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Molecular Identification, Antibiotics Sensitivity and Biofilm Formation of Pseudomonas Aeruginosa Isolated from Burn Infection Patients in Mosul, Iraq

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Abstract: Background: Burns are one of the most common and devastating forms of trauma. Pseudomonas aeruginosa is a gram- negative bacillus bacterium that is resistant to many antibiotics and able to form biofilms, which are the most important virulence factors of this bacterium. **The aim of this study:** Molecular identification, antibiotics sensitivity and biofilm formation of Pseudomonas aeruginosa isolated from burn infection patients in Mosul, Iraq **Materials and Methods:** 22 swabs were collected from patients suffering from contaminated burns in burn hospitals in Mosul city, during 4 months (August 2023-November, 2023). Swabs were cultured on MacConkey, Cetrimide and Blood Agar. Antibiotic sensitivity test and detection of biofilm formation by Congo Red Agar (CRA) and the Microtiter Plate method (MTP) were done. **Results:** 6 isolates (27.3%) of P. aeruginosa were isolated. Which is identified by biochemical tests, API 20E and confirms the identification with molecular techniques based on 16S rRNA. All isolates showed resistance (100%) to Cefotaxime and Cefixime, (83.3%) to Levofloxacin. Ceftriaxone, Ceftazidime, (50%) to Ofloxacin, Amikacin Aztreonam, Meropenem, Imipenem. Whereas they appeared low resistance to Norfloxacin, Gentamicin, Ciprofloxacin which was (33.3 %). According to biofilm formation, 50% of isolates showed positive results by CRA method, while all isolates showed positive results by MTP method. **Conclusion:** The study concluded that P. aeruginosa was resistant to many antibiotics, furthermore showed that the MTP method is more accurate than the CRA method in detection of biofilm formation.

Keywords: Pseudomonas aeruginosa, antibiotic susceptibility test, biofilm formation, 16S rRNA.

1. INTRODUCTION

One of the most prevalent and debilitating types of injuries is a burn. In order to lower morbidity and death, patients with effective thermal injury need to receive specialized care very away. Roughly 2 million fires were investigated annually, according to data from the National Center for Injury Avoidance and Control inside the United States, leaving 1.2 million people with burn injuries [1]. P. aeruginosa is one of the most widespread types of bacteria. It is found in various environments such as soil, water, animals, and plants, and it is one of the most pathogenic species for humans [2]. It is a gram- negative bacterium, bacillus, obligate aerobic, and motile. Because it possesses a single polar flagellum, this bacterium can be distinguished by the smell of the colonies when grown on the culture medium, as it has a distinctive grape-like odor. It is also characterized by having a metallic luster, and works to secrete many different colored pigments such as Pyocyanin. Pyocyanin, Pyorubin, Pyoverdine, and Pyomelanin. It is also characterized by its metallic luster. It has gained great importance at the present time as it is a major cause of infections and injuries in hospitals, Nosocomial Infections, and the infection may be fatal in people who suffer from weak immunity, such as patients infected with HIV, patients with cancer, or severe burns [3]. Due to the ability of bacteria p. aeruginosa adaptability and its high resistance to antibiotics, the known antibacterial treatments have become insufficient to eliminate it, leading to a high death rate [4]. The reason for the emergence of resistant strains of bacteria is due to the occurrence of mutations or due to horizontal transfer of genes between species, as well as excessive and indiscriminate use that leads to the emergence of resistance [5], and P. aeruginosa bacteria possesses many mechanisms that enable it to resist antibiotics. Such as the production of beta-

