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**ORIGINAL ARTICLE**

# Evaluation the Efficacy of Bacteriophage Against Pseudomonas Aeruginosa Isolated from Wound and Burn Infections

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## ABSTRACT

One Hundred clinical samples were collected from different skin infection, including wounds and burns from patients in several hospitals in Baghdad governorate. The phenotypic diagnosis of isolated was performed by culturing on MacConkey agar medium and selective cetrimide agar. After a microscopic and biochemical tests diagnosis, the diagnosis was confirmed using Viteck2 system, 42 bacterial isolates were isolated belonging to P. aeruginosa, with ages ranging from (4 days-75 years).

The results of sensitivity test with the Kirby–Bauer Disk diffusion technique found that 19 bacterial isolates only were highly resistant by 76.19% to Cefepime, while the resistance to Ciprofloxacin and Gentamicin was 45.2% for each of them. Moreover, it showed resistance to Aztreonem 33.33%, Ceftazidim and Piperacillin 28.5% each of them, while it also showed high sensitivity and low resistance to Imipenem 26.19%.

The results revealed that 3 bacteriophages were isolated with different phenotypic characteristics and had an effective effect on isolates resistant to antibiotics and were studied under different conditions such as the change in temperature and incubation period, as well as the difference in glycerol concentrations.

## INTRODUCTION

Pseudomonas aeruginosa is a Gram-negative bacilli, pathogenic and obligate aerobic and motile by using a single polar flagella and non-spore forming. It is distinguished by having a differential characteristic which is the release of a smell similar to the smell of grapes due to its production of an aromatic compound called 2-aminoacetophenone (1)(2).

Furthermore, P. aeruginosa has several virulence factors, and the most important of which is the exotoxin, which is one of the most productive virulence factors, as it is produced by up to 90% of bacterial isolates. In addition, it is characterized by its production of pyocynin and pyoverdin pigments, which are toxic to other bacterial species and human cells, as well as inhibiting the proliferation of lymphocytes, and most of them have a capsule that saves them from phagocytosis. Interestingly, this bacteria has the ability to form biofilm, which is closely related to its high resistance to antibiotics and its tolerance to inappropriate conditions (2)(32) (11).

These bacteria are considered as one of the most important opportunistic pathogens that infect a wide range of organisms including humans, animals and plants and spread in soil, water and even air (3). P.aeruginosa also is characterized by its high risk as it is the main cause of urinary tract infection (UTIs) in hospitals, and causes severe and fatal infections, especially in patients who have weak defense mechanisms, including eye infection, endocarditis, ear infection and bacteremia and septisemia. Moreover, it also causes inflammation of the lower and upper respiratory tract, such as cystic fibrosis, and it may lead to complete weakness in the lungs, in addition to being one of the main causes of soft tissue infection, wound and burn infection, especially in people with immunocompromised conditions (3)(4).
The high resistance of P. aeruginosa is due to its possession of major mechanisms of antibiotic resistance through impermeability of the outer membrane. (5), or to the presence of efflux pumps systems (6), or its alteration of the target site (7).

Bacteriophage is a virus that has the ability to infect and eliminate bacteria without causing any negative effect on host body cells (8). The using of bacteriophage therapy began since the twenties of the last century in the Soviet Union and is still used today in the countries of the former Soviet Union such as Poland, Russia and Georgia. Currently, bacteriophage therapy is being reconsidered as a possible alternative to antibiotics in Western countries. However, there are still some obstacles that may face them, such as choosing the most suitable phage against a specific infection, the risk of developing phages resistance and the immune response to the phage by the host (9). Thus, this study aims to study the effect of bacteriophage on multi-antibiotic resistant P. aeruginosa isolated from wounds and burns.

## MATERIALS AND METHODS

**The samples collection:** One hundred clinical samples were collected from different skin infection (wounds/burns) of patients hospitalized in Baghdad governorate hospitals, from 10/16/2021 to 15/1/2022. The samples were taken using a sterile transport media swab; they were classified according to gender, age, and place of sample collecting from patient.

**Isolation and identification of P.aeruginosa:** The samples were phenotypically diagnosed as they were cultured on MacConkey agar (Himedia, India) and selective Cetrimide agar (Himedia, India), while the microscopic diagnosis was performed using gram stain (Himedia, India) to observe the shape and aggregation of cells as well as the nature of their interaction with this dye. In addition, the biochemical tests were relied upon, and finally, its diagnosis was confirmed using Vitec2 system (BioMerieux, France).

