Antimicrobial Resistance and Detection Some Virulence Factors of Bacterial Isolated from Patients with Covid-19

Zahraa jasim Sattay[1], Waqas Saadi Mahmoud[2]
[1, 2]College of science / Tikrit University /Iraq
Email: zahraa.j.satay4422@st.tu.edu.iq

Abstract

For healthcare systems and doctors, the advent of the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has provided a daunting medical challenge. This study investigated the incidence of bacteria in patients with Covid-19 and their antimicrobial susceptibility pattern. One hundred ten throat swab samples were collected from Covid-19 patients suffering from acute pharyngitis. Bacterial identification was determined by colony morphology, microscopic, biochemical tests, and the VITKE2 system. Results showed 50 samples had positive culture 30(60%) isolates belonged to *S.auresu*, 10(20%) isolates belonged to *S.pyogenes,* 5(10%) belonged to *K.pneumonia*, 3(6%) isolates of *S.epidermids* and 2(4%) isolates *E.coli*, and male30(60%) more affected than female20(40%). The sensitivity of the bacterial isolates under study was tested to 11 antibiotics. The results showed that *S.aureus* were resistance to Tetracycline70%, Amoxicillin80%, Amoxicillin/Clavulanic acid63.3%, Cefotaxime60%, Ampicillin73%, and Penicillin80%. *S.pyogenes* were resistance to Tetracycline60%, Amoxicillin/Clavulanic acid70%, Ampicillin60%, and Penicillin60%. The results of the study showed some factors associated with the virulence of bacterial isolates and it was found that *S.aureus* were possesses four virulence factors hemolysin, protease, DNase, and lecithinase. *S.pyogenes* possesses three virulence factors hemolysin, protease, DNase. The identification of bacteria isolated from Covid19 patients with acute bacterial pharyngitis was performed in this study, which is one of the few of its kind in Iraq.

Keyword: COVID-19, S.aureus, S.pyogenes, S.epidermidis, E. coli, K.pneumonia

1. Introduction

Pharyngitis is a widespread upper respiratory tract disease characterized by pharyngeal inflammation (Artsimovich et al.,1994; Adeishvili et al.,2013). Pharyngitis is characterized by pain, tickling, discomfort in the throat, sleep disturbance, and complaints of constant runoff of mucus along the back wall of the pharynx ("lump" of mucus, coughing). These symptoms significantly worsen the quality of life of patients. In the case of recurrent Pharyngitis, different parts of the pharynx can be affected: nasopharynx, oropharynx, and larynx; often, the inflammatory process is descending. Morphological changes of the mucous membrane in Pharyngitis are predominantly localized in one of the anatomical parts of the pharynx, which allows for allocating individual nosology, for example, chronic nasopharyngitis (Rice et al.,1995).

Pharyngitis usually is classified according to the nature of the developed mucous changes: catarrhal (simple), atrophic or subtropic, and hypertrophic (hyperplastic, granulosa) (Snimshikova et al.,2018). A virus, which several diseases may produce, causes between 50% to 80% of pharyngitis or sore throat symptoms. The most prevalent pathogens include Rhinoviruses, Coronaviruses, Adenoviruses, Influenza virus, Parainfluenza virus, rare viruses' Respiratory syncytial virus, Herpes simplex viruses (types 1 and 2), Enteroviruses, Coxsackie virus, Epstein-Barr virus, Cytomegalovirus, Human immunodeficiency virus (HIV) (Nikolaev et al.,2007; Venezia et al., 2012). As previously stated, bacteria can also cause Pharyngitis, namely Staphylococcus aureus, 'Streptococcus pyogenes', E. coli, Proteus spp. Klebsiella spp., 'Pseudomonas aeruginosa etc (Moiranghem and Gurung., 2013; Abid Alkadem., 2018). 'Arcanobacterium haemolyticum' is an uncommon cause of Pharyngitis, and 'Neisseria gonorrhoeae' can cause acute Pharyngitis in sexually active teens. Other bacteria, such as 'Francisella tularensis', Yersinia enterocolitica, 'Corynebacterium diphtheria', and combination infections with anaerobic bacteria, are uncommon causes. 'Chlamydophila pneumonia' and 'mycoplasma pneumonia' have been rarely linked to pneumonia in humans. The causative agent of 'Lemierre syndrome', 'Fusobacterium necrophorum', can also cause uncomplicated Pharyngitis (Jensen et al., 2007).

