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Effect of Immunization of the Pili Protein 65.5 kDa *Klebsiella pneumoniae* on IFN-γ Levels of Spleen BALB/c Mice

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Abstract

Klebsiella pneumoniae is a Gram-negative bacterium that poses a threat to the global community. Currently, no vaccine for *K. pneumoniae* is licensed by the Food and Drug Administration (FDA). The delay in the manufacture of the *K. pneumoniae* vaccine was because many vaccine candidates failed at the clinical trial stage due to adverse cross-reactions. Pili can be used as a choice as a vaccine candidate. Pili *K. pneumoniae* is an immunogenic substance that triggers an immune response, one of which is the cytokine IFN-γ. Splenic splenocytes are the main source of IFN-γ-producing cells. The aim of this study is to determine the effect of immunization pili protein 65.5 kDa *K. pneumoniae* on IFN-γ levels from spleen BALB/c mice. There were 3 groups, K1 as control given PBS, K2 given pili protein 65.5 kDa + adjuvant, and K3 given adjuvant. IFN-γ was then measured by the ELISA method and analyzed by the ANOVA test. The results of measuring IFN-γ levels using one-way ANOVA showed that the total for all groups was 243.50 ± 43.7 with $p < 0.05$, the Post Hoc LSD test was continued. The Post Hoc test showed significant differences between K1 control and K2 groups, and between K1 and K3 groups, but not between K2 and K3 groups. It can be concluded that immunization with 65.5 kDa of pili protein does not affect the increase in IFN-γ levels in the spleen of BALB/c mice.

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INTRODUCTION

Klebsiella pneumoniae is a Gram-negative bacterium that is a threat to the global community. The rate of resistance to ciprofloxacin, a commonly used antibiotic, increased from 4.1% to 79.4%.[1] Increased isolation multi-drug resistant (MDR) *K. pneumoniae* narrowed the options for K. *pneumoniae* infections treatment.[2] According to data from Perhimpunan Dokter Paru Indonesia (PDPI), the most frequent cause of community pneumonia was *K. pneumoniae* (29%), followed by *Staphylococcus aureus* (16%) and *Streptococcus pneumoniae* (12%).[3] Riset Kesehatan Dasar (Riskesdas)[4] shows an increase in the prevalence of pneumonia caused by *K. pneumoniae* in Indonesia, increasing from 4.5% in 2013 to 5.0% in 2018. East Java has a high prevalence of 4.4%.

Currently, there is no vaccine approved by the FDA (Food and Drug Administration) against *K. pneumoniae* infections. The delay in the production of the *K. pneumoniae* vaccine was because many vaccine candidates failed at the clinical trial stage due to adverse cross-reactions. However, pili virulence factors have low antigenic variation and low cross-reactivity, making them suitable as a vaccine candidate.[5,6] Pili play a role in the attachment of bacteria to the surface of the human mucosa or epithelium. The advantage of pili is that the structure easily triggers the formation of antibodies compared to other virulence factors.[5,7]

Pili proteins *K. pneumoniae* from previous studies were known to have varying molecular weights. In research, Agustina et al. (2021)[8] tested the IgG response with Western Blot (WB) showing that the proteins that appeared were 85.6, 65.5, 46.9, and 29.4 kDa. Pili protein 65.5 kDa is band thickest. Pili *K. pneumoniae* is immunogenic and triggers immune responses, such as the production of cytokines, one of which is IFN-γ which is produced by T-helper (Th1) cells, natural killer (NK) cells, NKT, and CD8+ T cells.[9] Significant IFN-γ production occurs in the lungs, liver, and spleen after infection with *K. pneumoniae*. Splenic splenocytes are the main source of IFN-γ-producing cells.[10] IFN-γ will activate macrophages and B lymphocyte cells. B lymphocyte cells will stimulate the production of immunoglobulin G (IgG), which can prevent infection with *K. pneumoniae*.[11] Based on the above background, researchers are interested in testing the ability of the 65.5 kDa *K. pneumoniae* in inducing IFN-γ cytokines in the spleen of mice.

