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Research article

Immunogenic potential of 65.5 kDa pili protein of *Klebsiella pneumoniae*: Evaluation of IgG, IgG1, and IgG2a levels in BALB/c mice

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Abstract: *Klebsiella pneumoniae* is a significant etiological agent of both community-acquired and nosocomial infections, characterized by its pronounced vulnerability to drug resistance. Preventive measures, such as vaccine research, are essential since no effective and viable vaccine currently exists. Pili, a critical virulence component in *Klebsiella pneumoniae*, promote bacterial adherence and have very low variability relative to other virulence factors, rendering them a viable target for vaccination. The 65.5 kDa pili protein has been recognized as a possible antigen owing to its significant antigenicity. This study sought to assess the immunogenic response by quantifying IgG, IgG1, and IgG2a levels in BALB/c mice following the delivery of the 65.5 kDa pili protein. A total of 24 mice were categorized into three groups: PBS, adjuvant, and pili. Serum concentrations of IgG, IgG1, and IgG2a were assessed via ELISA. Statistical analysis was conducted utilizing the ANOVA test with a 95% confidence interval. The data indicate that vaccination with the 65.5 kDa pili protein elicits a robust humoral immune response characterized by increased IgG and IgG1 production, suggesting a Th2-biased response, while showing no significant impact on IgG2a levels, which are typically associated with Th1-mediated immunity. In summary, the 65.5 kDa pili protein shows promise as a vaccine candidate for *Klebsiella pneumoniae* by inducing a strong IgG and IgG1-mediated immunological response in BALB/c mice. Future studies should use flow cytometry to assess immune cell activation, cytokine

profiling for Th1/Th2 responses, and antigen-specific assays to explore T-cell activation. Research on dose optimization, adjuvant screening, immunity duration, and challenge studies with virulent *Klebsiella pneumoniae* is crucial. Proteomic analysis and epitope mapping can aid in refining vaccine design.

Keywords: antigen; immunoglobulin-G; immunoglobulin G1; immunoglobulin G2a; *Klebsiella pneumonia*; mice; pili; vaccine

1. Introduction

Klebsiella pneumoniae is a Gram-negative, non-motile, rod-shaped bacterium that is part of the Enterobacteriaceae family [1,2]. It is encapsulated and has facultative anaerobic traits [3]. *Klebsiella pneumoniae* is a prevalent opportunistic and nosocomial bacteria responsible for a range of diseases, including pneumonia, urinary tract infections, and meningitis. It is inherently found in the human microbiome but can become pathogenic, especially in immunocompromised individuals. The bacteria displays O and K antigens on its surface, enhancing its pathogenicity [1,2]. *Klebsiella pneumoniae* has become a notable issue in hospital environments due to its capacity to acquire multidrug resistance, including the synthesis of carbapenemases [4,5]. *Klebsiella pneumoniae* is a prominent pathogen responsible for nosocomial and community-acquired illnesses worldwide. Research indicates differing incidence rates in underdeveloped nations: 21.0% in a Nigerian hospital [6] and 9.72% as a uropathogen at a tertiary care facility in India [7]. A meta-analysis calculated the aggregated prevalence of nosocomial multidrug-resistant *K. pneumoniae* at 32.8% [8]. The prevalence rates are often elevated in females and older persons [6]. The rise of multidrug-resistant strains, such as ESBL-producing and carbapenem-resistant *K. pneumoniae*, presents a considerable public health risk, requiring heightened awareness and suitable treatment and preventative measures [7,8].

