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Toll-Like Receptor-4 Expression Level upon SARS-Cov2 Infection with and without Bacterial/Fungal Secondary and Co-Infection Among Iraqi Patients

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Abstract

Cytokine storms are an excessive immune response triggered by severe viral infections, such as SARS-CoV2. Toll-like receptor 4 (TLR-4) is a key player in these storms. Secondary and co-infections can exacerbate the immune response, leading to amplified cytokine release and potentially severe disease outcomes. This review aims to evaluate the expression of TLR-4 in fungal and bacterial infections that are associated with and secondary to SARS-CoV2. The study collected specimens from 70 patients admitted to a Baghdad hospital's ICUs and 35 healthy controls, aged 15-80 years, from June 2022 to April 2023. Real-time RT-PCR was used for infection detection, while conventional methods were used to isolate pathogenic microorganisms and the Vitec 2 system. The findings of this study suggest that TLR-4 may play a crucial role in secondary and co-infections in individuals with severe SARS-CoV2. The study implies that increased TLR-4 expression might contribute to the development of bacterial secondary and co-infections. When TLR-4 detects bacterial components, it triggers immune responses aimed at eliminating the bacteria. However, an overactive TLR-4 response may lead to an excessive release of cytokines, resulting in a cytokine storm. This excessive immune response can cause tissue damage and exacerbate the severity of SARS-CoV2. Furthermore, the TLR-4 expression may not significantly contribute to the immune response against co-fungal infections with SARS-CoV-2, despite its role in antiviral responses and bacterial defense.
Keywords: SARS-CoV2, TLR-4, secondary infections, co-infections, immune response.

Резюме

Цитокиновите бури са прекомерен имунен отговор, предизвикан от тежки вирусни инфекции, като SARS-CoV2. Toll-like receptor 4 (TLR-4) е ключов играч в тези бури. Вторичните и съпътстващите инфекции могат да изострят имунния отговор, което води до ускорено освобождаване на цитокини и потенциално тежки резултати от заболяването. Това изследване има за цел да оцени експресията на TLR-4 при гъбични и бактериални инфекции, които са свързани и вторични на SARS-CoV2. Проучването събира проби от 70 пациенти, приети в интензивните отделения на болница в Багдад и 35 здрави контроли на възраст 15-80 години от юни 2022 г. до април 2023 г. RT-PCR в реално време е използван за откриване на инфекция, докато конвенционалните методи са използвани за изолиране патогенни микроорганизми и системата Vitec 2. Резултатите от това проучване предполагат, че TLR-4 може да играе решаваща роля при вторични и съпътстващи инфекции при индивиди с тежък SARS-CoV2. Проучването предполага, че повишената експресия на TLR-4 може да допринесе за развитието на бактериални вторични и съпътстващи инфекции. Когато TLR-4 открие бактериални компоненти, той задейства имунни реакции, насочени към елиминиране на бактериите. Въпреки това, свръхактивен TLR-4 отговор може да доведе до прекомерно освобождаване на цитокини, което води до цитокинова буря. Този прекомерен имунен отговор може да причини увреждане на тъканите и да влоши тежестта на SARS-CoV2. Освен това, експресията на TLR-4 може да не допринесе значително за имунния отговор срещу ко-гъбични инфекции със SARS-CoV-2, въпреки ролята си в антивирусните реакции и бактериалната защита.

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Introduction

SARS-CoV-2 is a group of viruses that can cause respiratory tract infections in humans, causing mild symptoms to lethal outcomes. An outbreak of acute respiratory syndrome caused by the coronavirus SARS-CoV-2 first emerged in the city of Wuhan, China, towards the end of 2019. This disease, now commonly referred to as SARS-CoV2, quickly spread within Wuhan and eventually escalated into a global pandemic (Acter *et al.*, 2020; AL-Mashhadani and AL-Thwani, 2022). SARS-CoV-2, the virus responsible for SARS-CoV2, initiates infection by attaching itself to a specific receptor on the surface of human cells, known as the ACE2 receptor. This attachment is facilitated by the interaction between the S1 subunit of the virus' spike protein and the ACE2 receptor (Gadanec *et al*., 2021).

