PUBLIKASI JURNAL

Pili Protein 65.5 kDa of Klebsiella pneumoniae Induced a Decrease in IL-10 in Mice

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NIP. 198308012008122003

- Tenaga pengajar Mikrobiologi
  Fakultas Kedokteran Universitas Jember

KEMENTRIAN PENDIDIKAN, KEBUDAYAAN, RISET DAN TEKNOLOGI

UNIVERSITAS JEMBER

Karya Ilmiah di publikasikan pada:
Majalah Kedokteran Bandung (MKB)
2022; 54 (3): 143 – 147,
Faculty of Medicine Universitas Padjadjaran
pISSN 0126-074X / eISSN 2338-6223

pISSN 0126-074X
eISSN 2338-6223

Majalah Kedokteran Bandung

MKB

Bandung Medical Journal

Volume 54 Number 3
September 2022

MKB Majalah K

Publis

Vol 54, No 3 (2022)

Table of Contents

Articles

Case Report

Canaliculotomy as A Canalicular Obstruction Management Approach: a Case Series

Raden Angga Kartiwa, Firda Muthia Elsyanty, Ade Triyadi, Adessa Rachma, M. Rinaldi Dahlan, Shar Fitrianti Boesoirie, Niluh Putu Ayu Dewi

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This Journal indexed by

RESEARCH ARTICLE

pISSN: 0126-074X | eISSN: 2338-6223
https://doi.org/10.15395/mkb.v54n3.2690
Majalah Kedokteran Bandung. 2022;54(3):143–147

Majalah Kedokteran Bandung (MKB)

Received: February 18, 2022
Accepted: May 18, 2022
Available online: September 30, 2022

Pili Protein 65.5 kDa of *Klebsiella pneumoniae* Induced a Decrease in IL-10 in Mice

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Abstract

Klebsiella pneumoniae is one of the leading cause of nosocomial infection worldwide with clinical isolates mostly found in Multi-Drug Resistant (MDR) or Extensively Drug-Resistant (XDR). This makes therapeutic options due to this bacteria limited. Several studies have shown that the pili protein of *K. pneumoniae* with different molecular weights plays a role in the virulence factor, as they are hemagglutinin and adhesin proteins. Pili protein 65.5 kDa of *K. pneumoniae* can be considered as the antigen candidate for vaccines. This study aimed to determine the immune response based on the IL-10 level as induced by pili protein 65.5 kDa *K. pneumoniae*. This study was conducted from October 2020 to March 2021 at the Faculty of Mathematics and Natural Sciences and Faculty of Medicine of Jember University. This was true experimental with post-test only controlled group design on 27 male BALB/c mice which divided into 3 groups: K1, K2, and K3. The groups were induced with sterile PBS, pili protein and Freund's adjuvant, and Freund's adjuvant only, respectively. Samples were drawn through cardiac puncture, then serum was taken to measure the IL-10 levels using the ELISA method. Hemagglutination test showed a positive result with 1/8 as the highest titer. Results showed that the mean IL-10 levels were 290.92±45.33; 235.05±44.53; 218.54±64,81 for K1, K2, and K3, respectively. One-way ANOVA test results showed a statistical difference between groups (p=0,019). This research shows that pili protein 65.5 kDa *K. pneumoniae* decreases the IL-10 level. However, when it is provided with the Freund's Adjuvant, there is no proof of the immunogenicity when compared to the control group.

Keywords: Interleukin-10, immune response, *Klebsiella pneumoniae*, pili protein

Introduction

Klebsiella pneumoniae is one of the main bacteria causing nosocomial infections which still causes major morbidity and mortality worldwide.[1] Clinical isolate is often found in *Multi-Drug Resistant* even *Extensively Drug-Resistant* form so there are just a few therapeutic options for infections caused by this bacteria.[2] Interleukin-10 is an important cytokine cause it is associated to host survival during *Klebsiella pneumoniae* infection cases.[3,4] In several previous studies have proven that pili protein with different molecular weights as hemagglutinin and adhesin protein plays role in the virulence factor of *K. pneumoniae*.[5–7] From the electrophoresis that has been done, the thickest band contains protein 65.5 kDa from *K. pneumoniae* pili. According to Levinson, 2012 several factors determine molecular immunogenicity namely foreignness, molecular size, chemical-structural complexity, antigenic determinants; dosage, route, and timing of antigen administration; adjuvants.

