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Study the Effect of Bacteriophage on Biofilm Producer *Pseudomonas aeruginosa* causing Wounds and Burn Infections

Sarah A. Obaid , Rana M. Al-Shwaikh

Department of Biology, College of Education for Pure science (Ibn Al-Haitham), University of Baghdad, Baghdad, Iraq

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Abstract: *Pseudomonas aeruginosa* is one of the opportunistic bacterial pathogens that has a great tendency to develop resistance to many types of antibiotics, and contributes to its pathogenicity and danger to the host , its possession of several virulence factors such as The biofilm, the bacteria have a biofilm become highly resistant to immune system and antibiotic treatment, therefore, it is believed that treatment with phages can be effective for eliminating the biofilm and thus reducing the risk and virulence of bacteria. One hundred clinical samples of wounds and burns were collected from Baghdad city hospitals for the purpose of outwardly identification it and observing the bacteria on the culture in terms of its color, shape and distinctive smell in addition to its ability to produce pigment, and to identification it microscopically, it was stained with Gram stain and biochemical tests (Catalase, Oxidase, IMVC), and final identification by using VITEK2 system. In this study, 42 clinical isolates of *P. aeruginosa* were obtained after phenotypic, microscopic identification, using VITEK2 system to final identification. Their antibiotics resistance was tested by determining the Minimum Inhibitory Concentration (MIC). It showed different levels of resistance. The results showed the high sensitivity of the biofilm towards the bacterial phage and its ability to disperse its biomass, compared to the detection results before treatment. High inhibition rates were recorded for the biofilm. The results of detection before treatment were 42.85%. With strong composition, 50% with medium composition, and 7.14% with weak membrane formation, but it showed a difference in the percentages after treatment, as the percentage of 4.76%, that is, only two isolates, maintained its effectiveness in forming biofilm at a strong level, while the rest of the isolates ranged between 57.14% with medium composition. Also, 38.09% are weakly formed weak.

Keywords: *Pseudomonas aeruginosa*, Biofilm, Bacteriophage, Wounds and Burn infections.

Corresponding author: (Email: Sarra.ahmed1202a@ihcoedu.uobaghdad.edu.iq, rana.m.a@ihcoedu.uobaghdad.edu.iq).

Introduction

Pseudomonas aeruginosa is a Gram-negative, non-spore-forming aerobic bacillus (1,2). It is an opportunistic clinical pathogen with a high ability to develop resistance to antibiotics. It can cause many infections in major and different locations in the body, such as the respiratory system, urinary tract, ear infections, wound and burn infections, especially in immune compromised people (3). *P. aeruginosa* was ranked as the second common nosocomial pathogen as reported in the National Nosocomial Infection Surveillance System (NNIS) (4).

Biofilms are dense aggregations of bacteria embedded with a matrix of polysaccharide (alginate), intricately adhering to various surfaces including medical devices, tissues, plastics, and metals (5), and are known to be responsible for many clinical infections. Bacteria that possess these membranes have many ways to resist the host's defense systems and protect themselves from the immune system (6).

Pseudomonas aeruginosa adapts perfectly in the environment of the burn and wounds specially that bacteria have of biofilms, as it provides the advantage of survival and enormous resistance to tissue damage and a defect in the removal of dead white blood cells at the sites of infection of burn and wounds and accumulation of neutrophils in them is very excessive, all these are factors that promote the initial development of

P. aeruginosa to formation their biofilm within 72 hours of their bacterial growth because the high level of neutrophil products stimulates and accelerates the formation of membranes. Bacteria use these polymers to binding sites through biofilms (7).

Phage is a virus that infects and destroys bacteria. Felix d'Herelle took advantage of this phenomenon and formulated the idea of using phages as a treatment against bacteria and used them as a clinical treatment (8,9).

Phages are fully adapted to destroy biofilms by various mechanisms and methods such as breaking down the extracellular membrane and destroying it by stimulating the host bacteria to produce enzymes that destroying the Extracellular membrane material, disintegrating the sugars and proteins present within the matrix and the proteins present by the phages, in turn, inside the signaling cell. It depends on many factors, the most important of which is the age of the membrane, its thickness, and the bacterial strains present within it. The Phages have developed their mechanisms, such as their production: enzymes in the formation of biofilms such as Hydrolase, Endolysin, and depolymerases (10), as the recognize the sugars and destroy it in the bacterial walls (11). The aim of the study to assess the sensitivity of *P. aeruginosa* biofilms to the activity of the bacteriophage.

