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Detection of *Pseudomonas aeruginosa* with Biofilm Formation in Burn Patients: A Study in Al-Hussein Teaching Hospital of Iraq

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Abstract

A total of 100 clinical samples were collected from patients suffering from burn tissues in the burn unit at Al-Hussein Teaching Hospital, Karbala, Iraq, from January to July 2021. The obtained results indicated that 40% of isolates were *Pseudomonas aeruginosa* and the rest of the isolates (60%) were *non-Pseudomonas aeruginosa*. Various traditional and modern culturing methods along with biochemical tests were conducted to differentiate these isolates from other nosocomial infection bacteria. Bioluminescent assay and Congo Red Agar method were used to detect biofilm production. The Kirby-Bauer disc diffusion technique was implemented to investigate the antibiotic susceptibility of *Pseudomonas aeruginosa* bacteria according to CLSI standards. The bioluminescent assay approach was thought to be better than the Congo Red Agar method when analyzing a total of 40 clinical isolates. The bioluminescent assay identified 12.5% of the samples as high biofilm producers, 80% as moderate producers, and 7.5% as weak (non-biofilm) producers. In conclusion, *Pseudomonas aeruginosa* can form antibiotic-resistant biofilms.

Keywords: Biochemical tests, Burns, Biofilm formation, Culturing, *Pseudomonas aeruginosa*

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1. Introduction

Pseudomonas aeruginosa (*P. aeruginosa*) is a widespread Gram-negative bacterium that causes nosocomial and deadly infections in immunocompromised people, such as those suffering from infections caused by cancer, surgery, severe burns, or HIV [1]. In 2017, the World Health Organization identified *P. aeruginosa* as life-threatening and a priority pathogen for research and development of novel antibiotics [2]. Due to the adaptability and strong intrinsic antibiotic resistance of *P. aeruginosa*, common antimicrobial treatments, such as antibiotics, demonstrate inadequate efficiency leading to a higher rate of mortality [3]. In addition, *P. Aeruginosa* hinders the treatment of severe infections since it can produce biofilms, which protect it from environmental stressors, inhibits phagocytosis, and promotes colonization and long-term persistence [4]. Effective cell-to-cell communications within the microbial communities of *P. aeruginosa*, known as quorum sensing, boost this ability. Patients with chronic infections, such as chronic lung infection, chronic wound infection, and chronic rhinosinusitis, are frequently found to have biofilms with a highly organized morphology.

Most bacteria in nature may cling to various surfaces and create biofilms [5]. The biofilm is a complex aggregate of bacteria encased in a self-generated matrix of extracellular polymeric substances (EPS) and is one of the most important strategies for the survival of species during unexpected changes in environmental conditions, such as temperature fluctuation and nutrient availability [6]. Compared to their planktonic counterparts, biofilm-dwelling bacteria are 1000 times more resistant to antimicrobial treatments [7]. *Pseudomonas aeruginosa* is a well-known biofilm producer, making it an ideal model for biofilm formation research [8]. Its development cycle has six stages beginning with bacteria attachment to the surface and production of EPS, such as proteins, polysaccharides, lipids, and eDNA. This process is followed by cell division and irreversible adhesion. The next steps include generating microcolonies and growing them into mushroom-shaped structures. Cell-to-cell communication and virulence factor synthesis are required for biofilm formation and strength. The matrix cavity of the microcolony is then disrupted by cell autolysis, allowing the dispersed population to escape [9]. In about 2 hours, the discharged cells become planktonic and colonize the uncolonized regions.

2. Materials and methods

Pathological samples (burning skin) were collected from children and adults of both genders hospitalized at Al Hussein Teaching Hospital, Karbala, Iraq, from January to July 2021.

2.1. Ready Culture media

Nutrient agar, Nutrient broth, MacConkey agar, Brain hearth infusion agar, Amidst water Pepton, Triple sugar iron agar, Simmon citrate agar, and gelatin medium were purchased from the limited MLAB company (England, Figure 1).

2.2. Preparation of culture media

Blood agar medium, Citrilamid agar, King A, and King B agar were prepared according to the studies performed by Procop et al. [11] and Cappuccino et al. [12].