Proteins with molecular weights above 100 kDa are the most potent immunogen. Usually, molecules with molecular weights below 10 kDa are weakly immunogenic.[8] So there is the possibility of pili protein with molecular weights of 65.5 kDa being immunogenic. Clinical isolates of *K. pneumoniae* which are resistant to several types of antibiotics cause a critical need in searching for alternative treatment strategies for this bacterial infection.

The principle of immunity seems potential alternative strategy to control *K. pneumoniae* infections, one of them is the vaccine which is not yet licensed by Food and Drug Administration.[2] Pili protein 65.5 kDa *K. pneumoniae* which can be immunogenic can be considered as antigen for the vaccine candidate. Previously, it was necessary to conduct researches that aimed at

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determining the immune response which forms as IL-10 levels on induction of pili protein 65.5 kDa *K. pneumoniae*.

Methods

The research was conducted from October 2020 to March 2021 at the Faculty of Medicine and Faculty of Mathematics and Natural Sciences Jember University. *K. pneumoniae* isolate was obtained from doing a swab of the patients infected by *K. pneumoniae* in Syaiful Anwar Hospital, Malang. These isolates then were cultured in the Microbiology Laboratory of the Medical Faculty University of Jember. This cultured bacteria then through pili cutting. Pili protein was isolated by SDS PAGE from the pili fraction of *K. pneumoniae*. The gel obtained from SDS PAGE then been purified using electroelution and dialysis. The purified protein was measured using the Kingsley method hereafter the results were used for hemagglutination test and intraperitoneal induction in mice with ±25 grams weight, healthy and active. A hemagglutination test was carried out to identify bacterial protein adhesion to erythrocyte cells. If the result was positive at a certain titer, the protein dosage for induction can be adjusted according to the result. The intraperitoneal induction was carried out not only because the volume of antigen and adjuvant injected is quite large, but also because this method was safer for the administration of Freund's adjuvant which is dangerous for intravenous administration. The pharmacokinetics of substances administered intraperitoneally is nearly similar to oral administration because the primary route of absorption is into the mesenteric vessels, which can cause local and systemic effects.

The qualified mice, from each group of treatment, consisted of 9 mice, then were acclimatized for 7 days. Induction was carried out with a 50 µg antigen dose and Freund's Adjuvant with the same volume for the K2 group, PBS for the K1 group, while Freunds'd adjuvant and PBS for the K3 group. Priming was carried out, followed by the first and second booster, then termination with each 14 days interval. Sera were obtained by cardiac puncture with an open approach technique. The cardiac puncture was performed to obtain a large number of samples and the literature states that levels of pro-inflammatory and anti-inflammatory cytokines are higher in blood samples obtained from the heart compared with collection from the peripheral location. The blood obtained was then been waited for about 20–30 minutes before being centrifuged at 2,000-3,000 rpm for 30 minutes. Sera is stored after being separated from the pellet. Then, the measurement of IL-10 levels was carried out using the ELISA method.

All reagents have been brought to room temperature before use. The 120 µl of the standard (2400 pg/mL) was reconstituted with 120 µl of standard diluent to generate standard stock solution, and allowed to sit for 15 with gentle agitation to make dilution. Made 6 standard points, each been duplicated, by serially diluting the standard stock solution (1,200 pg/mL 1:2 with standard diluent to produce 600 pg/mL, 300 pg/mL, 150 pg/mL, and 75 pg/mL. Zero standards were obtained using standard diluent (0 pg/mL). The 20 mL wash buffer concentrate 25x was diluted into distilled water to yield 500mL of 1x wash buffer.[9]

The computer software used to analyze this data was IBM SPSS Statistics 25 Shapiro-Wilk test was performed, and the data was known to be in a normal distribution. Levene test was also performed, and the result was the samples have equal variances. The statistical analysis used in this study is the One-Way ANOVA test. The result of the One-Way ANOVA test was there was a statistically significant difference between the three treatment groups. This result was followed by The Post Hoc LSD test. Data showed that there was a significant difference between the antigen adjuvant treatment group with the control group, and between the adjuvant treatment group with the control group. However, there was no significant difference between the adjuvant and the antigen adjuvant groups.

The whole protocols related to experimental animals have been approved by the Ethics Committee of the Faculty of Medicine, Jember University (1553/H25.1.11/KE/2021). IL-10 levels performed a data normality test with the Shapiro-Wilk test before being analyzed by the One-Way ANOVA test, followed by the Post Hoc LSD after. The result of statistical analysis was considered statistically significant if $p<0.05$.