Materials and methods

Collection, isolation and identification of *P. aeruginosa*

One hundred clinical samples of wounds and burns swab were collected for inpatients and outpatient from Baghdad city hospitals for the purpose of outwardly from the period 2021 to 2022. the bacteria identification and observing the bacteria on the medium in terms of its color, shape and distinctive smell in addition to its ability to produce pigment , and to diagnose it microscopically, it was stained with Gram stain (Himedia, India), and biochemical tests (Catalase, Oxidase, IMVC), and finally the bacteria were identified with Vitek2 system (BioMerieux, France).

Antibiotic susceptibility test

The sensitivity of *P. aeruginosa* to antibiotics was tested and the value of the minimum inhibitory concentration (MIC) was determined. The Antibiotic Sensitivity Test Card (AST) with the number (AST-GN76) (BioMerieux Company) was used, which includes each of the following antibiotics:

Cefepime, Ceftazidime, Amikacin, Gentamycin, Ciprofloxacin, Levofloxacin, Tigecycline, Piperacillin/ Tazobactam.

Phage isolation, detection and purification

According to Beaudoin *et al*., (12) and Jameel(11). the phage was isolated from environmental sources (isolated from sewage and Tigris River water and stagnant water and bird feces) and centrifugation at a speed of 2500 revolutions per minute for 10-15 minutes for the purpose of separating the clear fluid, and then filtered with cellulosic filter paper, and then filtered with a Millipore filter With a diameter of 0.22 micrometers, the clear liquid, or what is called lysate, is then preserved in tightly closed tubes sterilized at a 4°C.

The purification by taking a plaque (i.e. the bacterial inhibition zone in the plate) and mixing it in a sterile tube with 4 ml of Lambda buffer (0.05g Gelatin, 0.726 Tris HCl, 2.46g $MgSO_4.H_2O$ in 1000 distill water) The tube was shaken periodically by the Vortex mixer every 10 minutes for one hour in order to release the phage from the agar layer. The tube was then shaken by centrifuge for 5 minutes at a speed of 2000 rpm. Per minute, the resulting clear liquid was filtered with a 0.22µl Millipore filter, a similar volume of Lambda buffer was added to it, and it was kept in the refrigerator at a temperature of 4°C and considered as Phage Stock (13).

The Double-Layer Agar technique (DLA)

Two hundred µl of phage recombinant samples were mixed with 200µl of *P. aeruginosa* in their log phase, grown on LB medium and incubated for 10 minutes. At a temperature of 37°C in order to ensure that Adsorption has occurred between the phage and the receptor, then added to 3 ml of Top agar (LB soft agar) at temperature 0.3 and mixed gently with a mixer (45°C) with each layer at a rate of 10 % glycerol. Vortex gently pour it onto the bottom agar and make sure it reaches all the edges of the dish. It was placed flat inside the implant chamber and waited for it to harden. It was turned over. It was incubated at a temperature of 37°C for 24 hours, and after the end of the incubation period, the presence of areas of inhibited growth or areas of diminished growth (plaque) indicating the presence of phage (14, 15).

Phage Spot Assay

Mix 3 ml of soft agar (at 45 °C) with 200 microliters of bacteria. *P. aeruginosa*, in its log phase, was grown for 18 hours on LB medium and was poured into dishes containing. The bottom layer and add directly a drop (10 microliters) of clearing samples Lysate. We wait for a period of time until it hardens and incubate at a temperature of 37°C for 24 hours after which Plaque purification in the presence of phage (13, 15).

The presence of the phage was also detected by the transfer of 200 µl of the *P. aeruginosa*. Transfer it logarithmically to a plate with 1.5% (LB) agar and spread it with a sterile cotton swab. Evenly, after ensuring that the dish is dry, add 10 µl of bacteriophage lysate samples and incubate directly at a temperature of 37°C for 24 hours. After the end of the incubation period, the presence of the phage will be indicated at Notice Plaque in the area of bacterial culture (16).

