

How to Cite:

Ali, Z. D., & Mohsin, M. I. (2022). Bacterial co-infection in a patient with COVID-19 pneumonia induce to release TNF-a. *International Journal of Health Sciences*, 6(S5), 5590–5600.
<https://doi.org/10.53730/ijhs.v6nS5.9931>

Bacterial co-infection in a patient with COVID-19 pneumonia induce to release TNF-a

Zaid Derbi Ali

Department of Laboratory Investigations, Faculty of Science, University of Kufa, Najaf, Iraq

Email: ziad.drbee@gmail.com

Muslim Idan Mohsin

Department of Laboratory Investigations, Faculty of Science, University of Kufa, Najaf, Iraq

Email: muslim.aljabri@uokufa.edu.iq

Abstract---The aim of study: to investigate which bacteria are the most common pathogen associated with COVID-19, and to investigate if the TNF-alpha cytokines at gene and protein levels is changed not only with Klebsiella infection but also change with the COVID-19 infection, using real-time qPCR and ELIZA method. Methodology: 145 specimens of sputum and blood were collected from different hospitals in Al-Najaf city to isolate Klebsiella pneumonia associated COVID-19 infection, and only COVID-19 during 4 months from 24st September 2021 to 24th January 2022). 10 ml of fresh venous blood samples were collected from COVID-19 infected patients for serological and immunological assay for measuring the levels of TNF-a at mRNA and protein levels. The results: We report 150 cases of a different-aged who presented with worsening shortness of breath in the context of COVID-19 complicated by Klebsiella pneumoniae var pneumonia. These case serves to highlight the possibility of bacterial superinfections. The present study has tested whether Klebsiella pneumoniae and COVID-19 is colonized in the lower respiratory tract of patients who were admitted in hospital with COVID-19 pneumonia. In this study, we have also assessed the levels of TNF-a in both K. pneumoniae infected patient's specimen and blood collected from patients who are suffered bacterial and COVID-19 infection at mRNA and proteins levels. We found that the most common bacterial co-infection with COVID-19 is Klebsiella pneumoniae, and the TNF-a was increased not only with COVID-19 patient but also with patients who are suffered with bacterial co-infection. Conclusion: Based on the results and findings that are reached in the present work, it can be concluded that: Klebsiella pneumoniae var pneumoniae has more virulent and most common pathogen in the lower respiratory tract

infection that is associated with the COVID-19. The TNF- α has been increased in response to *Klebsiella pneumoniae* var *pneumoniae* infection at mRNA and protein levels. It seems to have a vital role in co-bacterial infection associated with COVID-19 patients. Recommendation: It is highly recommended to investigate the role of selected pro-inflammatory in vivo, to test whether they have the same responses to others bacterial infection.

Keywords---SARS, *Klebsiella pneumoniae*, TNF- α , COVID-19 and mRNA.

1.1 Introduction

Immune response plays a vital role in protecting against infectious agents. It is the primary barrier to the spread of disseminated infections, which are usually associated with a high mortality rate. It is a well-known fact that the number of people who have been exposed to an infectious disease is far more than the number of people who have actually developed a disease. This indicates that the majority of people are capable of destroying these microorganisms and thereby preventing infection progression. Immune deficits, whether innate (phagocytic cell dysfunction or complement deficit) or adaptive (antibody production deficit or T-cell function deficiency), are significantly linked to infection susceptibility [1]. Although immune response is critical for guarding against most infectious agents, an evidence has accumulated through time that the main pathological characteristics of many infectious diseases are not connected to the direct action of an aggressor agent, but rather to abnormal immune response [2]. Cytokines are proteins that have the pro-inflammatory or anti-inflammatory properties. Their interaction has the effect of facilitating bacterial pathogen phagocytosis. The Proinflammatory cytokines like the TNF- α and IL-1 β cause hemodynamic changes and increase leakage through the vascular endothelium to this goal. In the meantime, IL-6 causes the liver to produce acute phase proteins. IL-8 and IL-17 stimulate neutrophil chemotaxis at the infection site [3]. *Klebsiella pneumoniae* infection has been linked to number of particular virulence factors. The majority of published research on *K. pneumoniae* pulmonary infection has focused on innate immunity components involved in the host response to airway infection. The relative lack of immunogenicity and failure to generate the substantial proinflammatory response associated with clearance of other airway infections are two significant factors influencing *K. pneumoniae*'s capacity to remain within the airway and resist phagocytosis and death. The mechanisms of cellular death in response to *K. pneumoniae* are poorly understood, but a small number of studies suggests that pyroptosis plays an important role in bacterial clearance [4]. Covid 19 Over the past 2 decades, coronaviruses (CoVs) have been associated with significant disease outbreaks in East Asia and the Middle East. The severe acute respiratory syndrome (SARS) and the Middle East respiratory syndrome (MERS) began to emerge in 2002 and 2012, respectively. Recently, a novel coronavirus, severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), causing coronavirus disease 2019 (COVID-19), emerged in late 2019, and it has posed a global health threat, causing an ongoing pandemic in many countries and territories [5]. Overall, many mistakes with using broad-spectrum antibiotics