MATERIALS AND METHODS

Identification of *Klebsiella pneumoniae* Pili Protein Molecular Weight

The Laemmli research method is used to identify the pili protein weight using Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE).[12] The gel components used are separating 12.5% and stacking 4%. Samples were run for 10 wells with nine wells containing samples and one well-containing protein marker of 45 µl, and buffer samples in the same volume. The color used was bromophenol blue. After the sample was added sample buffer, it was heated to 95°C for four minutes, and then it was ready to be inserted into each well after the gel was submerged in buffer. The voltage used was 120 mV, the current 400 mA, and run for 60 minutes. SDS-PAGE results were determined using a linear equation according to protein markers using MS Excel curve fitting so that the molecular weight of the pili protein can be read.

***Klebsiella pneumoniae* Pili Protein Purification**

Weight of the protein that forms the band resulting from electrophoresis was cut according to the required weight. The results of the cut bands sheet nitrocellulose were then mixed with sterile PBS for electroelution in an electroelution chamber filled with buffer. The nitrocellulose sheet was clamped tightly on both sides until the two sides were tight. The power supply was set to 125 mV, 0.3 A for 120 minutes. A beaker glass containing 1 liter of sterile PBS with a pH of 7.4 was prepared for dialysis of the electroeluted sample. The magnetic stirrer was inserted into the glass beaker, ensuring that the magnetic stirrer continued to rotate after being put in refrigerator for 2 x 24 hours. Every 24 hours sterile PBS was replaced with new sterile PBS. Dialyzed target protein samples were taken and stored in a refrigerator at -85°C. Protein content of purified proteins was determined by the Kingsley method.[8]

Mice Acclimatization

Acclimatization was carried out by keeping mice for seven days at the Experimental Animal Laboratory, Faculty of Medicine, Jember University. After seven days, the mice were randomized before being immunized according to the treatment group.

Immunization of Mice with Pili Protein 65.5 kDa

Immunization of white male mice with BALB/c strain aged 6-8 weeks was administered intraperitoneally three times with an interval of 14 days. There were three treatment groups, K1 (control) was given 0.2 ml of sterile PBS, K2 was given 50 µg of pili protein and Freund's adjuvant with the same volume as the diluted antigen volume, which was 0.1 ml, and K3 was given of Freund's adjuvant and 0.1 ml of PBS. In priming, Complete Freund's Adjuvant (CFA) was used, while Incomplete Freund's Adjuvant was used for booster.[13,14]

Termination of Mice

Fourteen days after the third immunization, the mice were terminated with cotton soaked in ether. An incision was made in the abdomen of the mice, then the spleen was separated and taken. Spleen organs were taken, washed with PBS pH 7.4, and then weighed. After that, the organs were chopped using a mortar and stamper on ice and then homogenized with PBS. In the final step, the organ was centrifuged at 2000-3000 RPM for 20 minutes.

Measurement of IFN-γ using the ELISA Method

Measurement of IFN-γ method Sandwich ELISA kit Bioassay Technology Laboratory)® No. E0056Mo from Shanghai Korain Biotech Co Ltd, China.

Statistical analysis

A one-way ANOVA test was used to assess IFN-γ in each group in order to identify the differences in each sample group. Final data were processed using IBM SPSS Statistics 24.

RESULTS AND DISCUSSION

Identification and Isolation of Pili Protein 65.5 kDa *K. pneumoniae*

K. pneumoniae's SDS-PAGE results revealed that there was a band with a molecular weight of 65.5 kDa. The band with a molecular weight of 65.5 kDa was thicker, so this band was isolated to be immunized in experimental animals. The results of protein purification were then measured to see the concentration and the protein yield reached 0.65 g/dl.

The protein identification results can be seen in Figure 1.

