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The Role of Biofilm Formation of Secondary Bacterial Infections in Severity of COVID-19 Patients

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Abstract

The present study included One hundred and fifty-five specimens were collected from COVID-19 patients (male and female) admitted and hospitalized in ICU after confirming the infection by using Reverse transcription-polymerase chain reaction (RT-PCR) as well as X ray and CT scan at Baqubaa Teaching Hospital from the period of the first of October 2021 to the first to March 2022.The sources of the specimens include (100) pharyngeal swabs and (55) sputum. Selective and differential media, microscopic examination, biochemical tests and the Vitek-2 compact system were used in diagnose all the isolates. Forty-five (29%) isolates from a total of 155 sever COVID-19 patients showed positive growth at a percentage (30%) while 70% showed negative growth. The positive growth isolates were distributed as 155/10 (6.45%) isolates of gram-positive Staphylococcus aureus were isolated only from Pharyngeal swab at a percentage of 10 (10%) and two types of gram-negative isolates which was 155/22(14.19%) of Pseudomonas aeruginosa and 13(8.38%) Acinetobacter baumannii, both of them isolated from Pharyngeal swab 5 (5%) and 9 (9%), and from Sputum was 17 (30.9%), and 4(7.27%) respectively. Antibiotics sensitivity test revealed strong resistance against 12 antibiotics from different classes were used for each isolates, the results showed that there was numerous bacterium isolates with multiple drug resistance (MDR) and extensively drug resistance (XDR) the results indicated that from the total 10 isolates of *Staphylococcus aureus* showed resistance to Chloramphenicol (87%), Clarithromycin (85%), Trimethoprim- sulfamethoxazole (82%), Imipenem (81 %), Levofloxacin (79 %), Nitrofurantoin (78%), Penicillin and Doxycycline

(75%), Gentamicin (74 %), Azithromycin (73%), Tetracycline (66%), Ciprofloxacin (62 %) and Clindamycin (60%). The resistance of *P. aeruginosa* was variable as follows: 83%, 81%, 80% 79%, 78% and 76% of the isolates resistant to Ciprofloxacin, imipenem, Piperacillin, Gentamicin,Tetracycline, Amikacin and Meropenem respectively. Whereas the resistance against Tobramycin and Levofloxacin was 73% and 65% respectively. Concerning to *A. baumannii;* the resistance rate was 88%, 85%nad 81% against Gentamicin, piperacillin and cefatzidime and cefepime respectively, while the resistance against meropenem and imipenem was 78% and 75% respectively. About tobramycin, Amikacin, tetracycline and Levofloxacin the rate of resistance was 79%, 76%, 71% and 66% respectively. Biofilms formation of the bacteria isolated from severity cases of COVID-19 patients showed that 75% of *S. aureus* were strongly former, 20% moderately and 5% were non-former, whereas *A. baumannii* isolates (90%) were strongly former, 10% moderately and no isolate was former. About *P. aeruginosa* (85%) were strongly former, 15% moderately and no one was former.

Keywords: COVID-19, Secondary Bacterial Infections, Antibiotics Resistance, Biofilm formation.

دور تكوين الاغشية الحيوية للعدوى البكتيرية الثانوية في شدة الاصابة لمرضى COVID-19

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الخلاصة

تضمنت الدراسة الحالية مائة وخمسة وخمسين عينة تم جمعها من مرضى COVID-19 (ذكور وإناث) الذين تم إدخالهم إلى المستشفى في وحدة العناية المركزة بعد التأكد من الإصابة باستخدام النسخ العكسي لتفاعل البوليميراز المتسلسل (RT-PCR) وكذلك الأشعة السينية والأشعة المقطعية في مستشفى بعقوبة التعليمي من الفترة من الأول من تشرين الأول2021 إلى الأول من آذار 2022 وشملت مصادر العينات (100) مسحة بلعومية و (55) بصاق. تم استخدام الاوساط الانتقائية والتفاضلية والفحص المجهري والاختبارات البيوكيميائية ونظام Vitek-2 المضغوط في تشخيص جميع العزلات. أظهرت 45 (29%) عزلة من إجمالي 155 مريضًا COVID-19 نموًا إيجابيًا بنسبة (30٪) بينما أظهر 70٪ نموًا سلبيًا. توزع

النمو الإيجابي من إجمالي 155؛ 10 (6.45٪) عزلة من *Staphylococcus aureus* موجبة لصبغة جرام تم عزلها فقط من مسحات بلعومية بنسبة 10 (10٪) ونوعين من العزلات سالبة لصبغة جرام بنسبة 22 (14.19٪) من *Pseudomonas aeruginosa* 13 (8.38٪) *Acinetobacter baumannii* وكلاهما معزولان من مسحة بلعومية 5 (5٪) و 9 (9٪) ومن البلغم 17 (30.9٪) و 4 (7.27٪) على التوالي.