against the bacterial or viral infection. Scientists around the world try to understand the viral-immune response and/or secondary infection associated with viral infection, and to find a new therapeutical way by using body immune response include cytokines. The biggest problem is to approve that: it is hypothesised that testing of TNF- α signals after the bacterial infection, could be regulated as a negative or positive regulator at the first stage of infection that would suggest a good strategy to find a new therapy way

1.2 Methodolgy

1.2.1 Collection of the specimens and bacteriological processing

A total of 220 specimens have been collected from the patient suffering from LRT infections during the period from 1st of November 2021 to 27th of February 2022. The specimens represented by 30 of lower respiratory infections which have included both gender and different aged group. The specimens have inoculated on the culture media (MacConkey agar, Blood agar) and incubated aerobically at 37°C for 24-48 hr., for the biochemical tests have been processed to identify the bacteria according to [6].

1.2.2 The Identification of bacterial isolates

Single colonies has been isolated from primary positive cultures and identified according to the criteria of [7] and [6] the following tests: the colonial characteristics (shape, volume, color, borders and texture) and examining microscopically after staining with Gram-stain, the Biochemical tests, and the final identification of isolates is confirmed with the automated VITEK₂ compact system by using GN-ID cards. The system identifies an organism via a methodology based on the characteristics of the data and knowledge about the organism and reaction being analyzed.

1.2.3 Real time RT-qPCR

The GB-Bio RNA Mini Kits are used to extract the mRNA from the whole blood for samples which are -ve (control), +ve COVID-19 associated-*Klebsiella pneumoniae* and only +ve COVID-19 infection. Whole blood cells were centrifuged at 200xg for 5min. Complementary DNA (cDNA) was made for the qPCR by using a Reverse Transcription Kit. 2x qPCR SYBR green Master Mix from Primer Design Precision company was used to perform PCR. Primers and probe sets in plate for our interested gene and internal house keeping control. Then the plate of qPCR was placed into the machine which is 7900HT AbiPrism system and run by 40 cycles. qPCR technique allows the cycling point identification where the product is visible by means of fluorescence emission. The Ct value correlates to the quantity of target TNF- α . TNF- α primers are used f: GGACCGTATGTCTCCAGTCAC , r: ATGAGGTACAGGCCCTCTGAT and GAPDH primers are used f: TGCACCACTGCTTAGC and r: GCATGGACTGTGGTCATGAG. These primers were obtained from macrogen Company from south korea. The level of relative expression was normalized against suitable housekeeping genes. The $\Delta\Delta C_t$ method was used for comparing relative fold expression differences.

1.2.4 The Estimation of Human Tumor necrosis factor α (TNF- α)

The Tumor necrosis factor α (TNF- α) were measured in all types of the samples. In regards to the protocol, we prepared the samples. The serum is centrifuged, briefly to remove particulates and assay immediately. The standard with 1.0mL standard \sample diluent is reconstituted this reconstitute produces as stock solution of 1000pg\ml, pipette 500ul of standard \ sample diluent into 500pg\ml tube and remaining tubes . firstly, A 100 μ l of standard and /or sample were added per well. The plate was covered and incubated for 90 min at 37°C. Each well is aspirated and washed with 1 x wash buffer three times. Then, A100 μ l working solution of the Biotin- conjugation anti-human TNF-a antibody is added to each well ,and incubated for 30 min at 37°C. Each well is washed with 1x Wash buffer three times. A100 μ l of HRP-avidin working solution is added to each well, the micro titer plate covered with a new adhesive strip. And incubated for 30 min at 37°C, and washed with 1 x wash buffer three times again. A100ul TMB substrate is added to each well and mixed gently, protected from light and incubates at 37°C for 15-20 min. It was added 50 μ l of stop solution to each well to stop the color reaction. The optical density is determined of each well within 30 minutes, using a micro plate reader set to 450 nm. Finally, all results were caculated to determine the amount of the TNF- α in an unknown sample.

