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Research Article

Relationship between Antimicrobial-Resistant Bacterial Isolates and Biofilm Formation in Burn Patients

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Abstract

Background: Biofilms are a serious problem and responsible for death from burns, and antibiotic-resistant bacteria threaten global public health due to high rates of pathogen infection. ***Objectives***: To investigate the correlation between the formation of biofilms and the presence of antibiotic-resistant bacterial isolates in burn patients. ***Methods***: 100 samples of swabs were collected from burn patients from January 2023 to June 2023. The grown colonies were identified based on traditional methods and the Vitec system, and multidrug resistance was determined when the isolates were resistant in three categories. A quantitative microtiter method was used to determine the formation of biofilms using ELISA. ***Results***: From 100 burn samples, 83 bacterial isolates were obtained: *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *E. coli*, *Klebsiella pneumoniae*, and *Acinetobacter baumannii*. Infection rates were highest for *P. aeruginosa (67.5%)*, followed by *S. aureus (16.9%).* The results showed high resistance in the bacterial isolates, which showed 100% resistance to imipenem in *P. aeruginosa*. 100% of the *E. coli* and *K. pneumoniae* were MDR, followed by 83.92% for *P. aeruginosa*, 75% for *A. baumannii*, and 71% for *S. aureus*. All the isolates produced biofilm in varying proportions, with 80.35% in *P. aeruginosa*, followed by 100% moderate biofilm in *E. coli*, 100% weak biofilm in *A. baumannii* and *K. pneumoniae*, and moderate and weak biofilm in *S. aureus*. ***Conclusion***: *P. aeruginosa* is the primary cause of burn contamination in hospitals, and all the isolates produced biofilm and exhibited high multi-drug resistance.

Keywords: Biofilm, Burns, Emerging threats, Multidrug resistant bacteria.

العلاقة بين العزلات البكتيرية المقاومة لمضادات الميكروبات وتكوين الأغشية الحيوية لدى مرضى الحروق

الخلاصة

الخلفية: تمثل الأغشية الحيوية مشكلة خطيرة وهي مسؤولة إلى حد كبير عن الوفاة الناجمة عن الحروق، وتهدد البكتيريا المقاومة للمضادات الحيوية الصحة العامة بسبب ارتفاع معدلات الإصابة بمسببات الأمراض. **الأهداف**: دراسة معدل التهديدات الناشئة لدى مرضى الحروق المتمثلة في عزل البكتيريا المقاومة للمضادات الحيوية المتعددة ودور تكوين الأغشية الحيوية في مقاومتها. **الطرق**: تم جمع 100 عينة من مسحات الحروق من مرضى الحروق في الفترة من يناير 2023 إلى يونيو 2023. وتم تحديد المستعمرات المزروعة بناءً على الطرق التقليدية، vitek system وتم تحديد المقاومة للأدوية المتعددة. تم استخدام الطريقة الكمية المعروفة باسم طريقة العيارية لتحديد تكوين الأغشية الحيوية باستخدام قارئ ELISA . **النتائج:** من 100 عينة حروق، تم الحصول على 83 عزلة بكتيرية: Staphylococcus aureus, *P. aeruginosa*, *E. coli*, *K. pneumoniae* و *A. baumannii*. كانت معدلات الإصابة أعلى بالنسبة لـ *P. aeruginosa* بنسبة 67.5%، تليها *S. aureus* بنسبة 16.9%. أظهرت النتائج مقاومة عالية في جميع العزلات البكتيرية بنسبة 100% لمضاد imipenem ، في *P. aeruginosa*. أظهرت النتائج أن نسبة 100% من بكتيريا E. coli و *K. Pneumoniae* كانت مقاومة للأدوية المتعددة، تليها 83.92% لـ *P. aeruginosa*، 75% لـ *A. baumannii*، و71% لـ *S. aureus*. حيث كانت جميع العزلات منتجة للأغشية الحيوية بنسب متفاوتة، مما يشير إلى إنتاج قوي للأغشية الحيوية بنسبة 80.35% من *P. aeruginosa*، يليها غشاء حيوي متوسط 100% في E. coli، و100% غشاء حيوي ضعيف. في *A. baumannii* و *K. Pneumoniae* بينما متوسط وضعيف في *S. aureus*. **الاستنتاج:** المتصورة الزنجارية هي السبب الرئيسي للتلوث بالحروق في المستشفيات، وجميع العزلات أنتجت غشاء حيويا وأظهرت مقاومة عالية للأدوية المتعددة.