**The sensitivity test to antibiotics:** The Kirby–Bauer Disk diffusion technique was performed to test the resistance and sensitivity of bacteria to the following antibiotics: Cefepime, Ceftazidime, Aztreoname, Gentamycin, Ciprofloxacin, Piperacillin, Imipenem, and the result was compared with international standard tables (10).

**Collection and isolation of bacteriophage samples:** The samples were collected from different environmental sources including sewage water, Tigris river water, soil, stagnant water and bird feces), and then transferred directly to the laboratory. The supernatant was separated using centrifugation at 2500 rpm for 10-15 minutes. Then, the supernatant was filtered using filter paper to reduce the amount of impurities, and then filtered using a Millipore filter with a diameter of 0.22 µm, so that the lysate was kept in sterile sealed tubes at a temperature of 4° C until using (12)(13).

**Enrichment of bacteriophage samples:** One ml of P. aeruginosa with its logarithmic phase (18 hours) grown on Luria broth (10g Tryptone, 10g NaCl, 5g Yeast in 1000ml of distilled water) and mix it with 9 ml of Lysate. Then, 1 ml of LB (10×) was added and incubated in shaking incubator at 37°C at 180 rpm for 24 hours. After the incubation period, the samples were centrifuged at 3000 rpm for 10 minutes, and the bacterial cells were removed using a 0.22 µm diameter Millipore Filter paper and the predicted bacteriophages were kept at 4°C in sealed tubes to prevent contamination (12).

**Detection of Bacteriophages**

**A) The Double-Layer Agar technique (DLA)**

Five hundred µL of filtered lysate and 500 µL of P. aeruginosa were mixed in its logarithmic phase grown on LB medium, and this mixture incubated for 10 minutes at a temperature of 37° C in order

to ensure that adsorption occurs between bacteriophage and bacterial receptors. Then, it was added to 3 ml of top agar (LB with 0.7 of agar) or the so-called soft agar at 45°C with 5% of glycerol and mixed gently using vortex mixer, and poured gently over the bottom agar (LB with 1.5 agar) and making sure that it reaches all sides of the dish; after hardening, it is stirred and incubated at a temperature of 37° C for 24 hours and after the end of the incubation period when the inhibition areas are appeared (plaque) that indicating the presence of phage. Used this method According to (4) (14) with some modifications,

**B) Phage Spot Assay**

Three mL of soft agar (at 45° C) mixed with 200 µL of P. aeruginosa grown in its logarithmic phase (18 hours) grown on LB medium and poured into dishes containing the lower agar and immediately added a drop (10 µL) of the bacteriophage lysate samples, and after hardening, stir and incubate at temperature of 37° C for 24 hours, after which the plaques are purified in the event of the presence of phage (15) (16).

**Purification of bacteriophage:** The plaque is transfer to a sterile tube containing 4 ml of Lambda buffer (0.05g Gelatin, 0.726 Tris HCl, 2.46g $MgSO_4.H_2O$ in 1000 distilled water). To release the bacteriophage from the agar, the tube is shaken using vortex mixture for 30 seconds every 10 minutes and for a period of one hour; then the mixture is centrifuge at 2000 rpm for 5 minutes, and the supernatant is taken and filtered using a 0.22 microliter Millipore filter paper to get rid of bacterial cells suspended in the agar, then similar amount of Lambda buffer is added, and is considered as phage stock and is kept at 4°C (16) until using.

**The effect of temperature on bacteriophage activity:** Four temperatures (4°, 37°, 45°, 60°) C were adopted to study its effect on the bacteriophage activity and its heat tolerance. The lysate (contain bacteriophages) with appropriate dilution were treated at the approved temperatures for an hour (4)(25), after which the bacteriophage was detected using double agar technique (DLA) according to the method mentioned in (4) (14).

**The effect of glycerol concentrations on bacteriophage activity:** Different concentrations of glycerol (5%, 10%, 20%) were added to the culture medium of the bottom agar layer and the top agar layer before sterilization in addition to 0% concentration as a negative control; then the bacteriophage was detected using the double agar technique (DLA). According to method (14),

**The effect of incubation period on bacteriophage activity:** Five time periods (12, 24, 48, 72, 96) hours were chosen to study the extent of the bacteriophage ability to eradicate P. aeruginosa. The double agar technique (DLA) was adopted to detect the bacteriophage, and the dishes were examined according to studied periods.