The severe acute respiratory syndrome coronavirus 2 '(SARS-CoV-2) that causes the coronavirus disease 2019 '(COVID-19) was first discovered in December 2019 in Wuhan, China, and is presently spreading around the world (Wang et al.,2020). Respiratory viral infections frequently involve co-infections and superinfections (McArdle et al.,2018; Paget et al.,2019). In patients with viral infections, secondary or bacterial co-infections with other viruses can

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greatly increase the death risk, according to laboratory, clinical, and epidemiological investigations (Beadling et al.,2004; Metzger et al.,2013).

RTIs are a group of illnesses involving microorganisms such as viruses, bacteria, and fungi that are restricted to the upper respiratory tract (such as rhinitis, sinusitis, pharyngitis, or tracheitis) and lower respiratory tract (mostly bronchitis and pneumonia). The pathogens involved have a diverse and heterogeneous character that reflects a wide range of infection settings (Mandell et al.,2005). Superinfections are consecutive infections caused by two separate organisms, although co-infections might be concurrent infections. Following an initial viral infection, bacterial superinfections are the situation that has been studied the most in the literature (Bello et al.,2014).

2. Materials and Methods

Study including 110 throat swab samples were collected from Covid-19 patients suffering from pharyngitis of both sexes (males &females) and ages 20-75. These patients were admitted to the two leading hospitals in Salah Al-Din and Baghdad governorate during the period 1/11/2021 until 25/3/2022, and the samples are transferred directly to the microbiology laboratory, specimens are planted on Blood agar, MacConkey agar, Nutrient agar and Mannitol salt agar for growth bacteria species, and incubated at 37C° for 18-24hours, to explore secondary bacterial species linked with novel coronavirus, the bacteria were diagnosis through phenotypic and microscopic examination and through biochemical tests that included (Oxidase, Coagulase, Catalase, IMVIC test) (Forbes et al., 2002; Koneman et al., 1997). The diagnosis is confirmed by using VITKE2 system. Use this device to confirm the diagnosis most types after diagnosing them using the usual methods.

antibiotic sensitivity test was carried out using 11 types of antibiotics on Gram-positive and Gram-negative bacterial isolates under study on Muller Hinton-agar medium according to the Kirby-bauer method described by the World Health Organization (Vandepitte et al.,2003), which included Imipenem (IMP), Tetracycline (TE), Amoxicillin (AX), Amoxicillin\Clavulanic acid (AMC), Azithromycin (AZM), Ceftriaxone (CTR), Chloramphenicol(C), Gentamycin (GN), Amikacin (AK), Ampicillin (AMP), Penicillin(P) Considering that the Turkish business BIOANALYS produced the pills utilized, and that the outcomes were documented by measuring the width of the 'inhibition zone surrounding each tablet.

2.1 Investigation of the production of virulence factors

The ability of the isolates to analyze red blood cells, analyze proteins, lecithin and DNase was tested using assay (Atlas and Synder, 2013 & Macfaddin,2000).

1- Hemolysin Production test
2- Protease Production test
3- DNase test
4- Lecithinase test

3. Results & Discussion

A total of 110 throat swab samples are collected from patients confirmed to be infected with coronavirus SARS-Cov-19. It found that 50 samples were positive growth and 60 were negative growth. The results showed the number of males 30(60.0%) and females 20(40.0%). These results were consistent with (Mohammed et al.,2020) demonstrated that males are more than females. As shown in table (5), the age group (50-70) recorded the highest infection rates among COVID-19 patients (42%). This might be explained by having a weakened immune system, being overweight, and having some chronic illnesses, including diabetes, asthma, and heart disease. The result almost agreed with (Dhuaif,2021), who recorded that the age group (46-65) was the highest infection and recoded (43%) Table1.

Table 1: Distribution of diseases according to age for COVID-19 patients.

Age group	Number	Percentage
50-70	21	42%
40-50	15	30%
30-40	9	18%
Less than30	5	10%
Total	50	100%

The isolates are distributed into 43 isolates (86.0 %) belonging to Gram-positive bacteria and 7 isolates (14.0%) belonging to Gram-negative bacteria. Table (1) shows bacteria isolates from pharyngitis in Coronavirus SARS-Cov-19 patients. Of which 30(60.0%) were S.aureus , S.pyogenes 10(20.0%), K. pneumonia 5(10%), S. epidermidis 3(6%) and E. coli 2(4%).

