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The Effect of Pili Protein of *Klebsiella pneumoniae* 65,5 kDa on Enhanced IFN-Gamma Levels in Mice Liver

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Klebsiella pneumoniae develops antibiotic resistance by producing enzymes such as Extended-Spectrum Beta-Lactamase and Carbapenemase. Antibiotic resistance causes *K. pneumoniae* to have less antibiotic activity and have more virulence factors. Capsule polysaccharide, lipopolysaccharide, Outer Membrane Protein, siderophores, and pili are all virulence factors in K. pneumoniae. This study aims to demonstrate the possibility for a host immunological response to the pili protein *K. pneumoniae* 65.5 kDa by injecting it into mice and measuring the levels of IFN-gamma cytokines in the mice's liver. This study used mice liver samples taken from 21 mice aged 6-8 weeks in the experimental investigation with a randomized post-test only controlled group design. Phosphate buffer saline was given to K1, pili protein antigen 65.5 kDa + Freunds' adjuvant was given to K2, and Freunds' adjuvant was given to K3. IFN-gamma concentration was measured using the sandwich ELISA method. The average concentration of IFN-gamma in the mice liver in this study was 247.68±47.67 pg $m^{-1}L^{-1}$, 163.19±13.63 pg $m^{-1}L^{-1}$, and 182.41±41.70 pg $m^{-1}L^{-1}$. The p-value of the Welch ANOVA test was 0.005 ($p < 0.05$), hence the Post Hoc Games-Howell test was used. The Games-Howell test showed a statistically significant difference in the mean value of IFN-gamma in K1 compared to K2 and K3 of 0.007 and 0.046, respectively. There was no statistically significant difference between K2 and K3 with a p-value of 0.511. These findings revealed that intraperitoneal injection of *Klebsiella pneumoniae* pili protein 65.5 kDa did not increase IFN-gamma levels in the mice liver.

Key words: IFN-Gamma, *Klebsiella pneumoniae,* Liver, Pili Protein

Klebsiella pneumoniae mengembangkan resistensi antibiotik dengan memproduksi enzim seperti *Extended-Spectrum Beta-Lactamase* dan Carbapenemase. Resistensi antibiotik menyebabkan penurunan aktivitas antibiotik dan meningkatkan faktor virulensi *K. pneumoniae*. Kapsul polisakarida, lipopolisakarida, *Outer Membrane Protein*, *siderophores*, dan pili merupakan faktor virulensi *K. pneumoniae*. Penelitian ini bertujuan untuk menunjukkan kemungkinan respon imun *host* terhadap protein pili *K. pneumoniae* 65,5 kDa dengan menyuntikkannya ke mencit dan mengukur kadar sitokin IFN-gamma di hepar mencit. Penelitian ini menggunakan sampel hepar mencit yang diambil dari 21 ekor mencit berusia 6-8 minggu dalam penelitian eksperimental dengan rancangan *randomized post test only controlled grup*. K1 diberikan PBS, K2 diberi antigen protein pili 65,5 kDa + *Freunds's adjuvant*, K3 diberi *Freunds's adjuvant*. Konsentrasi IFN-gamma diukur menggunakan metode *sandwich* ELISA. Rata-rata konsentrasi IFN-gamma pada hepar mencit pada penelitian ini adalah 247,68±47,67 pg $m^{-1}L^{-1}$, 163,19±13,63 pg $m^{-1}L^{-1}$, dan 182,41±41,70 pg $m^{-1}L^{-1}$. Nilai p uji *Welch* ANOVA adalah 0,005 ($p < 0,05$), maka digunakan uji *Post Hoc Games-Howell*. Uji Games-Howell menunjukkan perbedaan yang signifikan secara statistik nilai rerata IFN-gamma pada K1 dibandingkan dengan K2 dan K3 masing-masing sebesar 0,007 dan 0,046. Tidak ada perbedaan yang signifikan secara statistik antara K2 dan K3 dengan *p-value* 0,511. Temuan ini menunjukkan bahwa injeksi intraperitoneal protein pili *Klebsiella pneumoniae* 65,5 kDa tidak meningkatkan kadar IFN-gamma pada hepar mencit.