## RESULTS AND DISCUSSION

After the bacterial samples were morphologically and microscopically diagnosed using biochemical tests as well as Vitek2 system (BioMerieux, France), 42 clinical bacterial isolates of P. aeruginosa were obtained, representing a percentage of 42% (Figure 1). The rate of infection with P. aeruginosa is very high in wounds and burns, and this is due to many reasons, including the ease of obtaining low-quality and ineffective antibiotics without a prescription, and it may be due to the contamination of the hospital environment and permanent crowding of patients (2).

The study indicated that the number of isolates of burn infections was 27 (64.28%), while the number of isolates of wound infections was 15 (35.71%) (Table 1). This increase in infection rates in burn patients, especially as a result of many reasons including the damage of the host tissues and disruption of defense mechanisms in patients (17), in addition to repeated manipulation and scraping at the site of the burn and the failure to exercise preventive instructions; all of them lead to an increase in the risk and spreading of infection (18). Furthermore, the results of this study clearly showed that females are more susceptible to infection with P. aeruginosa, where the number of infected female isolates was 26 (61.9%), while the number of isolates that infected males was 16 (38%); it is possible that the reason for this increase is related to females spending time more close to fire (19).

These results were recorded for the age group (4 days-75 years), and the highest percentage of infection with P. aeruginosa appeared in this study in the age group (19-36), followed by the group (1-18) (Table 2). The reason for the high rate of infection in these ages compared to others is due to they are the most active and mobile, which makes the possibility of them being exposed to infection and burns more (27); moreover this may explain this increase in the percentage among young people because they are the least abiding by the instructions during the infection, which leads to a worsening of their health case (20)

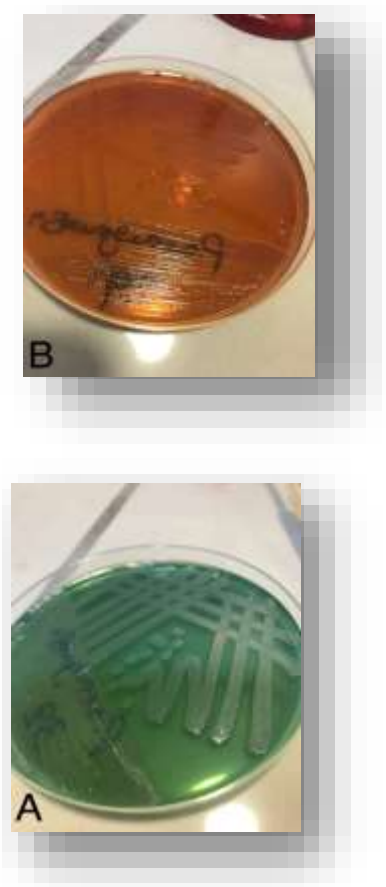


Figure 1: The growth of P. aeruginosa isolates. A: In Nutrient agar, (B) in MacConkey agar.

Table 1: Distribution of bacterial isolates according to the source and sex.

| Sex | Burns isolates n= 27 (64.28%) | Wounds isolates n= 15 (35.71%) |
|---|---|---|
| Females | 19 (70.4%) | 7 (46.66%) |
| Males | 8 (29.6%) | 8 (53.33%) |
| Total | 42 bacterial isolates | |

Table 2: Distribution of P. aeruginosa isolated from burns and wounds infections according to the age.

| Age groups | No. of patients | Percentage (%) |
|---|---|---|
| 1-18 | 13 | 30.9% |
| 19-36 | 18 | 42.8% |
| 37-54 | 7 | 16.6% |
| 55-75 | 4 | 9.5% |
| Total | 42 | 100% |

**The sensitivity test:** The results of the sensitivity test using Kirby–Bauer Disk diffusion technique that used 7 types of antibiotics for

42 P. aeruginosa isolates showed a clear variation in their resistance to antibiotics. The results revealed that P. aeruginosa was resistance to Cefepime by 76.19%, and resistance to Ciprofloxacin and Gentamicin by 45.2%, that is, to only 19 isolates. Furthermore, it was resistance to other antibiotics, such as Aztreonem by 33.33%, and Ceftazidim and Piperacillin by 28.5%, while it showed high sensitivity and low resistance to Imipenem by 26.19% (Fig. 2, Table 3).

Cefepime is effective against gram-negative bacteria, but this resistance that appeared in the isolates of this study may be due to the lack of permeability of the antibiotic through the plasma membrane of the bacteria or as a result of its secretion of cephalosporinase enzyme, which is expressed chromosomally. Additionally, the reason for resistance of the bacterial isolates may be due to its possession of beta-lactamase enzymes that affect and destroy the beta-lactam ring in the antibiotic, thus reducing its ability to bind to the proteins of the bacterial cell wall (29). The reason for the sensitivity of the isolates of this study to Ceftazidime is that it works to restrict and inhibit the cell wall construction of P. aeruginosa (21), while the reason for this low resistance may be attributed to the high affinity of Ceftazidime to PBPs enzymes, which preventing cell wall formation and thus bacterial cell death (28) (22) (31).

