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Detection of biofilm formation and antibiotic sensitive among *Pseudomonas aeruginosa* isolated from burn patients

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Abstract:

Burns are the most destructive condition, in which the outer covering layer of the skin disappears and a scab forms, which is colonized by various opportunistic microorganisms, *Pseudomonas aeruginosa* is a common isolate from burn infections,leading to various pathological conditions Burn swabs were collected from patients Iraqi hospitalized at Burns Specialist Hospital in the Medical City , Baghdad, Iraq by Levene technique or syringe technique, respectively. Standard laboratory protocols for identifying *P.aeruginosa* were followed. The Kirby-Bauer disk diffusion method was used to test the sensitivity of the isolates to antibiotics according to CLSI 2022 guidelines. Biofilm production was accessed by microtiter plate assay.Twenty five isolates of *P.aeruginosa* were isolated from Iraqi burn patients. Identified by Biochemical test and Vitek 2E System.Antibiotic susceptibility test was conducted to the isolates of *P. aeruginosa*by using disc diffusion method to 15 antibiotics from different classes.The antibiotics resistance rate was determined as follows: Trimethoprin/sulphamethhoxazole (100%) Ceftazidime and Tobtamycin (88%), Piperacllintazobactam and Cefotaxime (72%), Cefazolin and Meropenem (60%), Cefoperazone (52%) Gatifloxacin (%) and Aztreonam(20%). *Pseudomonas aeruginosa* isolates have the ability to form biofilms on microtiter plates .The results showed that only 56% of *P. aeruginosa* isolates were strong biofilm producers. Meanwhile, 36% and 8% of the isolates were intermediate and low productive, respectively. .The production of exopolysaccharides during biofilm formation makes the condition more severe. Furthermore, continuous monitoring of this type of burn wound infection using the antimicrobial profile of the isolates is essential not only for infection control but also for empiric treatment.

Keywords burn **,***Pseudomonas aeruginosa*, Biofilm formation , Antibiotic sensitive.

Introduction

The skin is the largest organ of the human body and acts as a physical and immunological barrier to disease-causing microorganisms. Skin lesions can serve as entry points for microbial contamination, leading to chronic wounds and other invasive infections. Chronic wounds are a serious public health problem because of their social, psychological, and economic consequences [1] . Wounds and burns are a common and major public health problem in many parts of the world. It is clear that patients with wounds and burns are at increased risk of acquiring nosocomial infections [2]. Bacteria have been found as major colonizers of burn wounds because they thrive on the moist surfaces of burns and can reach burn patients, usually through cross-contamination. Nosocomial infections in burn patients pose a major threat, as resistance to some antimicrobial agents often poses difficulties in treating bacterial infections and can lead to severe infections and increased mortality in these patients[3]. Therefore, the main key to treating burn patients is to control the infection of pathogenic bacteria on the wound surface [4].Patients with severe burns are more susceptible to infections caused by multidrug-resistant organisms (MDROs).Common examples include (MRSA) *Staphylococcus aureus* bacteria, (VRE) *Enterococcus spp*. bacteria, (MDR) *Pseudomonas spp*. bacteria and *Acinetobacter spp*. bacteria. The patronage and treatment of these patients was extremely difficult. If certain infection precautions are ignored, these pathogens can cause outbreaks in burn units. This infection results in prolonged hospital stay and high mortality rates in burn patients. Additionally, the cost of treating burn patients is prohibitive [5&6]. The prevalence of multidrug-resistant (MDR) bacteria in burn centers may lead to the empirical selection of antibiotics that target MDR bacteria, propagating a vicious cycle of increasing antimicrobial resistance [7]