2.3. Isolation of *Pseudomonas aeruginosa*

The samples were collected from the wounds by sterile swabs and were cultured in blood agar and MacConkey agar plates for 24 hours at 37ºC. Then, a colony was isolated and transferred to the liquid culture followed by culturing in blood agar and MacConkey agar for 24 hours of incubation at a temperature of 37ºC. The colonies that showed culturing characteristics similar to those of the bacteria under study were transferred to the Citrilimad agar medium and King A agar. They were then incubated at 37ºC for 24 hours to ensure that the isolates return to *P. aerogenosa* bacterium.

2.4. Identification of *Pseudomonas aeruginosa*

The planting characteristics of isolates of *P. aeruginosa* were studied by testing their growth ability. Different media, including aquarium media (MaConkey agar and blood agar), elective media (Cetrimide agar), and the media promoting pigment production, specially pyocyanin on a King A agar were used to identify the culture characteristics in terms of the shape and color of colonies and the nature of hemolysis [13]. The growing colonies were selected from the McConkey agar media and the blood agar media, which were characterized by fully fermented lactose- and hemolysis-type fermenters of hemolysis-β. They produced stain on a medium King A agar, which it is also uninhibited on citilimade agar. All isolates were preserved to perform a microscopic examination and following biochemical tests. IMViC tests were utilized to identify and differentiate *P. aeruginosa*. Three test tubes were inoculated with citrate, tryptone broth (for the indole test), and methyl red-Voges Proskauer broth (MR-VP broth) according to a study by Cappuccino et al. [12].

2.5. Biofilm formation

Biofilm production in *P. aeruginosa* was measured quantitatively using a microtiter test. In a glass tube containing 2 ml TSBglu, a single colony from each subcultured plate on blood agar was injected. Under aerobic conditions, the tubes were incubated overnight at 36°C . A flat-bottomed microwell plastic plate was used to transfer 200 µl from each of the inoculated TSBglu tubes. For optimal oxygenation, the infected microwell plastic plate was incubated overnight at 36°C, by inverting the plate and striking it on filter paper the next day, the contents were discarded. The microwell polycarbonate plate was washed once in 200 µl PBS (pH 7.2) and discarded. Then, for biofilm fixation, 200ul of newly produced sodium acetate (2%) was applied to each well for 10 minutes before being removed. Afterward, each well was stained with 200 IU crystal violet (0.1%). The stain was, then, eliminated after 30 minutes at room temperature. The washing procedure was repeated. After 1 hour of drying at room temperature, the absorbance was measured using a spectrophotometer at 620 nm OD (adapted from Mirani and coworkers; Figure 2).

3. Results and Discussion

Of 100 samples taken from burns, 45 samples indicated the bacterial growth of *P. aeruginosa* (Figure 3-1). The high percentage of *P. aeruginosa* being isolated from the burn unit may result from having poor maintenance and quality control of surgical instruments as well as not cleaning surgical instruments periodically due to the current conditions in the governorate's hospitals. In addition, an increase in the number of burned patients and the scarcity of disinfectants in these hospitals provide suitable conditions for the growth of this germ [14]. The distribution of infection for *P. aeruginosa* swabs isolated from burns was also recorded. Regarding body regions, the highest percentage of bacteria was found in the back area (66.6%), while the lowest percentage was 35% in the facial area (Table 1). The successive use of the same bed to withdraw fluids and clean burns from patients with different injuries by health staff contributes to the increase in transmission and spread of this germ. Motbainor et al. [15] indicated that the contamination of the clean surgical instruments with *P. aeruginosa* is one of the reasons for the spread and transmission of infection in the hospital environment. The discrepancies in the obtained results of various studies originate from differences in the source of isolation, cleanliness of hospitals, quality of sterilization materials and disinfectants used, and the number of samples under study.

Despite the frequent reports of multiply resistant *P. aeruginosa* infections in burn units [16] and its preference for the equipment used for burn wounds, most burn centers continue to use this method of wound care on a regular basis, and they rarely inspect equipment and its water supply for bacterial contamination [17]. The results of the microscopic examination of the bacterial smear stained with a cream dye indicated that the cells of this germ appeared in the form of small, negative bacilli of the cream dye. This finding was in line with the obtained result of a study conducted by Bjarnsholt et al [18].