Ciprofloxacin is effective against infections caused by P. aeruginosa infection, and this is what we found in the current study. It affects the nucleic acids of bacteria by inhibiting the enzyme Topoisomerase (DNA gyras), which leads to the destruction and killing of bacteria (28). On the other hand, Gentamicin is highly effective against skin infections, especially burns and wounds caused by P. aeruginosa infection, as it acts on protein synthesis sites and causes an imbalance in the amino acid sequence, thus improper protein synthesis (28). The reason for the resistance of some isolates may be attributed to their possession of aminoglycoside-modifying enzymes (AMEs) (23); furthermore, sometimes the resistance of bacteria is due to a mutation in the ribosomes or their possession of the permeability barrier (28). Aztreonem was indicated by (30) that it is an antibiotic similar in its action to beta-lactam antibiotics, as it has the ability to affect P. aeruginosa and degrades its cell wall and then its death permanently. The sensitivity of the isolated bacteria to Imipenem may be due to their lack of mineral beta-lactamase enzymes (MBLs), which work to impede the absorption of this antibiotic by bacteria and then analysis of it (24).

Table 3: The percentages of antibiotic resistance of P. aeruginosa isolates under study using Kirby–Bauer method.

| No. of isolate | Antibiotics | Sensitive isolates | | Moderate sensitive isolates | | Resistant isolates | |
|---|---|---|---|---|---|---|---|
| | | No. | Percentage (%) | No. | Percentage (%) | No. | Percentage (%) |
| 1 | Cefepime | 3 | %7.14 | 8 | %19.04 | 31 | %76.19 |
| 2 | Ceftazidime | 23 | %54.76 | 7 | %16.66 | 12 | %28.5 |
| 3 | Aztreoname | 18 | %42.85 | 10 | %23.80 | 14 | %33.33 |
| 4 | Gentamycin | 21 | %50 | 2 | %4.76 | 19 | %45.2 |
| 5 | Ciprofloxacin | 23 | %54.76 | 0 | %0 | 19 | %45.2 |
| 6 | Piperacillin | 21 | %50 | 8 | %19.04 | 12 | %28.5 |
| 7 | Imipenem | 23 | %54.76 | 8 | %19.04 | 11 | %26.19 |

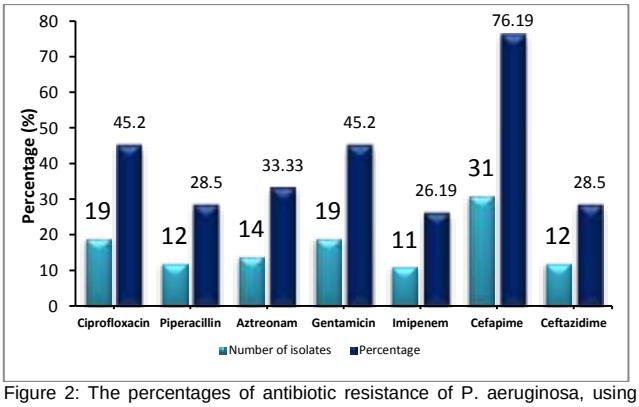


Figure 2: The percentages of antibiotic resistance of P. aeruginosa, using Disk diffusion method.

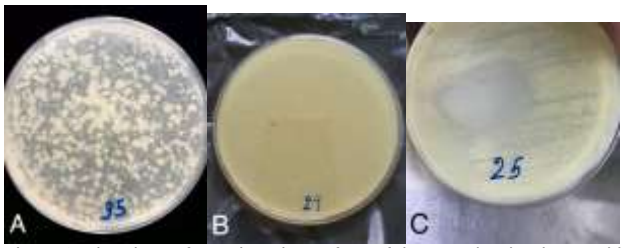


Figure 3: The plaque formed on the surface of the agar (A-B) using Double-Layer Agar technique (DLA), (C) by the Phage Spot Assay.

**Bacteriophage isolation:** The results of treatment of bacteriophage samples (isolated from sewage water, Tigris river water, stagnant water and bird feces) on P. aeruginosa showed that three bacteriophages have been found capable of infecting and destroying antibiotic-resistant bacteria, whereas the Phage1 was irregular, transparent, and clearer compared to the other isolated phages, while the Phage 2 appeared very small and circular (Fig. 3, Table 4). Through this study, it was found that hospital sewage was one of the optimal sources of bacteriophages isolation, which may due to it contained a large group of living organisms as a result of contamination with hospital feces and waste.