Detection of Biofilm formation

Pseudomonas aeruginosa isolates were culture on Brain Heart Infusion Broth (Himedia, India) and incubated at a temperature of 37 °C for 24 hours, after that, 10 microliters of bacterial culture were added to 1 ml of (BHIB) broth medium with a glucose ratio of 1% (1:100). 200 microliters of it were transferred to calibration plates (for each isolate duplicated) taking into account the placement of replicates for a culture medium and it was returned as a negative control for the purpose of comparison and incubated at a temperature of 37 °C for 24 hours . Washed with phosphate-buffered saline (PBS) to remove of non- adherent bacterial cells and left to dry at laboratory temperature then add 200 microliters of Crystal Violet at a concentration 1% to each hole for 15-20 minutes pour out the Crystal Violet and wash the calibration dish three times with a (PBS) solution to remove of the unrelated dyes and leave it to dry at the laboratory temperature then add 200 microliters of glacial acetic acid solution at a rate of 30% to each hole and measured the absorbance (optical density) directly with an ELISA device at a wavelength of 630 nm (17).

Sensitivity of biofilms to bacteriophages

Study the effect of the bacteriophage on *P. aeruginosa*, isolates that form biofilms were grown in calibration plates according to Toole 'O(17), then the effect of the bacteriophage on the biofilms of the isolates of this study was studied according to Cerca *et al*., (18) and Adnan *et al*., (19) with minor modifications in it, where after the growth of bacteria in the holes of the calibration dishes, the bacterial cells were removed from the holes and washed with a solution (PBS), 200 microliters of phage samples were added to each hole with two replicates for each bacterial isolate and incubated with the same Incubation conditions when biofilms are detected (for a period 24 hours at a temperature 37°C).

On the second day phage samples are poured and washed with (PBS) solution, 200 microliters of 1% crystal violet dye is added then left for 10-20 minutes and washed with (PBS) solution or water distilled and finally after making sure of its dryness 200 microliters of 30% glacial acetic acid solution were added and the absorbance was measured with an ELISA at a wavelength of 630 nm.

Results and Discussion

Isolation and identification of *P. aeruginosa*

The identification of the isolates was confirmed phenotypically by their appearance in a pale, colorless form on MacConkie agar as a result of not fermenting the sugar lactose, and they were distinguished by their odor similar to that of fermented grapes, and they grew on selective ceramide medium and nutrient agar, and changed the color of the culture medium, as some isolates gave a bluish-green glow as a result of their secretion The pyocyanin pigment, which is characteristic of its type, and some of them appeared in a yellowish-green color due to their secretion of the pyoverdin pigment. Gram-negative, and its diagnosis was confirmed by biochemical tests, and finally by the Vitek2 device, and we obtained 42 isolates of *P. aeruginosa*, with a percentage of 42%.

Phage isolation

The results of the study showed that three bacteriophages were isolated that have the ability to infect *P. aeruginosa*, which has multiple resistance to antibiotics, and they had different phenotypic characteristics.

Antibiotic sensitivity test

Pseudomonas aeruginosa showed high resistance against Tigecycline and ceftazidime, cefapime, Gentamicin, Ciprofloxacin, Levofloxacin, Amikacin and Imipenem and show a high sensitivity against Piperacillin / Tazobactam only, all result showed in Figure (1).

In this study, 42 clinical isolates of *P. aeruginosa* were obtained after phenotypic, microscopic identification and using Vitek2 system to final identification.

Pseudomonas aeruginosa showed high resistance against Tigecycline and Ceftazidime, Cefapime, Gentamicin, Ciprofloxacin, Levofloxacin, Amikacin and Imipenem and show a high sensitivity against Piperacillin / Tazobactam only**.** The study of Khorsheed and Al -Abdee (20)and Al-kazrage (21), showed high antibiotic resistance of their isolates, but another study by Hassan and Al-Nuaimi (22) and Al-Jubouri (23) gave different results, so it had a high resistance to these antibiotic.

As for the Cefapime, its resistance rate was also increased, which was similar to the resistance rates of Al-Jubouri's study (23). The high resistance of *P. aeruginosa* to cephalosporins such as Ceftazidime, and Cefapime may be due to their ability to produce beta-lactamase enzymes such as MBLs and ESBLs that help analyze these antibiotics by destroying the beta-lactam ring, and what increases of these enzymes is their carry-on genes. Plasmid or chromosomal in most bacteria, which leads to resistance to different classes of antibiotics.