lactamase enzymes, reducing the absorption of antibiotics, changing the target of the antibiotics, in addition to the active efflux of antibiotics. In addition, these bacteria have the ability to form biofilms, which protect them from harsh environmental conditions and prevent the process of phagocytosis [6], and the phenomenon of quorum sensing within bacterial communities works to enhance this ability [7]. The World Health Organization (WHO) has identified these bacteria as pathogenic and life-threatening, and a priority for research and development of new antibiotics [8]. These bacteria can be found in nature on various surfaces, including metals, plastics, medical devices, and tissues. The reason for this widespread spread and ability to cause infection is because they possess effective adhesion mechanisms and do not require complex nutritional requirements. They have features that differ from other bacterial species, such as their possession of a network of signals. The complex intercellular regulatory system that makes it able to transform from the free-swimming motile form to the static form represented by the biofilm, which is dense aggregates of bacteria embedded in a polysaccharide matrix that works to keep the bacteria alive during unexpected changes in environmental conditions, such as depending on temperature, nutrient availability, and pH [9], bacterial growth within biofilms enhances their survival and will be difficult to destroy and eliminate. These membranes also protect bacteria in the event of exposure to antibiotics, as they work to impede the spread of the antibiotic into the bacteria, which increases the risk of the bacteria and their pathogenicity [10], patients who suffer from chronic infections such as chronic wound infections, and lung infections. Chronic sinusitis and chronic sinusitis often have biofilms with a very regular shape, and it has been proven that bacteria that have the ability to form biofilms are highly resistant to antibiotics [11], 1000 times more than non-bacterial bacteria. Membrane forming [12].

Biofilm formation in P. aeruginosa goes through the following stages:

Bacteria adherence to the surface, Formation of microcolonies, Maturation of membrane structures and Proliferation and differentiation of biofilm [13].

MATERIAL AND METHOD

1. **Samples collections**: 22 swabs were collected from patients suffering from contaminated burns by using sterilized cotton swabs. Swabs were transferred directly to the laboratory using transport media for culturing and identification.
2. **Isolation and identification of *P. aeruginosa*:** Swabs were streaked onto MacConkey Agar, Cetrimide Agar and Blood Agar plates, then incubated for 24 hrs at 37° C. The following day, certain colonies were chosen and identified based on their morphological, cultural, and biochemical traits.
3. **Identification:** Bacterial isolates were identified based on bacteriological methods, such as characteristics and appearance of colonies on MacConkey agar, haemolysis on blood agar and producing pigments on Cetrimide Agar. Further identification was done by their gram stain reaction. Also, biochemical tests such as oxidase and catalase. Then, bacterial isolates were identified by API 20E Test and molecular method.
4. **Molecular Method:** Molecular method based on 16S rRNA was used to confirm the identification of bacterial isolates.

- **DNA Extraction**

The Genomic DNA Extraction Kit (Geneaid Biotech, Taiwan) was used to extract the whole genomic DNA of bacterial isolates, according to the manufacturer's instructions. The extraction was done in Dept. of Biology-College of Science-University of Mosul.

- **DNA Purification and Quantification:**

Using a nano-drop spectrophotometer, the concentration and purity of the extracted DNA were determined (Cambridge CB4, England), at wavelength 260-280 nm [14].

- **16S rRNA Gene Amplification - Polymerase Chain Reaction (PCR):**

This reaction done according to [15] by using universal forward primer 27- F (5'- AGAGTTTGATCMTGGCTCAG-3') / and reverse primer 1552- R (5'- AAGGAGGTGATCCARCCGCA -3'), supplied by (Geneaid Biotech, Taiwan). Conditions of the PCR Amplification Cycle are shown in **table (1).**

Table 1: Conditions of the PCR Amplification Cycle [15]

	Steps	**Temperature °C**	**Time**	**Cycle No.**
1	First denaturation	95	3 min.	1
2	Denaturation	95	30 sec.	30
3	Annealing	54	30 sec.	
4	Elongation	72	90 sec.	
5	Last elongation	72	3 min.	1

Amplification products were electrophoresed for 45 minutes at 100 volts alongside normal DNA on a 1% (w/v) agarose gel. Gel was stained for one hour with ethidium bromide solution, followed by UV- transilluminator photography at 320 nm (provided by Royal Biotech GmbH-Germany) [16].

- **16S rRNA Gene Sequence Analysis:**

The 16S rRNA PCR results were transmitted to the Samogen Company in the USA for sequencing. In order to identify the tested isolates, the sequences of the samples were compared with the genes of the sequences at the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov), by identifying the tested isolates with the use of the Basic Local Alignment Search Tool (BLAST).