أظهر اختبار الحساسية للمضادات الحيوية مقاومة عالية ضد 12 مضادًا حيويًا من فئات مختلفة تم استخدامها لكل عزلة ، وأظهرت النتائج وجود العديد من العزلات البكتيرية ذات المقاومة المتعددة للأدوية (MDR) والمقاومة الشديدة للأدوية(XDR), أشارت النتائج إلى أنه من إجمالي 10 عزلات من *Staphylococcus aureus* ، أظهر مقاومة لكلورامفينيكول (87٪) ، كلاريثروميسين (85٪) ، تريميثوبريم- سلفاميثوكسازول (82٪) ، إيميبينيم (81٪) ، ليفوفلوكساسين (79٪) ، نتروفورانتوين (78٪) ، بنسلين ودوكسيسيكلين (75٪).) ، الجنتاميسين (74٪) ، أزيثروميسين (73٪) ، التتراسيكلين (66٪) ، سيبروفلوكساسين (62٪) ، الكليندامايسين (60٪) ، وكانت مقاومة *P. aeruginosa* متغيرة على النحو التالي: 83٪ ، 81٪ ، 80 79٪ ، 78٪ و 76٪ من العزلات المقاومة للسيبروفلوكساسين ، الإيميبينيم ، البيبراسيلين ، الجنتاميسين ، التتراسيكلين ، الأميكاسين والميروبينيم على التوالي. بينما كانت المقاومة ضد توبراميسين وليفوفلوكساسين 73٪ و65٪ على التوالي. بشأن *A. baumannii*؛ كانت نسبة المقاومة 88٪ و85٪ و81٪ ضد الجنتاميسين والبيبراسيلين والسيفاتزيديم والسيفيبيم على التوالي، بينما كانت المقاومة ضد الميروبينيم والإيميبينيم 78٪ و 75٪ على التوالي. حول توبراميسين، أميكاسين، تتراسيكلين وليفوفلوكساسين كان معدل المقاومة 79٪، 76٪، 71٪ و 66٪ على التوالي. أظهر تكوين الأغشية الحيوية للبكتيريا المعزولة من الحالات الشديدة لمرضى COVID-19 أن 75٪ من بكتيريا *S. aureus* كانت مكونة قوية، و 20٪ كانت معتدلة و 5٪ غير مكونة للاغشية الحيوية ، في حين أن عزلات 90) *A. baumannii*٪(كانت مكونة قوية للاغشية الحيوية ، 10٪ بدرجة متوسطة ولا توجد اي عزلة مكونة للاغشية الحيوية. اما حول 85) *P. aeruginosa*٪(كانت مكونة بشدة للاغشية الحيوية و15٪ معتدلة ولم يكن اي عزلة غير مكونة للاغشية الحيوية.

الكلمات المفتاحية: كوفيد-19، العدوى البكتيرية الثانوية، مقاومة المضادات الحيوية، الغشاء الحيوي

Introduction

The severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), also known as COVID-19 was first discovered in Hubei Region, China, in December 2019, COVID-19, which is caused by the SARS coronavirus 2 (SARS Cov2), has caused 119.6 million infections and 2.65 million deaths worldwide as of 14 March 2021 [1].

The absence of natural immunity, viral replication in the superinfections, minor respiratory tract, secondary infections, or co-infections is thought to be some of the contributing factors to high occurrence of severe infection and humanity in COVID-19, which results in acute respiratory distress syndrome and severe lung injury (ARDS) [2]. Health care professionals have long expressed concern about secondary serious bacterial infection, and this worry has grown in the Covid-19. A correlation between COVID-19 disease and secondary bacterial infections has been demonstrated in several published studies [3].

