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Identification of Biofilm-Producing Isolates of *Pseudomonas aeruginosa* Isolated from Wound Infections

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Abstract:

Pseudomonas aeruginosa (P. aeruginosa) is the one of the most prevalence pathogenic bacteria especially in hospitals, and it is the most common bacteria causing wound infections. One of the most significant virulence factors it produces is biofilm, as it contributes to antibiotic resistance and persistent infections. The current study aims to detect biofilms formation for *P. aeruginosa* isolates collected from wound patients. A total of 51 isolates were obtained from 121 clinical sample of wound patients from different hospitals in Baghdad city, all isolates were submitted for cultural and biochemical tests for identification, then by using VITEK 2 system ،all isolates were confirmed as *P. aeruginosa*. Biofilm production was detected by microtiter plate (MTP) assay, according to the results, (17.64%) of the isolates were weak biofilm producers, (50.98%) were moderate, and (31.37%) produced strong biofilm. The results of this study revealed that all isolates showed a highly proportional capacity to form biofilm, biofilm is the most important virulence factor of *P. aeruginosa* and has a significant effect on chronic wounds and affects the wound healing process. Therefore, it is necessary to develop new strategies to eradicate it.

Keywords: *Pseudomonas aeruginosa*, Biofilm, Wound infections.

Introduction:

Wound infections account for one-third of nosocomial infection between surgery patients, which cause 70–80% of deaths. Regardless of the kind of wound, wound infections are linked to patient morbidity and mortality, particularly in developing nations [1]. It is commonly known that the growth

of bacteria in wounds causes infection and slows the healing process [2]. Among the Gram-negative aerobes that cause chronic wound infections, *P. aeruginosa* is one of the most well-known pathogens due to the scarcity of available treatments [3]. Biofilm formation is a significant factor that impedes wound treatment, which affects tissue regeneration, epithelialization, and the inflammatory response [4]. In multiple researches, the most common isolates from wounds, were *S. aureus* and *P. aeruginosa*, it may be found in both healing and non-healing wounds [5]. *P. aeruginosa* is a Gram-negative opportunistic bacteria that can result both acute and chronic infections [6]. It is mostly responsible for many infections, such as pneumonia, urinary tract, and wound infections [7]. For hospitalized patients, it is the most hazardous nosocomial pathogen, with a high rate of morbidity and mortality. It is a pathogen that poses a major threat to human life because of its capacity to adapt, endure, and build resistance to a wide variety of antibiotics [8]. Different virulence factors are produced from *P. aeruginosa* including flagella, pilli, and lipopolysaccharide (LPS) that aid in bacterial adherence and colonization, secretion systems that transport effectors and toxins to the host, as well as proteases and toxins that harm tissue [9]. This organism has the ability to produce biofilm, which results in a chronic infection [10]. Biofilms are groups of microorganisms that linked to surfaces via an extracellular polymeric substance (EPS) matrix [11], which made up of proteins, carbohydrates, and nucleic acids. EPS forms a three-dimensional, extremely complicated network that supports cellular adhesion serves as a barrier against traditional antibiotics [12]. *P. aeruginosa's* biofilm is one of its most notable virulence factors. Typically, it consists of three or more distinct exopolysaccharides, including Pel, Psl, and alginate. Alginate gives biofilms protection and structural integrity. Pel and Psl, the other two exopolysaccharides, are known to serve as structural scaffolds that are necessary to preserve the integrity of the biofilm [13]. So the bacteria could withstand 1,000 times the concentration of antibiotics compared to the same bacterium in a free-living, planktonic condition [14]. Biofilm production is a defining feature of chronic infections and a sign of the long-term persistence and progression of the illness [15]. Nonetheless, biofilm formation between clinical strains in vitro differs; clinical strains of *P. aeruginosa* can be categorized as strong, moderate, weak, or non-biofilm producers [16]. Wound infection is the colonization of bacteria and other microbes that can delay wound healing or worse, cause wound degeneration. The majority of infected wound instances are usually caused by bacterial contamination that comes

from the skin, other body components, or the outside environment [17]. Clinically, wounds can be categorized as either acute or chronic. Acute wounds heal on their own in 8–12 weeks, while chronic wounds take longer to heal (often months) because of ongoing inflammation. Chronic wounds can result from a variety of factors, including age, obesity, accidents, and long-term conditions like diabetes, cancer, and others [18]. Wound infections caused by pathogenic bacteria pose a global challenge to contemporary healthcare, particularly with the onset of the post-antibiotic era [19]. The most serious issues related to biofilms that their formation on wound surfaces [20]. The non-healing of chronic wounds is significantly influenced by biofilm. Only 6% of acute wounds are known to include the bacteria that produce biofilm, whereas 90% of chronic wounds indicate the production of biofilm [21]. This study aimed to detect biofilm formation among *P. aeruginosa* clinical isolates from wound infections for better management and therapy of wound patients.

