules can stimulate antibodies. TRF/BCGFII/BCDFμ was later renamed Interleukin 5 (IL-5). In humans, IL-5 has the ability to form antibodies, activate eosinophils to kill antibody-coated target cells, enhance opsonization, and produce Reactive Oxygen Species (ROS) (12).

T helper 2 (Th2) cells are the primary producers of IL-5, but this cytokine is also the initial factor released by ILC2. Innate Lymphoid Cells 2 (ILC2) are activated by IL-25 secretion in the lamina propria, which then secretes IL-13 and IL-5. Together with these cytokines, the T-helper 2 response undergoes clonal expansion and differentiation and reaches the tissue. Consequently, Th2 cells continue to produce IL-5. This interleukin is also produced by mast cells during inflammation in infected tissue (12). Interleukin 5 can stimulate B cells to induce antibody secretion and class switching. Another name for this interleukin is IgA Augmenting Factor, as research conducted by Takatsu in 2009 demonstrated that IgA was first identified in mice induced by IL-5 culture in B cells (17)

The acquired immune response is involved in the late stages of infection and the formation of immunological memory, mediated by a series of interactions between T cells, B cells, and Antigen Presenting Cells (APCs). T cell activation by antigenic peptides through specific T cell receptors (TCRs) enhances phagocytosis, induces the release of cytokines and chemokines that induce B cells and T cells, and induces humoral and cellular release. A factor that induces terminal differentiation of B cells into Antibody-Secreting Cells (ASCs) is T-cell Replacing Factor (TRF) or what we know as IL-5.

The body's immune response to *Klebsiella pneumoniae* is more influenced by Th1 cells by producing IFN-γ and Th17 cells, while Th2 cells are more specific towards the etiology of parasites and chronic infections. A study conducted by Darmadi in 2015 that reviewed IL-5 levels in children and adults infected with Ascaris Lumbricoides cross-sectionally involved the blood serum of 16 children (acute infection) and 16 adults (chronic infection). The results of this study showed that IL-5 levels were not significant in the blood serum of children and adults. In general, worm infections can trigger Th2 by expressing IL-4 and IL-5. The infection causes a modified Th2 response mechanism where the formed Th2 cells will inhibit the expression of IL-5 so that eosinophil cells are not formed. In chronic infections, there is the involvement of T regulation cells that produce IL-10 and TGF-β which play a role in suppressing Th1 and Th2 responses. Based on Darmadi's research, the Th2-specific immune response in the form of IL-5 levels associated with the etiology of parasitic infection can be insignificant due to the influence of the Modified Th2 response so that IL-5 in the etiology of *Klebsiella pneumoniae* bacteria can also not increase significantly. Study limitation study limitation as a comparison of IL-5 performance is explained in the study conducted by Widiatmaja in 2021 that discussed IL-4 levels immunized with RrgB 255-270 Streptococcus pneumoniae Protein by forming three treatment groups, namely the PBS group, the adjuvant group and the epitope group of RrgB 255-270 Streptococcus pneumoniae Protein showed insignificant IL-4 levels. This insignificant IL-4 level is caused by the level of mucosal antibodies, namely IgA produced quite high by the body. High enough IgA levels will affect the balance of cytokine levels in the body. This makes the body will do down regulation in the production or secretion of IL-4 so that the level of IL-4 in the body returns to a normal state. In the research that has been conducted, it may have almost the same mechanism as the study conducted by Widiatmaja where IL-4 does not rise significantly due to other immune response effects (15).

Suggestions for further research include using multiple doses to compare the potential effects of the 65.5 kDa pili protein antigen on IL-5. Other parameters, such as GM-CSF and IL-13, can be measured as indicators of the successful induction of the 65.5 kDa pili protein from *Klebsiella pneumoniae* as a vaccine candidate.

CONCLUSION

This study can conclude that there is no significant effect inducting pili protein 65.5 kDa *Klebsiella pneumoniae* to IL-5 in mice. However, more thorough follow-up studies with larger and more homogenous samples are needed to confirm whether the effectiveness and safety of pneumoniae vaccines can be applied to the entire population.

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