The results also showed that the isolates had resistance to the Gentamicin. This result is similar to Hassan and Al-Nuaimi(22), Saeed (24) and Khudair and Mahmood (25) , and unlike As a result, Azeez and Bakr (26) and Saleh (27) showed that their isolates had little resistance to this antibiotic. The Amikacin recorded a resistance rate increased so it showed similarity with the result of Al-kazrage (21). The reason for the resistance of some isolates to aminoglycosides may be attributed to the enzymatic inhibition of the antibody by possessing enzymes called aminoglycoside-modifying enzymes (AMEs) (28). As for its resistance to Ciprofloxacin, it was at a rate increased. Almost consistent with the study of Hassan and Al-Nuaimi (22), as it recorded a resistance rate increased to. As for the Levofloxacin belonging to the group of Fluoroquinolones, it also had a resistance rate completely similar to Ciprofloxacin, which was similar to a study in Baghdad Khudair and Mahmood (25), as the resistance rate of his isolates was decreased, but it differed completely with Azeez and Bakr (26), where the resistance rate was decreased.

Fluoroquinolones antibiotics known for their high effectiveness because they inhibit the manufacture of DNA for bacteria by stopping the action of DNA gyrase or the occurrence of a mutation in it responsible for winding the DNA

and killing bacteria, so it could explain the reason for resistance of some isolates to this antibiotic due to a mutation in the genes encoding the enzymes Topoisomerase and DNA gyrase (29) Finally, the antibiotic Imipenem, which showed a high resistance rate, which was lowest percentage when compared to antibiotics mentioned above, so it was consistent with the resistance rate of Saeed (24) and Khudair and Mahmood (25), but it is different with Azeez and Bakr (26) which was decreased resistance. Imipenem obtained the lowest rate of resistance among the tested antibiotics, as it is considered the most effective in treating infections infected with *P. aeruginosa* infection because of its ability to penetrate through the outer membrane of the bacterial cell, in addition to that it is an effective inhibitor of beta-lactamase enzymes because of its ability to persist in the hydrolysis caused by the antibiotic (30). Through the above results, it was found that all *P. aeruginosa* isolates in this study had multiple resistance to MDR antibiotics, so their ability to resist ranged from three or more antibiotics. This resistance can be explained by the high tolerance that *P. aeruginosa* possess to unsuitable environmental conditions and skin damage due to the loss of the defensive barrier against the bacteria. In addition, the excessive use of antibiotics and drugs and the polluted hospital environment are all factors that helped increase its resistance.