Results

The figure shows the result of the pili protein 65.5 kDa *K. pneumoniae* hemagglutination test on mice erythrocytes with the highest titer $\frac{1}{8}$. The table shows the result from the sera measurement of IL-10 levels in each group treatment. From the measurements, obtained

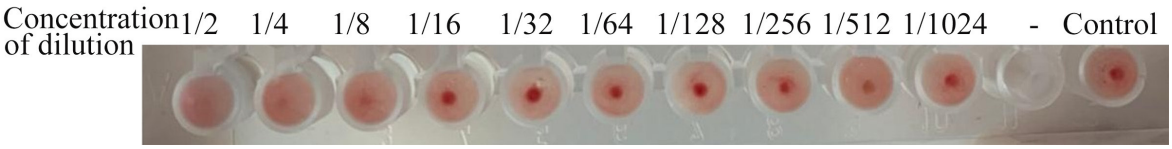

Figure Hemaggutination Test Result

the highest IL-10 levels in the control group and the lowest in the adjuvant group. Results from the *One-way* ANOVA test showed a p-value of 0.019 which means the treatments gave a statistically significant decrease in IL-10 serum levels (p=0.005). This result was continued with the Post Hoc LSD test and the results showed that there were significant differences between the K1-K2 (p=0.033), and K1-K3 (p=0.007). But there was no significant difference between K2-K3 (p=0.510).

Discussion

Hemagglutination test on pili protein 65,5 kDa *Klebsiella pneumoniae* with the concentration of 6.5 mg/mL in mice produced the highest titer$\frac{1}{8}$. This concentration of protein pili was measured by the Kingsley methods. The protein concentration at the highest or$\frac{1}{8}$titer was 36,1 µg/50 µl. This hemagglutination ability proves pili protein 65.5 kDa *Klebsiella pneumoniae* is a virulence factor that acts as hemagglutinin protein. It happens caused by proteins contained in pili that can be attached to sugar molecules that make up the membrane on the surface of the cell host.

The working principle of the hemagglutination test is the clumping which is visually observable caused by certain antibodies binding antigens thus forming the bigger complexes.[9] Lectin, a high-affinity protein, can bind specifically to carbohydrates on the host cell receptor. Generally, lectin can agglutinate erythrocytes.[10] The various hemagglutination titers to the pili protein with different molecular weights are influenced by the ability of each pilus to bind to the erythrocyte. The higher the hemagglutination titer, the stronger the adhesion ability of that pili protein.[11]

Table Mean of Result Serum IL-10 Levels (pg/mL)

Group	IL-10 Concentration
K1	290.92±45.33
K2	235.05±44.53
K3	218.54±64.81

K1=control group; K2=antigen and adjuvant group; K3=adjuvant group

The treatment of pili protein 65.5 kDa *K. pneumoniae* and Freund's adjuvant induction succeeded in decreasing cytokine IL-10 levels in mice within 14 days after the third induction. There are no similar previous studies that discuss the induction effect of pili protein 65.5 kDa *K. pneumoniae* along with Frend's adjuvant to IL-10 levels.

Protein-based antigens from extracellular bacteria not only produced antibodies as the main humoral immune response, but also activated cellular adaptive immune responses mediated by CD4(+) T cells that produce cytokines that induce local inflammation, increase phagocytosis, and microbicidal activity of macrophages and neutrophils. Th17 cells are also involved in monocyte and neutrophil recruitment thereby enhancing local inflammatory responses. In addition, it also induced Th1 cell immune responses that play a role in macrophage activation and the production of cytokines such as interferon-gamma (IFN-γ).[13]

Interferon-gamma cytokines produced by Th1 play an important role against immune evasion depending on IL-10 Klebsiella induced. This proved by IL-10 production was upregulated significantly in IFN-γ knockout mice. Simultaneously, this is followed by increasing bacterial burden and decreasing inflammatory mediators.[13]

Interferon-gamma alters Toll-Like Receptor 2 (TLR2)-induced signal transduction by increasing Glycogen Synthase Kinase 3 (GSK3) activity and suppressing Mitogen-activated Protein Kinase (MAPK) activation, leading to reduced IL-10 production. IFN-γ suppressed cAMP-responsive element-binding Protein (CREB) and cAMP Co-regulate Activator Protein 1(AP-1), a transcription factor that induces the expression of IL-10 and is regulated in part by MAPKs and GSK3. GSK3 and CREB are important players in integrating IFN-γ and TLR2 responses in innate immunity and inflammation.[15]