ACE2 receptors are present on the surface of various human cells, particularly in the respiratory tract, lungs, heart, blood vessels, kidneys, and intestines. When the virus encounters ACE2 receptors on the cell surface, the spike protein's RBD binds to the receptor, initiating the infection process (AL-Mashhadani and AL-Thwani, 2022), it can also utilize other proteins on the cell surface to aid in its entry. Two such proteins are transmembrane protease serine 2 (TMPRSS2) and glucose-regulated protein 78 (GRP78) (Balmeh *et al.*, 2020).

Secondary bacterial or fungal infections significantly influence SARS-CoV2 mortality. These infections are caused by various pathogens, including multidrug-resistant organisms. Monitoring antibiotic-resistant bacteria and rational use of antibiotics is crucial during the pandemic (Jayakumar *et al.*, 2023). The prevalence of hospital-acquired pneumonia, acquired during a hospital stay, is a contributing factor to the high incidence of secondary infections in SARS-CoV2 patients. Inadequate infection control measures, and the compromised immune system of patients due to the viral infection itself can increase the risk of acquiring these infections within healthcare settings (Rawson *et al*., 2020).

Co-infections refer to the presence of multiple infections simultaneously in an individual. In SARS-CoV2 patients, co-infections are relatively rare, with a prevalence of about 7% in hospitalized patients. This means that a small proportion of SARS-CoV2 patients may have additional infections alongside the primary SARS-CoV2 infection. It is important to note that co-infections can potentially complicate the clinical course of SARS-CoV2 and may require specific management strategies (Grasselli *et al*., 2021, Russell *et al*., 2021).

The hyper-inflammatory response and cytokine storms associated with severe SARS-CoV2 cases have posed a significant challenge in finding effective treatment options. The leading cause of mortality in these cases is often respiratory distress, resulting from widespread inflammation and lung damage (Aguida *et al.*, 2021)

The TLR-4 receptor is a key component of the innate immune system, responsible for detecting certain molecules produced when cells are damaged or undergo lysis due to host tissue injury or viral infection. These molecules, known as damage-associated molecular patterns (DAMPs), serve as molecular alarms, alerting the immune system to the presence of tissue damage or infection (Aboudounya and Heads, 2021; Fadhil and Saleh, 2023). When a primary infection occurs, TLR-4 recognizes and binds to specific molecules on pathogens, known as pathogen-associated molecular patterns (PAMPs). This recognition initiates a signaling cascade that leads to the activation of various immune cells and the production of inflammatory cytokines (Ebermeyer *et al*., 2021; Khalaf *et al*., 2022).

In conclusion, the prevalence of hospital-acquired pneumonia and the emergence of multidrug-resistant bacteria have added to the complexity of managing bacterial infections in SARS-CoV2 patients, particularly in countries like Iraq. Implementing comprehensive infection control measures and promoting responsible antibiotic use are vital in minimizing the impact of these secondary infections and ensuring better patient outcomes.

Superinfections (Secondary) can complicate the management and treatment of SARS-CoV2 patients, as they may require additional antimicrobial therapy. Severe or immunocompromised SARS-CoV2 patients are generally more susceptible to superinfections due to a weakened immune response. TLR-4, also known as Toll-like receptor 4, is indeed considered a significant player in the development of cytokine storms observed in secondary infections with SARS-CoV-2.

In a secondary infection with SARS-CoV-2, the presence of pre-existing anti-SARS-CoV-2 antibodies, acquired either through a previous infection or vaccination, can enhance the activation of TLR-4 and subsequently amplify the production of pro-inflammatory cytokines. This excessive immune response can lead to the drastic release of cytokines, causing tissue damage and contributing to the severity of the disease.