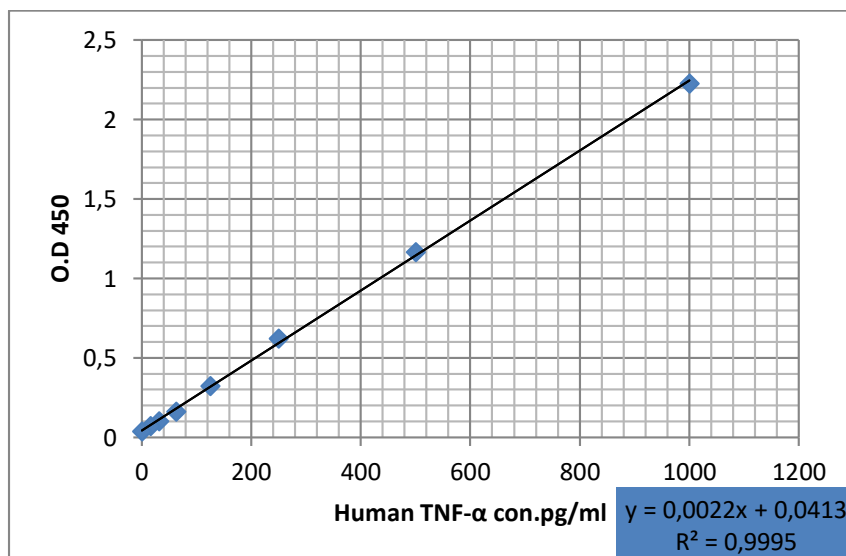


Figure 1: The standard curve of TNF- α concentration (mg/dl)

1.2.5 Statistical Analyses

GraphPadd prism.9 was used to make all graphs and analyse the data statistically. The significant differences between samples were determined using one and two-way ANOVA for multiple comparisons. Results are shown as mean $\pm$ SEM.

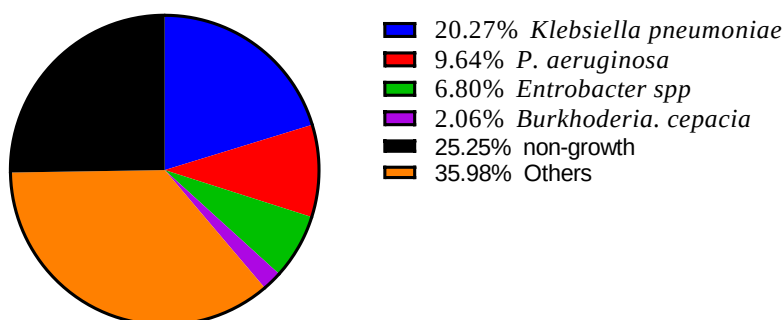
1.3 Results

1.3.1 Isolation and Identification

The aim was to examine which bacteria are the most common pathogen from the lower respiratory tract infection-associated with COVID-19 infection. 145 specimens of sputum and blood were collected from different hospitals in Al-Najaf city to isolate *Klebsiella pneumonia* associated COVID-19 infection, and only COVID-19 during 4 months from 24st September 2021 to 24th January 2022). To do this, sterile containers, serum and EDTA tubes have been used to collect sputum and blood from lower respiratory tract infection and blood stream. The samples have been diluted with normal saline and then culturing on different types of growth media. *Klebsiella pneumonia* is diagnosed depending on phenotyping, biochemical tests, morphology shape, selective media Table (1). Finally, all pure colonies were tested using Vitek₂ compact system to identify *Klebsiella pneumoniae var pneumoniae*. The results have been that most of specimens have bacteria but only 30 samples have *Klebsiella pneumoniae* which was about 30 (20.27%). *Burkholderia cepacia* is about 3 (2.06 %). The lower number is *Enterobacter* 10 (6.8%). However, *P. aeruginosa* was about 14 (9.64%), other pathogens were about (35.98) %. There is also no bacterial growth about (25.25%) as demonstrated in Table (1) and fig (1). It seems that *Klebsiella pneumoniae* is the main pathogen in lower respiratory tract infection as secondary infection with COVID-19 infection. Moreover, The Prevalence of *Klebsiella pneumoniae* isolates among gender and smokers patients with COVID-19 and without are shown in figs (3 and 4). The percentage of *Klebsiella pneumoniae* isolates is higher in males than females in regard to ages and smokers patients. In addition, the percentage of old patients in this screening data was higher in males with age more than 55 than 30 and 40 years old.