2. Methodology

2.1 Sample Collection and Bacterial Isolation

From October 2023 to January 2024, one hundred and twenty-one clinical samples were collected from wound patients from different hospitals in Baghdad city. Samples were taken from patients using sterile cotton swabs and sent directly to the microbiology laboratory under cooling conditions for bacterial isolation. All collected wound swabs were inoculated on blood agar and MacConkey agar and after that on Cetrimide agar as a selective medium for *P. aeruginosa*. Suspected isolates were identified using different morphological and biochemical tests according to the key of Bergey's Manual for Systemic Bacteriology (2001), isolates were subsequently confirmed as *P. aeruginosa* by using VITEK 2 compact system [22]. All participants agreed to provide the investigator with the specimens. The ethics committee of College of Science, Mustansiriyah University approved this work (Ref. BCSMU/1221/00018M). Informed consent according to the Declaration of Helsinki was obtained from all participants.

2.2. Biofilm Formation Assay

Quantification of biofilm formation of isolates was determined by microtiter plate (MTP) assay as described by Zhang *et al*. (2016) with some modifications [23].

1- The procedure involves inoculating bacteria obtained from fresh agar plate cultures on 5 ml of brain heart infusion broth (BHI) with 1% glucose and incubated for 24 hours at 37°C.

2- A 180 µl of brain heart infusion broth (BHI) were used to inoculate pre-sterilized 96-well polystyrene microtiter plates along with bacterial suspension (20 µl) from each isolate (equivalent to 0.5 McFarland standard) and then incubated for 24 hours at 37°C.
3- After incubation, all wells were washed with normal saline three times for the elimination of unattached cells.
4- A 200 µl of 99% methanol was added to each well for 15 minutes in order to fix the adherent cells. The plate was let to dry at room temperature for 30 minutes.
5- A 200 µl of 1% crystal violet was added to each well, and it was left for 15 minutes.
6- Following the removal of the dye solution and cleaned with sterile distill water, 96% ethanol was used to dissolve the attached dye. The optical density (OD) was then measured at 630 nm using ELISA reader.
The experiment was conducted in triplicate, and a cut-off value (ODc) was evaluated according to the Table 1.

Table 1: Microtiter plate evaluation for biofilm production

Optical density	Adherence
OD ≤ ODc	Non–adherent
2 ODc > OD > ODc	Weak
4 ODc > OD > 2 ODc	Moderate
OD > 4 ODc	Strong

OD= optical density, ODc= cut off value.

2.3. Statistical Analysis

Statistical Program for Social Science (SPSS) version 20.0 was applied to analyze the findings. Frequencies and percentages were used to express the qualitative data.

3. Results and Discussion

3.1. Sample Collection and Bacterial Isolation

The results of the study showed that out of 121 sample, 51 (42.1%) yielded growth of *P. aeruginosa* by using variety of diagnostic techniques, including cultural, biochemical tests, and VITEK 2 compact system. On MacConkey agar, the isolates appeared as pale colonies, indicating that the organism is a

non lactose fermenter, while on blood agar medium, the isolates showed growth of large gray colonies and surrounded by clear zone of β-hemolysis due to their production of hemolysin. The appearance of yellow to green colonies with a grape-like odour on Cetrimide agar indicates the presence of *P. aeruginosa*. Cetrimide agar inhibits bacterial growth with the exception of *P. aeruginosa* and promotes the synthesis of fluorescein and pyocyanin pigments, according to the method outlined by Holt *et al*. [24] (Figure 1). Biochemical tests was used for more determine, all isolates demonstrated positive oxidase test results when the color changes to purple/blue within 5–10 seconds, indicating the synthesis of oxidase. Additionally, the current study's findings showed that all the *P. aeruginosa* isolates gave positive results for catalase test through the formation of bubbles, which signifies the presence of the catalase enzyme. After diagnosing the isolates by the VITEK 2 system, according to the results, all isolates were confirmed as *P. aeruginosa.* VITEK 2 system is a rapid, fully automated system for bacterial identification and antimicrobial susceptibility test (AST) [25].