Understanding the role of TLR-4 in the cytokine storms associated with secondary SARS-CoV-2 infections is crucial for developing effective therapeutic interventions. Targeting TLR-4 or modulating it signaling pathway could potentially help control the exaggerated immune response and contribute to the management of severe cases of SARS-CoV-2. However, further research is necessary to fully comprehend the complex interactions between TLR-4, cytokine storms, and SARS-CoV-2 infection.

Materials and Methods

This descriptive study in patients in ICU SARS-CoV2 wards was collected in hospitals in Baghdad, Iraq, from July 2022 to April 2023.Both genders with ages ranging from 15 to 80 years. those who reported positive cases of SARS-CoV2 using nasopharyngeal swabs were diagnosed using a molecular test. An information sheet was filled out for each patient, and written consent was obtained. 204 Sputum or oral swab specimens were also collected and cultured on different media. Then, the microorganisms were isolated by culture on four culture media: MacConkey, Agar Blood Agar, Chocolate Agar, and Sabouraud Agar to support the growth of the bacteria. After incubation, the colonies formed on the medium were identified using Gram stain and other biochemical tests. Diagnosis was made by conventional methods in addition to the Vitec 2 systems. Additionally, 105 blood specimens were gathered. RNA has been extracted from each and every sample. The samples underwent rapid processing to convert RNA to cDNA following the extraction. Each component's necessary volume was computed. QRT-PCR (SYBR Green) was used to measure the target *TLR-4* gene.

Molecular diagnosis of SARS-CoV2

The study involved testing samples obtained from a patient through a nasopharyngeal swab and preserved in a VTM viral sampling tube. RNA was extracted manually and SARS-CoV2 RNA was detected using a one-step RT-PCR method using the SARS-CoV2 nucleic acid detection kit (Maccura, Biotechnology Co. Ltd. 16#. China). The PCR amplification parameters were prepared according to the manufacturer's recommendation. Results were analyzed and positive when the ct of three targets (FAM, ROX, and CY5) was < 38. Interpretation and annotation of results were based on the criteria provided by the groups.

Primer preparation

The study involved extracting RNA from samples and converting them to cDNA. The TLR-4 gene was quantified using qRT-PCR SYBR Green. The cDNA was used as a template for PCR. The RT-qPCR Biolab kit primers were used for detection. Thermal cycler steps of conditions cDNA Reverse Transcription: step 1, 42°C in 30 min, step 2, 85°C in 5 min, and step 3, 4°C. Alpha Company provided these primers in lyophilized form Detection of TLR-4 expression was done using RT-qPCR Biolab kit primers sequences used in this study Primer sequence: GABDH Forward:5'- ACAACTTTGGTATCGTGGAAGG-3′ and GABDH Reverse: 5'- GCCATCACGCCACAGTTTC-3′, Primer sequence: TLR4 Forward 5'-TGAGCAGTCGTGCTGGTATC-3′ and TLR4 Reverse: 5'- CAGGGCTTTTCTGAGTCGTC-3′, qPCR master mix, (SYBR):10 µl, forward and reverse Primer:0.5 µl, cDNA Template: 4 µl and Nuclease-Free Water up to 20 µl, The qPCR Reaction run; the cycling protocol was programmed according to the thermal profile. The mixtures were subjected to the following thermal profile of TLR-4 and CAPDH gene expression cycling parameters in a qPCR (Rotor-**Gene** Q): Initial denaturation at 95°C for 1 min, followed by (1 cycle) of amplification with 60°C for 30 sec, followed by (40 cycles). The samples were analyzed in triplicate and CAPDH was used as endogenous control for normalization

Statistical analysis

A statistical study was conducted using the Statistical Package for Social Sciences (SPSS version 26, Inc., Chicago, IL, USA) and the Microsoft Excel Worksheet. The results and examples of the current study were analyzed. P-value significance was determined when the value was less than 0.05 ($P<0.05$), and 95% Confidence Interval (Sorlie *et al.*, 1995) and use GraphPad Prism to plot the data.