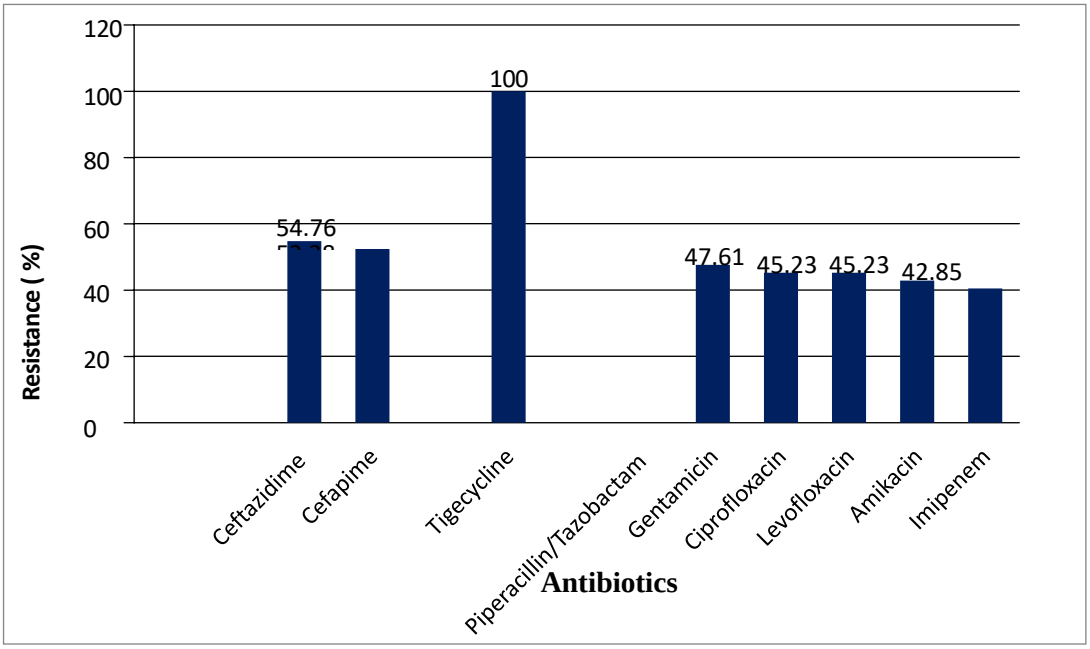

Figure (1): The percentages of antibiotic resistance by MIC method for *P. aeruginosa* isolated from wounds and burns.

Detection of Biofilm Formation

The results of the investigation on the ability of *P. aeruginosa* isolates to produce biofilm according to the changes in the optical density of the ELISA device showed that all of this study isolates under study were biofilm producers 100%, but with varying

intensity of formation at compare it with the reading rate of the negative control completely.

This study the results showed that 42.85% had strong biofilm formation (18 isolates), while 50% (21 isolates) had medium composition, while only 3 isolates were classified as having weak formation 7.14%, as shown in Figure (2).

This study isolates were producers biofilm 100%, but with varying intensity of formation at Compare it with the reading rate of the negative control completely agree with study by Al-Bayati(31) and Saeed (24), as the percentage of biofilm formation of their isolates was 100%, that biofilms affect the pathogenicity of bacteria and increase their resistance to antibiotics, because they were in an integrated environment within the host's cells, proteins, and microbial mucosa, which provides them with ideal growth potential and high antibiotic resistance (32, 33,34).

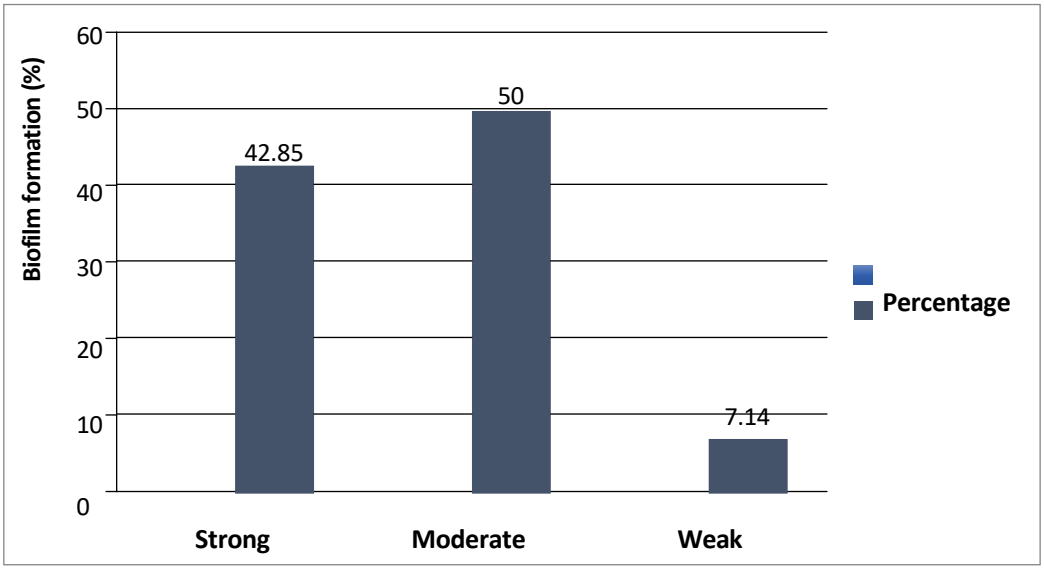

Figure (2): The percentages of biofilm formation by using the microtiter plate (MTP) method.

Biofilm Inhibition after treatment with bacteriophages

The phages in this study showed their effectiveness in dispersing and reducing the biofilm formation by a very large percentage, and this study is the first study in Iraq. That is, only two isolates maintained their effectiveness by forming biofilms at a strong level, while the rest of the isolates ranged between 57.14% Moderate and 38.09% Weak, all result showed in Figure 3 and Table (1).