Kata kunci: Hepar, IFN-Gamma, *Klebsiella pneumoniae*, Protein Pili

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Klebsiella pneumoniae is a Gram-negative bacterium that can be found on the mucosal surfaces of mammals, plants, water, and soil. Bacteremia, septicemia, urinary tract infections (UTIs), and pneumonia can all be caused by these bacteria. *K. pneumoniae* also contributes to the high prevalence of opportunistic infections in immunocompromised patients(Seifi *et al.* 2016). Apart from being the second most common cause of UTI, this bacterium possesses antibiotic-resistant strains in Indonesia whose numbers exceed in Japan, Europe, and America combined(Kitagawa *et al.* 2018)(Gharrah *et al.* 2017). Due to its high mortality rate, the pathogenic *K.*

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pneumoniae rapidly produces multidrug-resistant (MDR) strains that cause a major hazard to patients.

Previous research has demonstrated that the synthesis of enzymes like Extended-Spectrum Beta-Lactamase (ESBL) and Carbapenemase causes antibiotic resistance in *K. pneumoniae* (Munita and Arias, 2016). Antibiotic resistance causes *K. pneumoniae* virulence factors to rise and antibiotic action to decrease (Prestinaci *et al.* 2015). Virulence factors in *K. pneumoniae* play a function in biofilm development and defense against the host immune system (Li *et al.* 2014). Capsule polysaccharide, lipopolysaccharide (LPS), Outer Membrane Protein (OMP), siderophores, and pili are some of the virulence components found in *K. pneumoniae*. Pili have a role in bacterial conjugation and horizontal gene transfer, both of which are critical for the transmission of antibiotic resistance genes via plasmid transfer (Zheng *et al.* 2020). Furthermore, pili play a crucial function in the early stages of infection since they are required for the host's first colonization (Ares *et al.* 2016). Bacteria that successfully attach will be attacked initially by the innate immune system of the host (Wang *et al.* 2020). Pattern recognition receptors on immune cells recognize invasive microorganisms that enter the host (PRR). After being triggered by PRR, interferon regulatory factor (IRF) is activated, promoting the expression of type I interferons (Boxx and Cheng, 2016). Natural killer (NK) cells produce more IFN-gamma as a result of type I interferon produced by *K. pneumoniae* infection(Ivin *et al.* 2017). The purpose of this study was to see how exposure to the pili protein from *K. pneumoniae*, which has a molecular weight of 65.5 kDa, affected the increase in liver IFN-gamma levels in mice.

MATERIALS AND METHODS

Ethical Clearance. The Ethics Commission of the Faculty of Medicine, University of Jember, has approved this study with the number 1559/H25.1.11/KE/2021.

Preparation of Pili Protein. Pili protein antigen 65.5 kDa *Klebsiella pneumoniae* were obtained from the Laboratory of Microbiology, Faculty of Medicine, University of Jember. Mixing adjuvants with pili protein antigens that have been dissolved in PBS in a volume ratio of 1:1 to form a thick, white, and non-dispersed emulsion when dropped on a saline surface. Transfer the emulsion to the syringe.

Mice Induction. The mice were acclimatized for seven days before the treatment. The mice were randomized into treatment groups after 7 days before being inducted. PBS was administered to group 1, pili protein antigen 65.5 kDa + Freunds' adjuvant was administered to group 2, and Freunds' adjuvant was administered to group 3. Al Shoyaib *et al.(Al Shoyaib et al. 2020)(Care and Committee, 2017)* was conducted three intraperitoneal inductions at 14-day intervals. Each tail received 50 g of antigen, as well as the same volume of Freunds' adjuvant as the antigen diluted in PBS. CFA is used for priming, whilst IFA is utilized for the booster (Greenfield, 2020).

Sample Isolation. The mice were terminated with cotton soaked in ether fourteen days following the third induction. Then, using scissors, make an incision in the mouse's abdominal area until the peritoneum is exposed. Using tweezers, the liver is delicately removed. The liver organs were weighed and the weight was recorded after being cleaned with PBS. The organs were then minced and homogenized in PBS using an ice-cold glass homogenizer. For 20 minutes, spin at 2000-3000 RPM. The sample was placed in a falcon tube and kept at -80 °C in the fridge.

IFN-Gamma using Sandwich ELISA. Sample preparation and ELISA process were performed according to the ELISA kit E0056Mo from Bioassay Technology Laboratory®.

Statistical Analysis. The Shapiro Wilk normality test was used to assess the data because it was less than 50. Furthermore, statistical analysis was carried out using the ANOVA Welch comparison test to see if exposure to pili protein 65.5 kDa of *Klebsiella pneumoniae* had an influence on IFN-gamma levels in the liver of mice.