5. Antibiotic Susceptibility Test:

The test was carried out on Muller-Hinton Agar, following the recommendation given by CLSI depending on the Kirby-Bauer disc- diffusion method. The antibiotic agents used are described in table (2). The diameters of zones were measured by a ruler after 24hrs of incubation at 37˚C and were interpreted according to the CLSI recommendations [17].

Table 2: The Antibiotics Used for Sensitivity Test

	Antibiotics	Abbreviation	Concentration (μg)
1.	Aztreonam	ATM	30
2.	Meropenem	MEM	10
3.	Imipenem	IPM	10
4.	Ceftriaxone	CRO	10
5.	Cefotaxime	CTX	30
6.	Ceftazidime	CAZ	30
7.	Cefixime	CFM	5
8.	Gentamicin	CN	10
9.	Amikacin	AM	10
10.	Ciprofloxacin	CIP	5

11.	Ofloxacin	OFX	10
12.	Norfloxacin	NOR	30
13.	Levofloxacin	LEV	5

6. Detection of biofilm formation:

A.Using Congo Red Agar:

The medium was prepared by dissolving Brain Heart Infusion agar 37g/L in distilled water, adding sucrose 5g/L, agar 10g/L, and adding Congo Red dye 0.8g/L. After sterilization, isolates were cultured on Congo Red agar, incubated at 37°C for 24 hours [18].

B. Using Microtiter Plate Methods (MTP):

This method was designed by researcher Madilyn Fletcher and is one of the most widely used methods to detect or investigate the formation of biofilms [19], as follows:

A standard plate consisting of 96 wells was used, after preparing the bacterial inoculum in BHI broth. It was compared with McFarland tubes to give an approximate number of cells equivalent to (1.5×10^8) cells/cm^3. 200 µl of it was placed in the wells. The plate was covered with its own lid, incubated for 30 hours at 37 °C. After incubation, the plate was washed many times with normal saline to remove non-adherent bacterial cells. 200 µl of 1% Crystal Violet was added to each well for 45 minutes. The dye was removed with sterile distilled water, and the plate was left to dry for 45 minutes. 200 µl of 99% ethanol was added to each well. As control, we used 200 µl of bacteria-free BHI broth.

The optical density was read by ELISA reader, at a wavelength of 630 nm to determine the efficiency of the isolates that form biofilm. The cutoff value was calculated, and the strength of biofilm formation was determined.

When using the standard plate method, the result is read according to special equations, as follows:

- When the absorbance rate of the sample is higher than the Cutoff value, it is considered a weak positive.
- When the absorbance rate of the sample is twice the Cutoff value, it is considered moderately positive.
- When the absorbance rate of the sample is more than 4 * the Cutoff value is considered a strong positive.
- When the absorbance rate of the sample is equal to the Cutoff value, it is considered negative (not forming a biofilm).

RESULT

1. Identification :

In the present study, from 22 swabs collected from infected burns, 6 (27.3%) isolates of *P. aeruginosa* were recorded, according to the results of microscopic characteristics, which are short, gram-negative bacilli. Also based on phenotypic characteristics of the bacterial colonies, which they were non- lactose fermenter on MacConkey agar, produced β-hemolysis on blood agar, which appeared as a clear zone around colonies. and produced yellow to yellowish-green colonies on cetrimide agar. Furthermore, all isolates were positive to catalase and oxidase. Also, the identification was done by API 20E test.

2. Molecular Method

Confirm the identification by molecular method based on 16S rRNA. Results of the 16S rRNA for 6 isolates were sent to Samogen Company in the USA for sequencing. The sequence was analyzed at NCBI using BLAST. The results of 6 isolates showed they were belonging to the *P. aeruginosa*, and had been registered in NCBI: FS-1iq, FS-2iq, FS-4iq, 5iq, FS-6iq and FS-7iq under accession numbers: PP439490.1, PP439593.1, PP439629.1, PP439634.1, PP439635.1 and PP439637.1 respectively.

