

IMMUNODETECTION OF ADHESIN PILI PROTEIN 38.6 KDA *K. pneumoniae* USING WESTERN BLOT

Dini Agustina^{*}, Diana Chusna Mufida^{*}, Nurul Indah Saffanah^{**}

^{*}Department of Microbiology, Faculty of Medicine, University of Jember, East Java, Indonesia,
dini_agustina@unej.ac.id, chusna.fk@unej.ac.id

^{**}Medical student of Faculty of Medicine, University of Jember, East Java, Indonesia,
nurul.indah.saffanah@gmail.com

Email Correspondence : dini_agustina@unej.ac.id

Received : July 7, 2021

Accepted : December 10, 2021

Published : December 31, 2021

Abstract: *Klebsiella pneumoniae* is a pathogen that causes infections in communities and hospitals. These bacteria have many virulence factors that play an important role in the pathogenicity of infection and antibiotic resistance. Pili protein *Klebsiella pneumoniae* with 38.6 kDa as one of the virulence factors that have roles as hemagglutinin dan adhesin protein so potentially as a vaccine candidate. This research aimed to immunodetection pili protein 38.6 kDa using Western Blot. Western Blot was used to detect that protein with specific primary antibodies. These antibodies are obtained from mice serum-induced intraperitoneally with Pili protein 38.6 kDa *Klebsiella pneumoniae*. Protein bands that appear on the membrane of Western Blot results are proteins with a molecular weight of 85.6 kDa, 65.5 kDa, 46.9 kDa, and 29.4 kDa. This study concludes that Pili protein 38.6 kDa *Klebsiella pneumoniae* as the target protein does not appear in the Western Blot result.

Keywords: *Klebsiella pneumoniae*; pili protein; Western Blot

Abstrak: *Klebsiella pneumoniae* adalah patogen yang menyebabkan infeksi baik di komunitas maupun di rumah sakit. Bakteri tersebut memiliki banyak faktor virulensi yang berperan penting dalam patogenisitas terjadinya infeksi dan resistensi antibiotik. Protein pili *Klebsiella pneumoniae* dengan 38,6 kDa sebagai salah satu faktor virulensi, memiliki peran sebagai protein hemagglutinin dan adhesin sehingga berpotensi sebagai kandidat vaksin. Tujuan penelitian ini untuk imunodeteksi protein pili 38,6 kDa dengan menggunakan Western Blot. Western Blot digunakan untuk mendeteksi protein itu dengan spesifik antibodi primer. Antibodi ini diperoleh dari serum tikus yang telah diinduksi secara inteperitoneal dengan protein Pili 38,6 kDa *Klebsiella pneumoniae*. Band protein yang muncul pada membran hasil Western Blot adalah protein dengan berat molekul 85,6 kDa; 65,5 kDa; 46,9 kDa dan 29,4 kDa. Dari penelitian ini dapat disimpulkan bahwa protein Pili 38,6 kDa *Klebsiella pneumoniae* sebagai protein target tidak muncul.

Kata kunci: *Klebsiella pneumoniae*; protein pili; Western Blot.

Recommended APA Citation :

Agustina, D., Mufida, D. C., & Saffanah, N. I. (2021). Immunodetection of Adhesin Pili Protein 38.6 kDa *K. pneumoniae* Using Western Blot. *Elkawnie*, 7(2), 317-327. <https://doi.org/10.22373/ekw.v7i2.10127>

Introduction

Infectious diseases are still ranked as the top cause of morbidity and mortality in the world (WHO, 2020). Pneumonia is one of them, consisting of Hospital-acquired pneumonia (HAP), Ventilator-acquired pneumonia (VAP), and Community-acquired pneumonia (CAP). VAP and HAP are classified as nosocomial pneumonia, defined as an infection of the lung that develops in patients admitted to the hospital for ≥ 48 h. These infections affect a significant proportion of hospitalized patients and are characterized by poor clinical outcomes. (Ferrer & Torres, 2018; Torres et al., 2017; Vallecoccia et al., 2020) In the study in Sub-Saharan Africa, Gram-negative bacteria were reported as the most common cause of CAP (83.1%). Common Gram-negative bacteria isolated were *Klebsiella pneumoniae* (29.9%), *Pseudomonas aeruginosa* (11.7%), and *Escherichia coli* (9.1%), with 87.0% of *Klebsiella pneumoniae* (*K. pneumoniae*) isolates were resistant to ceftriaxone. In addition to being the most common cause of CAP, most *K. pneumoniae* are also resistant to antibiotics. (Kishimbo, Sogone, Kalokola, & Mshana, 2020; Paczosa & Meccas, 2016)

