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Agustina, Dini; Jiwangga, Bella; Nurdian, Yudha; Suswati, Enny; Mufida, Diana Chusna; Shodikin, Mohammad Ali Less

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Original Article

Effect of Exposure Pili Protein 65.5 kDa *Klebsiella pneumoniae* on Interferon Gamma Levels

Dini Agustina[1], *Bella Jiwangga*[2], *Yudha Nurdian*[3], *Enny Suswati*[1], *Diana Chusna Mufida*[1], *Mohammad Ali Shodikin*[1,4]

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Abstract

Introduction: *Klebsiella pneumoniae* is a Gram-negative bacterium that is mostly found as a nosocomial disease in hospitals. This bacterium is the largest contributor to the number of causes of pneumonia. Immune responses that play a role against *K. pneumoniae* is interferon-gamma (IFN-γ) as cellular immune responses, which function on the mucosal barrier in response to infection and contribute to the initial defense against various pathogens. The purpose of this study was to determine the effect of exposure to *K. pneumoniae* pili protein 65.5 kDa on IFN levels in mice. **Methods:** Thirty BALB/C mice were divided into three groups: K1 given phosphate buffer saline, K2 given pili protein and Freund's adjuvant, and K3 given Freund's adjuvant. The measurement of IFN-γ levels was carried out by enzyme-linked immunosorbent assay, the results of which were analyzed using the Kruskal–Wallis. **Results:** Pili protein 65.5 kDa *K. pneumoniae* showed a positive hemagglutination test with the highest titer of 1/8, which was indicated by the absence of red aggregate dots at the bottom of the well. Analysis test showed that the highest concentration of IFN-γ levels was in the control group and the lowest IFN-γ level was found in the adjuvant group. **Conclusion:** Pili protein 65.5 kDa *K. pneumoniae* had a role as a hemagglutinin protein and there was no effect of *K. pneumoniae* pili protein 65.5 kDa on IFN-γ levels in mice.

Keywords: *Interferon-gamma, Klebsiella pneumonia, pili*

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Introduction

Klebsiella pneumoniae is a Gram-negative bacterium that can cause pneumonia.[1-3] *K. pneumoniae* is most found as a nosocomial disease in hospitals.[4] *K. pneumoniae* infection has a high morbidity rate (63.3%) and a mortality rate of 28.3%. *K. pneumoniae* is the largest contributor to the number of causes of pneumonia, which is 56.25% compared to *Streptococcus pneumoniae* (18.25%) and *Staphylococcus aureus* (12.5%).[5] This bacterium has antibiotic-resistant strains, this condition can increase the virulence factor and decrease the work of antibiotics so that it will further complicate treatment.[6-8]

K. pneumoniae has virulence factors that can invade host cells, one of which is pili.[9] An important part in the development of infection by bacteria is the initial attachment of bacteria to the tissue surface (adhesion).[10] In the adhesion process, bacteria bind to host cell surface receptors mediated by proteins, namely hemagglutinin proteins which were able to increase the formation of antibodies as protection against infection by bacteria.[11,12] Previous studies have proven that the pili protein of *K. pneumoniae* 12.8, 38.6, and 96.4 kDa acts as an adhesion molecule.[13-15]

Immune responses that play a role against *K. pneumoniae* bacteria are humoral and cellular immune responses. Humoral immune response in the form of antibodies that can neutralize bacteria. Cellular immune response plays an important role in *K. pneumoniae* infection for the initial activation of the inflammatory response, namely interferon (IFN).

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IFN has been demonstrated to be an important signal in cell-mediated immunity against various infectious agents.[16] This cytokine is required for T-cell-mediated cellular immunity and functions on the mucosal barrier in response to infection and contributes to the initial defense against various pathogens.[17-19]

T helper 1 (Th1) cells contribute to the production of cytokines, such as IFN-γ. This IFN-γ plays a role in macrophage activation and induces the production of opsonizing antibodies by B cells. The Th1 cell response also activates cytotoxic T lymphocytes which induce cell death due to infection with viruses and other intracellular pathogens and activates natural killer cells which play a role in inducing apoptosis in tumors. Moreover, cells are infected by viruses.[20] There are two types of IFN, namely IFN Type 1 (IFN-α and IFN-β) which play a role in viral infections and IFN Type 2 (IFN-γ) which play a role in bacterial infections. Therefore, it is necessary to conduct a study to determine the effect of *K. pneumoniae* pili protein 65.5 kDa on IFN-γ levels in mice.