P.aeruginosa proliferates in the wound environment by suppressing the host's innate immune response in the wound tissue [8]. *P.aeruginosa* is lethal in immunocompromised patients and is a major cause of bacteremia and sepsis in neutropenic patients [9]. *P.aeruginosa* is a Gram-negative opportunistic pathogen that is aerobic, motile, and nutritionally versatile. Shows inherent resistance to many antibiotics. Therefore, infections caused by these bacteria are highly life-threatening, and patients may require longer hospital stays, further increasing morbidity and mortality [10&11]. Bacterial, biofilms are also important virulence factors and play an important role in

antibiotic resistance[12]. Although biofilms are primarily seen in device-related infections, biofilms have also been reported in burn injuries caused by *P.aeruginosa* [13]. Biofilms are in the form of dense aggregates of cells that produce components of the extracellular matrix that bind the community, and One of the most important things for developing biofilms for *P. aeruginosa* is for the bacterial cells to be in communication [14&15].

A report from Iraqi Kurdistan showed that the prevalence of *P. aeruginosa* infection in burn wounds was 23% [16], while a report published by a recent study from Nepal showed that the prevalence of *P. aeruginosa* infection in burn wounds was the prevalence has been shown to be 60% [17&18]. In India, it is approximately 32.1%, of which 73.8% of isolates contain biofilm-producing material [19].Burns are chronic, nonhealing, and difficult to treat due to development and changes in the antimicrobial properties of the associated pathogens. Therefore, this study can reveal biofilm formation and antimicrobial susceptibility patterns among *P. aeruginosa*, so that antibiotics are prescribed and taken accordingly, and the treatment of burn patients is controlled and their medical condition is improved.

Materials and Methods

Samples Collection

A total of 167 samples were collected from Iraqi burn patients hospitalized at Burns Specialist Hospital in the Medical City, Baghdad, Iraq, For the purpose of isolating and diagnosing *P.aeruginosa* bacteria, isolated from May 2023 to October 2023. Levine's wound swab technique was performed 48 h after burn injury, and pus samples were taken directly with a syringe after careful preparation of the skin [20]. The Samples collected were immediately transported to the laboratory and incubated aerobically for 24 h at 37 °C in blood agar for recognition hemolysin producing colonies[21].

Done for each sample were various tests Include : biochemical (IMViC tests, TSI tests, urease tests) such as characteristics of culture , Gram staining, catalase test, oxidase test, and growth at 42 °C have been performed to identify *P. aeruginosa* [17&22].

2.3.Identification of *P. aeruginosa* by Vitek 2 compact system

All isolates suspected of being *P. aeruginosa* by phenotypic identification were included in this assay. The bacterial of suspensions were organized according to the McFarland standard tube of 0.5/2.5 mL of 0.45% Sodium chloride (NaCl) solution using a VITEK-2 apparatus (bioMerieux,

France). The vaccine is always ready within 30 minutes of filling out the card. The Gram-positive detection card is a completely closed system that does not use any detectors. The cards are placed on strips intended for use with the VITEK-2 system, housed within a device that is periodically filled into a sealed vacuum chamber, and incubated at 37 °C for 15 min. Chromatography (new read peak) is performed mechanically every time. (Incubation time up to 8 hours). Informative analysis is based on the VITEK-2 database, allowing detection of organisms in motile mode starting 180 minutes after culture initiation [23].

Antibiotic susceptibility testing:

The disk diffusion method as described by the Clinical Laboratory Standards Institute was used to perform antibiotic susceptibility testing [CLSI] 2022 [24]. Antibiotic tablet Trimethoprin / sulphamethhoxazole (SXT) (25mg) , Piperacllintazobactam(TPZ)(110 mg),Cefoperazone (CEP) (75mg), Ceftazidime (CAZ)(10mg),Aztreonam(ATM)(30mg),Tobtamycin(TOB)(10mg),Gatifloxacin (GAT)(5 mg), Cefotaxime (CET)(5 mg),Cefazolin (CZ)(30 mg) , Meropenem (MEM)(10 mg) were used for antibiotic susceptibility testing "AST". Isolates that exhibit resistance to more than three different classes of anti-pseudomonas antibiotics have been certified as multi-drug resistant (MDR) [25]. In this study, *Escherichia coli* ATCC strain 25922 used as control