Table (1): Biofilm formation results before and after phage treatment.

The state of biofilm	Before treatment	After treatment
Strong isolates	42.86%	4.76%
Moderate isolates	50%	57.14%
Weak isolates	7.14%	38.09%

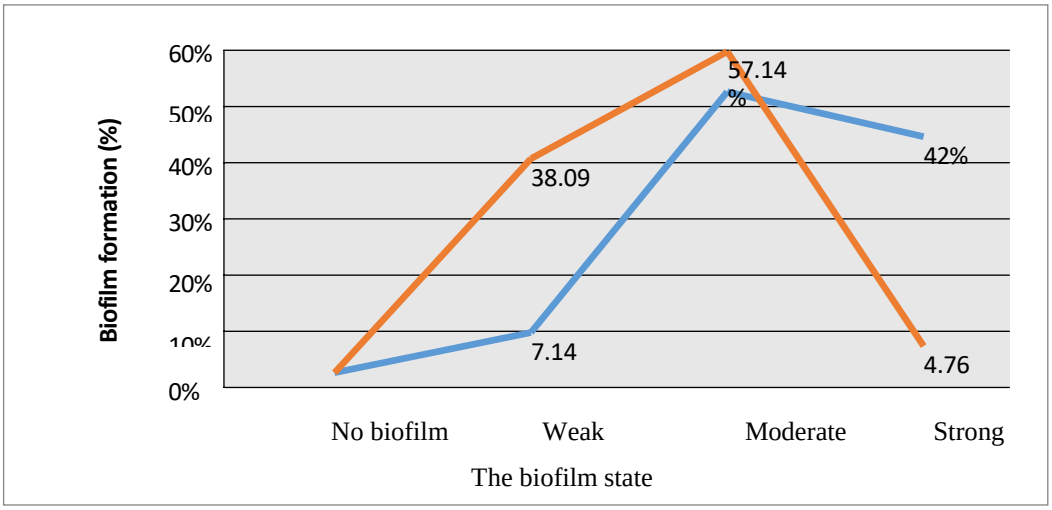

Figure (3). The percentages of biofilm detection results for *P.aeruginosa* before and after treatment with bacteriophages.

Through treatment of bacterial with bacteriophage samples (isolated from sewage and Tigris River water and stagnant water and bird feces) on the *P. aeruginosa*, it was isolate and identify three of the bacteriophages. Through these experiments,sewage water and Tigris River water were the best isolation sources for phages.This may be because they contain a large number of living organisms as a result of their contamination with hospital waste. On the other hand, the results of this study showed a difference in the transparency and clarity of the lysis area bacteria by phages differed according to the detection methods, as the phage plaque was clearer with the phage spot assay methode than it is with the Double-Layer Agar technique (DLA) . The reason may be that the plaque formed is a result of inhibiting bacterial growth. Therefore, it is possible that the bottom agar in the double agar technique helps support the growth of at a higher level, plaques appear paler, less clear, and fade over time. (35).

The isolates from this study were 100% biofilm producer with different formation densities, but their treatment with the phage differed in formation density results and recorded high inhibitory percentages indicating the ability of the phage to penetrate and destroy them.

Yuan *et al*., (36) found that phages were able to reduce the percentage of biofilm formation during 48 hours of incubation, while Adnan *et al*.,(19) noted that the total biofilm mass of *P.aeruginosa* decreased by 2.1, 2.5, and 3.2 fold. When incubated for a period of 24, 48,72 hours, respectively. Also his study attributed to the fact that biofilms can form on all surfaces, whether live or non-living, and provide an effective defense mechanism for bacteria so that they cannot be eliminated with traditional disinfectants and sterilizers. But the phage was able to damage to it by dismantling its structural components. And helps the phage in causing infection is the Depolymerize enzyme that is derived from the phage itself, as it works to break down extracellular materials, polysaccharides, LPS, and peptidoglycans, in addition to helping the bacteriophage to reach its

receptors in the bacterial cell (37).

Conclusion

Isolated three phages capable to infecting *P. aeruginosa* that was antibiotic-resistant. The phages showed success in reducing the ability of bacteria for biofilm formation.

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