Multiple studies have reported a correlation between viral infections (e.g., the influenza virus) and bacterial pneumonia as a secondary bacterial coinfection in patients admitted to the ICU [4]. Transmission of bacterial infections within hospitals occurs by direct or indirect contact among hospitalized patients, health care workers (HCWs) and hospital equipment [5]. Ventilators and catheters are widely used during hospitalization and are a well-known risk factor for nosocomial infections [6]. *Acinetobacter* species, *Enterobacter* species, *Enterococcus* species, *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas* species and *Staphylococcus* species are linked with hospital acquired infections [7]. Prolonged hospitalizations in the ICU may be linked with increased likelihood of developing bacterial coinfection in critically ill COVID-19 patients [8]. Bacterial species transmitted in hospitals are high likely to be multi-drug resistant (MDR), which is a major challenge in managing ICU patients, leading to approximately 700,000 deaths worldwide in 2019 [9]. Co-infection and superinfection are two terms to define the detection of a second pathogen in COVID-19 patients at time of diagnosis or hospitalization, respectively [10].

Global health authorities are concerned about the widespread use of antibiotics during the ongoing coronavirus disease 2019 (COVID-19) pandemic and the potential emergence of bacteria that are resistant to them [11]. Several antimicrobial medications have lost their effectiveness even before the COVID-19 pandemic, and are no longer effective against life-threatening infections. Since the worsening of antimicrobial resistance [12]. The COVID-19 pandemic is likely to cause resistance to be yet another casualty [13].

Secondary bacterial infections have virulence factors that acquire antibiotic resistance, especially multiple drug resistance [14]. Worldwide, several studies have reported high incidence of infections due to methicillin resistant *S. aureus*, carbapenem-resistant *A. baumannii*, carbapenem-resistant Enterobacteriaceae, and *P. aeruginosa* among COVID-19 patients admitted to ICUs [15].

A biofilm is a community of stable microorganisms encapsulated in an extracellular matrix produced by themselves. Many types of microorganisms that are found on living hosts or in the environment can form biofilms. These include pathogenic bacteria that can serve as a reservoir for persistent infections, and are culpable for leading to a broad spectrum of chronic illnesses and emergence of antibiotic resistance making them difficult to be treated [16]. Common bacteria that form biofilms include *Pseudomonas aeruginosa, Staphylococcus aureus* [17,18,19], Biofilm recalcitrance [20,21,22] is the potential of the microbes to sustain even in high concentrations of antibiotics, which leads to recurrence of infections and collapse of treatment [23,24,25]. It's a prevalent source of persistent infection, and QS systems keep track of it [26]. Antibiotics resistance in COVID-19 patients who suffer from infection with secondary bacterial infection because these bacteria have virulence factors that acquire antibiotic resistance, especially multiple drug resistance. COVID-19 individuals who take a lot of broad-spectrum antibiotics are more likely to get bacterial infections and develop multidrug resistance [27]. The COVID-19 clinical spectrum ranges from asymptomatic to symptomatic, with the first symptoms typically appearing after five to six days [28]. In addition to pneumonia, sneezing, malaise, diarrhea, headache, and conjunctivitis, common symptoms include fever, fatigue, dry cough, sore throat, and dyspnea [29]. Loss of taste and smell [30] has also been reported. due to its typical process of progression and aggravation. Around the world, 80% of COVID-19 cases that were reported had mild respiratory symptoms, 15% of cases necessitated hospitalization, and 5% of cases were critical [31]. COVID-19 has a variety of effects on the human body. Although the virus is well known for having a severe impact on the respiratory systems, [32] other bodily functions are also affected, such as those of the heart, CNS, kidneys, liver, and gastrointestinal tract. [33, 34] Despite vaccinations, individuals with immunosuppressed and underlying conditions frequently have a high risk of a severe COVID-

19 outcome [35]. The severity of COVID-19 results, however, varied among subgroups based on age, sex, race, gender, and other factors [36]. As the pandemic progresses, health researchers never stop trying to understand which symptoms are more closely related to severe cases. So, the current study aimed to determine the secondary bacterial infection and biofilm formation related to antibiotic resistance and the severity of COVID19 to gain a more thorough understanding of the symptoms and severity of COVID19 infection.