2.5.Biofilm formation assay

Two approaches were followed to detect biofilm production: the Congo Red Agar (CAR) method and the second method, examination using a microtiter plate (MPA). For the (CAR) method described by [26] was used to investigate the ability of *P. aeruginosa* bacteria to produce biofilm. A 24-hour-old colony of *P. aeruginosa* was taken and grown on plates containing (CRA) medium prepared by dissolving 37 grams of brain heart infusion broth (Oxoid). , UK), 50 g of sucrose, 10 g of agar No.1 (Oxoid, UK), 8 g of CongoRed indicator dye (Oxoid, UK) in a liter of distilled water and sterilize in an autoclave at 121°C for 15 minutes, The plates were incubated at 37°C for 24 hours. All isolates were then grown on Congo red agar (CRA) for 24 h/37°C. Colonies change color to black, indicating biofilm productivity.

For the MPA method, 96-well polystyrene microtiter plates with coverslips were used. Inoculate 20 microliter of fresh bacterial suspension [at a concentration of 5 x 10 CFU/ml] into 180 microliter.Mueller-Hinton broth

containing 1% glucose to give approximately 5 x 10 CFU/ml as the final inoculum in a microtiter plate.Cultures incubated aerobically at 37° C. overnight .And then were washed thoroughly three times with phosphate buffered(PBS) in pH (7.2). Adherent bacteria were then fixed by baking at 60°C for 60 min, and the wells were then stained with 150 µM 2% crystal violet for 15 min at room temperature. The blot was aspirated, the microtiter plate was washed appropriately with PBS, and the air-dried plate was redissolved in 150 µM 95% ethanol to separate the fixed cells from the wells. Leave the plate at room temperature without shaking and cover .To estimate biofilm density, absorbance at 630 nm was measured with a microplate reader An ELISA plate reader [Ascension Software (version 2.6)] was used. To measure the optical density of each well.Cut-off values and the ability of isolates to form biofilms distinguish them as non-biofilm producers and biofilm producers, and are further differentiated as described elsewhere[27]classifction was as follows:strong,moderate and weak.

Results and Discussion

Twenty five isolates of *P. aeruginosa* bacteria were obtained from people with burns.Burns Specialist Hospital in the Medical City , Baghdad, Iraq, identified by Biochemical test Table (1).

Table 1: Biochemical tests of the *P. aeruginosa* isolates

Biochemical Test	**Result**
Gram reaction	Negative (-)
Citrate test	Positive **(+)**
Oxidase test	Positive **(+)**
Catalase test	Positive **(+)**

The VITEK 2 system is an easy-to-use system that provides a fast (4-15 hour) and reliably accurate method for repeatable identification and identification of *P. aeruginosa*. Reliably detect orphan species. A successful identification study of *P.aeruginosa* was carried out by[28]using the VITEK 2 system .

Kunwar[17] found that the isolation rate of *P.aeruginosa* from burn exudates was higher than other studies, and samples from our hospital were more common than samples from other leaves. This pathogen can attach to a variety of surfaces via biofilms. In addition to these basic defenses, biofilms provide a haven where microorganisms can acquire antibiotic resistance [29].

Antibiotic susceptibility test was conducted to the isolates of *P. aeruginosa* by using (disc diffusion method) to 15 antibiotics from different classes.The resistance percentages of *P. aeruginosa* isolates as follows: Trimethoprin / sulphamethhoxazole(100%), Ceftazidime and Tobtamycin (88%), Piperacllintazobactam and Cefotaxime (72%), Cefazolin and Meropenem (60%), Cefoperazone (52%) , Gatifloxacin (44%) and Aztreonam(20%) as can be seen in (Figure 1 and Table 2). *P.aeruginosa* onresponse to antibiotics especially, trimethopurine/sulfamethoxazole(100%), ceftazidimeand(88%), and piperacillin (72%), is a major concern for healthcare professionals.Therefore,as it is a nosocomial pathogen in burn centers, appropriate infection control measures must be implemented to prevent the spread of infection

Table 2 Profile of Antibiotic SusceptibilityTest of *Pseudomonas aeruginosa*.