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INTRODUCTION

Burns are one of the most prevalent types of excruciating damage. Improving intensive care is yielding progressively excellent effects. Infection is still a significant source of morbidity and mortality, whether limited to the wound site or systemic [1]. Problems in modern health care have included controlling the spread of diseases caused by microorganisms' resistance to antibiotics. These multidrug-resistant (MDR) organisms cause significant public health problems [2]. Gram-positive and Gram-negative bacteria, which are commonly drug-resistant [3], Burn wound colonization by *Pseudomonas aeruginosa*, *Acinetobacter baumannii*, and *Staphylococcus aureus*, which are reputed to be opportunistic pathogens, has caused outbreaks of disease in burn units globally, as these pathogens in particular are commonly found in the hospital environment [4]. *Pseudomonas aeruginosa's* ability to produce biofilms is recognized as a significant virulence feature critical to its pathogenic success [5]. Microorganisms are capable of producing biofilms [6]. Biofilm is a complex consortium of microorganisms residing in self-produced or acquired extracellular polymeric substances (EPSs) and adhering to biotic or abiotic surfaces [7]. The polymeric materials include extracellular DNA, proteins, exopolysaccharides, and amyloidogenic proteins [8]. In addition, bacteria produce deteriorating enzymes, lessen the permeability of their outer membranes, employ efflux pumps, and change their targets to fend off the harmful effects of antibiotics [9]. Pathogenic infections can result in growths that pose a threat to the public's health on a global scale, including the issue of antibiotic-resistant bacteria. As a result, there are higher rates of mortality and morbidity due to this threat [10]. Some studies have been done on Pseudomonas aeruginosa, but little is known about the role of other pathogenic bacteria in biofilm formation and its relationship to antibiotic-resistant bacteria in burn patients. Therefore, this study aimed to investigate the existence of multidrug-resistant pathogenic bacteria in burn patients and evaluate the role of biofilm formation in causing resistance, in addition to the rate of emerging threats from them. This study aimed to determine which results may provide important insights to improve patient outcomes.

METHODS

Samples collection

One hundred samples (burn swabs) were collected from burn patients at the burn center of Al-Imam Ali Hospital in Baghdad Province, Iraq. The samples were collected from January 2023 to June 2023. The samples were grown on three types of agar: blood agar, MacConkey agar, and chocolate agar. They were then incubated at 37 ºC with and without oxygen in a jar with an Oxoid gas pack. The colonies were recognized based on many characteristics, such as colony shape, Gram stain, catalase test, and other biochemical tests. Furthermore, using Vitek 2 gram-positive and gram-negative identification cards, some agents, such as viruses and anaerobic bacteria, were excluded. This manuscript does not contain animal testing, only bacterial isolates.

Antibiotic susceptibility test

The antibiotic sensitivity was performed on all isolates; the antibiotics chosen were Amikacin (30 µg), Imipenim (10 µg), Cefixime (5 µg), Ceftriaxone (30 µg), Ceftazidime (30 µg), Augmentin® (20/10 µg), Ticracillin (75 µg), Clarithromycin (15 µg), Clindamycin (2 µg), Erythromycin (15 µg), Tetracycline (30 µg), Cefoxitin (30 µg), Azithromycin (15 µg), and Ciprofloxacin (5 µg) (Bioanalyse, Turkey) using the disc diffusion method. Isolate suspension was prepared at 0.5 McFarland, and 100 µl of suspension was spread onto plates with Mueller Hinton agar, followed by incubation at 37°C for 18 h. After incubation, the inhibition zone around the disc was measured in millimeters. The resistance of all isolates against antibiotics was measured according to the Clinical and Laboratory Standard Institute [11].

Detection of multidrug-resistant bacteria

According to a new standardized international document presented by the Center for Disease Control and Prevention (CDC), MDR isolates were defined as acquired non-sensitivity to one or more antibiotics in ≥ 3 antibiotic categories.