Materials and Methods

The Definitions COVID19 Patients and Specimens Collection

One hundred and fifty-five specimens included: 100 specimens of pharyngeal swabs and 55 of sputum's specimens, were collected from COVID-19 patients (male and female and different ages) admitted ICU from the period from the first of October 2021 to the first to March 2022; the infections were confirmed by reverse transcription-polymerase chain reaction (RT-PCR) as well as X ray and CT scan in epidemiological isolation Baqubah Teaching Hospital. All the patients suffering the symptoms as fever, loss of smell and taste, headache, vomiting, cough, shorting of breath as well as the patients need ventilation.

Isolation and Identification of the Secondary Bacterial infections.

Plate streaking methods were used for culturing the bacteria from the total (55) sputum and (100) pharyngeal swab specimens on Blood agar, MacConkey agar, Mannitol salt agar, Nutrient agar and pseudomonas agar [37], followed by incubation at 37 ◦C for 24 h. The purity of the causative bacteria was then identified by microscoping morphological characters; Gram stain reaction and cell arrangement were seen under a microscope. Cultural morphological were used to identify the isolates. Biochemical tests such as; oxidase, urease and motility were conducted as well as oxidation–fermentation (O-F) tests, catalase tests, IMViC tests (methyl red tests, voges-proskauer tests and citrate utilization tests, triple sugar iron Agar and carbohydrate fermentation tests), these tests prepared according [38,39]. VITECK system was used to confirm identification of the isolates.

Quantitative Assay for Biofilm Formations

A microtiter plate assay was used by the protocol to find the development of the biofilm [40]. For 24 hours, the bacteria were incubated at 37°C after being injected into the brain heart infusion broth medium. Then, 200 µl of the isolate suspension was added to each of the three wells of a 96-well flat-bottom polystyrene plate using the same medium as the diluent, and the plate was incubated for 24 hours at 37°C. Each well was then vigorously shaken and washed three times with distilled water before being completely dried. The adherent bacterial cells were fixed in 200 l of absolute ethanol altogether. After that, 200 µl of 0.5 per cent crystal violet was used to dye each well for 15 minutes. In line with [40], The test was made in triplicates, and the absorbance of wells filled with bacteria-free brain heart broth served as a negative control. The amount of crystal violet removed by 95% ethanol in each well was quantified by measuring the OD 630 nm using an ELISA reader according to what was stated by [41]. Because of this, the absorbance values represented the intensity of the biofilm formed by well-studied isolates on the surface of the microtiter. The results obtained were categorized into three groups as shown in table (1).

Table 1: Classification of the isolates according to biofilm formation

"OD" ≤ "ODC"	NON-BIOFILM PRODUCER
"ODc < OD ≤ 2 x ODc"	Moderately biofilm producer
"2 x ODc < OD"	strong biofilm producer

OD= optical density reader of isolate, ODc= optical density reader of control.

Antimicrobial susceptibility test

The aantimicrobials susceptibility test were done as recommended in Clinical and Laboratory Standards Institute's (2022). Disk diffusion method on Mueller-Hinton agar was employed to evaluate each isolates of gram negative bacteria (*A. baumannii* and *P. aeruginosa)* susceptibility to various classes of antibiotics testing including meropenem (10 µg), pipracillin (100µg), levofloxacin (5 µg), ceftazidime (30 µg), Amikacin (30 µg), ciprofloxacin (5 µg), impenem (10 µg), cefepime (30 µg), tetracycline (30 µg), Gentamicin (10 µg), Tobramycin (30 µg) and cefoxitin (30µg), as well as the sensitivity of gram positive bacteria (*S. aureus)* tested

against Clindamycin(2µg), Doxycycline(30µg), Nitrofurantoin(30µg), Azithromycine(15µg), Trimethoprim- sulfamethoxazole (30 µg), Clarithromycin(15µg), and chloramphenicol (30µg) in addition to some antibiotic as mentioned above.. After 24 hours, the inhibition zone diameters around each disc were compared according to CLSI [42].

Results

Clinical Features of COVID-19 Patients

Based on medical examination and diagnosis, all patients participating in this study were suffering from COVID-19 infection and admitted to ICU. The clinical symptoms of the patients as shown in table (2); (77%) were suffering from headache, (45%) nausea, (39%) vomiting, (81%) shortness of breath, (19%) diarrhea, (93%) loss of smell, (87.5%) fever, (93%) couch, (65%) using the artificial respiration and (77%) treated with antibiotics. All of the factors had highly significant differences, according to the statistical analysis.