Antibiotics used	Number of isolates Sensitive (%)	Number of isolates Resistance (%)	Total
Trimethoprin / sulphamethhoxazole	0(0%)	25(100%)	25
Ceftazidime	3(12%)	22(88%)	25
Tobtamycin	3(12%)	22(88%)	25
Piperacllintazobactam	7(28%)	18(72%)	25
Cefotaxime	7(28%)	18(72%)	25
Cefazolin	10(40%)	15(60%)	25
Meropenem	10(40%)	15(60%)	25
Cefoperazone	12(48%)	13(52%)	25
Gatifloxacin	14(56%)	11(44%)	25
Aztreonam	16(64%)	9(36%)	25

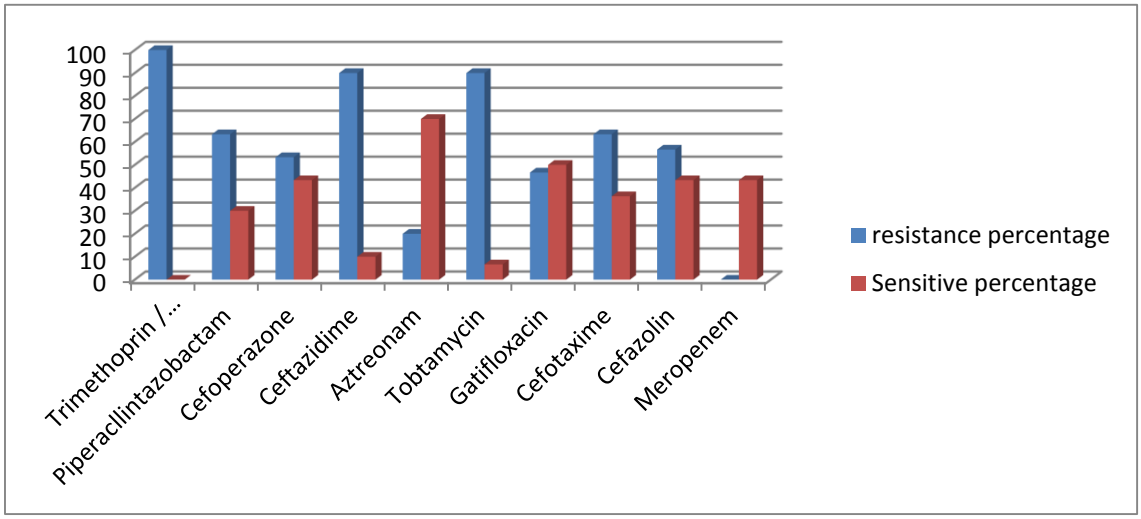


Figure (1). Antibiotics susceptibility test results of *Pseudomonas aeruginosa* isolates.

Pseudomonas aeruginosa isolates have the ability to form biofilms on (CRA) Table (3) and the isolates have ability to form biofilms on microtiter plates (Table 4 and Figure 2). The results showed that only 56% of *P. aeruginosa* isolates were strong biofilm producers. Meanwhile, 36% and 8% of the isolates were intermediate and low productive, respectively .