Diverse vaccination techniques have been investigated, encompassing whole-cell, outer membrane vesicles, and polysaccharide-based methods [9]. Protein-based subunit vaccinations aimed at conserved antigens or virulence factors have demonstrated potential in animal models [10]. Lipopolysaccharide (LPS) has been recognized as a prospective vaccine target, with antibodies against core and O-antigen components possibly providing protection [11]. Notwithstanding these gains, there is currently no licensed vaccination for *K. pneumoniae*, underscoring the necessity for ongoing research and development in this domain. Factors contributing to the pathogenicity of *Klebsiella pneumoniae* include adhesion factors, notably the hemagglutinin proteins present in pili. The hemagglutinin proteins in *Klebsiella pneumoniae* have adhesin characteristics, particularly the 12.8 kDa pili protein. These adhesins, functioning as virulence factors, can elicit an immunological response in the host, including immunoglobulin G (IgG), which plays a crucial role in neutralizing and eliminating microorganisms [12]. Several studies have highlighted the immunomodulatory effects of bacterial components, including pili, in enhancing specific antibody responses such as IgG, IgG1, and IgG2a [13,14]. The role of *K. pneumoniae* antigens, including its pili structures, in driving Th1/Th2 immune polarization has been explored in various infection and vaccine models [15,16]. Moreover, the adjuvant-like properties of bacterial surface structures in stimulating humoral immunity are well-documented, supporting the relevance of studying pili 65.5 as a potential immunogenic target [17]. Proteins with a molecular weight of 65.5 kDa are regarded as the thickest and most antigenic. This study seeks

to examine the vaccine induction impact of the 65.5 kDa pili protein of *K. pneumoniae* on serum IgG, IgG1, and IgG2 levels in BALB/c mice.

2. Materials and methods

2.1. Ethical clearance

This research has received ethical approval by the Ethics Commission of the Faculty of Medicine, University of Jember, based on the consideration of 3 ethical principles, 7 standards, and 25 items of ethical guidelines for human research by CIOMS-WHO and based on the Guide For The Care And Use Of Laboratory Animals (1996). Ethical approval is evidenced by the issuance of the ethical approval letter with the number 1638/UN.25.1.10.2/KE/2024, dated April 25, 2024.

2.2. Bacterial culture and pili isolation of Klebsiella pneumoniae

A culture of *Klebsiella pneumoniae* was conducted on MacConkey, BHI, and TCG media. Following harvest, TCA was used to achieve a concentration of 3%, followed by the centrifugation of the suspension. The resultant pellet was resuspended in PBS (1:10), again subjected to centrifugation, and subsequently mixed with PBS (1:1). The bacterial pili were cleaved using a pili cutter (5000 rpm, 4 ℃, 1 min) and subsequently centrifuged (12,000 rpm, 4 ℃, 30 min). The supernatant obtained from centrifugation was transferred to a separate tube [18,19].

2.3. Identification of the molecular weight of Klebsiella pneumoniae *pili protein by SDS-PAGE*

The gel employed in this technique comprised a 12.5% separating gel and a 4% stacking gel. Protein samples, previously stored in a deep freezer, were initially thawed at ambient temperature. Thereafter, samples were subjected to electrophoresis. The dye employed was bromophenol blue. A protein having a molecular weight of 65.5 kDa was discovered in this SDS-PAGE investigation [19,20].

2.4. Purification of 65.5 kDa pili protein

The protein purification process started with the molecular weight of the protein generating a gel band by electrophoresis, which was subsequently excised based on the desired weight. The excised band was thereafter exposed to electroelution within an electroelution chamber. Thereafter, the protein underwent dialysis, and its concentration was quantified utilizing the Kingsley technique [19,21].

2.5. Administration of 65.5 kDa pili protein to mice

Mice underwent acclimatization for seven days prior to the therapy. The experimental animal samples were randomized by basic randomization. Research samples were categorized into three groups, each subjected to distinct treatments. The control group received PBS; the adjuvant group was administered a mixture of Freund's adjuvant and PBS; and the antigen group was administered a combination of 65.5 kDa *Klebsiella pneumoniae* pili protein at a dosage of 50 μg, Freund's adjuvant, and PBS. Administration was conducted by intraperitoneal injection. The induction occurred on

days 0, 14, and 28 [22,23]. Termination and serum sampling occurred 14 days following the third induction. The sample size for this investigation was determined using the degrees of freedom calculation, supplemented by a 10% correction factor to account for potential attrition, yielding 6 mice per group and a total of 18 mice for the study [24].

2.6. Sample isolation

Fourteen days post-third induction, mice were euthanized. Intraperitoneal anesthesia was administered using a combination of ketamine at a dosage of 100 mg/kg and xylazine at 10 mg/kg [25]. After the administration of anesthesia, mice were dissected in the abdominal region until the peritoneum was revealed. Blood was collected through cardiac puncture with a syringe and subsequently transferred to an Eppendorf tube. Sample preparation adhered to the protocols specified in the ELISA kit manual. Blood samples were incubated at room temperature for 10–20 min prior to centrifugation at 3000 rpm for 20 min. The supernatant may be utilized for testing immediately or preserved at −20 ℃ for one month or at −80 ℃ for three months.