Methods

This study was conducted at the Faculty of Medicine and Faculty of Mathematics and Natural Sciences at the Universitas Jember between October 2021 and March 2022. The study was approved by the Faculty of Medicine Universitas Jember Ethics Committee (Reference 1572/H25.1.11/KE/2022).

This study used an *in vivo* posttest control group design on experimental animals BALB/C mice (*Mus musculus*) which were divided into three groups, K1 given phosphate buffer saline, K2 given pili protein and Freund's adjuvant, and K3 given Freund's adjuvant. This experiment used some methods, including bacterial sample, *K. pneumoniae* pili protein isolation, pili protein purification *K. pneumonia*, hemagglutination (HA) test, experimental animal induction, and measurement of IFN-γ levels.

Bacteria sample

The samples of this study were *K. pneumoniae* pili protein found in the Laboratory of Microbiology, Faculty of Medicine, University of Jember, and erythrocyte cells of male BALB/C mice aged 6–8 weeks weighing ±25 g in good health and able to eat and drink well.

Klebsiella pneumoniae pili protein isolation

Identification of the molecular weight of *K. pneumoniae* pili protein was carried out by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) using the Laemmli research method. The protein sample was removed from the refrigerator until it melted. Protein isolation using 4% stacking gel and 12.5% separating. The running process is carried out for 60 min with a voltage of 120 mV.[21,22]

Pili protein purification *Klebsiella pneumoniae*

The SDS-PAGE gel that forms the protein weight band is cut and then put into a nitrocellulose sheet, after that, it is put into a horizontal electrophoresis chamber which already contains running buffer for electroelution. The running process is carried out for 2 h with a voltage of 125 mV, 300 mA. Next, prepare 1 L of sterile PBS in a glass beaker with a pH of 7.4 for sample dialysis, the result of electroelution. Then, put the magnetic stirrer into the beaker glass and make sure to always spin for 2 h × 24 h in the refrigerator. The sterile PBS is replaced every 24 h with a new one. After 48 h, the electroeluted and dialyzed samples can be used for the HA test.[22]

Hemagglutination test

HA test was performed to determine which pili cut had the highest HA titer to be used in the next protocol. Serial dilutions of samples were carried out on V microplate with a volume of 50 µl. For 1 min, place the V microplate on top of the rotator plate. Then placed at room temperature and observed for 1 h, by observing the presence of erythrocyte agglutination from the highest to the lowest dilution can determine the amount. The interpretation of the results of the HA test is the formation of a red ring of erythrocytes (negative) and the absence of a red ring of erythrocytes at the bottom of the well (positive) titer.[23,24]

Experimental animal induction

The mice used were male BALB/C minutes in healthy condition, aged 6–8 weeks, and weighed ±25 g. Mice were acclimatized for 7 days. After acclimatization, mice were randomly selected for induction according to the treatment group. There are three groups, namely K1 (control group) given PBS immunization with a total volume of 200 µl intraperitoneal, K2 (antigen and adjuvant group) given immunized with the volume of 200 µl, in the first treatment 92 µl PBS, 8 µl antigen, and 100 µl complete Freund's adjuvant (CFA) and for the final booster, immunization was given 184 µl PBS, 16 µl antigen, and 100 µl incomplete Freund's adjuvant (IFA), and K3 (adjuvant group) given 100 µl CFA and 100 µl PBS immunization in the first treatment and given 100 µl IFA and 100 µl PBS for the final booster. Induction was carried out intraperitoneally, three times (first dose with two repetitions/boosters) with an interval of 2 weeks. The dose of pili protein used was 50 g in each mouse.[25-29]

Measurement of interferon-γ levels

The measurement of IFN-γ levels was carried out according to the procedure in the enzyme-linked

immunosorbent assay (ELISA) kit. The mice were terminated after 2 weeks from the third treatment. Termination in mice using ether, then the blood of mice was taken by means of cardiac puncture. Serum sample preparation was carried out by allowing whole blood to stand in Eppendorf at room temperature for 20–30 min, then centrifuging at 3000 rpm for 20 min, and then separating the serum into a new Eppendorf. In this study, the Bioassay Technology Laboratory (Mouse IFN-γ ELISA kit) was used with catalog number No.E0056Mo.