Table 4: The bacteriophage characteristics of P.aeruginosa isolates

| No. of phage | Plaque size | Plaque Shape | Margin cut | Plaque Clearly |
|---|---|---|---|---|
| Phage 1 | / | Irregular | Irregular | Clear |
| Phage 2 | 0.5-1 mm | Circular | Regular | Clear |
| Phage 3 | 2 mm | Oval | Regular | Clear |

**The effect of glycerol concentrations on bacteriophage activity:** The results observed that the addition of glycerol improves the appearance of plaques, as the transparency and clarity of the decomposition areas resulting from the bacteriophage were directly and positively proportional to the concentrations of glycerol, and the concentration of 10% was the most efficient of them in analyzing the bacteria by the bacteriophage (Figure 4) and this result is in agreement with (14) study.

**The effect of temperature on bacteriophage activity:** The bacteriophage was able to maintain its activity and effectiveness with a high infection rate at temperatures 4° C, 25° C and 37° C, while its activity began to decrease at a temperature of 45° C and was ineffective at a temperature of 60° C and boiling point, and this is consistent completely with (33) (26) studies which revealed that disabling bacteriophage activity at high temperatures may be due to a change in the nature of the chemical composition of the phage (33).

**The effect of incubation period on bacteriophage activity:** The results of the current study found that the incubation period significantly affects both bacteria and phages, as the bacterial

inhibition and phage fading gradually increased with the increase in the incubation period (Figure 5).

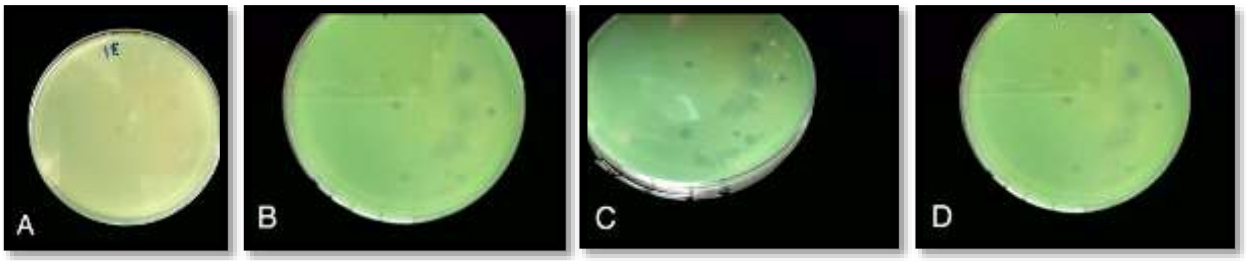


Figure 4: The optimum glycerol concentration for the appearance of bacteriophages. (A) without glycerol Control, (B) glycerol 5%, (C) glycerol 10%, (D) glycerol 20%

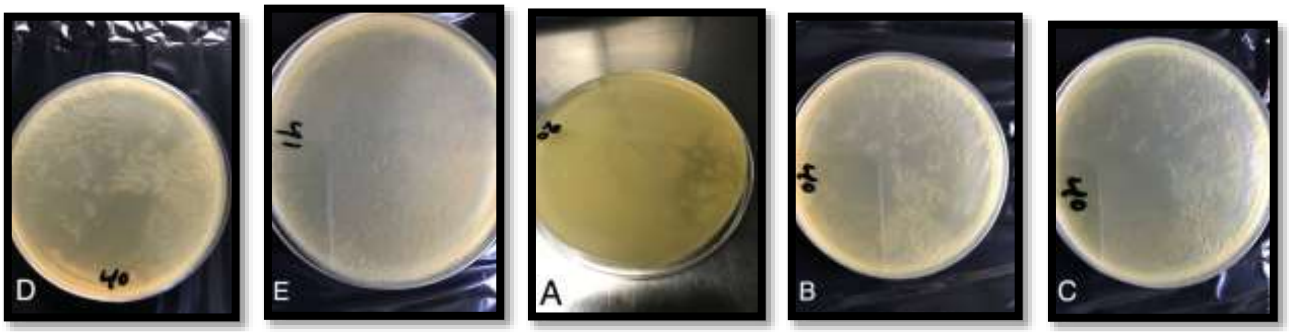


Figure 5: The effect of incubation period on bacteriophage activity (A) after 12 hours of incubation, (B) after 24 hours, (C) after 48 hours, (D) after 72 hours, (E) after 96 hours of incubation

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