2.7. Measurement of immunoglobulin levels in serum using the ELISA method

2.7.1. Immunoglobulin G level measurement methods

IgG levels were measured using the competitive ELISA method. Before initiating the test, serum samples and the ELISA kit, which were stored in the refrigerator, were permitted to acclimate to room temperature. The reagents were carefully prepared following the instructions outlined in the ELISA kit; 120 μL of standard diluent reagent was added into an Eppendorf labeled 5, contents were homogenized, and then 120 μL of the resulting mixture from Eppendorf 5 was transferred to Eppendorf 4, continuing this process as required. To create a 25× dilution of the wash buffer concentrate, 20 mL of the concentrate was combined with 500 mL of double-distilled water to achieve the final solution. The next phase entailed organizing the ELISA mapping to determine the necessary quantity of strips. First, 50 μL of standard was added to each well. Next, 50 μL of the sample was added to the specified sample wells. Finally, 50 μL of biotinylated antigen was added to each well. A sealer was administered and incubation was performed for 60 min at 37 ℃. The sealer was then removed, the liquid was discarded, the wells were washed three times with the buffer, and then the plate was dried. Following the drying process, 50 μL of avidin-HRP was added into each well; wells were sealed and incubated at 37 ℃ for 60 min. The sealer was removed, and wells were cleaned three more times. Then, 50 μL of substrate A and 50 μL of substrate B were added to each well. A sealer was applied, and wells were incubated for 10 min at 37 ℃ while ensuring no exposure to light. 50 μL of stop solution was added to each well, which caused a color change from blue to yellow. The optical density of each well was assessed with the ELISA reader at a wavelength of 450 nm within 10 min after the addition of the stop solution. The IgG1 ELISA kit from BT-LAB (catalog number: EA0027Mo) was employed in this study.

2.7.2. Immunoglobulin G1 and immunoglobulin G2α measurement methods

Levels of IgG1 and IgG2a were quantified utilizing the sandwich ELISA technique. The preparation of reagents started with the placement of all components from the ELISA kit at room

temperature, specifically within the range of 18–25 ℃. The necessary preparations involved the wash buffer, standard working solution, biotinylated detection antibody working solution, and HRP conjugate working solution. Each standard, blank, and sample was introduced into the respective well at a volume of 100 μL and incubated for 90 min at 37 ℃. Following the incubation period, the liquid was removed, and 100 μL of the biotinylated detection antibody working solution was introduced to each well, with a subsequent incubation of 60 min at 37 ℃. The liquid was removed, and the plate underwent washing by adding 350 μL of wash buffer, repeating this procedure three times. Following the washing step, 100 μL of the HRP conjugate working solution was introduced, and the mixture was incubated for 30 min at 37 ℃. Upon completion of the incubation period, the liquid was removed, followed by five washing cycles. Following the fifth wash, 90 μL of substrate reagent was introduced to each well, and the plate was incubated for 15 min at 37 ℃. The subsequent procedure involved the addition of 50 μL of stop solution. The concluding phase required the utilization of an ELISA reader to analyze the results at a wavelength of 450 nm. The IgG1 ELISA kit and the IgG2a ELISA kit utilized in this study were sourced from Elabscience (catalog numbers: E-EL-M3035 and E-EL-M0694).

2.8. Statistical analysis

The Shapiro–Wilk normality test was employed to evaluate the data due to its sample size being less than 50. The Levene's test was employed to assess the homogeneity of the data. The one-way ANOVA test is applicable when the data is normally distributed and homogeneous. Statistical analyses were conducted utilizing SPSS v26 software. The statistical results were presented in a table and bar chart.

3. Results

The Shapiro–Wilk normality test showed that the IgG, IgG1, and IgG2a concentration data for K1, K2, and K3 were normally distributed, with p-values higher than 0.05 ($p > 0.05$). The Levene's homogeneity test showed that the IgG, IgG1, and IgG2a concentration data were homogeneous (p-value for all variables higher than 0.05). As normality and homogeneity tests proved that the data were normally distributed and homogeneous, statistical analysis could proceed with a one-way ANOVA. Details for the normality and homogeneity tests are available in Tables S1–S4.