Table 2: Percentage of bacteria species isolated from COVOD-19 patients.

Bacterial isolates	Number	%
S.aureus	30	60%
S.pyogenes	10	20%
K.pneumonia	5	10%
S.epidermidis	3	6%
E. coli	2	4%
Total	50	100%

The results show 43 isolates of Gram-positive bacteria, where it showed 30 isolates of S.aureus with a percentage of 60% where the most frequent bacteria among isolated bacteria, The capacity to manufacture coagulase and DNase makes S.aureus the most clinically significant species of Staphylococci. It is an opportunistic pathogen that commonly exists in the human microflora and causes illness when the immune system is weakened (Danielsson,2002; Oliveira et al.,2002). Where these results agreed with (Naher and Al-Charrakh.,2021), who found that S.aureus were one causative agent of pharyngitis with Coronavirus SARS-Covid-19 with a percentage(18.5%), S.pyogenes were 10 isolates with percentage 20% is one of the bacterial causes secondary bacterial infection, where this result is consistent with(Abdul-Hadi and Al-Charrakh,2021) which founded that S.pyogenes isolates are the principle causative of pharyngitis with Coronavirus SARS-Covid-19 with percentage 44%,

(Musher et al.,2021) which demonstrated that S. pyogenes bacteria have a variety of virulence factors that aid in their ability to propagate by destroying connective tissues and resisting the body's defenses, thus increasing its virulence, such as M protein, Capsule and Toxin, Where showed (Brooke et al.,2001) is that these bacteria cause of the sinusitis, acute pharyngitis, tonsilitis and pneumonia, where Fiverr discovered during the influenza outbreak in 1918 the S.pyogenes bacteria is a prominent bacterial organism in influenza, Also, (Shakoor et al.,2019), where discussed in his research the critical role that this bacterium capsule has in the pathogenesis of bacteria. , as it acts as a physical barrier that helps it survive for as long as possible by resisting the body's immunity in addition to its role in resisting the process of phagocytosis.

As for S.epidermidis, the infection rate was 3 isolates with percentage 6%, these result was agreed with (Naher and Al-Charrakh.,2021) which demonstrated that S.epidermidis is considered one of the factors that cause pharyngitis, these bacteria have the ability to infect respiratory tract (Kloos, 1994), it is considered opportunistic pathogens that take advantage of the weak immune system of the host and the length of stay in the hospital to cause disease (Brooks et al, 2013). As for the number of Gram-negative bacteria, shown in Table (1), that five isolates of K.pneumonia are (10%), these result was consistent with(Abdul-Hadi and Al-Charrakh,2021), who found that K.pneumonia were one causative agent of pharyngitis with coronavirus SARS-Covid-19 with percentage (7%), Klebsiella pneumonia belongs to the bacterial species that cause opportunistic infections, and its pathogenicity is attributed to the fact that it contains several important virulence factors, especially the capsular virulence factor (Ssekatawa et al., 2021). K. pneumonia bacteria are found in healthy people, comprising 5-38% in the stool and 1-6% in the nasopharynx. Outbreaks of hospital-acquired infections occur through the contaminated hands of health workers and the use of harmful artificial respiratory devices and newborns (Para et al., 2017; Ergul et al., 2018).

Where the results showed that E. coli 2 isolates with a percentage (4%). These results close match what was found (De Lastourrs et al.,2015) We hypothesize that prolonged supine position, inhalation from the stomach, altered gastric pH caused by routine use of a proton inhibitor, multiple interactions with medical personnel, or possibly a disturbed local immune system, are all factors that promote E. coli oropharyngeal colonization. Additionally, salivary composition may have an impact on oropharyngeal content because it affects bacterial adhesion and causes reversible bacterial changes that enhance bacterial growth. These elements might account for the relatively low levels of E. coli observed in the oropharynx.

3.1 Antibiotic susceptibility test

To determine the antibiotic resistance of bacterial isolates in this study, 14 antibiotics were tested based on (Kirby Bauer, 1966), and the results of this study found that most isolates of bacteria from COVID-19 with secondary bacterial infection were resistant to common antibiotics (Table 2).