Ethical approval

The study was conducted in accordance with the ethical principles that have their origin in the Declaration of Helsinki. The study protocol, the subject information and the consent form were reviewed and approved by a local ethics committee according to document number 1316 A.

Results and Discussion

A study of cases of SARS-CoV2 in Iraqi hospitals and ICUs from July 2022 to April 2023, aged 15-80, involved real-time PCR tests to confirm health status and distinguish between infected and control samples. The study used a multiplex reverse transcription PCR system targeting ORF 1ab, E, and N genes, and a control gene to prevent

false negative test results. RT-PCR tests are considered the gold standard for detecting the genetic material of the SARS-CoV-2 virus, which causes SARS-CoV2. This test can accurately identify the presence of the virus in a person's respiratory sample. CT scans of the chest can also be helpful in visualizing any abnormalities in the lungs, aiding in the diagnosis of SARS-CoV2 (Al-Hashimi *et al*., 2023). A study conducted by Krishnaraj *et al.* (2022) highlighted the widespread RT-PCR (Reverse Transcription Polymerase Chain Reaction) is widely utilized for this purpose all over the world.

As shown in Fig.1, the distribution of SARS-CoV2 infection is displayed based on gender and age groups. Among the total number of patients, 26 (36.1%) were females, while 44 (61.1%) were males. To further analyze the age factor, the patients were categorized into three age groups: 15-45 years, 46-65 years, and 66-80 years. The number of patients in each age group was 20 (27.8%), 35 (48.6%), and 15 (20.8%) respectively.

individual's ability to fight off the virus effectively (Zyara *et al.*, 2023; Khudhr and Shehab, 2022).

In this study, 204 sputum and throat swab samples were collected from both severe and moderate SARS-CoV2 patients at two different time points - at the beginning of infection and after infection. The purpose of the study was to identify the pathogens present in these samples using selective media, as well as conventional methods and the Vitec 2 system.

The incidence rate of secondary infections following a primary infection with SARS-CoV2 is generally higher compared to the overall co-infection rate with SARS-CoV2. Primary infections occur when an individual first contracts the virus, while secondary infections refer to subsequent infections that may occur after the initial recovery from SARS-CoV2. Additionally, the respiratory system may be compromised following a primary infection, making it easier for other respiratory pathogens to establish an infection. Covid-19 can cause inflammation

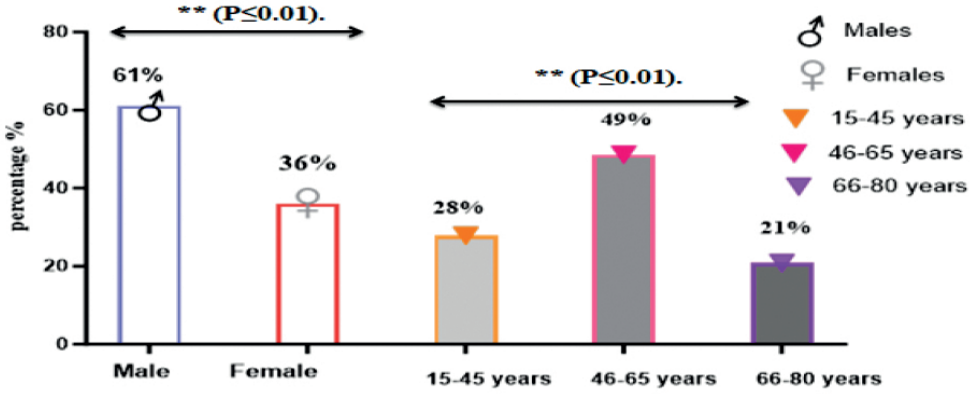

Fig. 1. Distribution of SARS-CoV2 according to age and gender (n=70)

A research study has found that there is a higher incidence of male SARS-CoV-2 patients compared to females. Therefore, it can be hypothesized that higher ACE2 expression levels in these individuals may contribute to an increased susceptibility to infection. Extensive research has shown that individuals who are sick, male, and older tend to exhibit higher levels of ACE2 expression even before being infected with the SARS-CoV-2 virus (Salah and Mehta 2021; Ghazzi *et al*., 2023).