In general, antibiotic resistance in *K. pneumoniae* consists of two major types, extended-spectrum β -lactamases (ESBLs) and carbapenem-resistant *Klebsiella pneumoniae* (CRKP). ESBLs can hydrolyze oxyimino cephalosporins third-generation cephalosporins are ineffective against treatment. Due to this resistance, carbapenems became a treatment option for ESBL. If CRKP is diagnosed, several antibiotics are needed to treat it. Combination therapy of two or more agents may decrease the mortality rate of this case. Related to this resistance problem, a study in Mansoura University demonstrates the correlation between ESBL production and several virulence factors that play an important role in the pathogenicity of infection. (Ashurst & Dawson, 2018; Atmani et al., 2015; Cienfuegos-Gallet et al., 2019; Eichenberger & Thaden, 2019; Paczosa & Meccas, 2016; Tsereteli, Sidamonidze, Tsereteli, Malania, & Vashakidze, 2018; Zhu, Yuan, & Zhou, 2020)

Klebsiella pneumoniae has many virulence factors such as capsular polysaccharides, siderophores, aggregate adhesions, and fimbriae, also known as pili, types 1 and 3. Pili allows the microorganism to adhesive itself to the host cell. This is because pili have proteins that can bind to the constituent sugars of the host cell membrane, called hemagglutinin proteins, and proteins that can bind to cell surface receptors in which are called adhesin proteins. (Khairuzzaman, 2016; Khater et al., 2015; Paczosa & Meccas, 2016; Thanassi, Bliska, & Christie, 2012; Vuotto, Longo, Balice, Donelli, & Varaldo, 2014) In previous research, some adhesion protein factors, both pili and outer membrane (OMP) *K. pneumoniae* have been shown to act as hemagglutinin and adhesion. These proteins include OMP 20 kDa, pili 12.8 kDa, and pili 38.6 kDa, which means they are potential as a vaccine candidate. (Agustina et al., 2020; Agustina, Sumarno, & Noorhamdani,

2014; Finka et al., 2019) One of technique that can be used to separate and identify proteins is Western blot.

The Western blot technique separates the protein mixture by gel electrophoresis based on molecular weight and type. These results are then transferred to a membrane which produces a band for each protein. The membrane is then incubated with an antibody label specific to the desired protein. The unbound antibody is washed away, leaving only the antibody bound to the desired protein. The bound antibody is then detected by expanding the film. Since the antibody binds to only the desired protein, only one band should be visible. Band thickness according to the amount of protein present; so doing the standard can show the amount of protein present. (Blancher & Jones, 2003; Gallagher, Winston, Fuller, & Hurrell, 2008; Madamanchi & Runge, 2001)

An immune agent to be used as a vaccine candidate, in this case, the 38.6 kDa pili protein, needs to be immunologically detected using Western Blot. One of the tests chosen in this study is the western blot

Methods

Ethical Clearance

This research has received an ethical approval letter from the Ethics Committee of the Faculty of Medicine, University of Jember, with the number 1493/H25.1.11/KE/2020 dated October 20th, 2020.

Protein Immunization

Five female mice strain BALB/C age 6-8 weeks were used for this study. The production of polyclonal antibodies was done for 1 month. Immunization was done four times in 1 month, with 7 days interval injected intraperitoneally. For the first immunization, the antigen was emulsified with Complete Freud Adjuvant (CFA) for 1:1 with a dose of antigen was 50 µL/mL diluted in PBS. The last immunization was done with antigen and Incomplete Freud Adjuvant (IFA) for the same dose and diluted in PBS as well. The serum will be taken 10 days after the last immunization. (Agustina et al., 2014; Drenckhahn, Jöns, & Schmitz, 1993).