As showed in table (3) S.aureus were resistance to Tetracycline(70%), Amoxicillin (80%), Cefotaxime (60%), Ampicillin (73.3%) and Amoxicillin/Clavulanic acid (63.3%) . In humans, this may be brought on by community members' lack of health knowledge and the frequent usage of antibiotics. These results were disagreed with (Naher and Al-Charrakh,2021) reported S.aureus were resistance for Ampicillin 11%, Amoxicillin/Clavulanic acid 11%,Cefotaxim45%,and Tetracycline50%. Although these bacteria were also resistant to penicillin, Staphylococcal resistance to penicillin is caused by the Blaz' gene, which codes for the β-lactamase enzyme. When staphylococci are exposed to 'β-lactam antibiotics, this mostly extracellular enzyme hydrolyzes the β-lactam ring and renders the β-lactam inactive. Two nearby regulatory genes, the repressor blaI and the antirepressor blaR1, regulate Blaz (Kernodle,2000). Recent research has shown that sequential cleavage of the regulatory proteins BlaR1 and BlaI is necessary for the signaling pathway that leads to the creation of β-lactamases. 'BlaR1, a transmembrane sensor-transducer, cleaves itself after being exposed to β-lactams (Gregory et al.,1997; Zhang et al.,2001). (Zhang et al.,2001) Hypothesize that the cleaved protein acts as a protease that either directly or indirectly cleaves the repressor BlaI (BlaR2, another protein, may be implicated in this route), allowing Blaz to produce the enzyme. The current study results show that S.pyogenes were resistant to Tetracycline, Amoxicillin/clavulanic acid, Ampicillin, and Penicillin. These results were consistent with results obtained by several authors (Jubeh et al.,2020). The current investigation discovered that secondary bacterial infections in COVID-19 individuals had isolated bacteria extremely resistant to classical antibiotics. Since antibiotics are losing their effectiveness due to the target species' development of resistance mechanisms, antimicrobial resistance is a significant worry in the healthcare industry. Also, these results were compatible with (Naher and Al-Charrakh,2021) demonstrated that these bacteria were resistance Tetracycline 50%, Ampicillin 50%, and Amoxicillin/clavulanic acid50%, and bacterial resistance is brought on by the possibility of multidrug-resistance (MDR) infections in elderly and immunocompromised individuals.

As *S.epidermidis* were completely resistant to Tetracycline, Amoxicillin, Azithromycin, Ampicillin and Penicillin with a percentage 100%.

However, Amoxicillin\Clavulanic acid, Cefotaxime, Chloramphenicol, and Gentamycin showed moderate resistance for these bacteria as a showed in table (3). *S.epidermidis*, antibiotic penetration, and permeability are reduced by using the exopolysaccharide matrix or the bacteria's ability to form biofilms as a defense mechanism. Antibiotics such as tetracycline, aminoglycosides, cephalosporins, fluoroquinolones, penicillin, and macrolides are frequently ineffective against these bacteria (Schaefler,1997; Duran et al.,2012). Antibiotic resistance is caused by specific genes for antibiotic resistance (Schaefler,1997; Duran et al.,2012). Antimicrobial resistance to aminoglycosides, tetracycline, macrolides, and penicillin is caused by the genes aacA-D, tetK, ermC, and mecA (Dehkordi et al.,2017; Schaefler,1997; Duran et al.,2012). These outcomes closely match what was obtained (Chabi &Momtaz,2019) reported S.epidermidis were resistant to Penicillin95.65%, Tetracycline 91.30%,and Azithromycin60.86%,and (AL-Samraey,2021) show that S.epidermidis were resistant to Gentamycin75% and Azithromycin 75% .

As for K.pneumonia, the results show complete resistance to Tetracycline, Amoxicillin/clavulanic acid, Cefotaxime, Penicillin, and Ampicillin table(3). K. pneumoniae evolved a variety of antimicrobial agent resistance mechanisms, including an efflux pump, altered membrane permeability, inactivating enzyme synthesis, target site alteration, and acquisition of alternative metabolic pathways that are resistant to antibiotics (Munoz et al.,2007),Cephalosporinase and penicillinase, β-lactamase enzymes may have a role in K.pneumonia resistance to the cephalosporin and penicillin groups by cleavage the drugs β-lactam ring Pencilline and cephalosporin can be rendered inactive(Stock and Wiedmenn, 2001; Pagani et al., 2006). These results closely match what was obtained (Al-Obaidy,2014) reported K.pneumonia were resistance to Amoxicillin/clavulanic acid97.5%,Ampicillin97.5%,Cefotaxime 90%,and Tetracycline35%.