RESULTS

Pili protein antigen 65.5 kDa *Klebsiella pneumoniae* were obtained from the Laboratory of Microbiology, Faculty of Medicine, University of Jember. The results of the protein isolation are available in Figure 1. The results showed that the control group (K1) had the greatest mean IFN-gamma concentration, while the antigen + adjuvant group had the lowest mean IFN-gamma concentration (K2) are available in Table 1. The Post Hoc Games-Howell test was continued after the statistical analysis of the Welch ANOVA test revealed a significant difference in the mean concentration of IFN-gamma between groups. The Games-Howell test revealed a statistically significant difference between the mean IFN-gamma in the control group and the mean IFN-gamma in the

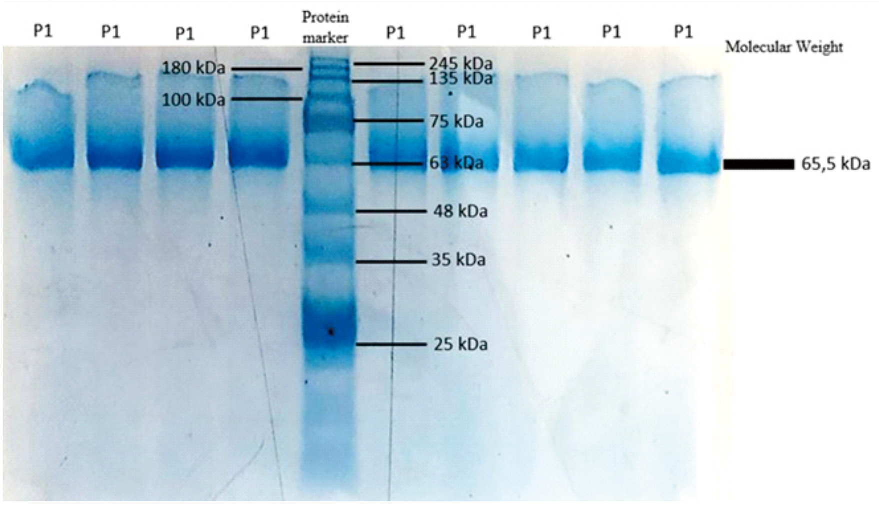

Fig 1 The isolation result of *Klebsiella pneumoniae* pili protein. Each well represent replication from P1 which means the protein from the first piece of the *Klebsiella pneumoniae* pili.

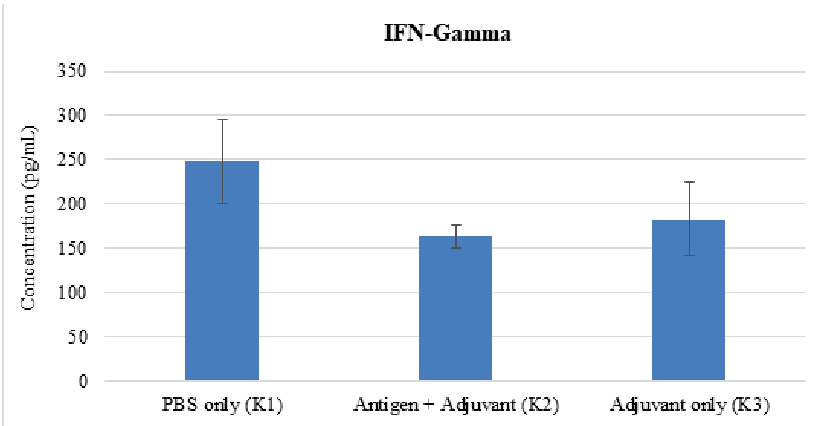

Fig 2 IFN-Gamma concentration from each experimental group. The results showed that the control group (K1) had the greatest mean IFN-gamma concentration (247.68 ± 47.67), while the antigen + adjuvant group (K2) had the lowest mean IFN-gamma concentration (163.19 ± 13.63).

other treatment groups, but not between the antigen + adjuvant group and the adjuvant group are available in Table 2 and Figure 2. These findings demonstrated that mice liver IFN-gamma levels were unaffected by exposure to *Klebsiella pneumoniae* pili protein 65.5 kDa.