Table 2: Clinical Characterize of COVID-19 Patients under the study

CLINICAL SIGNS	PATIENTS NO (%)		P- VALUE
Headache	Yes	120 (77%)	0.0001 **
	No	35(23%)	
Nausea	Yes	70 (45%)	
	No	85 (55%)	
Vomiting	Yes	60 (39%)	
	No	95 (61%)	
Bronchial breathing and Shortness of breath	Yes	125 (81%)	
	No	30 (19%)	
Diarrhea	Yes	30 (19%)	
	No	125 (81 %)	
Loss of smell and tasting	Yes	140 (93%)	
	No	15 (7%)	
Fever	Yes	135 (87.5%)	
	No	25 (12.5%)	
Couch	Yes	140 (93%)	
	No	15 (7%)	
Use of artificial respiration	Yes	100 (65%)	
	No	55 (35%)	
Treated antibiotics or not	Yes	120 (77%)	
	No	35 (23%)	

Isolation and Identification of Secondary bacterial infections isolates

According to the methods which were used for isolation and identification of the secondary bacterial infection from severity COVID19 patients, forty-five (29%) isolates from a total of 155 COVID-19 patients showed positive growth at a percentage (30%) while 70% showed negative growth as appeared in figure 1. The positive growth isolates were distributed as 155/10 (6.45%) isolates of gram-positive *Staphylococcus aureus* which were isolated only from Pharyngeal swab at a percentage of 10(10%) and two types of gram-negative isolates which was 155/22(14.19%) of *Pseudomonas aeruginosa* and 13(8.38%) *Acinetobacter baumannii*, both of them isolated from Pharyngeal swab 5(5%) and 9(9%), and from Sputum was 17 (30.9%), and 4(7.27%) respectively as shown in table (3).

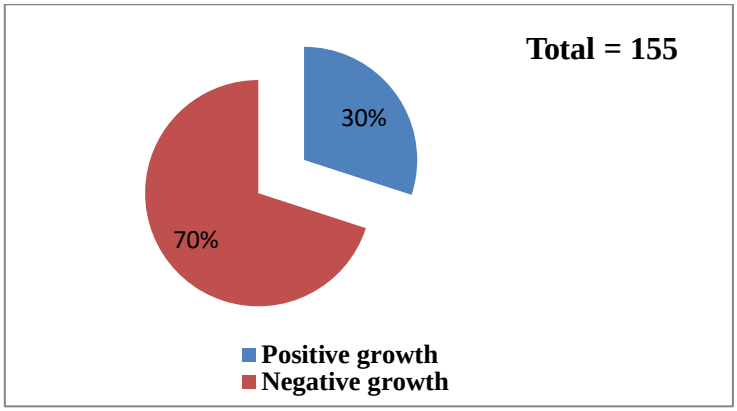

Figure 1: The percentage of positive and negative growth

Table 3: Isolation of Secondary Bacterial Infections According to the Sources

Types of Bacteria	No. of Pharyngeal Swab (100) NO. (%)	No. of Sputum (55) NO. (%)	Total No of Specimens (155) NO. (%)
Staphylococcus aureus	10 (10%)	0 (0%)	10 (6.45)
Pseudomonas aeruginosa	5 (5%)	17 (30.9%)	22 (14.19%)
Acinetobacter baumannii	9 (9%)	4 (7.27%)	13 (8.38%)
Total	24 (24%)	21 (38.18%)	45 (29%)

Antimicrobial Susceptibility Test

Using the disk diffusion method, all bacterial isolates were tested against 12 antibiotics, the results in figures 2 and 3 revealed that there was numerous bacterium isolates with MDR and extensively drug resistance (XDR).Figure 2 indicated that from the total 10 isolates of Staphylococcus aureus showed resistance as follow; to Penicillin (75%), Ciprofloxacin (62 %), Clarithromycin (85%),Clindamycin (60%), Doxycycline (75%),Gentamicin (74%),Imipenem (81 %), Levofloxacin (79%), Tetracycline (66%), Nitrofurantoin (78%), Azithromycin (73%), Trimethoprim - sulfamethoxazole (82%) and Chloramphenicol (87%) .