Serum Preparation

Blood was collected from the heart as much as 0,3-0,5 mL. The blood was put in a sterile container and incubated for 30 minutes at 37°C. Then, the blood was transferred to a refrigerator with a temperature of 4°C for 10 min. The blood was taken out and centrifuged at 10.000 rpm for 5 min. Supernatants were taken and put in a sterile container, then stored in a refrigerator with -86 °C. (Wright, 1989)

SDS-PAGE

SDS-PAGE procedure was used to determine protein's molecular weight. The separating gel concentration chosen was 12% containing Acrilamid 30%, 1,5

M Tris-HCl pH 8,8, SDS 10%, H₂O, APS 10%, and TEMED while stacking gel was 4% containing same as the separating gel but used 0,5 M Tris-HCl ph 6,6. Protein samples before being put into wells, given buffer sample containing 62.5 mM Tris-HCl (pH 6.8), 10% glycerol, and 0.001% bromophenol blue with 5% mercaptoethanol heated in 95°C for 5 min. 15 – 20 µL marker and samples was inserted into each well and run for 1 h with 110 V. Coomassie Brilliant Blue R-250 was used as a dye and as a pre-stained protein marker. (Agustina et al., 2014)

Western Blotting

Western blot procedure was done using semidry blotter Bio-Rad. The samples were run on SDS-PAGE blotted to a PVDF membrane. Before the samples were blotted, SDS-PAGE was soaked in buffer transfer, along with PVDF membrane and Whatman paper. The membranes were stacked like a sandwich and blotted for an hour with 100mA. After blotting, membranes were rinsed with TBS and blocked for 1 h in 5% skimmed milk in 10 mL TBS with gentle shaking. The membrane was then washed with TBS three times for five minutes each. The primary antibody (1:250) was added in 10 mL skimmed milk and incubated overnight at a low temperature (4°C) in a dark place. The membrane was washed three times for five minutes each with TBS, and a secondary antibody (alkaline phosphatase rabbit anti-mouse immunoglobulin G) was added for 1:2500 in 10mL skimmed milk. The membrane was incubated for 2 h with gentle shaking. Finally, the membrane was washed with TBS, and the substrate (NBT-BCIP) was added. After 5 min, the membrane was taken out, rinsed with aquadest, and dried. (Hoppe, Bier, & von Nickisch-Roseneck, 2012)

Results and Discussion

Determination of protein molecular weight using SDS-PAGE

SDS-PAGE procedure was used to determine protein's molecular weight. The separation of proteins is influenced by their molecular weight, electric charge, and the isoelectric point (pI). Smaller proteins pass through the pores easier and faster than large proteins. Acrylamide concentration will determine gel resolution; therefore, a higher concentration makes a better resolution for low molecular weight proteins. Conversely, a lower concentration will be better for high molecular weight proteins. (Anaya, Shoenfeld, & Rojas-Villarraga, 2013)

The result of protein pili identification can be seen in figure 1. In this study, the antigen used during the SDS-PAGE procedure was a crude protein of *K. pneumoniae* antigen so that more than 1 molecular weight appeared. In figure 1, it can be seen that the 38.6 kDa pili protein does not appear, whereas only 34.6 kDa and 11.6 kDa pili proteins appear. The molecular weight is directly proportional to the concentration of protein, so it can be stated that the 38.6 kDa pili have a low concentration, so it does not appear on the SDS membrane. In contrast, 34.6 kDa and 11.6 kDa proteins have concentrations high enough that both bands appear.

The bacteria that have been identified are then cut into piles using a pili cutter for 5 repetitions of cutting. The cutting results were then electrophoresed using the SDS-PAGE method, the results of which can be seen in lanes 3-7 (figure 2). These results show that the 1st pili slices (lane 3) are more visible in color than the other pili pieces (rows 4-7). This shows that the protein concentration of the 1st pili slice is higher than the other pili slices. In the first pili section, it can be seen that the protein with a molecular weight of 38.6 kDa has a darker color than the other molecular weights, indicating that the concentration of the protein is higher than the others. 38.6 kDa protein may be the protein that most plays a role in the pathogenicity caused by pili *K. pneumoniae*.