In this study, significant results were obtained in IgG and IgG1. This is evidenced by the p-value of 0.00 ($p < 0.05$) in the one-way ANOVA. Regarding the IgG variable, the control group presented a mean and standard deviation of 2722.43 ± 947.29, which were 4910.65 ± 616.13 for the antigen group. Regarding the IgG1 variable, the mean and standard deviation were 662.82 ± 43.91 for the control group and 736.24 ± 22.00 for the antigen group. The ANOVA test results for the IgG and IgG1 variables showed significant differences between the control group and the group that received the antigen (IgG $p = 0.000$; IgG1 $p = 0.009$). Significant differences were also found between the control group and the adjuvant group (IgG $p = 0.02$; IgG1 $p = 0.02$). The ANOVA test between the adjuvant group and the antigen group showed no significant differences between the two groups (IgG $p = 0.124$; IgG1 $p = 0.85$). As for IgG2a, no significant differences were found between groups; however, the highest mean was obtained for the antigen group at 34.84 ± 0.45. These results suggest that while both adjuvant and antigen groups induce immune responses compared to the control, the antigen group did not significantly outperform the adjuvant alone in this setting. This may indicate that the observed effect

could partially be attributed to the adjuvant. Detailed values of each parameter and group can be seen in Table 1. Visualization of the average of each parameter from each group can be seen in Figure 1.

Table 1. One-way ANOVA result test.

		IgG (ng/mL)			
Groups	Mean ± SD	SE	Min.	Max.	95% CI
PBS	2722.43 ± 947.29	386.73	1790.87	4197.72	1728.31–3716.56
Adjuvant	4107.83 ± 1060.84	433.09	2942.97	5851.71	2993.75–5220.32
Pili	4910.65 ± 616.13	251.53	4140.68	5737.64	4264.06–5557.23
		IgG1 (ng/mL)			
Groups	Mean ± SD	SE	Min.	Max.	95% CI
PBS	662.82 ± 43.91	17.92	614.94	713.59	616.75–708.90
Adjuvant	733.94 ± 30.93	12.63	693.58	773.61	701.48–766.40
Pili	736.24 ± 22.00	8.98	710.49	774.65	713.16–759.33
		IgG2a (ng/mL)			
Groups	Mean ± SD	SE	Min.	Max.	95% CI
PBS	34.24 ± 0.93	0.38	32.91	35.13	33.27–35.22
Adjuvant	33.97 ± 0.61	0.25	33.34	35.11	33.35–34.61
Pili	34.84 ± 0.45	0.20	34.28	35.56	34.32–35.36