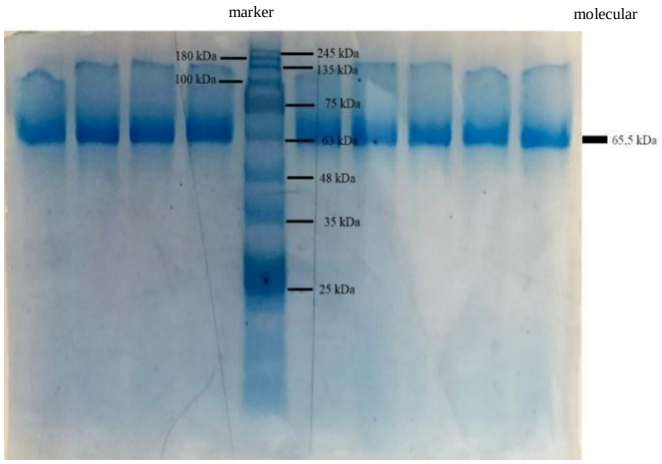

Figure 1. The results of SDS-PAGE protein pili *K. pneumoniae.* The band with a molecular weight of 65.5 kDa is thicker.

IFN-γ

The results of measuring IFN-γ cytokine levels using ELISA showed the highest average IFN-γ levels in the adjuvant group, followed by the adjuvant+antigen group, and the control group showed the lowest average IFN-γ levels. The results of the measurement of IFN-γ cytokine levels are presented in Table 1. The normality test was used in the study using the Shapiro-Wilk test. In the test results, it was found that the data had a normal distribution with all groups having a significance value of $p > 0.05$.

Table 1. Results of Measuring Levels of IFN-γ

	N	IFN-γ (*Mean* ± *SD*)
K1 (Control)	7	208.16 ± 24.69
K2 (Antigen + Adjuvant)	7	252.07 ± 32.36
K3 (Adjuvant)	7	270.27 ± 48.29
Total	21	243.50 ± 43.71

Results of Data Analysis

One-way ANOVA was used to assess IFN-γ levels, as shown in Table 2. The one-way ANOVA analysis of variance yielded $p < 0.05$ as its results. Between the groups in this study, there were notable variances or significantly different.

Table 2. Comparative Test Results One-way ANOVA

	Sum of Squares	Df	Mean Square	F	Sig.
Between Groups	14272.708	2	7136.354	5.367	.015
Within Groups	23932.410	18	1329.578		
Total	38205.118	20			

One-way ANOVA revealed significant differences between the group means, and Post Hoc LSD tests were continued as shown in Table 3. Post Hoc LSD tests revealed significant differences between control and antigen+adjuvant groups ($p = 0.037$), and between control and adjuvant groups ($p = 0.005$), but not between the antigen+adjuvant group and adjuvant group ($p = 0.363$). The significant difference can be seen from the asterisk (*) behind the number in the mean difference.

IFN-γ levels were significantly different ($p < 0.05$) between the groups, according to the results of the statistical test using one-way ANOVA. This shows that the administration of antigen+adjuvant or adjuvant treatment alone can increase IFN-γ levels. Similar to the study of Ramsugit et al. (2016)[15] using pili protein *Mycobacterium tuberculosis*, IFN-γ levels differed significantly with a one-way ANOVA showing $p<0.05$. Post Hoc LSD analysis showed that IFN-γ levels in control and antigen + adjuvant groups were significantly different ($p < 0.05$). This was

Table 3. Post Hoc LSD Test Results

Groups	Groups	Mean Difference	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Control	Adjuvant + Antigen	-43.911565*	19.490498	.037	-84.85958	-2.96355
	Adjuvant	-62.108844*	19.490498	.005	-103.05686	-21.16083
Adjuvant + Antigen	Control	43.911565*	19.490498	.037	2.96355	84.85958
	Adjuvant	-18.197279	19.490498	.363	-59.14530	22.75074
Adjuvant	Control	62.108844*	19.490498	.005	21.16083	103.05686
	Adjuvant + Antigen	18.197279	19.490498	.363	-22.75074	59.14530

* The mean difference is significant at the 0.05 level.

in line with the research of Udin et al. (2014)[16] which showed significantly higher IFN-γ secretion in the group injected with *M. tuberculosis* protein compared to the control group with $p < 0.05$. According to the theory, pili protein can trigger immune responses, one of which is IFN-γ. When the host is injected with *K. pneumoniae* pili protein, the host cell will elicit an immune response in the form of T-helper. T lymphocytes will activate inflammatory cytokines, one of which is IFN-γ.[7,17] When pili antigen is combined with Freund's adjuvant, it will stimulate the immune system more strongly. These adjuvants contain immunoreactive molecules that induce IFN-γ.[17,18] This indicates that in this study there was an effect of the antigen+adjuvant administration on increasing IFN-γ levels compared to PBS alone in the control group.