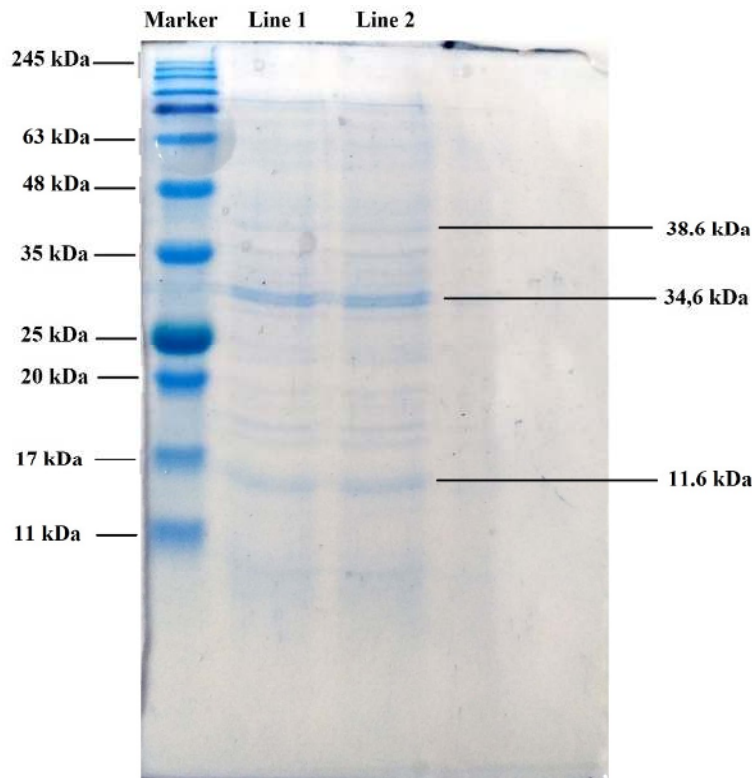


Figure 1. Results of SDS Page crude protein *Klebsiella pneumoniae* pieces of pili 4

Immunogenic detection of proteins using Western Blotting

The results of Western Blotting can be seen in figure 2. The reason that the target protein, the 38.6 kDa band, did not appear can be caused by various factors such as antibodies, antigens, or buffers. If the wrong primary or secondary antibody is used, the band may not appear. In addition, the antibody concentration must also be appropriate; the signal may not be visible if the concentration is too low. Another reason could also be the absence of an antigen. Antigens can confirm whether the problem lies with the sample or the antibodies. Washing for too long can also lower the signal. Buffers can also be a factor. It must be ensured that buffers such as transfer buffer, TBST, running buffer, and ECL are all new

and uncontaminated. Buffers that have been contaminated with sodium azide will inactivate HRP. (Mahmood & Yang, 2012)

There are several reasons why the 38.6 kDa band does not appear, possibly due to insufficient protein to be transferred, too low antibody concentration, antibody incompatibility, membrane overwashing, too much blocking, inactive substrate, insufficient film exposure, or the presence of small proteins that moving across the transfer membrane. The disadvantage of the western blot is the method needs specific antibodies to target a protein, so many target proteins cannot be studied and researched due to the lack of available antibodies. (Gilda et al., 2015)

In figure 2, four molecular weights of antibody-antigen bonds appear, with molecular weights of 85.6 kDa, 65.5 kDa, 46, 9 kDa, and 29.4 kDa. The results on western blotting indicate that the four proteins are immunogenic. Immunogenic protein is a protein in which stimulates the host immune response and creates antibodies. (Baker, Reynolds, Lumicisi, & Bryson, 2010) This study found that the target protein, 38.6 kDa, did not appear in the Western blotting results, but other bands emerged from crude protein. This proves that the 38.6 kDa *K. pneumoniae* pili antigen is immunogenic because it induces antibodies so that the antibody can bind to other antigens in crude protein. Still, the protein is likely to have been degraded, thus showing the little affinity and too low concentration. In addition, there can be cross-reactivity in crude protein because the protein has the same epitope so that antibodies bind with stronger antigens.

