Pharmacy Education (2023) 23(4) 304 - 310
https://doi.org/10.46542/pe.2023.234.304310

ICMHS 2022 SPECIAL EDITION

RESEARCH ARTICLE

Increased levels of IL-10 in the spleen after induction of pili protein 65.5 kDa *Klebsiella pneumoniae*

Dini Agustina[1], Diana Chusna Mufida[1], Enny Suswati[1], M. Ali Shodikin[1], Bagus Hermansyah[3], Ajeng Samrotu Sa'adah[2], Tio Wisnu Pradana Putra[2], Samudra Ayu[2]

[1] Department of Microbiology, Faculty of Medicine, Universitas Jember, Jember, Indonesia
[2] Faculty of Medicine, Universitas Jember, Jember, Indonesia
[3] Department of Parasitology, Faculty of Medicine, Universitas Jember, Jember, Indonesia

Keywords
IL-10
Klebsiella pneumoniae
Protein pili
Spleen

Correspondence
Dini Agustina
Department of Microbiology
Faculty of Medicine
Universitas Jember
Jember
Indonesia
dini_agustina@unej.ac.id

Abstract

Background: One of the gram-negative bacteria often found as the cause of nosocomial infections is *Klebsiella pneumoniae*. This bacterium has a mortality rate of 28.3% due to ESBL strains that cause resistance to several antibiotics and the absence of a vaccine as a preventive measure. **Objective:** To determine the level of IL-10 in the spleen after induction of the protein pili *K. pneumoniae* 65.5 kDa in mice. **Method:** This study used mice spleen samples from 21 mice aged six to eight weeks in an experimental investigation with a randomised posttest-only control group design. There are three groups, K1 as a PBS-given control, 65.5 kDa antigen protein pills + Freund's adjuvants are given to K2, and Freund's adjuvants are given to K3. Interleukin-10 concentrations were measured using the ELISA and analysed by a one-way ANOVA assay. **Result:** The research showed significant differences in each group at IL-10 levels after administration of the pili protein *K. pneumoniae* using one-way ANOVA (p = 0.036). The treatment group had the highest average levels of IL-10, so there was an increased association between exposure to pili protein and IL-10 levels in rats. A significant increase in IL-10 levels was found in the treatment group compared to the control and adjuvant groups. **Conclusion:** Induction of protein pili *Klebsiella pneumoniae* 65.5 kDa increases IL-10 levels in spleen mice.

Introduction

Klebsiella pneumoniae is a rod-shaped gram-negative bacterium that is opportunistically pathogenic. This bacterium is the leading cause of healthcare-related (nosocomial) infections, primarily affecting individuals with low immune systems or suffering from concurrent bacterial infections. However, with the dramatic increase in hyper-virulence strains and the emergence of new strains resistant to antibiotics, the mortality rate and incidence of infections due to these bacteria are increasing, especially in low- and middle-income countries, one of the cases whose main cause of this bacterium is neonatal sepsis. These microorganisms account for neonatal sepsis in 4-9% of cases in developed countries and 16-28% in developing countries. In addition to hypervirulence strains, in K. *pneumoniae*, there is also a "classic" strain (classic K. pneumoniae/c-KP). This non-virulent strain is usually associated with pneumonia, urinary tract infections, nosocomial infections, and neonatal sepsis in immunocompromised patients. C-KP can also cause neonatal sepsis outbreaks in hospitals and ICUs (Camacho-Gonzalez *et al.,* 2013; Shon *et al.,* 2013; Haller *et al.,* 2015; Zea-Vera & Ochoa, 2015; Khaertynov *et al.,* 2016; Verma *et al.,* 2017; Khaertynov *et al.,* 2018; Arato *et al.,* 2021).;