DISCUSSION

The results of the mean IFN-gamma levels in Table 1 demonstrated that K1 as a control group has relatively higher mean IFN-gamma levels than K2 and K3. This is in contrast to the research hypothesis that group 2 antigen+ adjuvant had greater predicted IFN-gamma levels than groups 1 and 3. Several factors can influence the above results, one of which is the speed with which the host's immune system responds to viruses. Antigens in the body will be eliminated more quickly if the host immune response is strong. After seven days of acclimation, the mice were found to be in good health, with active activity and a decent appetite, indicating that the host's immunological response to antigens injected intraperitoneally into experimental animals had begun to work after the initial injection. After the third injection on day 28, the experimental animals were killed 14 days later. IFN-gamma levels were low from day 28 to day 42 before termination, indicating that the body is in good shape or that the cytokines produced by the body were sufficient to combat antigens in the body, perform bacterial cleansing, and prevent further damage to host tissues. This research is similar to that of Widiatmaja *et al.* who investigated the effect of intranasal RrgB protein epitope 255-270 *Streptococcus*

Table 1 IFN-Gamma concentration from each experimental group

Experimental Group	IFN-gamma concentration (pg m^{-1}L^{-1}) (Mean+SD)	95% CI	P-value
PBS only (K1)	247.68 ± 47.67	203.59 – 291.77	0.005
Antigen + Adjuvant (K2)	163.19 ± 13.63	150.59 – 175.79	
Adjuvant only (K3)	182.41 ± 41.70	143.84 – 220.97	

Table 2 Post Hoc Gomes-Howell test for IFN-Gamma results

Experimental Group	IFN-gamma concentration (pgm^{-1}L^{-1}) (Mean+SD)	p-value
PBS only (K1)	Antigen + Adjuvant (K2)	0.007
	Adjuvant only (K3)	0.046
Antigen + Adjuvant (K2)	PBS only (K1)	0.007
	Adjuvant only (K3)	0.511
Adjuvant only (K3)	PBS only (K1)	0.046
	Antigen + Adjuvant (K2)	0.511

pneumoniae immunization on IL-4 levels (Widiatmaja *et al.* 2021).

The findings of this study contrast with those of Lin *et al.* who found that the host response to K. pneumoniae invading the liver in diabetic and non-diabetic mice could boost IFN-gamma production 72 hours after injection or three days later (Lin *et al.* 2013). McNab *et al.* discovered that type I IFN signaling, which promotes the immunosuppressive cytokine Interleukin-10 (IL-10) during Mycobacterium tuberculosis infection in mice, suppresses macrophage production of proinflammatory cytokines, particularly Interleukin-12 (IL-12) (McNab *et al.* 2014). Another study conducted by Setiawan and Nugraha at the Karang Tembok Lung Hospital in Surabaya found that tuberculosis patients' peripheral blood mononuclear cells secreted more IL-10, which reduced the host immune response and limited tissue damage by inhibiting the production of proinflammatory cytokines like IFN-gamma Setiawan and Nugraha, 2016). Namakae *et al.* found that IL-10 inhibits the protective immune response against Plasmodium parasite infection in another comparable investigation (Nakamae *et al.* 2019).

The initial line of defense against invading microorganisms is the innate immune system. To infect humans*, Klebisiella pneumoniae* must first overcome mechanical and chemical barriers (Wang *et al.* 2020). Immune cells recognize invasive pathogens that enter the host through pattern recognition receptors (PRR). After PRR stimulation, the interferon regulatory factor (IRF) is activated (Boxx and Cheng, 2016). The transcription factor IFN regulatory factor (IRF) controls type I IFN expression. Type I interferon generated by K. pneumoniae infection causes NK cells to produce more IFN-gamma. IFN-gamma will then give macrophages feedback, causing them to produce more Interleukin-12 (IL-12) and destroy bacteria (Ivin *et al.* 2017). When proinflammatory cytokine production is suppressed, macrophages become insensitive to IFN-gamma feedback from Th1 cells and other IFN-gamma sources such as NK cells. Interferon-gamma plays a crucial function in infection protection, but if not managed properly, the immune response might cause further damage to host tissues. Interleukin-10 has the ability to protect the host from disease produced by an overactive immune response.

In conclusion, ased on the results of research on the effect of exposure to *Klebsiella pneumoniae* 65,5 kDa on enhanced IFN-gamma levels in mice liver, it can be concluded that immunization of *Klebsiella pneumoniae* pili protein 65.5 kDa intraperitoneally did not increase IFN-gamma levels in mice liver.

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REFERENCES

Ares, M. A. et al. 2016. H-NS nucleoid protein controls virulence features of *Klebsiella pneumoniae* by regulating the expression of type 3 pili and the capsule polysaccharide, Frontiers in Cellular and Infection Microbiology, 6(13), pp. 113. doi: 10.3389/fcimb.2016.00013.

Boxx, G. M. and Cheng, G. (2016) The Roles of Type i Interferon in Bacterial Infection, Cell Host and Microbe, 19(6), pp. 760769. doi: 10.1016/j.chom.2016.05.016.