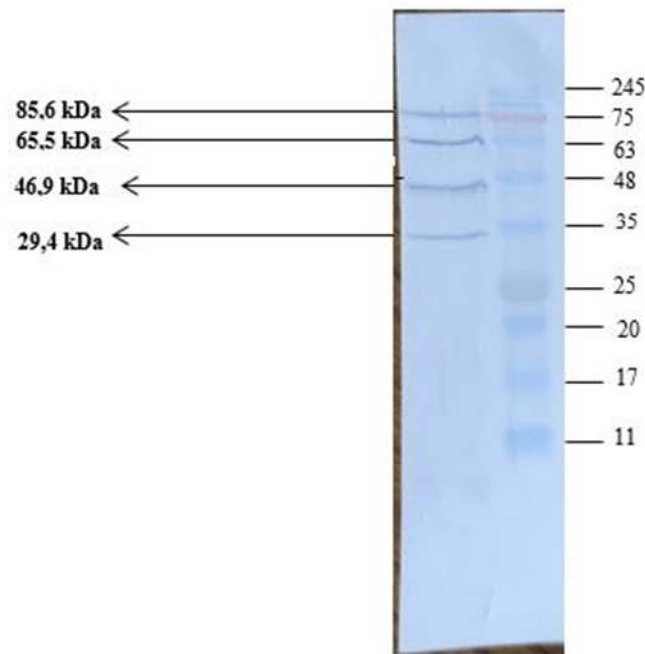


Figure 2. Results of *Western Blotting* pili protein 38.6 kDa *Klebsiella pneumoniae* against mice serum IgG antibody-induced intraperitoneally

Even though the procedure for western blot is simple, several issues and possibilities can arise, resulting in surprising results. The matter is classified into six categories: (1) high background on the blot, (2) no bands, (3) faint bands or weak signal, (4) uncommon or unexpected bands, (5) uneven or uneven spots on the blot and, (6) no bands may arise thanks to many reasons involving protein, antigen, or buffer used. If an improper antibody is used, the band will not show either primary or secondary. (Mahmood & Yang, 2012)

Furthermore, the band did not show up if the concentration was simply too low. The signal might not be visible. Weak signals are caused by a low concentration of protein or antigen. It is vital to recollect some antibodies that are not used for western blot. Another excuse for no visible bands is the lowest concentration or absence of the substance. During this case, antigens from another supply cannot assure whether or not the matter lies with the sample or with alternative elements reminiscent of the antibody. In addition, prolonged laundry may decrease the signal. Buffers can also contribute to the problem. It should be ensured that buffers like the transfer buffer, TBST, running buffer, and ECL, are all new and non-contaminated. If the buffers are contaminated with metal azide, it can inactivate HRP. Another excuse might be nonfat powdered milk masking the antigen. Increasing exposure time may facilitate forming the band clearer (Mahmood & Yang, 2012)

The band(s) at the molecular weight are slightly higher than expected. The protein can be glycosylated or modified at one or more amino acid residues for the solution to allow enzymes to remove the suspected modification and return the molecular weight closer to the expected one. Then it is necessary to re-examine the amino acid sequence and the reference literature. The same condition can also occur because the sample has not been completely reduced and denatured, so dimers, multimers, or protein-protein interactions occur. Solutions that can be done include adding fresh DTT or bME to the sample and reheating before repeating the experiment using a new sample/ fresh with fresh loading buffer. (Bio Rad, 2020)

Mahmood and Yang also explained that the appearance of unusual or unexpected bands could be caused by protease degradation, which results in bands at unexpected positions, so it is recommended to use fresh samples that have been stored on ice or by changing antibodies. If the protein appears to be too high, then reheating the sample can help break down the quaternary protein structure. (Mahmood & Yang, 2012) The point is that the main cause of multimers is disulfide, so reduction and alkylation are the ways to overcome them. The hope is that the process can break bonds and stop them from forming again. However, some proteins form multimers that cannot be broken under any circumstances due to very strong ionic interactions, and this protein could be one of them.

Conclusion

Pili protein 38.6 kDa *Klebsiella pneumoniae* as the target protein does not appear in the Western Blot result because the protein of samples had low concentration and low affinity, glycosylated or modified at one or more amino acid residues, not been completely reduced and denatured or very strong ionic interactions.

Acknowledgements

We thank the Research Group Project of the University of Jember for the funding (Grant No. 11872/UN25/LT/2020) and thank all the medical students for participating in the study.