A risk factor for neonatal sepsis is ineffective therapy in nosocomial infections due to antibiotic resistance (Al-Hasan *et al.,* 2011; Weiner, 2016). Antibiotic resistance of the *K. pneumoniae* strain is associated primarily with the Enzyme Extended Spectrum β-Lactamase (ESBL) production. In recent decades,

isolates of K. pneumoniae ESBL have been found worldwide, especially in intensive care units (ICUs). The prevalence of ESBL-producing strains of K. *pneumoniae* is 23% in the US and up to 85-100% in some European countries (The state of the World's Antibiotics, 2015 - Center for Disease Dynamics, Economics & Policy (CDDEP), n.d.). In 2017 the World Health Organisation included ESBL-producing K. *pneumoniae* in its list of the most dangerous superbugs along with acinetobacter baumani and Pseudomonas aeruginosa (WHO Releases List of World's Most dangerous superbugs - scientific American, n.d.). The dramatic increase in microbial resistance to antimicrobial agents and the lack of new antimicrobials have limited therapeutic options against these pathogens, further exacerbating the disease burden and resulting in an increase in morbidity and mortality (Assoni *et al.,* 2021; Choi et al., 2020). The right solution to solve this problem is a vaccine.

Vaccines can reduce the incidence of infection, reducing the need for antimicrobials and not being affected by antimicrobial resistance mechanisms. No vaccine has been licensed by the Food and Drug Administration (FDA) (Choi et al., 2020; Lundberg et al., 2013a). Although there is a lot of evidence that supports the protection of antibodies against polysaccharide capsules, the large number of types causes vaccines from this virulence factor to be less feasible, so it is necessary to develop vaccines from other virulence factors such as fimbriae/pili (Arato et al., 2021; Choi et al., 2020; Lundberg et al., 2012). Our previous research showed that the pili protein K. *pneumoniae* 65.5 kDa exhibits immunogenic properties, which can hypatinate erythrocytes and can act as adhesin (Agustina *et al.,* 2014, 2020; Kurniawati *et al.,* 2021; Thanassi *et al.,* 2012), as well as being able to induce cytokines as a sign of the emergence of an immune response (in editing). The cytokine used in this study was interleukin-10 (IL-10). Interleukin-10 is an anti-inflammatory cytokine produced during a bacterial infection, acting as an immunomodulator that affects the innate and adaptive immune systems. These cytokines are necessary for bacterial clearance, reduction of tissue damage, and survival of hosts (; Duell et al., 2012; Ng et al., 2013; Peñaloza et al., 2018; Decker *et al.,* 2019).).

In addition to IL-10, this study also used the spleen; in contrast to previous studies, the examination of serum shows that pili protein 65.5 kDa K. *pneumoniae* decreases the IL-10 level (Agustina *et al.,* 2022). IL-10 functions as a mediator of immune responses, with NK (Natural Killer) cell intermediaries as the leading primary producers of IL-10. This immune reaction can be measured by looking at increased levels of Interleukin-10 with an Enzyme-linked immunosorbent assay (ELISA). Based on the above statement, researchers want to determine the levels of IL-10 in the spleen after the induction of the pili protein K. *pneumoniae* 65.5 kDa in mice.

Methods

Design

This study is purely experimental, with a post-control-only group design and random sampling techniques. In this study, 21 mice spleen samples aged 6-8 weeks with a body weight of approximately 25 grams were used, which were divided into three groups. The first group was the control group with Phosphate Buffer Saline (PBS) administration, the second group was the treatment group with pili protein administration, and the third group was the adjuvant group with the administration of CFA (Complete Freund's Adjuvant) and IFA (Incomplete Freund's Adjuvant). The K. *pneumoniae* bacteria used are the culture stocks of the microbiology laboratory, Faculty of Medicine, University of Jember.

This study's working procedure consisted of identifying the K. *pneumoniae* 65.5 kDa pili protein using SDS-PAGE electrophoresis based on the Laemmli method (Blancher & Jones, 2003; Brunelle & Green, 2014; Laemmli, 1970; SCHÄGGER, 2003). After identification of the desired protein band, propagation and purification are then carried out and measured using the Kingsley method (Kingsley, 1939; Sukarjati *et al.,* 2018). The purified protein was induced intraperitoneally in group 2 mice three times with an interval of 14 days. The adjuvant that was first given was CFA, then as a booster, was given IFA. Mice are terminated and taken spleen after 14 days of administration of the second booster (Care & Committee, 2017; Greenfield, 2020, 2019). The prepared spleen is washed, crushed, and homogenised with PBS; the last step is centrifugation in 2000-3000RPM for 20 minutes.

Assessment

IL-10 measurements were carried out by the ELISA method with the Bioassay Technology Laboratory kit. The measurement results are then analysed using one-way ANOVA (CUSABIO, n.d.; Setiawan & Nugraha, 2016). The steps in the study can be seen in Figure 1.