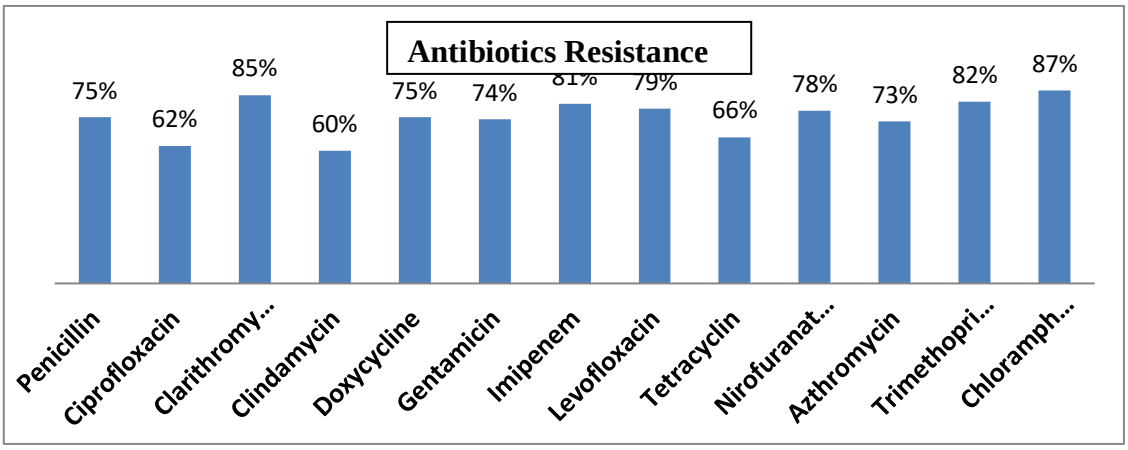

Figure 2: Resistance of Gram positive (*S. aureus)* against antibiotics

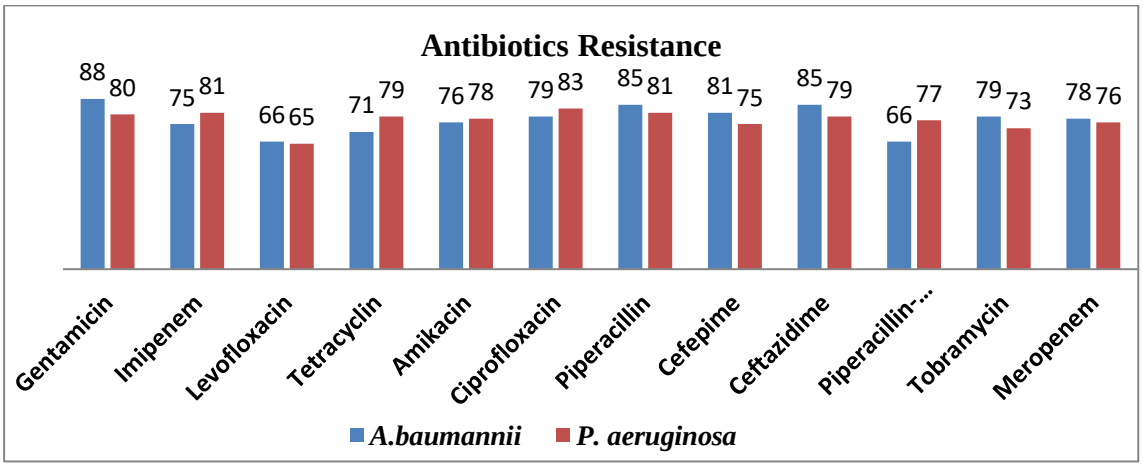

Figure 3: Resistance of Gram-negative bacteria against Antibiotics

Biofilm formation of Secondary Bacterial Infections associated with COVID-19

The results in figure 4 showed the biofilm formation of SBIs in severe cases of COVID-19 patients as follows: *S. aureus* 75% strongly, 20% moderately, 5% non-former, about *A. baumannii* 90% strongly, 10% moderately, 0% non-former and *P. aeruginosa* 85% strongly, 15% moderately, 0% non-former.