References

- Agustina, D., Nadyatara, K., Mufida, D. C., Elfiah, U., Shodikin, M. A., & Suswati, E. (2020). Faktor Virulensi Outer Membrane Protein 20 kDa *Klebsiella pneumoniae* sebagai Protein Hemagglutinin dan Adhesin. *EJournal Kedokteran Indonesia*, 7(3), 200–204. <https://doi.org/10.23886/ejki.7.10425>.
- 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* | Agustina |

- Journal of Tropical Life Science. *Journal of Tropical Life Science*, 4(1), 19–25.
- Anaya, J., Shoenfeld, Y., & Rojas-Villarraga, A. (2013). Autoimmunity - NCBI Bookshelf. Retrieved September 22, 2020, from PubMed website: <https://www.ncbi.nlm.nih.gov/books/NBK459447/>
- Ashurst, J. V., & Dawson, A. (2018). Pneumonia, Klebsiella. In *StatPearls*. StatPearls Publishing.
- Atmani, S. M., Messai, Y., Alouache, S., Fernández, R., Estepa, V., Torres, C., & Bakour, R. (2015). Virulence characteristics and genetic background of ESBL-producing *Klebsiella pneumoniae* isolates from wastewater. *Fresenius Environmental Bulletin*, 24(1), 103–112.
- Baker, M. P., Reynolds, H. M., Lumericis, B., & Bryson, C. J. (2010, October). Immunogenicity of protein therapeutics: The key causes, consequences and challenges. *Self/Nonsel - Immune Recognition and Signaling*, Vol. 1, pp. 314–322. <https://doi.org/10.4161/self.1.4.13904>
- Bio Rad. (2020). Western Blot Troubleshooting: Unusual or Unexpected Bands | Bio-Rad. Retrieved November 30, 2021, from Bio-Rad Laboratories, Inc. website: https://www.bio-rad-antibodies.com/western-blot-unusual-unexpected-bands-western-blotting.html?JSESSIONID_STERLING=87077389C9241CBCD37EB2D5931A0369.ecommerce2&evCntryLang=ID-en&cntry=ID&thirdPartyCookieEnabled=true
- 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>
- Cienfuegos-Gallet, A. V., Ocampo De Los Ríos, A. M., Sierra Viana, P., Ramirez Brinez, F., Restrepo Castro, C., Roncancio Villamil, G., ... Jiménez, J. N. (2019). Risk factors and survival of patients infected with carbapenem-resistant *Klebsiella pneumoniae* in a KPC endemic setting: A case-control and cohort study. *BMC Infectious Diseases*, 19(1), 830. <https://doi.org/10.1186/s12879-019-4461-x>
- Drenckhahn, D., Jöns, T., & Schmitz, F. (1993). Production of Polyclonal Antibodies against Proteins and Peptides. *Methods in Cell Biology*, 37(C), 7–56. [https://doi.org/10.1016/S0091-679X\(08\)60242-3](https://doi.org/10.1016/S0091-679X(08)60242-3)
- Eichenberger, E. M., & Thaden, J. T. (2019, June 1). Epidemiology and mechanisms of resistance of extensively drug-resistant gram-negative bacteria. *Antibiotics*, Vol. 8, p. 37. MDPI AG. <https://doi.org/10.3390/antibiotics8020037>
- Ferrer, M., & Torres, A. (2018). Epidemiology of ICU-acquired pneumonia. *Current Opinion in Critical Care*, 24(5), 325–331. <https://doi.org/10.1097/MCC.0000000000000536>
- Finka, R., Agustina, D., Rachmawati, D. A., Suswati, E., Mufida, D. C., &