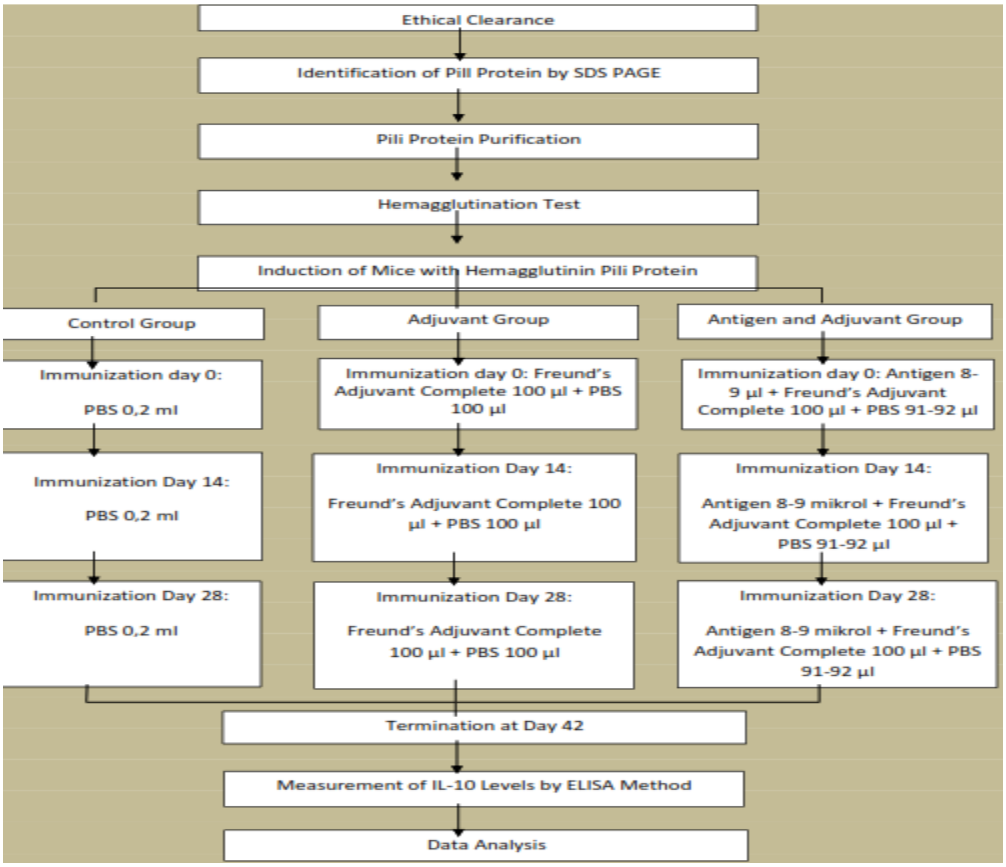

Figure 1: Research procedure

Results

In the Sodium Dodecyl Sulphate Poly Acrylamide Gel Electrophoresis (SDS-PAGE) gel, the thickest protein. The band is a protein band with a molecular weight of 65.5 kDa (see Figure. 2), while the results of measuring the purified protein reach 0.65 g/dl. After the induction procedure and preparation of the spleen sample, standardisation is conducted first before measurements with ELISA, with the results seen in Figure 3.