Biofilm formation assay

Using a microtiter plate, the adherence of all isolates was evaluated as described by Stepanović *et al*. (2007) [12], with minor modifications. Pure colonies from all bacterial isolates were grown for 24 hours in brain-heart infusion broth and incubated at 37 °C. After that, the bacteria were suspended at a McFarland concentration of 0.5. Then, an amount equal to 200 ul of each bacterial isolate suspension by three replications was inoculated into a polystyrene microtiter plate well with a negative control of sterile BHIB. Then, the plate was covered and incubated for 24 hours at 37 °C. After the incubation period ends, the plates are covered and incubated at 37 ◦C for 24 h. After incubation, the plates were washed with deionized water and left to dry. Then, 200 µL of crystal violet 0.1% was added to all wells and incubated for 15 min. After that, the plates were washed to remove the excess stain and left to dry, and the wells were dried. To determine the biomass of biofilm, 200 µL of 99% ethanol was added to all wells to solubilize the crystal violet (Figure 1). The optical density was read for all wells at 630 nm using an ELISA reader. Biofilm-production ability was considered positive compared with the equations as shown in Table 1.

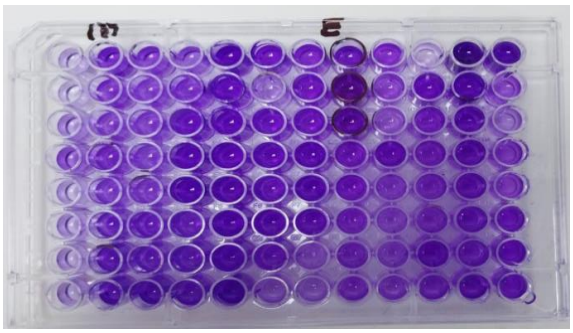

Figure 1: Measurement of biofilm formation by the MTP method.

Table 1: strength of biofilm production according to the optical density

Optical density at 630 nm	Biofilm production
ODc* ≥ ODs**	non formation
ODc < ODs ≤ 2ODc	Weak
ODc< ODs ≤ 4ODc	moderate
ODs > 4ODc	Strong

*Optical density of negative control; **Optical density of sample.

Ethical consideration

The ethics committees of the Iraqi University, Faculty of Medicine approved the study protocol (No.: FM.SA.154, dated: 22/8/2023).

Statistical analysis

IBM SPSS version 28.0 was used to analyze the results [13]. The mean, standard error, and chi-square test were employed to determine the statistical differences at a p-value < 0.05.

RESULTS

The current study included 100 isolates collected from burn patients. Of those samples, 17% showed no growth and 83% showed bacterial growth, as shown in Table 2, and the results revealed statistically significant differences.

Table 2: The number and percentage of bacterial isolates isolated from burn patients

Samples	Number (%)
No growth	17(17)
Bacterial growth	83(83)
p-value	1.0×10^{-20}

After being diagnosed by various traditional methods and confirmed by the vitec 2 system, five bacterial species were isolated, as shown in Figure 2 and Table 3. *Pseudomonas aeruginosa* had the highest percentage of infections and burn contamination (67.5%), followed by *Staphylococcus aureus* (16.9%), *Acinetobacter baumannii* (9.6%), *Escherichia coli* (3.6%), and *Klebsiella pneumonia* (2.4%).

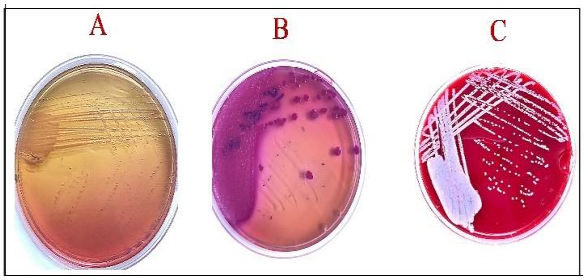

Figure 2: Growing colonies of bacteria species on a different media; A: *P. aeruginosa* on MacConkey agar; B: *E. coli* on MacConkey agar; C: *S. aureus* on blood agar.

Table 3: Number and percentage of bacterial species

Bacteria	Isolates n(%)
Pseudomonas aeruginosa	56(67.5)
Staphylococcus aureus	14(16.9)
Acinetobacter baumannii	8(9.6)
Escherichia coli	3(3.6)
Klebsiella pneumoniae	2(2.4)
p-value	0.00001