- Shodikin, A. (2019). The Role of Pili Protein 38,6 kDa *Klebsiella pneumoniae* as a Hemagglutinin and Adhesin Protein which Serves as a Virulence Factor. *Journal of Agromedicine and Medical Sciences*, 5(2), 9. <https://doi.org/10.19184/ams.v5i2.9558>
- Gallagher, S., Winston, S. E., Fuller, S. A., & Hurrell, J. G. R. (2008). Immunoblotting and immunodetection. *Current Protocols in Molecular Biology*, 83(SUPPL. 83), 10.8.1-10.8.28. <https://doi.org/10.1002/0471142727.mb1008s83>
- Gilda, J. E., Ghosh, R., Cheah, J. X., West, T. M., Bodine, S. C., & Gomes, A. V. (2015). Western blotting inaccuracies with unverified antibodies: Need for a Western Blotting Minimal Reporting Standard (WBMRS). *PLoS ONE*, 10(8). <https://doi.org/10.1371/journal.pone.0135392>
- Hoppe, S., Bier, F. F., & von Nickisch-Rosenegk, M. (2012). Microarray-based method for screening of immunogenic proteins from bacteria. *Journal of Nanobiotechnology*, 10(1), 12. <https://doi.org/10.1186/1477-3155-10-12>
- Khairuzzaman, M. Q. (2016). *Hemagglutinin*. 4(1), 64–75.
- Khater, F., Balestrino, D., Charbonnel, N., Dufayard, J. F., Brisse, S., & Forestier, C. (2015). In Silico Analysis of Usher Encoding Genes in *Klebsiella pneumoniae* and Characterization of Their Role in Adhesion and Colonization. *PLOS ONE*, 10(3), e0116215. <https://doi.org/10.1371/journal.pone.0116215>
- Kishimbo, P., Sogone, N. M., Kalokola, F., & Mshana, S. E. (2020). Prevalence of gram-negative bacteria causing community-acquired pneumonia among adults in Mwanza City, Tanzania. *Pneumonia*, 12(1), 7. <https://doi.org/10.1186/s41479-020-00069-0>
- Madamanchi, N. R., & Runge, M. S. (2001). Western blotting. *Methods in Molecular Medicine*, 51, 245–256. <https://doi.org/10.1385/1-59259-087-X:245>
- Mahmood, T., & Yang, P. C. (2012). Western blot: Technique, theory, and troubleshooting. *North American Journal of Medical Sciences*, 4(9), 429–434. <https://doi.org/10.4103/1947-2714.100998>
- Paczosa, M. K., & Mecsas, J. (2016). *Klebsiella pneumoniae*: Going on the Offense with a Strong Defense. *Microbiology and Molecular Biology Reviews*, 80(3), 629–661. <https://doi.org/10.1128/mubr.00078-15>
- Thanassi, D. G., Bliska, J. B., & Christie, P. J. (2012, November 1). Surface organelles assembled by secretion systems of Gram-negative bacteria: Diversity in structure and function. *FEMS Microbiology Reviews*, Vol. 36, pp. 1046–1082. Oxford Academic. <https://doi.org/10.1111/j.1574-6976.2012.00342.x>
- Torres, A., Niederman, M. S., Chastre, J., Ewig, S., Fernandez-Vandellos, P., Hanberger, H., ... Wunderink, R. (2017, September 1). International ERS/ESICM/ESCMID/ALAT guidelines for the management of hospital-

- acquired pneumonia and ventilator-associated pneumonia. *European Respiratory Journal*, Vol. 50, p. 1700582. European Respiratory Society. <https://doi.org/10.1183/13993003.00582-2017>
- Tsereteli, M., Sidamonidze, K., Tsereteli, D., Malaria, L., & Vashakidze, E. (2018). Epidemiology Of Carbapenem-Resistant Klebsiella Pneumoniae In Intensive Care Units Of Multiprofile Hospitals In Tbilisi, Georgia. *Georgian Medical News*, (280–281), 164–168.
- Vallecoccia, M. S., Dominedò, C., Cutuli, S. L., Martin-Loeches, I., Torres, A., & De Pascale, G. (2020, September 30). Is ventilated hospital-acquired pneumonia a worse entity than ventilator-associated pneumonia? *European Respiratory Review*, Vol. 29, pp. 1–8. European Respiratory Society. <https://doi.org/10.1183/16000617.0023-2020>
- Vuotto, C., Longo, F., Balice, M., Donelli, G., & Varaldo, P. (2014). Antibiotic Resistance Related to Biofilm Formation in Klebsiella pneumoniae. *Pathogens*, 3(3), 743–758. <https://doi.org/10.3390/pathogens3030743>
- WHO. (2020). WHO reveals leading causes of death and disability worldwide: 2000-2019. Retrieved December 12, 2020, from <https://www.who.int/news/item/09-12-2020-who-reveals-leading-causes-of-death-and-disability-worldwide-2000-2019>
- Wright, K. (1989). Antibodies a Laboratory Manual. *Biochemical Education*, 17(4), 220. [https://doi.org/10.1016/0307-4412\(89\)90165-9](https://doi.org/10.1016/0307-4412(89)90165-9)
- Zhu, W. M., Yuan, Z., & Zhou, H. Y. (2020). Risk factors for carbapenem-resistant Klebsiella pneumoniae infection relative to two types of control patients: A systematic review and meta-analysis. *Antimicrobial Resistance and Infection Control*, 9(1). <https://doi.org/10.1186/s13756-020-0686-0>