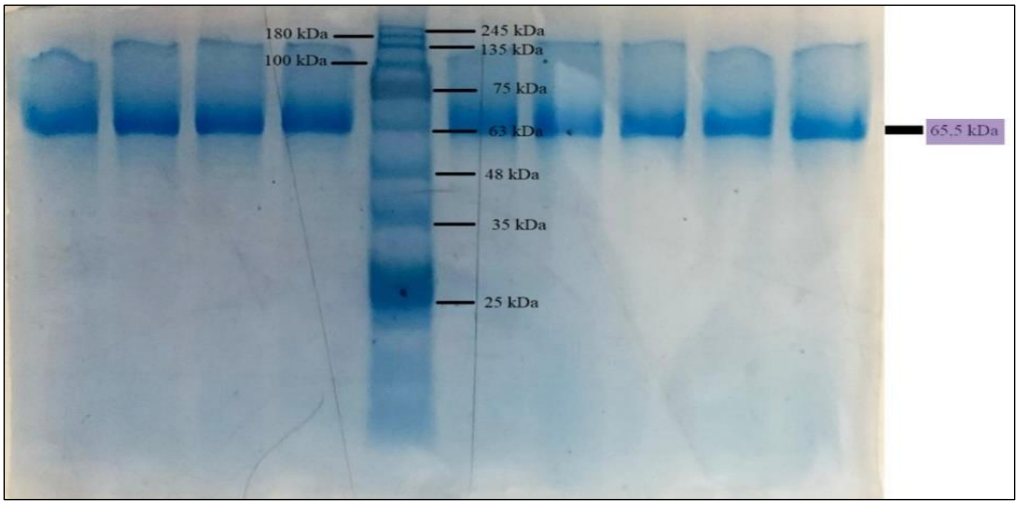

Figure 2: Identification of 65.5 kDa protein with SDS-PAGE electrophoresis

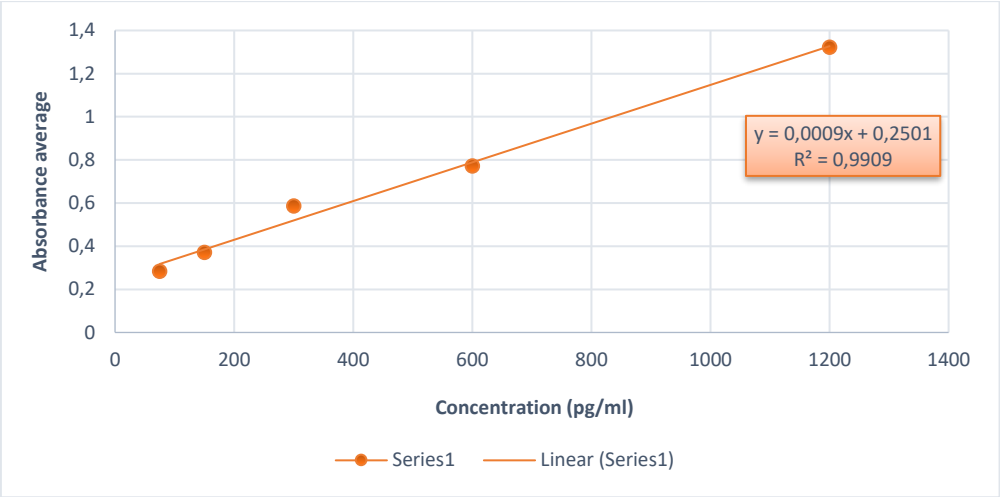

Figure 3: IL-10 standard curve results (for ELISA)

The results of measuring IL-10 levels using ELISA can be seen in Figure 4. The figure showed an increase in IL-10 levels in the treatment group given 65.5 kDa pili protein *K. pneumoniae*, with mean and standard deviation for each group shown in Table I. In addition, there were significant differences in each group at IL-10 levels after administration of the pili protein *K. pneumoniae* using one-way ANOVA (p = 0.036). The treatment group had the highest mean IL-10 levels, so there was an increased relationship between exposure to pili protein and IL-10 levels in mice. A significant increase in IL-10 levels was found in the treatment group compared to the control group. It was also found in the treatment group compared to the adjuvant group (see Table II).

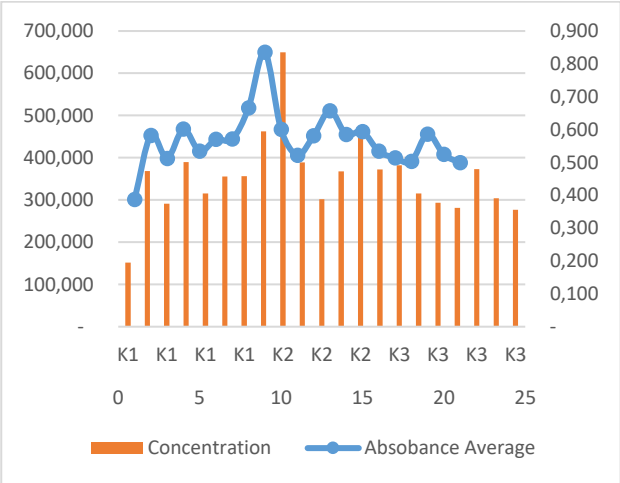

Figure 4: Results of measuring IL-10 levels with ELISA

Table I: Mean and standard deviation of each group

	N	Mean	Standard deviation
Control	7	318.46032	80.506918
Adjuvant + Antigent	7	427.82540	111.899846
Adjuvant	7	317.98413	42.921664
Total	**21**	**354.75661**	**95.166181**