Care, I. A. and Committee, U. (2017) Policy for Adjuvant Use with Special Emphasis on Complete Freunds Adjuvant, UNMC Animal Care and Use Program, pp. 15.

Gharrah, M. M., Mostafa El-Mahdy, A. and Barwa, R. F. (2017) Association between Virulence Factors and Extended Spectrum Beta-Lactamase Producing *Klebsiella pneumoniae* Compared to Nonproducing Isolates, Interdisciplinary Perspectives on Infectious Diseases, 2017, pp. 114. doi: 10.1155/2017/7279830.

Greenfield, E. A. (2020) Standard Immunization of Mice, Rats, and Hamsters, Cold Spring Harbor Protocols, 2020(3), pp. 8284. doi: 10.1101/pdb.prot100297.

Ivin, M. et al. (2017) Natural killer cell-intrinsic type I IFN signaling controls *Klebsiella pneumoniae* growth during lung infection, PLoS Pathogens, 13(11), pp. 129. doi: 10.1371/journal.ppat.1006696.

Kitagawa, K. et al. (2018) International comparison of causative bacteria and antimicrobial susceptibilities of urinary tract infections between Kobe, Japan, and Surabaya, Indonesia, Japanese Journal of Infectious Diseases, 71(1), pp. 813. doi: 10.7883/yoken.JJID.2017.233.

Li, B. et al. (2014) Molecular pathogenesis of *Klebsiella pneumoniae*, Future Microbiology, 9(9), pp. 10711081.

Lin, Y. C. et al. (2013) Activation of IFN-γ/STAT/IRF-1 in hepatic responses to *Klebsiella pneumoniae* infection, PLoS ONE, 8(11), pp. 112. doi: 10.1371/journal.pone.0079961.

McNab, F. W. et al. (2014) Type I IFN Induces IL-10 Production in an IL-27Independent Manner and Blocks Responsiveness to IFN-γ for Production of IL-12 and Bacterial Killing in *Mycobacterium tuberculosis* Infected Macrophages , The Journal of Immunology, 193(7), pp. 36003612. doi: 10.4049/jimmunol.1401088.

Munita, J. M. and Arias, C. A. (2016) Mechanisms of Antibiotic Resistance, Microbiology Spectrum, 4(2), pp. 124.

Nakamae, S. et al. (2019) Role of IL-10 in inhibiting protective immune responses against infection with heterologous Plasmodium parasites, Parasitology International, 70(January), pp. 515. doi: 10.1016/j.parint.2019.01.003.

Prestinaci, F., Pezzotti, P. and Pantosti, A. (2015) Antimicrobial resistance: A global multifaceted phenomenon, Pathogens and Global Health, 109(7), pp. 309318. doi: 10.1179/2047773215Y.0000000030.

Seifi, K. et al. (2016) Evaluation of biofilm formation among *Klebsiella pneumoniae* isolates and molecular characterization by ERIC-PCR, Jundishapur Journal of Microbiology, 9(1), pp. 16. doi: 10.5812/jjm.30682.

Setiawan, H. and Nugraha, J. (2016) Analisis Kadar IFN-γ dan IL-10 pada PBMC Penderita Tuberkulosis Aktif, Laten dan Orang Sehat, Setelah di Stimulasi dengan Antigen ESAT-6, Jurnal Biosains Pascasarjana, 18(1), p. 50. doi: 10.20473/jbp.v18i1.2016.50-63.

Al Shoyaib, A., Archie, S. R. and Karamyan, V. T. (2020) Intraperitoneal Route of Drug Administration: Should it Be Used in Experimental Animal Studies?, Pharmaceutical Research, 37(12), pp. 117. doi: 10.1007/s11095-019-2745-x.

Wang, G. et al. (2020) The characteristic of virulence, biofilm and antibiotic resistance of *Klebsiella pneumoniae*, International Journal of Environmental Research and Public Health, 17(6278), pp. 117. doi: 10.3390/ijerph17176278.

Widiatmaja, D. T., Mufida, D. C. and Febianti, Z. (2021) Pengaruh Pemberian Imunisasi Intranasal Epitope Protein RrgB 255-270 *Streptococcus pneumoniae* Terhadap Kadar IL-4, Sriwijaya Journal of Medicine, 4(1), pp. 6773. doi: 10.32539/sjm.v4i1.155.

Zheng, W. *et al.* (2020) 'Cryoelectron-Microscopic Structure of the pKpQIL Conjugative Pili from Carbapenem-Resistant *Klebsiella pneumoniae*', *Structure*, 28(12), pp. 1–8. doi: 10.1016/j.str.2020.08.010.