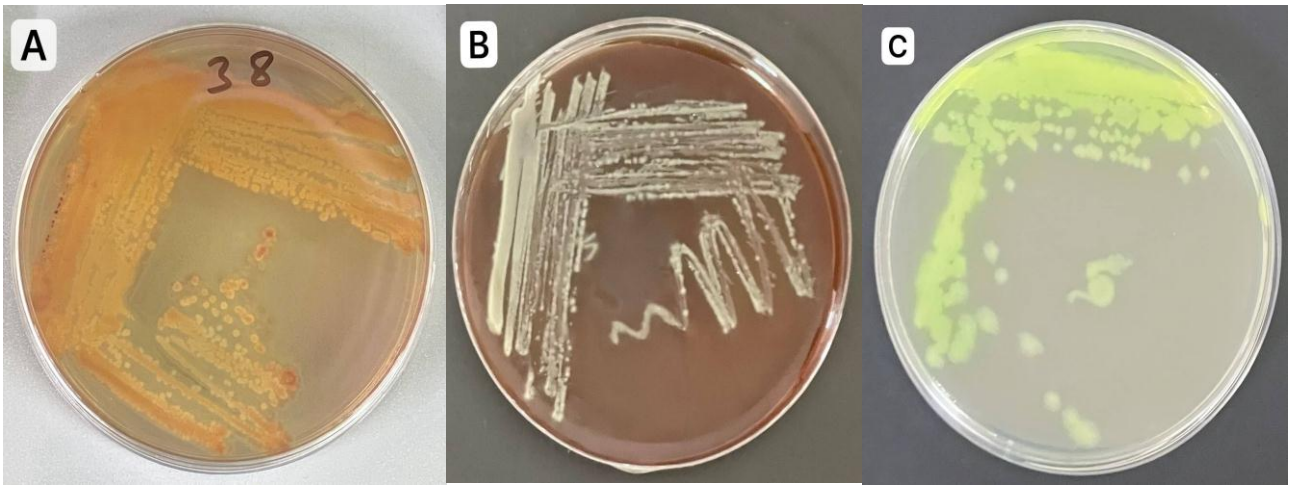

Figure 1: Growing colonies of *P. aeruginosa* on:
A- MacConkey agar (flat, smooth, pale and non-lactose fermenting colonies)
B- Blood agar (large gray-white colonies with uneven spreading margins)
C- Cetrimide agar (colonies with a green-yellow fluorescent pigment).

3.2. Biofilm Formation Assay

In this study, the ability of *P. aeruginosa* to form biofilm was estimated quantitatively using the colorimetric microtiter plate assay in the 51 *P. aeruginosa* isolates. The study's findings showed that every isolates were able

to form biofilm and they ranged between weak, moderate, and strong biofilm producers (Figure 2). Out of 51 isolates, nine (17.64%) form weak biofilm, twenty-six (50.98%) form moderate biofilm, and sixteen (31.37%) form strong biofilm as shown in Table 2. The ability of bacteria to form biofilms is known as a key virulence characteristic essential to its pathogenic success [26]. A number of previous studies stated varying rates of biofilm formation by *P.aeruginosa* isolates. A local study conducted by Al-Wasfi *et al.* [27] who reported that among 30 wound isolates of *P. aeruginosa*, 4 isolates produced weak biofilm, 11 produced moderate biofilm, 12 produced strong biofilm, and 3 isolates non-producer. In another recent study, Chimi *et al.* [28] who found that among 38 isolates, 5 isolates were weak biofilm producers, 14 isolates were moderate producers, and 19 isolates were classified as strong biofilm producers of *P. aeruginosa* isolated from infected wounds. In another study, Ghasemian *et al*. [29] reported that biofilm formation was detected in 40/40 (100%) *P. aeruginosa* isolates from burn wounds, 22.5%, 35%, and 42.5% of isolates were weak, moderate, and strong biofilm producers, respectively. Biofilm production efficiently promotes *P. aeruginosa* colonization, enhancing the bacteria's resistance to antibiotics and thwarting the host immune system [30]. In our study, we observe that all *P. aeruginosa* isolates that isolated from wound infections have the ability to produce biofilm. This is compatible with study of Motevasel *et al.* [31] who found that 100% of wound *P. aeruginosa* isolates were biofilm producers. The variations in the intensity of biofilm between the study isolates may be related to sensitivity of the microtiter plate method in assessing the amount of formed biofilm. Ahmed *et al.* [32] reported that there was a great deal of variation in the study isolates' ability to form biofilms, this variation may have been caused by the varying genetic compositions of isolates as well as by the gene expression of their biofilm and quorum sensing genes. *P. aeruginosa* is a common bacterium causing wound infections. Biofilm is the most important virulence factor of *P. aeruginosa* and has a significant effect on chronic wounds and affects the wound healing process. Therefore, it is necessary to develop new strategies to eradicate it. The rate of biofilm production by bacteria was high in this study, so it is recommended to take the appropriate precautions to control wound infections and to develop alternative non-antibiotic treatments to eliminate *P. aeruginosa* in the early stages of wound infections before biofilm formation.