Table II: Result of One-Way ANOVA

	Sum of squares	Df	Mean square	F	Sig.
Between groups	56060.788	2	28030.394	4.034	0.036
Within groups	125071.252	18	6948.403		
Total	**1811132.040**	**20**			

Discussion

Klebsiella pneumoniae infection, which causes an increase in morbidity and mortality so that it becomes a heavy burden on the economy, especially in developing countries, causes the development of vaccines to prevent this infection to be necessary. The development of the *Klebsiella* vaccine has focused on five virulence factors: capsules, lipopolysaccharide (LPS), siderophores, adhesions, and exotoxin. Among the five types of vaccines that protein-based vaccines focus on, immunogenic proteins are outer membrane proteins and fimbriae/pili proteins (Lundberg *et al.*, 2013b). The pili protein *K. pneumoniae* generated a host immune response shown by an increase in mean

levels of IL-10 in the treatment group, which exceeded the mean value of the control group. This can happen because the pili protein triggers T cells and B cells to opsonize the pili protein and causes an increase in levels of IFN (Interferon) as a pro-inflammatory. Humoral responses (antibodies) are important in controlling *K. pneumoniae* infection. In mice that have been infected with *K. pneumoniae,* anti-K antibodies pneumoniae mice cause a protective response in the form of helper T cells that eventually become IL-10 (Pennini et al., 2017). Infection is considered non-existent if there is no increase in IL-10 levels, and the administration of the pili protein K. *pneumoniae* as a trigger for the formation of the immune system is considered to have failed (Sudiono, 2014).

The Post Hoc test showed significant differences in the treatment group compared to the adjuvant group. This result is because the immunogenic levels of pili proteins are much higher than adjuvants. Pili protein triggers the immune system specifically by forming an anti-K. *pneumoniae*. Adjuvants using Freund's adjuvant trigger the immune system thoroughly and nonspecifically. This makes the difference in mean IL-10 levels between the treatment and adjuvant groups. There is a significant difference (Gregoriadis *et al.,* 2018).

Another study by Won-Hee Lee (2015) obtained a significant increase in IL-4 levels in a family with IL-10. IL-4 functions as an anti-inflammatory, producing memory B cells that will be circulated throughout the body. Won-Hee Lee's study used extracellular vesicles from *K. pneumoniae* injected intra-peritoneally into mice to find the best vaccine candidate. However, in this study, the mortality rate from mice was still too high with the administration of a lethal dose of *K. pneumoniae* (1x108 CFU), so further research is still needed (Lee *et al.,* 2015). A similar study by Gaowei Hu (2022) compared the effectiveness of the administration of *K. pneumoniae* by IWB (inactivated whole bacteria) with the outer membrane phosphoporin with 3x intraperitoneal administration. This study used a strain with a molecular weight of 38 kDa and obtained an increase in IL-4 3x higher than normal, and immunised mice were given *K. pneumoniae* at a lethal dose. IL-4 is still a family with IL-10 with almost the same effect. Mice immunised with IWB and outer membrane phosphoporin have a high survival rate with minimal damage to mice. This research proves outer membrane protein be used as a vaccine candidate (Hu *et al.,* 2022).

Previous studies on mice serum proved that 65.5 kDa *K. pneumoniae* protein pills that lowered IL-10 levels when administered together with the Freund adjuvant did not prove to be the expected immunogens compared to the control group (Agustina *et al.,* 2022). So, the results of this study can contribute to efforts to make protein-based vaccines against K. *pneumoniae*, especially in proving the ability of these proteins to induce cytokines, especially IL-10.

The limitation of this study was not differentiating doses between and within the group and not conducting a microscopic assessment to see whether there was damage and specific protein expression in spleen mice infected by *Klebsiella pneumoniae*.