Table 1 The percentage of the isolation and identification of different types of bacteria from lower respiratory tract infection

Type of sample	Number of specimens	<i>Klebsiella pneumoniae var pneumonia</i>	Others
Sputum	145	30 (20.27)	<i>Pseudomonas spp</i> 9.64% <i>Burkholderia cepacia</i> 2.06% <i>Enterobacter spp</i> 6.8% Non-growth 25.25% and Others (35.98%),



Total=100

Figure 2: The Percentage of the different types of bacteria isolated from lower respiratory tract infection (LRT)

The data shows that the number and percentage of isolates from 145 samples of the LRT infection from different hospitals in Al-Najaf province (Alsadar Medical city and Alforat hospital). Pure colonies were isolated and placed in the VITEK₂ microbial identification. For more validation, manual diagnostics methods have been performed including biochemical and culturing tests.

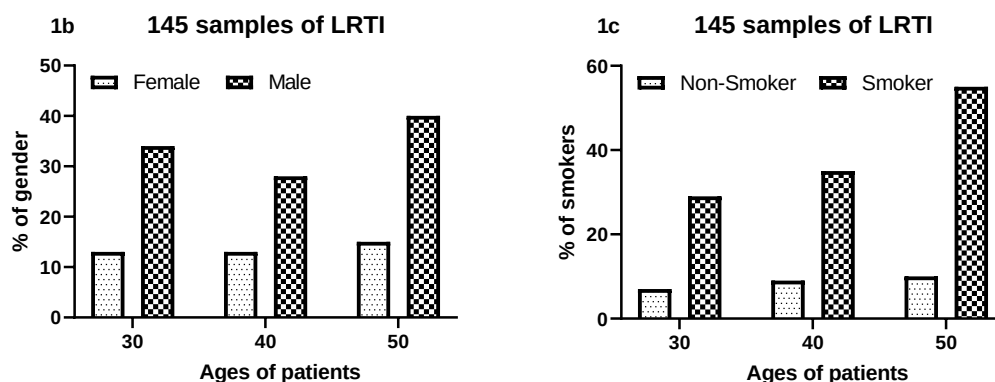


Figure 3: The Age and gender wise distribution of infection among male and female. Figure (4) Smokers and non-smokers patients among male and female

1.3.2 The Changes of Pro-inflammatory genes in response to COVID-19 and *Klebsiella pneumoniae* infection

This study was to investigate the gene expression of TNF- α at the mRNA level in the Polymorphonuclear cells at different cases of patients. This study used the qPCR method to measure the mRNA level of Pro-inflammatory. According to the criteria for primer design for real-time qPCR, we designed human TNF- α and GAPDH primers. The expression of the mRNA is determined by the accumulation of a fluorescent signal, using SYBR green fluorescent dye. The number of PCR cycles required for the fluorescent signal to cross the threshold is the cycle

threshold (Ct). The Ct value was used to calculate the relative amount of RNA. The relative expression of Pro-inflammatory at mRNA is calculated against a suitable housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH) for polymorphonuclear cells human. the results show the relative expression of TNF- α is slightly low at the normal cases.