Data analysis

The data are presented in the form of a table of the results of concentration measurements for each group along with the mean standard deviation. The Shapiro–Wilk test was used to find out the distribution of the data, and the Levene test was used to find out whether the data used were homogeneous or not. Because the data are not homogeneous, Kruskal–Wallis test was used as a comparative test in this study. Significant results are shown if $P < 0.05$. Whole data were analyzed using the International Business Machines (IBM) Statistical Package for Social Science (SPSS) IBM Armonk, New York, United States of America.

Results

Hemagglutination test

The HA test was conducted in the treatment group. The concentration of pure pili protein 65.5 kDa *K. pneumoniae* from dialysis and electroelution was 0.65 g/dL or 6500 g/ml. HA test was carried out to prove that pili 65.5 kDa is a hemagglutinin protein, by serial dilution of the sample on V microplate. HA test results can be read and interpreted after 1 h by observing the presence of erythrocyte deposition. The HA test results obtained in this study were positive, indicated by the absence of a red aggregate point at the bottom of the well, which indicated that the pili protein 65.5 kDa *K. pneumoniae* had the ability to agglutinate erythrocytes with the highest titer of 1/8. The results of the HA test are shown in Figure 1 and Table 1.

Measurement of interferon-γ levels by enzyme-linked immunosorbent assay

The results of measuring IFN levels using the ELISA method with twice repeated are shown in Table 2. It is known that the highest concentration of IFN-γ levels is in K1 and the lowest levels of IFN-γ are in K3.

Discussion

HA test was conducted to determine the tested protein acts as a hemagglutinin protein. In this study, the readings of the HA test results can be interpreted after 1 h by showing the highest HA titer of 1/8. These results prove that the pili protein 65.5 kDa *K. pneumoniae* is a hemagglutinin protein capable of agglutinating mouse erythrocytes. The higher the titer from the hemagglutinin test results, the higher the bacterial pathogenicity in infecting host cells.[30] Previous studies have proven that the pili protein of *K. pneumoniae* 12.8 kDa acts as an adhesion molecule.[13] In addition, pili protein with a molecular weight of 38.6 kDa *K. pneumoniae* is also a hemagglutinin and adhesin protein that acts as a virulence factor.[24]

Pneumonia is the leading cause of death in children worldwide. *K. pneumoniae* bacteria can easily colonize human mucosal surfaces including the gastrointestinal tract and oropharynx. There are two main types of antibiotic resistance in *K. pneumoniae*, which involve the expression of extended-spectrum β-lactamases and the expression of carbapenemases.[31,32] Therefore, a vaccine approach against *K. pneumoniae* is urgently needed.

Based on the results of the study in Table 1 above, the highest concentration of IFN-γ levels occurred in K1, namely the control group. In this study, it is expected that the levels of IFN-γ in K1 are lower than in K2 and K3. This study is in line with the research of Widiatmaja *et al*.[33] which showed the mean result of the control group was higher than the other groups. This can occur due to several factors such as the different immune responses of each experimental animal. The immune response of each individual to vaccines can be influenced by several things, namely the route of immunization (oral or injection), the form of antigen (live, killed, soluble, peptide subunit, and particular), dose, host, genetics of the pathogen, and already has immunity.[34,35] IFN-γ levels decreased before termination due to increased IFN-γ levels occurred 72 h after inoculation or injection.[18] In T cells that were in the resting phase (not activated), IFN-γ cytokines were not expressed so that the protein could not be detected. After

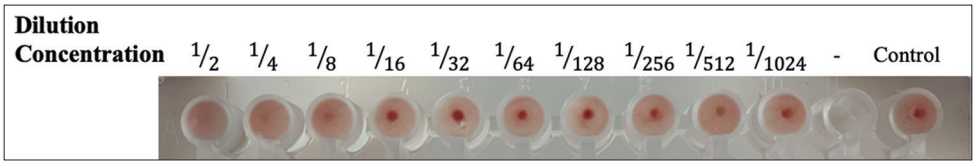

Figure 1: Hemagglutination test results for pili protein 65.5 kDa *Klebsiella pneumoniae*