Conclusion

Induction of the protein pili *Klebsiella pneumoniae* 65.5 kDa intraperitoneally increases the levels of IL-10 spleen mice.

Acknowledgement

We thank the Faculty of Medicine at the University of Jember for the support of our research.

Source of funding

This research was conducted on a 2021 Keris group grant by the University of Jember.

References

Agustina, D., Aisyah, S. M., Sutejo, I. R., & Mufida, D. C. (2020). Characterization of pili protein with the molecular mass of 85 kDa Escherichia coli as an adhesin and a hemagglutinin. *JKKI : Jurnal Kedokteran Dan Kesehatan Indonesia*, **11**(3), 241–249. https://doi.org/10.20885/JKKI.VOL11.*ISS3*.ART5

Agustina, D., Sumarno, & Noorhamdani. (2014). Inhibition of Klebsiella pneumoniae adhesion in mice enterocytes by antibodies of hemagglutinin pili protein with MW 12.8 kDa of Klebsiella pneumoniae.*Journal of Tropical Life Science*, **4**(1), 19–25.

Agustina, D., Wati, M. L., Wisudanti, D. D., Shodikin, M. A., Mufida, D. C., & Suswati, E. (2022). Pili protein 65.5 kDa of Klebsiella pneumoniae induced a decrease in IL-10 in mice. *Majalah Kedokteran Bandung,* **54**(3), 143–147. https://doi.org/10.15395/mkb.v54n3.2690

Al-Hasan, M. N., Huskins, W. C., Lahr, B. D., Eckel-Passow, J. E., & Baddour, L. M. (2011). Epidemiology and outcome of Gram-negative bloodstream infection in children: A population-based study. *Epidemiology and Infection*, **139**(5), 791–796. https://doi.org/10.1017/S0950268810001640

Arato, V., Raso, M. M., Gasperini, G., Scorza, F. B., & Micoli, F. (2021). Prophylaxis and treatment against Klebsiella pneumoniae: Current insights on this emerging antimicrobial resistant global threat. *International Journal of Molecular Sciences,* **22**(8). https://doi.org/10.3390/IJMS22084042

Assoni, L., Girardello, R., Converso, T. R., & Darrieux, M. (2021). Current stage in the development of Klebsiella pneumoniae vaccines. *Infectious Diseases and Therapy,* **10**(4), 2157–2175. https://doi.org/10.1007/s40121-021-00533-4

Blancher, C., & Jones, A. (2003). SDS-PAGE and Western Blotting Techniques. In *Metastasis research protocols* (Vol. 57, pp. 145–162). Humana Press. https://doi.org/10.1385/1-59259-136-1:145

Brunelle, J. L., & Green, R. (2014). One-dimensional SDS-polyacrylamide gel electrophoresis (1D SDS-PAGE). *Methods in Enzymology,* **541**, 151–159. https://doi.org/10.1016/B978-0-12-420119-4.00012-4

Camacho-Gonzalez, A., Spearman, P. W., & Stoll, B. J. (2013). Neonatal infectious diseases: Evaluation of neonatal sepsis. *Pediatric Clinics of North America*, **60**(2), 367–389. https://doi.org/10.1016/J.PCL.2012.12.003

Care, I. A., & Committee, U. (2017). Policy for adjuvant use with special emphasis on complete freund's adjuvant. UNMC animal care and use program, 1–5.

Choi, M., Hegerle, N., Nkeze, J., Sen, S., Jamindar, S., Nasrin, S., Sen, S., Permala-Booth, J., Sinclair, J., Tapia, M. D., Johnson, J. K., Mamadou, S., Thaden, J. T., Fowler, V. G. J., Aguilar, A., Terán, E., Decre, D., Morel, F., Krogfelt, K. A., Tennant, S. M. (2020). The diversity of lipopolysaccharide (O) and capsular polysaccharide (K) antigens of invasive Klebsiella pneumoniae in a multi-country collection. *Frontiers in Microbiology*, **0**, 1249. https://doi.org/10.3389/FMICB.2020.01249

CUSABIO. (n.d.). Mouse interleukin 10 (IL-10) ELISA Kit. Catalog Number. CSB-E04608m, **17**, 1–14.