To analyse which gene expression levels are changed during the *Klebsiella pneumoniae* infection, qPCR was used. Pro-cytokines genes expression changes were analysed in polymorphonuclear cells in response to infection by *Klebsiella pneumoniae* isolated from lower respiratory tract infection. The fold change was calculated using the equation $2^{(-\Delta\Delta Ct)}$. In brief, the RNA has been extracted and cDNA was prepared using a reverse transcriptase kit. The primer specificity was checked using melting curves as an extra step in a real-time PCR machine (Figure 5). Amplification was performed on cDNA samples. using reverse and forward primers for Pro-cytokine (human) using a real-time PCR machine. TNF- α of polymorphonuclear cells had detectable mRNA levels in normal cases, COVID-19 and COVID-19 associated *Klebsiella pneumoniae* patients but only the mRNA levels of TNF- α has increased with COVID-19 associated *Klebsiella pneumoniae* patients and COVID-19 patients compared with control, as shown in (Fig 5). To analyse which cytokines expression are changed during the *Klebsiella pneumoniae* and COVID-19 infection, ELIZA technique was used. Pro-cytokines expression changes have been analysed in polymorphonuclear cells in response to infection by *Klebsiella pneumoniae* and COVID-19 detected from pneumonia. TNF- α had detectable at protein levels in normal cases, COVID-19 and COVID-19 associated *Klebsiella pneumoniae* patients.

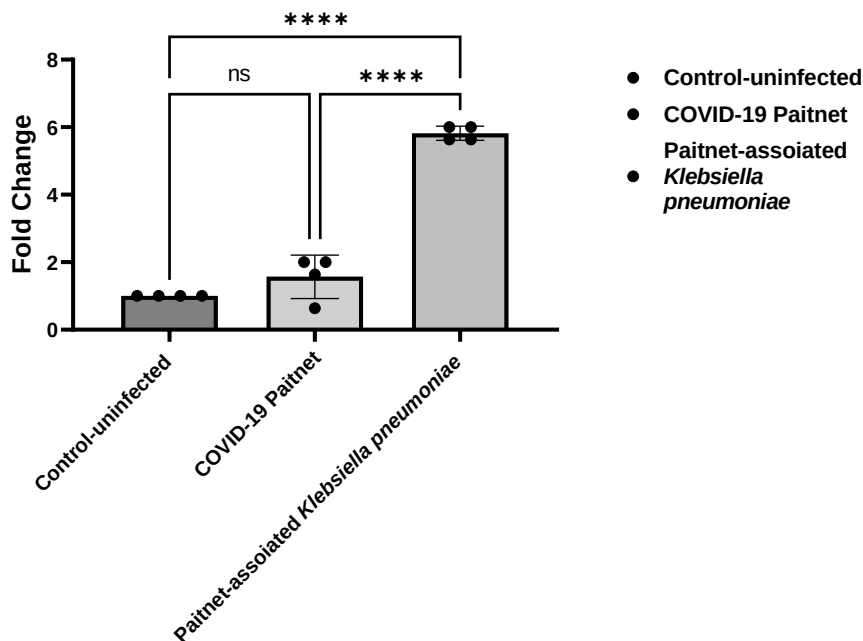


Figure 5 The TNF- α gene expression has changed in response to *Klebsiella pneumoniae* var *pneumoniae* infection

The TNF- α expression has been measured by qPCR in patients have infection with COVID-19 only and with COVID-19 association *Klebsiella pneumoniae var pneumoniae*. Expression of these Pro-inflammatory in non-infected and infected Polymorphonuclear cells was calculated using the $2^{-\Delta\Delta C_t}$ method following estimation of the house keeping gene **GAPDH**. **TNF- α** showed changes in Polymorphonuclear cells. The significance of differences has been tested by one-way ANOVA, where **** $p < 0.0001$ significant; ns=non-significant. The data are the means of 3 separate experiments with duplicate.

The levels of TNF- α has increased with COVID-19 associated *Klebsiella pneumoniae* patients and COVID-19 patients compared with control, as shown in (Fig 6). It seems that the TNF- α have a vital role with secondary bacterial infection, not viral infection. Interestingly, *K. pneumoniae* can play a vital role to upregulate GM-CSF, shielding the airway epithelium from harm, which is consistent with these findings [18]. Capsule of *K. pneumoniae* has role to inhibit the production of epithelial IL-8 and intracellular adhesion molecule, preventing phagocyte recruitment [19]. There is another study the has found that the stimulation of IL-12, IL-23, and IL-17 by DCs in host defenses against *K. pneumoniae* lung infection has been found to be important for host defenses against this pathogen [16] [13].