The study emphasizes that individuals with pre-existing chronic conditions are more vulnerable to severe outcomes when infected with the virus. As people age, their immune system naturally declines, making it harder for the body to mount a strong defense against pathogens. Combined with chronic illnesses, the immune system's response becomes even weaker, further compromising the and damage to the lungs, creating an environment conducive to the growth of other microorganisms.

The findings from the study indicated the presence of different microbial species in the specimens. Bacterial infections were detected in a total of 34 specimens, with *Staphylococcus aureus* being the most prevalent at 10 cases (29.4%). This was followed by *Streptococcus pneumoniae* with 2 cases (5.9%), *Escherichia coli* with 3 cases (8.8%), *Pseudomonas aeruginosa* with 4 cases (11.8%), and *Klebsiella pneumoniae* with 6 cases (17.6%). Other bacterial pathogens identified were *Acinetobacter baunmanni* with 2 cases (5.9%) and *Moraxella* spp. (pathogenic strain) with 2 cases (5.9%). Apart from bacterial infections, fungal infections were also observed in the samples. Candida spp was found in 5 cases (14.7%). These results are summarized in Fig. 2.

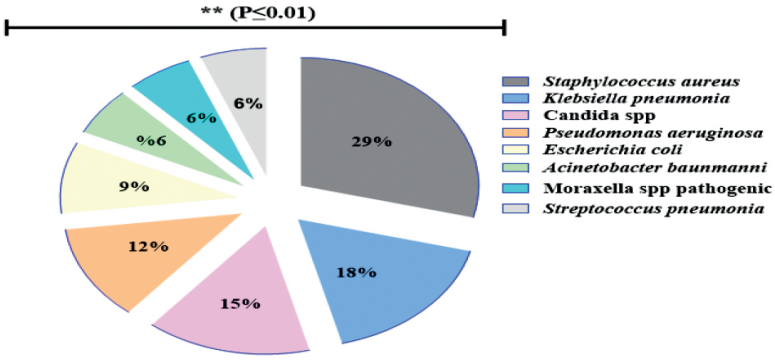

Fig. 2. Secondary and Co-infection isolate with SARS-CoV2(n=34)

It is worth noting that a considerable number of patients, 57 (28%), showed no co-infection. Additionally, the majority of the specimens, 112 (55%), were categorized as having normal flora, indicating the presence of non-pathogenic microorganisms commonly found in the body.

Based on the data presented, it can be concluded that the percentage of bacterial and fungal secondary and co-infections in individuals with SARS-CoV2 is approximately 16.6 %. On the other hand, the percentage of individuals with no secondary and co-infection is estimated to be around 28%. Additionally, it appears that the normal flora accounts for approximately 55% of the cases. There is supporting evidence for the significance of bacterial secondary and co-infections in viral respiratory diseases, this aspect remains understudied in the context of COVID-19. Detecting bacterial co-infections in COVID-19 patients poses a challenge as these bacteria can be part of the commensal bacteria normally present in the body, associated with an underlying chronic disease, or acquired during hospitalization (Boutin *et al*., 2021).

Secondary and co-infections can significantly impact the health outcomes of individuals with SARS-CoV2, particularly if these infections go undiagnosed. This is particularly important to consider during the administration of immunosuppressive drugs, as these can further weaken the immune system and make individuals more susceptible to co-infections.

Nosocomial pneumonia, also known as hospital-acquired pneumonia, is a significant concern in intensive care units (ICUs) and can pose a major risk to patients' health. While the associated deaths of SARS-CoV2 have primarily affected the elderly with pre-existing health conditions, the presence of lower respiratory tract infections, such as nosocomial pneumonia, can exacerbate the condition of patients (Velavan and Meyer, 2020).