Figure 2: Screening of *P. aeruginosa* isolates for biofilm formation by MTP method.

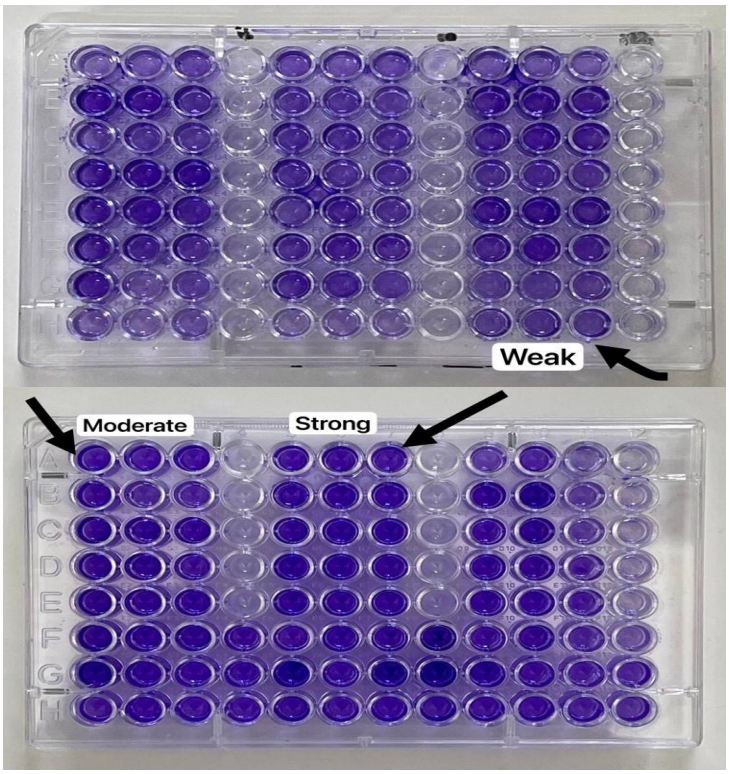

Table 2: Strength of biofilm produced by *P. aeruginosa* isolates.

Biofilm formation	No. of isolates	The percentage (%)
Non-biofilm producer	0	0
Weak	9	17.64%
Moderate	26	50.98%
Strong	16	31.37%

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تحديد العزلات المكونة للأغشية الحيوية لبكتريا *Pseudomonas aeruginosa* المعزولة من إصابات الجروح

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مستخلص البحث:

Pseudomonas aeruginosa (*P. aeruginosa*) هي احد اكثر انواع البكتريا المرضية انتشاراً خاصة في المستشفيات، وهي البكتيريا الأكثر شيوعاً المسببة لأصابات الجروح. يعتبر الغشاء الحيوي احد اهم عوامل الضراوه التي تنتجها وذلك لمساهمته في مقاومة المضادات الحيوية والعدوى المستمرة. تهدف الدراسه الحالية الى الكشف عن تكوين الأغشية الحيوية لعزلات الزائفة الزنجارية التي تم جمعها من مرضى الجروح. إجمالي 51 عزله تم الحصول عليها من 121 عينه سريرية تم جمعها من مرضى الجروح من مختلف المستشفيات في مدينة بغداد، جميع العزلات تم تقديمها للاختبارات الزرعية والكيميائية الحيوية للتعرف عليها، ثم بأستخدام نظام الفايتك جميع العزلات تم تأكيدها على انها *P. aeruginosa* . تم الكشف عن انتاج الأغشية الحيوية عن طريق اختبار لوحة المايكروتيتر، وفقاً للنتائج، (17.64%) من العزلات كانت منتجة ضعيفة للأغشية الحيوية، (50.98%) كانت متوسطة، و(31.37%) أنتجت غشاء حيوي قوي. وقد أظهرت نتائج هذه الدراسة أن جميع العزلات أظهرت قدرة متناسبة عالية على تكوين الأغشية الحيوية، حيث تعد الأغشية الحيوية أهم عامل ضراوة لبكتيريا *P. aeruginosa* ولها تأثير كبير على الجروح المزمنة وتؤثر على عملية التئام الجروح، لذا من الضروري تطوير استراتيجيات جديده للقضاء عليها.

الكلمات المفتاحية: *Pseudomonas aeruginosa* ، الغشاء الحيوي، إصابات الجروح.