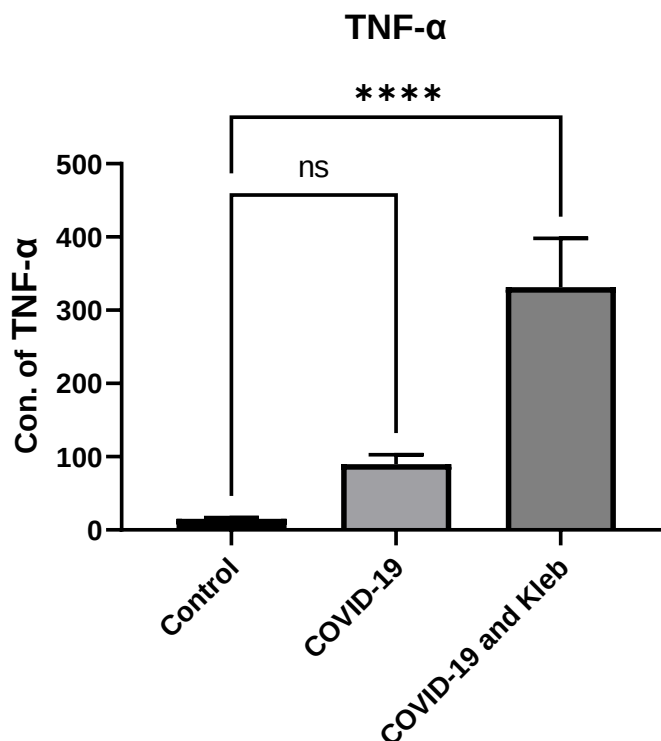


Figure 6 The TNF- α protein levels in the patients with normal, COVID-19 and COVID-19 with *K. pneumoniae* patient. The bars showed changes TNF- α in serum. The significance of differences was tested by one-way ANOVA, where ****

p<0.0001 significant; ns=non-significant. The data are the means of 145 samples of each panels.

1.4 Discussion

As shown in (Fig 1 and table 1), the results seem that *Klebsiella pneumoniae* is the main pathogen in lower respiratory tract infection as secondary infection with COVID-19 infection. These findings have been compared with the other researchers findings which reported that *Klebsiella pneumoniae* was one of the main causative agents in lower respiratory tract infection. Our results have shown similar results [8]; [9] and [10]. However, El-Gamasy (2017) and Mohammed *et al.* (2017) have found that *Klebsiella pneumoniae* was recorded in 15% and 3% respectively [11] and [12]. These different results could be because the condition of collection and source of infection. However, it needs further investigation.

In recent publication, it has been demonstrated that the inflammatory features started with cell induction to produce large amounts of several cytokines including tumor necrosis factor (TNF) alpha. These cytokines exert a wide variety of effects to accelerate inflammation. The results have been compared with the other researches in the same scope to more understand the mechanism of regulating these pro-inflammatory in response to the *K. pneumonia* infection [13]. Craig and his co-workers have demonstrated that the TLR9 activation by CpG improved *K. pneumoniae* clearance by increasing cytokine expression (TNF, IL-12, and CXCR3 chemokines) [14]. Also, other researchers have found that TLR9-dependent recruitment and maturation of DCs has occurred after CpG ODN recruited numerous inflammatory cells into the lungs (T cells, NK cells, and neutrophils) [15]. Furthermore, TLR4 and TLR9 signaling cooperate in the activation of macrophages and the production of adaptive immunity (IL-23 and IL-17) [16]. According to the research, it is unclear why *K. pneumonia* fail to generate a robust and protective immune response in an immunocompetent host [17].

Ethical Clearance: All experimental protocols were approved under the Faculty of science.

Acknowledgements

Research facilities provided by different hospitals in alnajaf city acknowledged.

Reference

1. Janeway, C.A., *How the immune system works to protect the host from infection: a personal view*. Proceedings of the National Academy of Sciences, 2001. **98**(13): p. 7461-7468.
2. de Sousa, J.R., P.F.D.C. Vasconcelos, and J.A.S. Quaresma, *Functional aspects, phenotypic heterogeneity, and tissue immune response of macrophages in infectious diseases*. Infection and drug resistance, 2019. **12**: p. 2589.
3. STEPHEN, L. and G. MARTIN, *Pathogenesis and pathophysiology of bacterial infections*. Infections of the central nervous system, 2004: p. 331.