Through the results of biofilm formation by the CAR and MPA methods, the ability of each isolate to produce was evaluated, as the isolates showed their ability to produce greater production using the CAR method compared to the MPA method. On the contrary, the productivity of isolates for biofilms was moderate and weak in the MPA method. The number of isolates producing biofilms was moderate 9 out of a total of 25 isolates compared to the CAR method, in which the number of isolates was 3. One isolate was moderately productive. (Figure 3,Table 4)shows the comparison between the MPA and CRA methods**.**

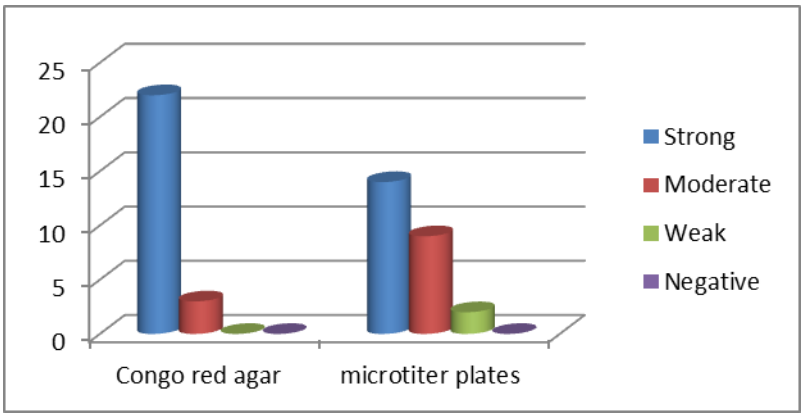
Table (3) :Biofilm formation capacity of *P. aeruginosa* isolates use Congo red agar (CRA)

Biofilm formation.	Number of *P. aeruginosa* isolates (25)	Percentage (100%)
Strong	22 (25)	**88%**
Moderate	3 (25)	**12%**
weakly	0 (25)	0
Negative	0 (25)	0

Table (4) :Biofilm formation capacity of *P. aeruginosa* isolates use microtiter plate (MPA)

Biofilm formation.	Number of *P. aeruginosa* isolates (25)	Percentage (100%)
Strong	14 (25)	**56%**
Moderate	9 (25)	**36%**
weakly	2 (25)	**8%**
Negative	0 (25)	0

Figure(2): Biofilms on microtiter plates Discussion



Figure(3): Comparison between Congo red agar method and Microtitre plate assay

Burn infections remain a main public health problem . This is a severe form of shock whose exacerbation causes various metabolic and inflammatory changes. As a result, it is a main cause of morbidity and estimated 180,000 deaths per year, most of which occur in low and middle economy countries[17].In Nepal, according to[17] it is the third most common cause of injury and ten years ago it was estimated that out of 55,000

burns, 2,100 resulted in death. All age groups are affected and the severity of the injury varies. From minors who do not require medical treatment to very serious conditions that require immediate medical attention [17].

Among the results, it is ambiguous whether gentamicin is the most effective drug compared to other antibiotics, and this may be because these isolates contain different resistance mechanisms to those antibiotics. It shows that it is possible. *Pseudomonas aeruginosa* exhibits various forms of resistance to various antibiotics, especially those belonging to the minoglycosides. Quinolones and beta-lactam drug classes [30]. Another study by[31] showed that *P.aeruginosa* contains a large number of resistance genes, including extended-spectrum ones.It was discovered by [32]show that they can rapidly mutate and acquire drug resistance to adapt to the environment and spread resistant bacteria. The number of antibiotics used is increasing year by year. The most important biofilm-forming factor that synthesizes polysaccharides through intercellular adhesion *in vivo* [33&34].Antibacterial agents have been developed, and the rise in drug resistance has become a problem. A study by[35] showed that biofilm formation and antibiotic resistance are strongly linked and that biofilms are a resistance mechanism for various antibiotics.

Burn patients are immunosuppressed hosts. An example of a typical opportunistic, biofilm-producing organism is *P.aeruginosa*. Biofilm production by *P.aeruginosa* not only contributes to antibiotic resistance but also serves as an important determinant of virulence [36].Naturally, these bacteria are resistant to many antibiotics because they constitutively secrete a variety of enzymes. Furthermore, apart from chromosomal mutations and extrachromosomal DNA acquisition, it enhances resistance to antibiotics. Furthermore, biofilms also play an important role in antibiotic resistance [37].