IFN-γ levels between control and adjuvant groups were significantly different ($p < 0.05$). These results follow a study conducted by Udin et al. (2014)[16] which the adjuvant-only injected group had significantly higher IFN-γ secretion in the group than the control group, $p = 0.000$ ($p < 0.05$). The increase in IFN-γ could be due to the choice of adjuvant used in this study. Freund's adjuvant consists of N-acetylmuramil-l-alanyl-d-isoglutamine (muramyl dipeptide) and trehalose 6,6'-dimycolate, which are immunoreactive molecules that can induce IFN-γ thereby polarizing T-helper (Th1) cells.[19] Freund's adjuvant has several disadvantages, namely that it can cause granulomas, inflammation, and lesions.[20] Lesions may lead to inflammation, and it can boost the release of cytokines, such as IFN-γ.[21] This shows that the adjuvant group can affect increasing levels of IFN-γ compared to the control group.

IFN-γ levels between the antigen+adjuvant group and the adjuvant group showed no significant difference ($p > 0.05$). This shows that pili protein immunization does not affect increasing IFN-γ levels in the spleen. These results follow a study conducted by Ayu.[22] The results showed that even in livers injected with pili 65.5 kDa, IFN-γ levels did not significantly differ between the adjuvant and antigen+adjuvant groups, $p = 0.511$ ($p > 0.05$). *K. pneumoniae.* The absence of a significant difference in IFN-γ levels could be due to the sample being measured in this study being tissue, not blood circulation. IFN-γ is produced by T lymphocytes (Th1) cells produced by lymphoid organs and then flows through the blood circulation and empties into the organs that have dendritic cells, one of which is the spleen. Then it will migrate into the lymphatic tissue, then migrate back into the blood circulation.[11,23] IFN-γ which will migrate back to the blood circulation causes IFN-γ levels to be more

measured in the blood circulation. This is consistent with the findings of Martínez-Orellana et al. (2022)[24], who showed that the concentration of IFN-γ measured in blood serum increased after immunization of the Leishmania infant. This shows that the increase in IFN-γ levels when given *K. pneumoniae* will be more or significantly measurable in the circulation than in the tissue.

The absence of significant differences in IFN-γ levels between the antigen+adjuvant group and the adjuvant group could also be due to the type of adjuvant used in this study. Freund's adjuvant is an adjuvant based on mineral oil emulsions. Vaccines containing oil emulsion-based adjuvants such as Freund's adjuvant can cause the antigen release process to be slower, but the immune response formed can last longer in the body.[19,25] When the adjuvant is combined with the antigen, it can slow down the release of the antigen to induce IFN-γ, so that the level of IFN-γ measured is lower than the adjuvant group alone. This could be the reason that caused the level of IFN-γ in the antigen+adjuvant group (252.07 pg/ml) to be lower than the adjuvant group (270.27 pg/ml) in this study, due to the length of IFN-γ response formed in the antigen+adjuvant group.

STRENGTH AND LIMITATION

The strength of this study is that this *K. pneumoniae* pili protein is available as a vaccine candidate, which will be useful in the development of future vaccines for *K. pneumoniae* infections. The limitation of this research pertains to there are no groups for only antigen without adjuvant. For future research, it is hoped that there will be only antigen groups without adjuvant in research, so that maximum and unbiased results are obtained.

CONCLUSIONS

From the results of this study, it can be concluded that immunization with 65.5 kDa of pili protein does not affect the increase in IFN-γ levels in the spleen of BALB/c mice.

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ETHICAL CLEARANCE

Ethical approval letter (No. 1565/H25.1.11/KE/2022) was obtained from the Ethics Committee of the Faculty of Medicine of the Jember University for this study.

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CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

AUTHOR CONTRIBUTION

Writer, literature searcher, collecting data from literature : AS, concept and supervision : DA, review and supervision: DCM and PWP.

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