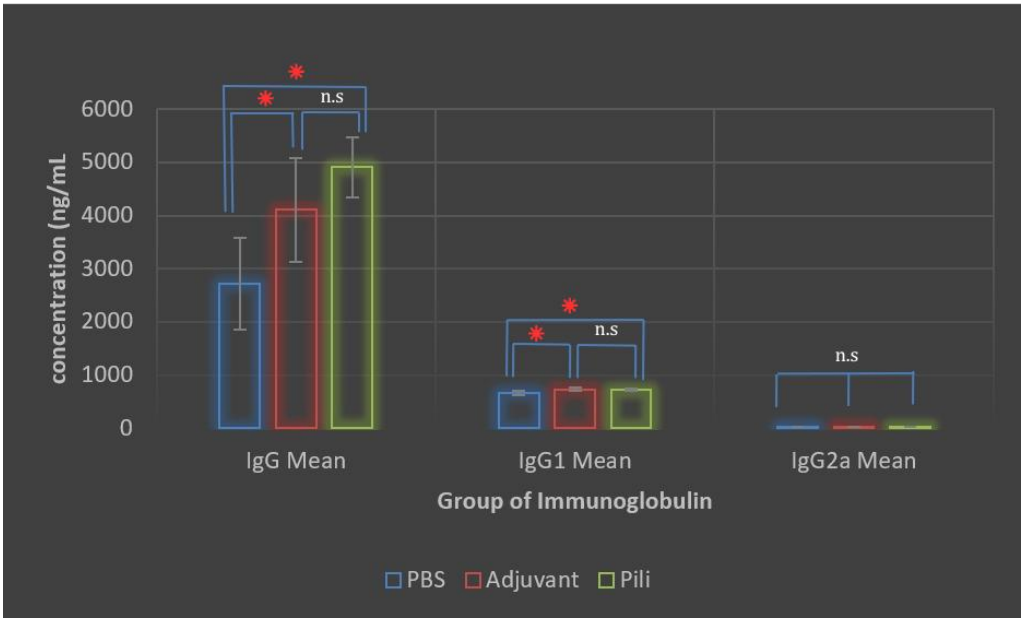

Figure 1. Bar chart showing mean ± SEM serum levels of IgG, IgG1, and IgG2a in the PBS, adjuvant, and pili groups. Statistical comparisons were performed using ANOVA and Tukey's post hoc test. Significant increases in IgG and IgG1 levels were observed in both the adjuvant and pili groups compared to the PBS group. No significant differences were found between the adjuvant and pili groups. (red asterisk mark*: Significant at $p < 0.05$; n.s.: Not significant).

4. Discussion

This study demonstrated a significant increase in IgG and IgG1 levels in mice following induction with *K. pneumoniae* pili. The elevation of IgG and IgG1 levels in the treatment group resulted from the administration of *K. pneumoniae* pili protein 65.5 kDa, which has the capacity to stimulate the host immune system. *K. pneumoniae* pili play a crucial role in the initial stages of infection by facilitating adhesion to host cells. *K. pneumoniae* pili proteins interact with macrophages, which function as antigen-presenting cells (APCs). These will display antigens via the major histocompatibility complex II (MHC II), subsequently presenting these antigens to T-helper 2 (Th2) cells. Th2 stimulates B cell differentiation into plasma cells that produce IgG1, a significant component of the IgG subclass [26–28].

Immunoglobulin G (IgG) in mice consists of several subclasses, namely IgG1, IgG2a, IgG2b, and IgG3. IgG1 is the most dominant subclass, with a proportion reaching 50% in BALB/c mice. IgG2a and IgG2b each have a proportion of about 20%, while IgG3 is about 10% [29]. Through this mechanism, *K. pneumoniae* pili proteins can induce the formation of an immune response in the host, which mainly consists of IgG1 [30]. The results of this study are consistent with the research by Agustina (2017), which showed that subcutaneous induction of *K. pneumoniae* pili protein in BALB/c mice can induce the formation of IgG immunoblotting. The 65.5 kDa pili protein of *K. pneumoniae* has been identified as antigenic through western blot analysis using IgG serum of mice induced intraperitoneally [31]. Research by Uta (2024) also showed that *K. pneumoniae* pili protein with a molecular weight of 65.5 kDa can increase IL-4 levels in the spleen of BALB/C mice [32]. The results of this study support the theory that Th2 produces IL-4, which can stimulate B cells to differentiate into IgG1-producing plasma cells [33]. IgG2a was selected as the research variable to confirm that IgG1, produced by the Th2 immune response, exhibits antagonistic properties against Th1, which is responsible for the production of IgG2a. Complete Freund's adjuvant contains activated mycobacterium, while incomplete Freund's adjuvant consists only of an oil and water emulsion without mycobacterium. The administration of complete Freund's adjuvant and incomplete Freund's adjuvant has a significant role in immune system activation. Freund's adjuvant stimulates Th2 induction, which can induce the differentiation of B cells into IgG1-producing plasma cells. Adjuvants are administered to trigger a vaccine response in the population, thereby creating a protective immune state [33–36].