Nosocomial infections are typically defined as infections acquired during hospitalization within 48-72 hours after admission. These infections can be transmitted through various means, including person-to-person contact, contaminated devices, and instruments. The proximity of patients and healthcare personnel in ICUs provides an environment conducive to the spread of nosocomial infections (Agaba *et al*., 2017).

TLR-4 is an important component of the innate immune system responsible for recognizing and initiating an immune response against viral pathogens. Also, plays a critical role in the innate immune response to pathogens, including bacteria and fungi. This review aims to evaluate the expression of TLR-4 in fungal and bacterial infections that are associated with and secondary to SARS-CoV2.

TLR-4 recognizes lipopolysaccharides (LPS), a component of the outer membrane of Gram-negative bacteria. TLR4 forms a complex with the co-receptor MD-2 to recognize LPS. This recognition also activates the NF-κB pathway and leads to the production of pro-inflammatory cytokines. Additionally, TLR4 activation induces the production of type I interferons, which play crucial roles in antiviral responses I (Stenzel *et al.,* 2008). as shown in Table 1. Fold of TLR-4 gene expression and relations secondary and co-infection with SARS-CoV2 and no secondary and co-infection with SARS-CoV2.

The dysregulation of TLR-4 in SARS-CoV2 patients may be postulated to be a contributing factor to the increased susceptibility to bacterial and fungal infections. TLR-4 expression acts as an important mediator of the immune response against these pathogens, initiating an inflammatory cascade and activating immune cells.

The attachment of bacterial pathogens to the airway epithelial cells is further facilitated by the dysregulated TLR4 and TLR5 pathways. This altered signaling disrupts the normal immune response, allowing bacteria to evade immune detec-

Table 1. TLR-4 gene expression upon SARS-CoV2 with and without secondary and co- infection(n=105)

Groups	Fold of TLR-4 gene expression against GAPDH
Healthy control	1.00 ±0.00 b
SARS-CoV2infection	2.21 ±0.28 a
SARS-CoV2 with co-infection	0.66 ±0.28 a
SARS-CoV2 with secondary infection	1.69 ±0.73 ac
LSD	1.57 **
(P-value)	0.002

Means having with the different letters in column differed significantly, ** (P≤0.01)

tion and clearance. Consequently, secondary bacterial pneumonia can develop and complicate the initial viral infection, leading to increased morbidity and mortality (Elabbadi *et al*., 2021).

Also, when the virus genome enters the cell, the pattern-recognition receptors PRRs on the surface of the infected cell (such as TLR-4), endosomal Toll-like receptors TLR3 and TLR7, and cytosolic receptors (MDA5 and RIG I) recognize the SARS-CoV2 RNA. Thus, these transcription factors lead to an activity-inducing gene transcription for (α) and (β) IFN and pro-inflammatory cytokines, the production of pro-inflammatory cytokines, on the other hand, helps regulate the immune response and recruit immune cells to the site of infection.

This coordinated immune response is crucial for the eventual clearance of the virus and resolution of the infection (De Wit *et al.,* 2016). Despite their crucial role as bactericidal, pro-inflammatory cytokines such as TNF-α produced in response to infection could be detrimental to the host cells. During a viral infection, TLR and RIG-I-like receptor activation induces the production of type I IFNs, which can augment the inflammatory response to TLR ligands, including lipopolysaccharides (Didierlaurent *et al.,* 2008). According to data in Table 2, the fold of TLR4 expressions according to pathogen type of co-infection.

Preliminary findings demonstrate that SARS-CoV2 patients with secondary bacterial and fungal infections often exhibit altered TLR-4 expression compared to individuals without co-infections. Bacterial and fungal PAMPs can directly activate TLR-4, triggering an immune response. Dysregulation or impaired expression of TLR-4 in SARS-CoV2 patients may compromise the clearance of pathogens and exacerbate the severity of secondary and co-infections (Bourgeois and Kuchler, 2012).