Decker, M. D., Greenberg, D. P., Johnson, D. R., & Pool, V. (2019). Randomised study of immune responses to two Tdap vaccines among adolescents primed with DTaP compared with results among adolescents primed with DTwP. Vaccine, **37**(35), 5003–5008. https://doi.org/10.1016/J.VACCINE.2019.07.015

Duell, B. L., Tan, C. K., Carey, A. J., Wu, F., Cripps, A. W., & Ulett, G. C. (2012). Recent insights into microbial triggers of interleukin-10 production in the host and the impact on infectious disease pathogenesis. *FEMS Immunology and Medical Microbiology*, **64**(3), 295–313. https://doi.org/10.1111/j.1574-695X.2012.00931.x

Greenfield. (2020). Standard immunisation of mice, rats, and hamsters. Cold Spring Harb Protocol, **3**(2).

Greenfield, E. A. (2019). Preparing and using adjuvants. Cold Spring Harbor Protocols, **2019**(1), 73–78. https://doi.org/10.1101/pdb.prot100214

Gregoriadis, G., Popovic, M. O., Centar, L., & Perrie, Y. (2018). Vaccine adjuvants preparation methods (Issue February). https://doi.org/10.1385/1-59259-083-7

Haller, S., Eller, C., Hermes, J., Kaase, M., Steglich, M., Radonić, A., Dabrowski, P. W., Nitsche, A., Pfeifer, Y., Werner, G., Wunderle, W., Velasco, E., Sin, M. A., Eckmanns, T., & Nübel, U. (2015). What caused the outbreak of ESBL-producing Klebsiella pneumoniae in a neonatal intensive care unit, in Germany from 2009 to 2012? Reconstructing transmission with epidemiological analysis and whole-genome sequencing. BMJ Open, **5**(5), e007397. https://doi.org/10.1136/BMJOPEN-2014-007397/-/DC1

Hu, G., Chen, X., Chu, W., Ma, Z., Miao, Y., Luo, X., & Fu, Y. (2022). Immunogenic characteristics of the outer membrane phosphoporin as a vaccine candidate against Klebsiella pneumoniae. Veterinary Research, **53(**1), 5. https://doi.org/10.1186/s13567-022-01023-2

Khaertynov, K. H. S., Anohin, V. A., Nikolaeva, I. V., Semenova, D. R., Lyubin, S. A., Agapova, I. V., Muginova, A. I., & Khasanova, G. R. (2016). Neonatal sepsis caused by Klebsiella. *Medical News of North Caucasus,* **11**(1), 82–86. https://doi.org/10.14300/MNNC.2016.11004

Khaertynov, K. S., Anokhin, V. A., Rizvanov, A. A., Davidyuk, Y. N., Semyenova, D. R., Lubin, S. A., & Skvortsova, N. N. (2018). Virulence factors and antibiotic resistance of Klebsiella pneumoniae strains isolated from neonates with sepsis. *Frontiers in Medicine,* **5(**AUG), 225. https://doi.org/10.3389/FMED.2018.00225/BIBTEX

Kingsley, G. R. (1939). The determination of serum total protein, albumin, and globulin by the biuret reaction. *Journal of Biological Chemistry*, **131**(1), 197–200. https://doi.org/10.1016/S0021-9258(18)73494-7

Kurniawati, L. R., Shodikin, M. A., Agustina, D., & Sofiana, K. D. (2021). Protein pili 96,4 kDa Klebsiella pneumoniae sebagai protein hemaglutinin and. *Indonesian Journal for Health Sciences*, **5**(1), 25. https://doi.org/10.24269/ijhs.v5i1.2700

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 1970 227:5259, **227**(5259), 680–685. https://doi.org/10.1038/227680a0

Lee, W. H., Choi, H. Il, Hong, S. W., Kim, K. S., Gho, Y. S., & Jeon, S. G. (2015). Vaccination with Klebsiella pneumoniae-derived extracellular vesicles protects against bacteria-induced lethality via both humoral and cellular immunity. *Experimental & Molecular Medicine,* **47**(9). https://doi.org/10.1038/EMM.2015.59

Lundberg, U., Senn, B. M., Schuler, W., Meinke, A., & Hanner, M. (2012). Identification and characterisation of antigens as vaccine candidates against *Klebsiella pneumoniae. Human* Vaccines & Immunotherapeutics, **9**(3), 497–505. https://doi.org/10.4161/HV.23225