IgG2a levels exhibited no significant differences among the groups, as the immune response in this study was primarily Th2-dominated. *K. pneumoniae* is recognized as an extracellular pathogen. The immune response to extracellular pathogens is primarily mediated by Th2 cells [37–39]. IL-4 is an anti-inflammatory cytokine synthesized by Th2 cells, which can promote the production of IgG1. IL-4 inhibits the expression of TBX21, a regulatory gene for transcription factors critical in Th1 differentiation. This inhibition results in reduced IFN-γ production, which may influence IgG2a levels [33,40–42]. The findings align with the study by Agustina, et al. (2023), which demonstrated that the *K. pneumoniae* pili protein, with a molecular weight of 65.5 kDa, did not elevate IFN-γ levels in mouse serum [20]. Sa'adah, et al. (2024) found that the 65.5 kDa *K. pneumoniae* pili protein did not elevate IFN-γ levels in the spleen of mice [43]. Furthermore, Z. Chen, et al. (2024) demonstrated that the *K. pneumoniae* trivalent vaccine primarily elicited a Th2 response, marked by a significant elevation in IgG1 levels [44]. Babu, et al. (2024) demonstrated that the *K. pneumoniae* outer membrane protein vaccine primarily induces a Th2 response, marked by a significant increase in IgG1 [45]. The

findings of Hussein, et al. (2018) indicated that the outer membrane proteins OmpK17 and OmpK36 are associated with a predominance of Th2 responses [46].

The low house temperature (<22 ℃) observed in this study may induce cold stress, potentially leading to an increase in norepinephrine levels. Elevated norepinephrine levels can induce a transition in the immune response from Th1 to Th2, resulting in a preference for IgG1 production over IgG2a in mice [33,40,47,48]. Research by Bucsek, et al. (2017) indicated that mice housed in experimental animal facilities at 22 ℃ exhibit significantly higher norepinephrine levels compared to those maintained at 30 ℃ [49]. Estrada, et al. (2016) demonstrated that elevated norepinephrine levels can decrease cytokine production by type 1 helper T cells [50]. The reduction in IgG2a levels may be affected by the 14-day interval between the final injection and serum collection, given that the half-life of IgG2a is approximately 4–5 days [51].

This study demonstrated the immunogenic potential of the 65.5 kDa pili protein, as evidenced by the induction of IgG, IgG1, and IgG2a antibodies in BALB/c mice. The protein was purified using a denaturation-based method (TCA precipitation, SDS-PAGE, and dialysis), which may affect its native conformation and antigenicity; although non-denaturing approaches such as native-PAGE would be preferable, access to such methods is limited in our region. The purification procedure was carefully controlled to minimize potential structural loss, and the observed antibody response suggests that key immunoreactive epitopes were retained. We also acknowledge the absence of *in vitro* neutralization assays, such as bacterial adhesion inhibition, which are important for evaluating the functional capacity of the antibodies. Due to limitations in both time and funding, these assays could not be included in the present study. Future research should incorporate native protein purification methods and *in vitro* neutralization assays to more comprehensively assess the immunogenicity and protective potential of the pili protein.

5. Conclusions

The 65.5 kDa pili protein of *K. pneumoniae* exhibits considerable promise as a vaccine candidate by markedly increasing IgG and IgG1 levels, indicating a vigorous humoral immune response in BALB/c mice. Although IgG2a levels were unchanged, the targeted immune activation underscores the protein's function in facilitating a Th2-biased response. The findings establish a potential basis for the advancement of a targeted vaccination against *K. pneumoniae*, responding to the pressing necessity for effective preventative tactics against this multidrug-resistant organism.

Use of AI tools declaration

The authors declare they have not used Artificial Intelligence (AI) tools in the creation of this article.

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Conflict of interest

The authors declare no conflict of interest.

Author contributions

All authors made substantial contributions to the research. Dini Agustina was accountable for ideation, methodological design, data collection, analysis, paper preparation, and final endorsement. Diana Chusna offered oversight, project management, thorough text evaluation, and final endorsement. Yunita Armiyanti facilitated resource procurement, oversaw laboratory operations, and ensured data verification. M. Ali Shodikin conducted statistical analysis, interpreted data, and performed critical article review. Enny Suswati offered oversight, help on experimental design, and manuscript editing. Muhammad Farhan Hibatulloh participated in experimental protocols, data acquisition, and preliminary data analysis. Shafrizal Aufal Ikhsani provided assistance with laboratory activities, sample processing, and technical support. Virly Kania Mitananda executed laboratory experiments, data input, and preliminary analysis, whilst Priska Liana Lutianto oversaw data organization, did the literature review, and edited the report. All authors reviewed and endorsed the final version of the text.

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