The results of study done [38]are a local finding that biofilm formation tests showed that *P. aeruginosa* isolates were biofilms associated with the transition from acute to chronic infection. It is consistent with 47.6%, 23.8%, and 14.2% of *P. aeruginosa*-producing isolates were strong, moderate, and weak isolates, respectively, and 14.2% were non-biofilm-forming isolates,while in [39], clinically isolated *P. aeruginosa* from various diseases It was found that 68.33% of the strains had the potential to form biofilm streams. A study by [40] when bacteria were isolated from Covid-19 patients, the results showed that 100% of the isolates were *P.aeruginosa* and were

positive for biofilm formation, with 73.3% and 26.7% of the isolates being strong and moderate biofilm producers, respectively producers. As discovered by[41] showed that *P.aeruginosa* is an important biofilm-forming species and also a model bacterium for studying biofilms. According to [42&43], It has been reported that 65% to 80% of nosocomial infections were associated with biofilms. Mortality from *P. aeruginosa* infection increases with the product of various virulence factors that help *P. aeruginosa* evade the host immune response and cause pathogenic damage . Quorum sensing (QS), an important aspect of biofilm communication, can be used to prevent the occurrence of some developmental processes such as initial attachment, microcolonization, and maturation. . A study by [44] showed that the production, release, and accumulation of extracellular signaling molecules are mediated by the QS process, a chemical exchange between bacterial cells.

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الكشف عن تكوين الأغشية الحيوية وحساسية المضادات الحيوية بين بكتيريا *Pseudomonas aeruginosa* المعزولة من مرضى الحروق العراقيين

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مستخلص البحث:

جمعت مسحات الحروق من المرضى باستخدام تقنية Levene أو تقنية المحقنة على التوالي. اتبع بروتوكولات المختبر القياسية لتحديد *Pseudomonas aeruginosa*. استخدامت طريقة انتشار القرص Kirby-Bauer لاختبار حساسية العزلات للمضادات الحيوية وفقًا لإرشادات CLSI 2022. توصولت الدراسة إلى إنتاج الأغشية الحيوية من خلال اختبار لوحة الميكروتيتر. عزلت (25) عزلة من *Pseudomonas aeruginosa* من مرضى الحروق العراقيين. شخصت من خلال الاختبار الكيميائي الحيوي ونظام Vitek 2E. اجري اختبار حساسية المضادات الحيوية لعزلات *Pseudomonas aeruginosa* باستخدام طريقة انتشار القرص لـ 15 مضادًا حيويًا من فئات مختلفة. اختبرت حساسية عزلات *Pseudomonas aeruginosa* للمضادات الحيوية، وحدد معدل المقاومة على النحو التالي: تريميثوبرين / سلفاميثوكسازول (100٪)، سيفتازيديم وتوبتاميسين (88٪)، بايبراكلينازوباكتام وسيفوتاكسيم (72٪)، سيفازولين وميروبينيم (60٪)، سيفوبيرازون (52٪)، جاتيفلوكساسين (٪) وأزتريونام (20٪). تتمتع عزلات Pseudomonas aeruginosa بالقدرة على تكوين أغشية حيوية على ألواح الميكروتيترزأظهرت النتائج أن 56٪ فقط من عزلات *Pseudomonas aeruginosa* كانت منتجة قوية للأغشية الحيوية. في غضون ذلك، كانت 36% و8% من العزلات متوسطة الإنتاجية ومنخفضة الإنتاجية على التوالي. إن إنتاج عديدات السكاريد الخارجية أثناء تكوين الأغشية الحيوية يجعل الحالة أكثر شدة. علاوة على ذلك، فإن المراقبة المستمرة لهذا النوع من عدوى الجروح الناتجة عن الحروق باستخدام ملف مضادات الميكروبات للعزلات أمر ضروري ليس فقط لمكافحة العدوى ولكن أيضًا للعلاج التجريبي