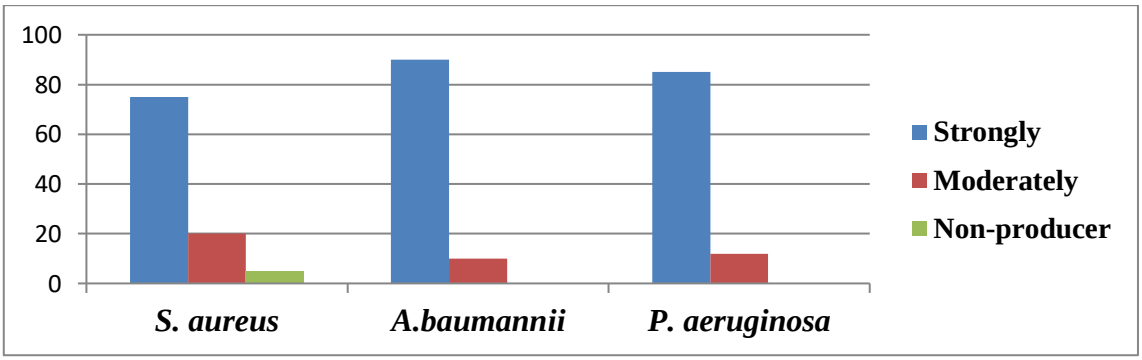

Figure 4: Percent of Biofilm formation degrees of secondary bacterial infection

Discussion

The current results shown in table 2 showed clinical features of COVID-19 patients, several studies agree with current results. The number of patients infected with COVID-19 had risen considerably since the epidemic, and some have died as a result of the progression of the disease [43]. Several signs and symptoms were reported in this study as shown in table 2. In Tehran, Iran, [44] found that 83.0% of 5,057 confirmed SARS-COV-2 individuals had a fever and 65.2% had a cough. In Saudi Arabia, the most common clinical sign was fever 67.7%, cough 60.6 %, dyspnea 43.4 %, upper respiratory symptoms 27.3%, fatigue 26.3 %, diarrhea 19.2 %, and loss of smell 9.1 % among 99 hospitalized SARS-COV-2 patients [45].

The most prevalent symptoms were fever (87%) and cough (93 %), this result corresponds with the results of [46] who showed that fever is the most prevalent symptom (88.7%), cough is the second most common symptom (67.8%), and diarrhea is uncommon (3.8%). The frequency of diarrhea in Chinese patients ranged from 2% to 10% [47]. In every previous study, pneumonia

and abnormal chest imaging were found in the majority of patients upon admission. According to four studies, bilateral patchy shadowing and ground-glass opacity were the two most frequent findings on chest computed tomography (CT) images [48,49]. The results from the CT were almost identical to those from the studies' reported CXR. Acute respiratory distress syndrome (ARDS) [50] and hypertension and diabetes [51] were the most common comorbidities, and a significant portion of patients with these conditions deteriorated more quickly and developed multi-organ disorders.

The current results showed positive growth at 30% from the total 155, a positive growth distributed between one Gram-positive bacteria; *S. aureus*, (10 isolates) were isolated from pharyngeal swabs, whereas. Gram-negative bacteria; *P. aeruginosa* 22 (14.19%) isolates and *A. baumannii* 13 (8.38%) isolates were isolated from Pharyngeal swabs (5%,9%) and Sputum (30.9%, 7.27%) respectively. A study done by [52] showed most COVID-19 patients developed a secondary bacterial infection, which included *A. baumannii*, and *S. aureus* isolated from pharyngeal and sputum, these results agree with our results. Another study was close to the present study [53] identified secondary bacterial infection in 25 per cent of COVID-19 patients. Secondary bacterial infections (SBIs) can develop in COVID-19 patients, resulting in a significant fatality rate. The severity of the illness at the time of admission was linked to the occurrence of SBIs and the resistance levels of the principal isolated bacteria were usually high [54].

The prevalence of bacterial infections in COVID-19 patients admitted to the ICU and hospitals has been reported in several studies [55]. A recent systematic review and meta-analysis study evaluating co-infections among patients infected with COVID- 19 performed by Langford et al. reported that the rate of secondary bacterial infection is 14.3% [56]. Recent studies reported 41 and 28% rates of co-infection among patients admitted to North American and French ICUs [55]. Respiratory sites were the most common sites of bacterial infection in COVID-19 patients. Gram-negative pathogens were predominant in respiratory infections. Herein, we report from the total 45 isolates 10(18.18%) 13(23.6%) and 22(40%) rates of bacterial superinfection mostly due to *S. aureus, P. aeruginosa* and *A. baumannii* respectively. This observation was

similar to the findings reported by previous studies that investigated bacterial co-infections in patients with COVID-19, particularly ICU partners [54].