A decrease in IL-10 levels in the K2 group is

possibly due to a similar reason to the case of antigen induction from *M. tuberculosis* which stimulates protective Th1 cells. Interleukin-10 is an anti-inflammatory cytokine produced by Th2 cells. IL-10 in dealing with germs will reduce immune response and limit tissue damage by inhibiting the excessive inflammatory response. These cytokines have also been identified as the other cytokines inhibitor since it inhibits the myeloid cells' ability such as macrophages and dendritic cells to activate Th1 cells so that the cytokines production by Th1 cells is blocked.[12]

Another possible cause of the decrease in IL-10 levels is the microorganisms burden, in this study was formed as pili protein along with adjuvant induction, was not high enough to increase the production of IL-10. As other studies explained that infections caused by gram-negative bacteria, the microorganism burden can affect the IL-10 response. The high bacterial burden will increase the IL-10 production, while a low bacterial burden can trigger the pro-inflammatory response. [13]

In this study, we used 50 µg for antigen dosage and 0,2 mL for the total mixture volume of antigen adjuvant according to the immunization protocol.[18] The 50µg of antigen dosage was also appropriate to the result of the highest titer from the hemagglutination test as the preliminary study proved that the antigen in the concentration of 36.1 µg/50 µl was bound to the erythrocyte cells.

Another study presented a different result. As a virulence factor, the protein contained in pili is the potential to increase IL-10 production. No study specifically discusses the effect of pili protein from *Klebsiella pneumoniae* on IL-10 cytokine levels. But, live flagellated *Salmonella*, as a gram-negative bacteria, can induce Th-1 activity while *flagella Flic* protein which is a major component of flagella, the soluble form can induce the host Th-2 response.[13]

An increase in IL-10 levels was found in a study using mice in which the vein tail was injected with bacteria solution. The *Klebsiella pneumoniae* injection group will increase IL-10 levels 1 hour after bacterial injections. This may indicate that IL-10 levels increased in suspected bloodstream infection cases and may positively be correlated to gram-negative bacterial infections.[14]

Interleukin-10 parameters were measured considering its role in bacterial infection cases, in which IL-10 as a pleiotropic, immunoregulatory cytokine that is important to protect the host from infection-associated immunopathology.[20] This cytokine acts as a moderator that balances the responses between Th-1 and Th-2 in the dynamics of the host environment during infection. As the anti-inflammatory cytokine which inhibits the production of various pro-inflammatory factors, IL-10 may be moderating the immunopathology in associated infections with a strong Th-1 response. In addition, IL-10 is also promoting the secretion of microorganism-specific IgA and the activation of B lymphocytes.[13]

A decrease in IL-10 levels occurred on antigen and adjuvant induction compared with adjuvant induction is known to have been not statistically different. This is caused by proteins 65.5 kDa contained in *K. pneumoniae* pili are not a potent enough immunogen. As it is known, the most potent immunogens are proteins with molecular weights of more than 100 kDa while the weak or poor are with molecular weights of less than 10 kDa.[8]

As the pili proteins are not the most potent immunogens, the use of pili protein for stimulating immune response needs to be mixed with an adjuvant. Complete Freund's Adjuvants (CFA) which were injected at priming were known for strongly triggering Th1 cell responses.[14,15] However this adjuvant is not approved for use in humans since it was too toxic and produced excessive local side effects.[15] CFA has generally been used to assess antigen immunogenicity in mice and the induction of autoimmune disease.[16] Meanwhile, Incomplete Freund's Adjuvant (IFA) was injected at booster CFA triggering Th2 cell responses.[14]

The limitation of this study was the dose of antigen in the treatment group was all same without any different doses to find out which was the most effective for inducing an immune response. Another study is needed to conduct with various dosages of this antigen and adjuvant to find out the effective dose, which increases the IL-10 levels.

In conclusion, pili protein 65,5 kDa *Klebsiella pneumoniae* which decreases IL-10 levels if given along with Freund's adjuvant is not proven to be an expectedimmunogen compared to the control group.

Acknowledgement

Authors would like thank to the Department of Microbiology teams, the Medical Faculty University of Jember for excellent technical assistance and LP2M University of Jember for the

KeRis Research Group funding program based on the decree of the Chancellor of the University of Jember number 9268 /UN25/LT/2021.

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