3. Antibiotic susceptibility test results:

Table (3) displays the *P. aeruginosa* resistance profile to the antibiotics being investigated.

Table 3: Resistance Profile of *P. aeruginosa* to the Antibiotics

	Antibiotics	**Resistance** %
1.	Aztreonam	50
2.	Meropenem	50
3.	Imipenem	50
4.	Ceftriaxone	83.3
5.	Cefotaxime	100
6.	Ceftazidime	83.3
7.	Cefixime	100
8.	Gentamicin	50
9.	Amikacin	50
10.	Ciprofloxacin	33.3
11.	Ofloxacin	50
12.	Norfloxacin	33.3
13.	Levofloxacin	83.3

All isolates appeared resistance (100%) to Cefotaxime and Cefixime, (83.3%) to Levofloxacin, Ceftriaxone, Ceftazidime, (50%) to Ofloxacin, Amikacin Aztreonam, Meropenem, Imipenem. Whereas they appeared low resistance to Norfloxacin, Gentamicin, Ciprofloxacin which was (33.3 %). One of a clinician's biggest challenges is nosocomial infection in burn patients. According to estimates, infections were a factor in 75% of all burn patient fatalities. Extended usage of antibiotics causes multidrug resistant (MDR) bacteria to grow and become more prevalent, which exacerbates problems and makes therapy unsuccessful. Therefore, knowledge of the microbiological flora and the most recent trends of antibiotic susceptibility are crucial for the clinician managing burn sepsis [20]. The process of horizontal gene transfer via transposon genes or resistant plasmids (R-plasmids), as some bacteria have structural characteristics that make them naturally resistant to certain medications Gram-negative bacteria are characterized by the absence of permeability in their outer cell wall envelope, which serves as a barrier separating them from Gram-positive bacteria. Numerous antigens adhere to the cell, giving it the ability to fend against these [21].

4. Biofilm formation:

Two methods were used to detect biofilm formation, namely the Congo red agar method CRA and the microtiter plate method MTP, to determine the ability of bacterial isolates to form biofilms.

A. Congo Red Agar test result:

When using the Congo red agar method, the result can be identified according to the colors of the colonies. The positive result was represented by the appearance of black or grayish-black colonies, evidence of abundant production of the slime layer. The appearance of brown or pink colonies with the appearance of a dull black color in the center of the colony is evidence of weak or moderate production of the slime layer. 3 isolates showed positive results, while 3 isolates showed negative results, represented by the appearance of dark red or pink colonies. As shown in **Figure (1).**

Previous study showed that *P. aeruginosa* appeared 40% positive results for Congo red agar method [22], whereas [23] showed 55% of isolates were positive. Congo red dye is used in experiments used to detect the formation of biofilms, such as its use in dyeing exo glycemic substances rich in glucose produced by some types of bacteria, as the sugary substance can interact with this dye, causing a change in its color. To varying degrees depending on the bacteria isolates production of the sugary substance or the sticky layer [24].

A

Figure (1):

A – Black colonies indicative of biofilm formation (positive result)
B – Red colonies indicate the inability to form biofilm (negative result)

B. Microtiter Plate Method Test:

The results of detecting the ability of isolates to form biofilms based on the MTP method according to changes in the optical density of the ELISA device showed that (100) % of the isolates have the ability to form biofilms with varying intensity of formation when compared with the control reading rate. These high percentages indicate a high rate of resistance to all antibiotics [25] indicated that biofilms increase the pathogenicity of bacteria and also increase their resistance to antibiotics.The results showed that the percentage of isolates forming biofilms was (100%), (3) bacterial isolates (50%) could be considered strong biofilm- forming isolates, and one isolate (16.6%) was of moderate formation, while two isolates were (33.3%) weak formation. **Table (4)** show the intensity of biofilm formation of isolates in MTP method.

Table 4: The intensity of biofilm formation of isolates in MTP method

%	Intensity of biofilm formation	Total number of isolates n=6
50 %	+++	3
16.7 %	++	1
33.3 %	+	2

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