The results showed that there were highly statistically significant differences p=0.00001. Eighty-three samples were examined to identify antibiotic-resistant species. Antibiotics were selected according to the CLSI for each isolate; there is a variation in resistance rates according to the species of bacteria and the antibiotics used. The results showed a higher percentage of antibiotic resistance was imipenem (100%), followed by ticarcillin and ceftriaxone in all species. Multiple antibiotic-resistant bacteria were found for more than three drug groups. All of the E. coli and K. pneumoniae bacteria were MDR, followed by 83.92% of the P. aeruginosa bacteria, 75% of the A. baumannii bacteria, and 71% of the S. aureus bacteria (Table 4). Table 5 showed that all the isolates were biofilm producers, including strong, medium, and weak isolates. The mean level in *P. aeruginosa* was 80.35% (0.554±0.008) and moderate 29.65% (0.368±0.0016), and the results exhibited statistically significant differences. In *Staphylococcus aureus*, the highest percentage of biofilm formation was weak (0.298±0.005, 42.86%) and moderate (0.182±0.005, 57.14%) biofilm production. Whereas, all the bacterial isolates of *A. baumannii* and *K. pneumoniae* had the ability to form a biofilm that was weak (0.149±0.0057, 100%) and 0.174±0.002, 100%) and moderate in *E. coli* (0.442±0.01, 100%). Antibiotic resistance and biofilm formation were the emerging threats, and almost all biofilm-forming species displayed multiple resistances, as shown in Figure 3.

DISCUSSION

Microorganisms with the ability to produce biofilms are considered to be one of the main factors leading to antibiotic resistance. Therefore, many attempts have been made to overcome these serious problems by finding new drugs that can suppress biofilm formation [6].

Table 4: Percentage of resistance to antibiotics and multi-resistance among bacterial species

Antibiotics	*P. aeruginosa*	*S. aureus*	*A. baumannii*	*E. coli*	*K. pneumoniae*
	n (%)				
Amikacin	47(83.92)	11(78.57)	6(75)	3(100)	2(100)
Imipenem	56(100)	14(100)	8(100)	3(100)	2(100)
Cefixime	30(53.57)	---	---	1(33.33)	---
Ceftriaxone	54(96.42)	10(71.42)	8(100)	3(100)	1(50)
Ceftazidime	53(94.64)	8(57.14)	8(100)	3(100)	---
Amoxicillin-Clavulanic acid	13(23.21)	6(42.85)	---	---	2(100)
Ticarcillin	55(98.21)	3(21.42)	8(100)	2(66.66)	---
Clarithromycin	---	5(35.71)	---	---	2(100)
Clindamycin	---	12(85.71)	----	---	---
Erythromycin	---	2(14.28)	---	---	---
Tetracycline	---	1(7.14)	---	---	1(50)
Cefoxitin	---	4(28.57)	---	---	---
Azithromycin	11(19.64)	---	---	---	---
Ciprofloxacin	---	2(14.28)	---	---	1(50)
MDR	47(83.92)	10(71.42)	6(75)	3(100)	2(100)

There were 83 of the developing isolates used to test the resistance of antibiotics to more than three antibiotics and to see how much biofilm they could make using a microtiter plate, which is a precise quantitative method. The finding demonstrated the highest infection rate for *P. aeruginosa* and started a decline in the remaining isolates. *P. aeruginosa* is listed by the World Health Organization as a pathogen, so it is considered one of the most life-threatening bacteria and a priority for research and development of new antibiotics [14].

Table 5: Results of biofilm formation in all isolates using ELISA reader

Bacterial isolates	Biofilm formation (OD)	Biofilm formation *n*(%)	*p*-value
P. aeruginosa	Strong 0.55±0.008	45(80.35)	$1.3x10^{-10}$
	Moderate 0.37±0.002	11(19.65)	
S. aureus	Moderate 0.3±0.005	6(42.86)	0.450
	Weak 0.18±0.005	8(57.14)	
A. baumannii	Weak 0.15±0.006	8(100)	-
E. coli	Moderate 0.44±0.01	3(100)	-
K. pneumoniae	Weak 0.174±0.002	2(100)	-

Values are presented as mean±SD.

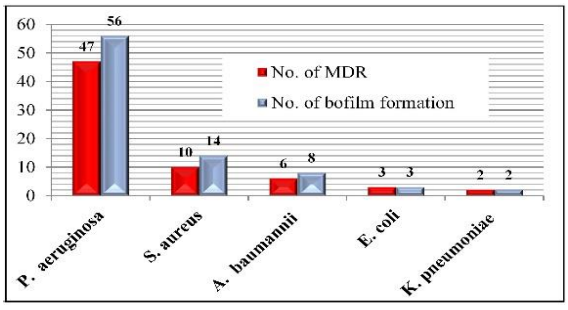

Figure 3: The rate of emerging threats represented by multiple antibiotic resistance bacteria and biofilm formation.