TLR4 expression is likely to be upregulated in secondary bacterial infections, as it is crucial for recognizing LPS from Gram-negative bacteria, activating the immune response, and promoting bacteria clearance (Stenzel *et al.*, 2008; Thajel *et al*., 2023). Also, TLR4 expression can be altered during viral secondary infections, such as respiratory syncytial virus (RSV) infection, which can upregulate it in the airway epithelium, enhancing immune responses against viral infections (Agac *et al*., 2023).

In the case of secondary fungal infections, TLR4 expression may not play a significant role, as TLR4 primarily recognizes bacterial LPS. Instead, other pattern recognition receptors, such as TLR2 or TLR5, might be more involved in the recognition and response to fungal pathogens like *Candida albicans* or Aspergillus (Bruno *et al*., 2020). According to data presented in Table 3, the fold of

Table 2. TLR4 expressions according to pathogen type of co-infection

Groups	Fold of TLR-4 gene expression
Healthy Control	1.00 ±0.00 b
SARS-CoV2 infection	2.21 ±0.28 a
SARS-CoV2 co-infection with *S. pneumonia*	1.12 ±0.14 c
SARS-CoV2 secondary and co-infection with *Moraxella* spp. (pathogenic strain)	0.76 ±0.30 cd
SARS-CoV2 co-infection with *Candida* spp.	0.64 ±0.25 de
LSD	0.85 *
(P-value)	(0.01)

This means having with the different letters in column differed significantly, *(P≤0.005)

Table 3. TLR4 expressions according to pathogen type of secondary infection

Groups	Fold of TLR-4 gene expression
Healthy Control	1.00 ±0.00 h
SARS-CoV2 infection	2.21 ±0.28 g
SARS-CoV2 secondary infection with *K. pneumonia*	2.46 ±0.25 a
SARS-CoV2 secondary infection with *S. aureus*	1.94 ±0.72 ab
SARS-CoV2 secondary infection with *P. aeruginosa*	1.22 ±0.3 cd
SARS-CoV2 secondary infection with *E. coli*	1.14 ±0.36 de
SARS-CoV2 secondary infection with *A. baunmanni* 0.99 ±0.35 ef	
SARS-CoV2 secondary infection with *Candida* spp.	0.55±0.14 fg
LSD	1.102 **
(P-value)	(0.0001)

Means having with the different letters in column differed significantly, ** (P≤0.01).

TLR4 expressions according to pathogen type of secondary infection.

Conclusion

The incidence rate of secondary infections following a primary infection with SARS-CoV2 is generally higher compared to the overall co-infection rate with SARS-CoV2. This can be attributed to factors such as a weakened immune response, compromised respiratory system, and ongoing exposure to infected individuals. In this study, we observed that not all patients had a co-infection with the SARS-CoV-2 virus, which could either be a secondary infection or a side effect of taking antibiotics without consulting. antibiotics, which are frequently prescribed for COVID-19, are ineffective against SARS-CoV-2 infection. This adds another layer of complexity in detecting bacterial co-infections since conventional culture-based detection methods may have reduced sensitivity due to prior antibiotic usage. It is important to note that the use of high-dose antibiotics may increase the likelihood of secondary and co-infections with drug-resistant bacteria.

Furthermore, our results confirmed an increase in the expression of TLR-4 in cases where there was a bacterial co-infection with SARS-CoV2 among patients. Interestingly, certain bacterial infections such as *K. pneumoniae* and *S. aureus* had a higher impact on TLR-4 expression compared to other microbial species. These findings highlight the importance of considering bacterial/fungal secondary and co-infections in SARS-CoV2 patients, as they can potentially worsen the symptoms and complicate the treatment. Understanding the role of TLR-4 in these co-infections can provide insights into the immune response and help in the development of targeted therapies for managing SARS-CoV2 infections. TLR4 role in the immune response to certain pathogens may be less prominent against co-fungal infections with SARS-CoV-2, necessitating further studies to understand immune response mechanisms and potential therapeutic targets.

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