4. Webster, S.J., et al., *Distinct cell death programs in monocytes regulate innate responses following challenge with common causes of invasive bacterial disease*. The Journal of Immunology, 2010. **185**(5): p. 2968-2979.
5. Lai, C.-C., et al., *Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) and coronavirus disease-2019 (COVID-19): The epidemic and the challenges*. International journal of antimicrobial agents, 2020. **55**(3): p. 105924.
6. MacFaddin, J., *Biochemical Tests for Identification of Medical Bacteria*, Williams and Wilkins. Philadelphia, PA, 2000: p. 113.
7. Collee, J., et al., *Enterobacteriaceae-Escherichia, Klebsiella, Proteus and other genera*. 137-149. Collee, JG, JP Duguid, AG Froser and BP Marmion (ed 5.), Practical medical microbiology, 14th eds. Hong Kong: Mackie and MacCorthey, Lonyman Group UK Ltd, 1996.
8. Ahmed, S.S., et al., *Uropathogens and their antimicrobial resistance patterns: Relationship with urinary tract infections*. International journal of health sciences, 2019. **13**(2): p. 48.
9. Kadhim, A.K., S.A. Kadhum, and A.M. Al-Jbouri, *Demonstration of the effects of virulence factor and antibiotic resistance on the plasmids of Proteus mirabilis and Proteus vulgaris*. Journal of Pharmaceutical Sciences and Research, 2019. **11**(4): p. 1304-1312.
10. AL-Oqaili, N.A.D., M.K. AL-Shebli, and A.N. Almousawi, *Antimicrobial susceptibility and molecular characterization for some virulence factors of Proteus Mirabilis isolated from patients in Al-Qadisiyah Province/Iraq*. Al-Qadisiyah Journal of Veterinary Medicine Sciences, 2017. **16**(2): p. 1-7.
11. El-Gamasy, M.A., *Prevalence of infective organisms of infections of urinary tract in a sample of Arab infants and children*. Journal of Integrative Nephrology and Andrology, 2017. **4**(4): p. 136.
12. Karzan Mohammed, K., O. Faeza Burhan, and Y. Shahida Nooruldeen, *Isolation and Identification of Urinary Tract Infectious Bacteria and Exploring their Anti-drug Potential against Some Common Antibiotics*. J Microb Biochem Technol, 2017. **9**: p. 6.
13. Zhang, J., et al., *Outer membrane vesicles derived from hypervirulent Klebsiella pneumoniae stimulate the inflammatory response*. Microbial Pathogenesis, 2021. **154**: p. 104841.
14. Craig, A., et al., *Neutrophil recruitment to the lungs during bacterial pneumonia*. Infection and immunity, 2009. **77**(2): p. 568-575.
15. Deng, J.C., et al., *CpG oligodeoxynucleotides stimulate protective innate immunity against pulmonary Klebsiella infection*. The Journal of Immunology, 2004. **173**(8): p. 5148-5155.
16. Happel, K.I., et al., *Cutting edge: roles of Toll-like receptor 4 and IL-23 in IL-17 expression in response to Klebsiella pneumoniae infection*. The Journal of Immunology, 2003. **170**(9): p. 4432-4436.
17. Bhan, U., et al., *TLR9 is required for protective innate immunity in Gram-negative bacterial pneumonia: role of dendritic cells*. The Journal of Immunology, 2007. **179**(6): p. 3937-3946.
18. Glaser, L., et al., *Airway epithelial derived cytokines and chemokines and their role in the immune response to respiratory syncytial virus infection*. Pathogens, 2019. **8**(3): p. 106.
19. Regueiro, V., et al., *The uptake of a Klebsiella pneumoniae capsule polysaccharide mutant triggers an inflammatory response by human airway epithelial cells*. Microbiology, 2006. **152**(2): p. 555-566.

20. Khidoyatova, M. R., Kayumov, U. K., Inoyatova, F. K., Fozilov, K. G., Khamidullaeva, G. A., & Eshpulatov, A. S. (2022). Clinical status of patients with coronary artery disease post COVID-19. *International Journal of Health & Medical Sciences*, 5(1), 137-144. <https://doi.org/10.21744/ijhms.v5n1.1858>
21. Suryasa, I. W., Rodríguez-Gámez, M., & Koldoris, T. (2021). The COVID-19 pandemic. *International Journal of Health Sciences*, 5(2), vi-ix. <https://doi.org/10.53730/ijhs.v5n2.2937>