As for E. coli shows complete resistance to Tetracycline, Amoxicillin, Cefotaxime, Amoxicillin\Clavulanic acid, Ampicillin, and Penicillin (see table 3). In order to inactivate the antibiotics, lactamases hydrolyze the β-lactam ring, which is the main mechanism by which E. coli develops resistance to lactam antimicrobial agents (Livermore,1995). Four pathways, including efflux, ribosomal protection, enzymatic inactivation, and target modification, mediate tetracycline resistance (Chopra and Roberts,2001). These results closely match what was obtained (Olorunmola et al.,2013; Abdulsahib,2015).

3.2 Detection of Hemolysin Production

Hemolysin is a virulence factor possessed by bacteria and can destroy the cell membrane of red blood cells (Arthur et al.,2002). The results of this study are shown figure (1) S.aureus (100%), S.pyogenes (100%), and E.coli (50%) showed positive results for hemolysin production. The ability of bacteria to produce hemolysin varies according to several factors, the most important of which is the source of red blood cells used in the medium through which the ability of bacteria to produce hemolysin is detected. It is also affected by the test method used and the presence of serum and cholesterol in the blood, which leads to inhibition of the hemolysis process (Hellerstein,2002). These results are close to what was found (Al-Doori,2018; Al-khalidi,2016; Abbas,2021).

3.3 Detection of Proteas production

Cell survival and communication are significantly influenced by extracellular protease. According to some researchers, clinical isolates are extremely pathogenic and cause many human illnesses since they release different toxins (Olchowik-Grabarek et al.,2020). figure (1) results indicate that the bacteria S.aureus, S.pyogenes, K.pneumonia, E.coli With percentage 100%, 100%,60% and 50% respectively. These results closely match what was obtained (Naher and Al-Charrakh,2021; Karlsson and Arvidson, 2002).

3.4 Detection of Lecithinase production

The term lecithinase refers to a heterogeneous group of enzymes that participate in the breakdown of one or more Aster linkage in the glycero-phosphorylated lipids of the cell membrane (Ansell and Hawthrone,2008). The results show in figure (1) S.aureus, S.epidermidis, and K.pneumonia with percentage 90%, ,100%, and 100% respectively. These results align with what was discovered (Al-Doori,2009; Hullur et al.,2022).

3.5 Detection of DNase production

Extracellular DNases / nucleases, produced by a variety of bacteria, are necessary for virulence, biofilm formation, extracellular DNA use as a nutrient, and (NETs) (Dang et al., 2016). The study results are shown in table (1) S.aureus and S.pyogenes with 100% and 100% percentages, respectively. These results were consistent with (Remmington and Turner,2018; Khwen et al.,2021).

Table 3: shows percentage of antibiotic resistance for bacterial isolates from throat swab sample patients with COVID-19.

Antibiotic	S.aureus	S.epidermidis	S.pyogenes	K.pneumonia	E.coli
Imipenem	0	33.3	10	0	0
Tetracycline	70	100	60	100	100
Amoxicillin\Clavulanic acid	63.3	66.7	70	100	100
Amoxicillin	80	100	40	80	100
Azithromycin	40	100	10	20	50
Cefotaxime	60	66.7	40	100	100
Chloramphenicol	30	66.7	20	20	0
Gentamycin	43.3	66.7	30	20	50
Amikacin	13.3	0	20	0	50
Ampicillin	73.3	100	60	100	100
Penicillin	80	100	60	100	100

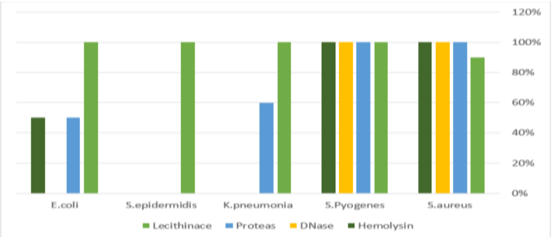

Figure 1: The ability of isolates to produce virulence factors

4. Conclusion

This study is considered the first study of its kind in Iraq for isolating and characterizing bacteria isolated from Covid-19 patients infected with pharyngitis. The study concluded that the high rate of Staphylococcus aureus followed S.pyogenes , K.pneumonia, S.epidermidis, and E.coli , where males were more affected than females. These bacteria were resistant to Tetracycline, Amoxicillin, Cefotaxime, Ampicillin, and Penicillin except Imipenem, Azithromycin, Chloramphenicol, Gentamycin, Amikacin, Amoxicillin\Clavulanic acid and more virulence when detected some virulence factors.

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