Multiple microorganism's as bacteria and fungi were identified in severe cases of COVID-19 [57]. Following viral infection, mechanical or immunological mechanisms can weaken the host's respiratory tract defense against bacteria. Some of these mechanisms were derived from animal studies of multiple infections with respiratory viruses and bacterial pathogens [58]. Figures 2 and 3, showed the antibiotics susceptibility test for both gram-positive and negative SBIs and our results revealed that most secondary bacterial infections have strong resistance to antibiotics. These results agreed with the data published by [59]; they recorded that *S. aureus* was highly resistant to different types of antibiotics under their studies. Both beta-lactam penicillinase and the *mecA* gene contribute to S. aureus resistance to beta-lactam drugs. The first mechanism necessitates the production of beta-lactamases or penicillinases, which are found on plasmids and are encoded by blaZ. The beta-lactam ring in the structure of a beta-lactam antibiotic is broken down by this enzyme, rendering it inactive [60]. The second defense mechanism requires the acquisition of the *mecA* gene, which encodes PBP2a protein, and assists in bacterial cell wall synthesis even in the presence of beta-lactam antibiotics [61]. The current outcomes are consistent with [62], who in his paper noted the great prevalence of carbapenem and aminoglycoside resistance in long-term nosocomial infections caused by *A. baumannii* at the main campus hospital, particularly in Covid-19 patients. On other hand, the isolates of *A baumannii* and *P. aeruginosa*, which accounted for 90.4% of all cases and exhibited high resistance to antibiotics at 89%, were also identified as the most prevalent causes of bacterial infection and were strongly associated with death [63].

It has been established that the contaminated environment contributes significantly to nosocomial infections [64]. Since the main identified bacteria have high antimicrobial resistance rates, more precise antibacterial agent administration for SBIs in COVID-19 patients is necessary [54]. SBIs had become a covert threat that existed behind COVID-19. The use of an effective antibiotic regimen is one of the most crucial elements in the successful treatment

of COVID-19. In severe COVID-19 patients with SBIs, a brief guide recommends empiric antibiotic treatment for all potential microorganisms [65,66].

Aetiology and antimicrobial resistance may change as a result of the increased use of antibacterial drugs. Additional microbiological data should be used to guide the treatment of secondary bacterial infections in COVID-19 patients [67]. Certain instances of bacterial infections were noted in studies on the clinical characteristics of COVID-19; however, there were no systematic studies on the causes of SBIs, and the overall number of positive cultures was small [68].

The extensive use of antibiotics in ICUs restricts the options for the following therapy, which may cause the emergence of bacteria that are multidrug resistant. Patients in intensive care units (ICUs) are at a higher risk of infection for a variety of conditions, including underlying illnesses, immunosuppression, and various medical treatments like the use of mechanical ventilation, central venous catheters, and urinary tract catheters. According to several studies [69], the rates of medical infection in ICUs are 5–10 times higher than those in general hospital wards.

The virulence factors such as biofilm formation play an important role in their infections, our results revealed most secondary bacterial infections for all species in the current study produce strong biofilm formation in moderate and severe cases of COVID-19 shown in figures 4,5. The relationship between biofilm formation and disease persistence was first studied several centuries ago [68].

A biofilm is a 3-dimensional structure made up of microorganisms that are embedded in a polymeric matrix that they have secreted. An exopolymeric substance makes up more than 90% of a biofilm. In addition to the epithelial cells, blood vessel linings, bones, and teeth of hosts, pathogens can also form biofilms on catheters, various implants, and artificial organs that are used in dentistry and medicine [70]. Biofilms of *A. baumannii* are more than 1,000 times more resistant to disinfectants than planktonic bacteria, the humidity in the ICU's medical environment makes it easier for them to form and become difficult to remove [71].

When antibiotics are intended to treat biofilm infestation, they must have the ability to cross the biofilm matrixome to target the cells embedded within. Although this is not the case most of the time as the antibiotics fail to cross the biofilm extracellular matrix due to surface modification of the biofilm causing decreased influx. The mechanisms by which antibiotic resistance develops are a crucial determinant factor in the survival of biofilm microbes. The microbes that form biofilms inherently undergo high mutation that allows them to evolve resistant mechanisms providing fortuity for genes to develop enzymes that inactivates the antibiotics or extrudes the antibiotics by efflux pumps [72,73].

Conclusions

Biofilm formation of secondary bacterial infections play important role in defense and gives high resistance against antimicrobial agents. The high percentage of biofilm formation by SBIs in severe cases of COVID-19 patients.

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