Table 1: Hemagglutination test results

Mice red blood cells	Concentration									
	1/2	1/4	1/8	1/16	1/32	1/64	1/128	1/256	1/512	1/1024
Pili 65.5 kDa	+	+	+	–	–	–	–	–	–	–

Table 2: Concentration of interferon-γ

Group	IFN-γ concentration (pg/mL)
K1 – Control	147.66±17.38
K2 - Antigen+adjuvant	143.57±4.92
K3 – Adjuvant	138.77±25.37

Statistical analysis test using Kruskal–Wallis test. Kruskal–Wallis test results obtained P=0.546. IFN: Interferon

T-cell activation occurs, IFN-γ cytokines will be detected at 6–8 h and the level reaches a maximum at 12–24 h and then decreases to the baseline value.[36]

K. pneumoniae as extracellular bacteria has protein antigens that can activate cellular adaptive immune responses mediated by $CD4^+$ T cells, Th17, and Th1. Th1 cells contribute to the production of cytokines, such as IFN-γ. These bacteria have pili as virulence factors, which will invade host cells and an adhesion process will occur, which will be responded to by pattern recognition receptors (PRRs) by binding to bacterial components and initiating immune cells. Upon stimulation of PRR ligation recruit's adapter, molecule signals and activates IFN regulatory factor kinase through toll-like receptors which predominantly induce proinflammatory cytokines from IFN.[16,37]

Type II IFN or also called IFN-γ which acts as a modulator of the immune system. IFN-γ acts on epithelial cells and neutrophils residing in tissues, dendritic cells, macrophages, B cells, and plasmacytoid dendritic cells in the blood. These cytokines can increase the permeability of airway epithelial cells, which is thought to contribute to the spread of bacteria from the airways to the spleen. IFN-γ cytokines produced by epithelial cells then act on virus-infected epithelial cells to suppress viral replication and prevent the spread of virus through the epithelium.[36,38]

The results of the data analysis test using Kruskal–Wallis method showed $P > 0.05$, which was 0.546. These results indicate that there is no effect of *K. pneumoniae* pili protein 65.5 kDa on IFN-γ levels in mice. This may be due to the activity of anti-inflammatory cytokines that cause IFN-γ levels to decrease. Interleukin (IL-10) can regulate the inflammatory process and can be characterized by its increased levels to suppress the activation of proinflammatory cytokines, one of which is IFN-γ.[39] Another study conducted by Setiawan and Nugraha[40] showed that in tuberculosis patients, there was an increase in the secretion of IL-10 in peripheral blood mononuclear cells, this can cause the production of proinflammatory cytokines to decrease and limit tissue damage. IL-10 can also inhibit the proliferation of Th1 cells so that it can reduce the immune response. IL-10 can inhibit the proliferation and synthesis of $CD4^+$ T-cell cytokines including the production of IFN-γ and IL-2 by Th1. It can be concluded that pro-inflammatory cytokines (Th1) with anti-inflammatory cytokines (Th2) have antagonistic properties, namely IL-10 will inhibit IFN-γ, and vice versa.[41]

Although the research results are less potential, in theory, IFN-γ plays an important role in immunoregulation.[42] IFN-γ works to activate immune cells and induces major histocompatibility complex molecules, which are important in the next stage of the immune response. Therefore, these cytokines have an important role as an immune response to antivirals and antimicrobials for a long time and can coordinate the transition from innate immunity to adaptive immunity.[43] IFN-γ functions in early prevention, because IFN-γ is found before macrophages are activated or antibodies are formed that appear in response to infection.[44] During *K. pneumoniae* infection, IFN-γ is essential for the control of primary infection and the spread of infection from the lungs.[31] Limitation of this study is the small amount of serum obtained from the results of treatment in mice meant that more repetitions were not possible when measuring cytokine levels with ELISA.

Conclusion

Pili protein 65.5 kDa *K. pneumoniae* had a role as a hemagglutinin protein and there was no effect of *K. pneumoniae* pili protein 65.5 kDa on IFN-γ levels in mice's spleen.

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Conflicts of interest

There are no conflicts of interest.

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