Pseudomonas aeruginosa was positive for the oxidase test, the catalase test, and the urease test. However, all isolates were negative for tests of Indole, Methyl Red, and Voges proskauer. All isolates indicated a positive result of the citrate consumption test, used to investigate the susceptibility of bacteria to consumption. Regarding stearates as the sole carbon source, the isolates were also grown on a medium of triglyceride-sugar-iron agar (TSI). The findings indicated no fermentation by any of the three types of sugars (glucose, lactose, sucrose). Moreover, CO2 gas and hydrogen sulfide gas were not produced, which was in the same line with the

findings of a study by Dykstr et al. [19]. The phenotypic examination was carried out using a bioluminescent assay with crystal violet dye and Congo Red Agar to determine the ability of bacterial isolates to produce biofilm. The results showed that 5 bacterial isolates could be considered strong biofilm-forming isolates, while the rest of the isolates (n = 3) were unproductive and weak. On the other hand, 42 samples of *P. aeruginosa* were moderate biofilm-producing isolates (Figure 3). These results were consistent with a study conducted by Toole et al. [20]. *Pseudomonas aeruginosa* bacteria isolated from burn patients indicated a high resistance to many antibiotics. The resistance rate reached 100% to beta-lactam antibiotics (Table 2), which could be due to the permeability barrier represented by the outer membrane layer. Bacterial resistance to antiseptics is an important type of resistance that occurs as a result of the reduced permeability of the outer membrane, and consequently affects the rate of absorption of antibiotics [21].

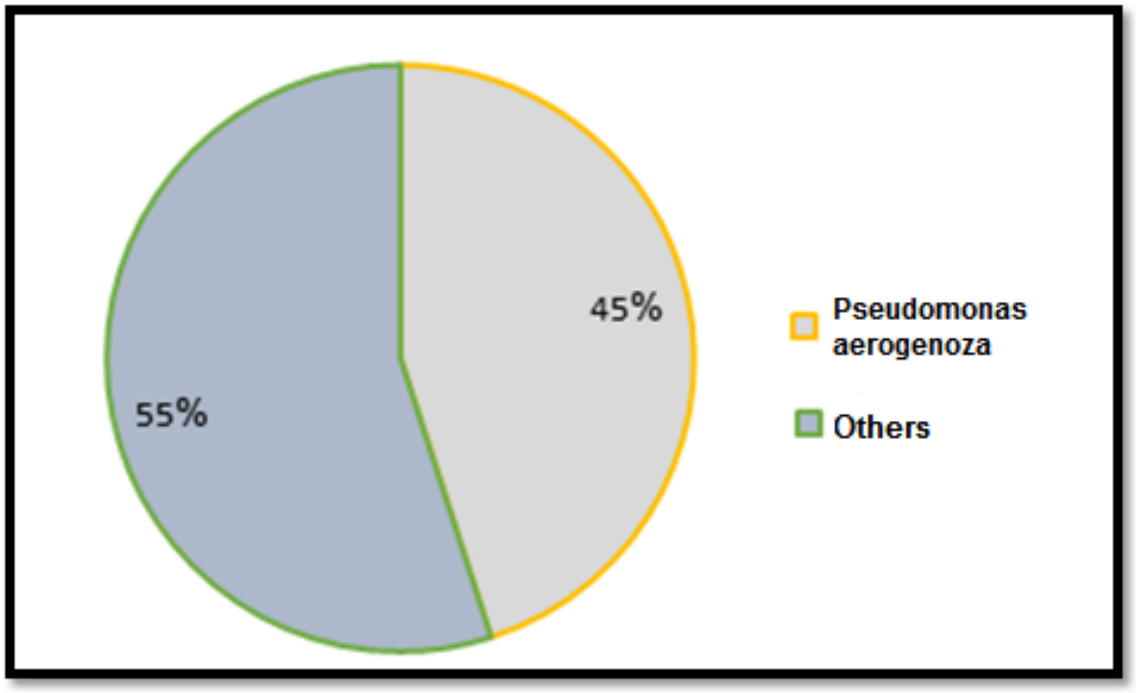

Figure 2. Biofilm formation in culture.