Most of the bacteria were resistant to antibiotics; the highest rate of antibiotic resistance was imipenem in all species, and this may be due to the excessive administration of this antibiotic and other available antibiotics, and thus the increase in resistance to all isolates over time. which is non-compatible with other studies that showed the isolates had a lower rate of resistance to imipenem but agreed with the rate of biofilm formation (100%) in *P. aeruginosa* [15]. According to the number of isolates, this study discovered that *P. aeruginosa*, *A. baumannii*, and *S. aureus* had the most multiple antibiotic resistance. But *E. coli* and *K. pneumonia* had 100%. Also, antimicrobial resistance is built into bacterial biofilms, which may make treating patients even harder [16]. This is because the number of clinical strains that are becoming antimicrobial resistant is growing. In the current study, all isolates were biofilm producers, including strong, medium, and weak isolates. A strong biofilm in P. aeruginosa and a moderate biofilm in *E. coli* represented the highest production rate, respectively, according to the values for bacterial isolates. This study agrees with a study in India that revealed biofilm formation, but at a lower rate, which was common in *P. aeruginosa*, followed by Klebsiella spp. [17]. The current study demonstrated that *A. baumannii* and *K. pneumonia were* biofilm producers but were weak, and most of the bacterial isolates resistant to antimicrobials were biofilm producers. Biofilm-mediated infections account for approximately 60–80% of all bacterial infections. The results of the current study are consistent with the Mishra *et al.* study [6]. Therefore, the microbial biofilm is the main reason for the failure of any antibiotic to kill microbial pathogens [18]. New research shows that burns can be affected by many things, such as how infections are managed and controlled, how these isolates continue to contaminate hospitals, and how biofilm production rises. This can make bacterial isolates more resistant to multiple antibiotics over time, leaving burns untreated and causing severe inflammation. Biofilms are complex communities of microbes that cling to surfaces and are enclosed in an extracellular matrix

that can defend against antimicrobial agents in addition to the host immune system [19]. They looked at antimicrobial resistance and the ability of each strain to form biofilms and found that MDR phenotypes were more common in bacteria that could form biofilms [20]. In addition to the fact that biofilm is made of host proteins and a mucous layer, biofilm is essential for pathogenicity because it provides the ideal environment for bacteria to grow and develop therapy resistance [21]. *P. aeruginosa* is also a good biofilm producer that aggravates burn patients by slowing or not responding to antibiotics [22]. Biofilm formation is associated with increased drug tolerance and resistance, as well as persistent inflammation. Gram-negative bacteria, such as Pseudomonas aeruginosa, are commonly found in burn wound infections and are known to form biofilms. The prevalence of biofilm formation varies among different bacterial species, with A. baumannii showing the highest rates [23,24]. Gram-positive and Gram-negative bacteria can adhere to and create biofilms on device surfaces; however, Staphylococcus aureus and Pseudomonas aeruginosa are the most frequently found biofilm-forming microorganisms [25]. Therefore, by inhibiting cell attachment and coating surfaces with substances that don't encourage adhesion [26], biofilms are a major issue with burns, with 60% of burn deaths attributed to biofilms [27]. Gram-negatives obtain resistance against multiple antibiotics due to impaired influx and efflux pumps in membranes [28]. *S. aureus* bacteria quickly colonize burns present on the patient's skin as well as environmental surfaces that are infected. The wound is colonized by gram-negative bacteria, especially *P. aeruginosa* and *A. baumannii*, within hours to a few days. Treating burns early is critical to preventing colonization by many bacteria, especially *P. aeruginosa* and *A. baumannii* [29]. Thus, we need strategies to combat and prevent biofilm formation and multidrug resistance in burn infections. There are some differences in the results of the studies, which may depend on the number and type of isolates diagnosed, the treatments used in the country, and the degree of burning of the patient.

Conclusion

P. aeruginosa is the primary cause of burn contamination in hospitals, and all the isolates produced biofilm and exhibited high multi-drug resistance. The findings disclosed the highest infection rate for P. aeruginosa in burns and marked the beginning of a decline among the remaining isolates.

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Conflicts of interest

There are no conflicts of interest.

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Data sharing statement

Supplementary data can be shared with the corresponding author upon reasonable request.

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