Lundberg, U., Senn, B. M., Schuler, W., Meinke, A., & Hanner, M. (2013a). Identification and characterisation of antigens as vaccine candidates against Klebsiella pneumoniae. Human Vaccines & Immunotherapeutics, 9(3), 497–505. https://doi.org/10.4161/HV.23225

Lundberg, U., Senn, B. M., Schuler, W., Meinke, A., & Hanner, M. (2013b). Identification and characterisation of antigens as vaccine candidates against Klebsiella

pneumoniae. *Human Vaccines and Immunotherapeutics*, **9**(3), 497–505. https://doi.org/10.4161/hv.23225

Ng, T. H. S., Britton, G. J., Hill, E. V., Verhagen, J., Burton, B. R., & Wraith, D. C. (2013). Regulation of adaptive immunity: The role of interleukin-10. *Frontiers in Immunology*, **4**(MAY), 129. https://doi.org/10.3389/FIMMU.2013.00129/BIBTEX

Peñaloza, H. F., Noguera, L. P., Riedel, C. A., & Bueno, S. M. (2018). Expanding the current knowledge about the role of interleukin-10 to major concerning bacteria*. Frontiers in Microbiology,* **9**(SEP), 1–8. https://doi.org/10.3389/fmicb.2018.02047

Pennini, M. E., De Marco, A., Pelletier, M., Bonnell, J., Cvitkovic, R., Beltramello, M., Cameroni, E., Bianchi, S., Zatta, F., Zhao, W., Xiao, X., Camara, M. M., DiGiandomenico, A., Semenova, E., Lanzavecchia, A., Warrener, P., Suzich, J., Wang, Q., Corti, D., & Stover, C. K. (2017). Immune stealth-driven O2 serotype prevalence and potential for therapeutic antibodies against multidrug-resistant Klebsiella pneumoniae. *Nature Communications*, **8**(1), 1–12. https://doi.org/10.1038/s41467-017-02223-7

Schagger, H. (2003). SDS electrophoresis techniques. In Membrane protein purification and crystallisation, 85–103. https://doi.org/10.1016/B978-012361776-7/50005-X

Setiawan, H., & 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), 50. https://doi.org/10.20473/jbp.v18i1.2016.50-63

Shon, A. S., Bajwa, R. P. S., & Russo, T. A. (2013). Hypervirulent (hypermucoviscous) Klebsiella pneumoniae, **4**(2), 107–118. https://doi.org/10.4161/VIRU.22718

Sudiono, J. (2014). *Sistem kekebalan tubuh*. Penerbit Buku Kedokteran EGC, June, 1–86.

Sukarjati, S., Amilah, S., & Sudjarwo, S. (2018). No Title. *Folia Medica Indonesiana*, **54**(2). https://doi.org/10.20473/fmi.v54i2.8866

Thanassi, D. G., Bliska, J. B., & Christie, P. J. (2012). Surface organelles assembled by secretion systems of gram-negative bacteria: Diversity in structure and function. In *FEMS Microbiology Reviews* (Vol. 36, Issue 6, pp. 1046–1082). Oxford Academic. https://doi.org/10.1111/j.1574-6976.2012.00342.x

The State of the World's Antibiotics. (2015). Center for Disease Dynamics, Economics & Policy (CDDEP).

Verma, P., Berwal, P. K., Nagaraj, N., Swami, S., Jivaji, P., & Narayan, S. (2017). Neonatal sepsis: Epidemiology, clinical spectrum, recent antimicrobial agents and their antibiotic susceptibility pattern. *International Journal of Contemporary Pediatrics,* **2**(3), 176–180. https://doi.org/10.18203/2349-3291.IJCP20150523

Weiner, L. (2016). Vital signs: Preventing antibiotic-resistant infections in hospitals—United States, 2014. *Am. J. Transpl*., **16**, 2224–2230.

WHO Releases List of World's Most Dangerous Superbugs - Scientific American. (n.d.).

Zea-Vera, A., & Ochoa, T. J. (2015). Challenges in the diagnosis and management of neonatal sepsis. *Journal of Tropical Pediatrics,* **61**(1), 1–13. https://doi.org/10.1093/TROPEJ/FMU079