Table 1. Number and percentage of *P. aeruginosa* in different regions of patients' body

Isolation in the body regions	Total	Number of Positive	Percentage
Back area	15	10	66.60%
Abdomen area	30	13	43.30%
Upper extremities	15	7	46.60%
Lower extremities	20	8	40%
Face area	20	7	35%
Total	100	45	45%

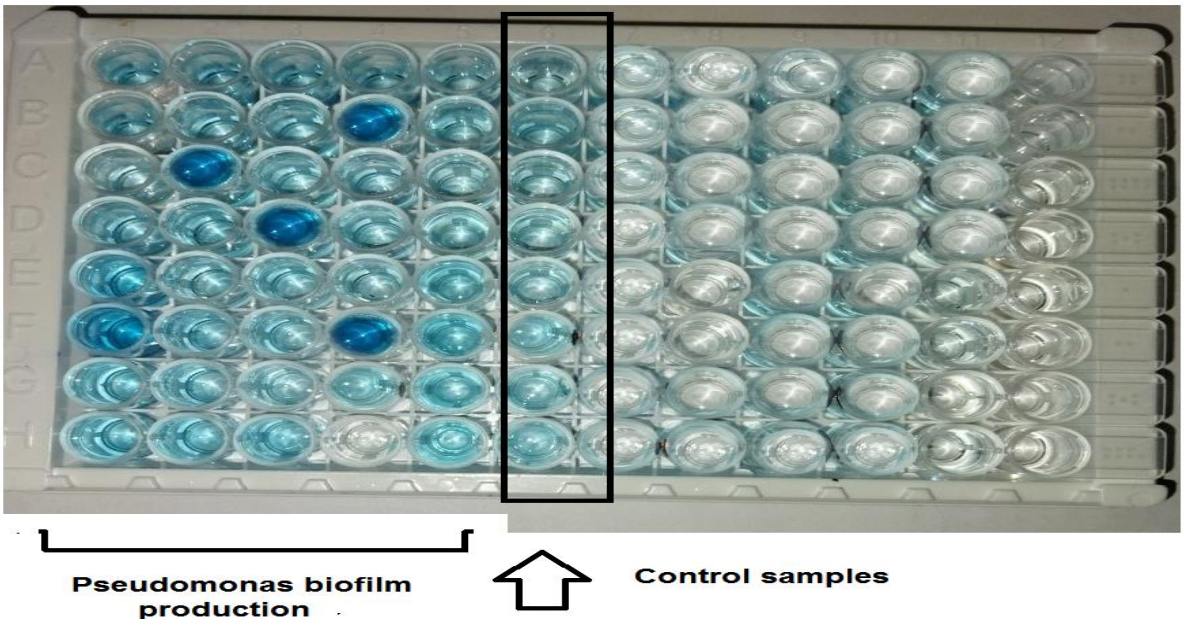

Figure 3. Bioluminescent assay for *Pseudomonas aeruginosa* producing biofilm production

Table 2. Antibiotics susceptibility test

Antibiotic	**Total**	**R**	**%**	**S**	**%**
Ticarcillin	40	19	38	21	62
Cefepime	40	22	44	18	56
Ceftazidime	40	31	62	9	38
Piperacillin	40	24	48	16	52
Ticarcillin/Clavulanic Acid	40	22	44	18	56
Tobramycin	40	15	30	25	70
Gentamicin	40	17	34	23	66
Amikacin	40	25	50	15	50
Meropenem	40	18	36	22	64
Imipenem	40	13	26	27	74
Trimethopim/Sulfamethoxazole	40	26	52	14	48
Minocycline	40	23	46	17	54
Pefloxacin	40	14	28	26	72
Ciprofloxacin	40	20	40	20	60

The bioluminescent assay identified 12.5 % of the samples as high biofilm producers, 80 % as moderate producers, and 7.5% as weak/non-biofilm producers. Present study shows *Pseudomonas aergenoza* have ability to form antibiotic-resistant biofilms in hospital environments.

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