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Experimental therapy of bacteriophage on multiantibiotic resistant local isolated *pseudomonas aurogenosa*

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The wide spread of multi-drug resistant *Pseudomonas aeruginosa,* is the most reason of death in burn patients. A new approach is needed to target bacterial pathogens that evolve resistance to therapeutic treatments. In this study we prepared lytic bacteriophage of Pseudomonas aeruginosa that isolated from burn Iraqi patients and shows high resistance to the traditional antibiotics. The strategy of using phages as antibacterial agents, could be potentially reducing the severity of bacterial infections. The results of susceptibility of ten isolates (Ps1, Ps2, Ps3, Ps4, Ps5, Ps6, Ps7, Ps8, Ps9 and Ps10) of *P.aeruginosa* to 8 antibiotics (Ceftazidime, Cefotaxime, Piperacillin, Erythromycin, Vancomycin Amikacin, Ciprofloxacin and Meropenem) was (10%, 0%, 10%, 0%, 0%, 100%, 90% and 80%) respectively. While the lytic activity of bacteriophage was evaluated on P. aeruginosa, It was found that five isolates of MDR S. aureus were susceptible to phage five isolates were resistant. Thus, bacteriophage could be a safe and efficient strategy in the treatment of infections caused by P. aerogenosa.

Keywords: Bactriophage, P. aerogenosa, therapy, Multiantibiotic, Resistant.

INTRODUCTION

Inappropriate consuming of antibiotics resulted in to multi-drug resistant microorganisms (MDR), that may present frequently in human infections contributing significantly to death (Maragakis et al.,2008) Many bacteria show resistance to antibiotics leading to in emerging novel strains with pan-drug-resistant (Falagas et al.,2008)

The Gram-negative bacterium, *Pseudomonas aeruginosa*, is an opportunistic infectious agent that becomes a common pan-drug-resistant disease problem*. P. aeruginosa* can make biofilm-mediated infections, such as, ventilator associated pneumonia, grafts, also catheter associated UTI (Cole et al., 2014)

Patients suffering from severe burns under risk for *P. aeruginosa* infections that acquired from hospitals (Ohmagari, 2005). *P. aeruginosa* infections are not easy to cure due to the low antibacterial permeability of its outer membrane and the antibiotic resistance which led to cross resistance to different antibiotic classes (Masuda, 2000)

Patients suffering from serious burns are at higher risk to bacterial infections. Recently, emerging of resistant agents did forced burn care providers all over the world to search for alternative type of treatment? Multidrug-resistant *Pseudomonas aeruginosa*is the major cause of the increasing morbidity and mortality (Cosgrove, 2006)

The burn injury is a major public health issue all over the world. *P. aeruginosa* is the major cause of burn wound infections in patients with burns due to its resistant to many drugs. *P. aeruginosa* still the main cause of nosocomial infections in most burn centers. Septicemia may

occur early in post burn after days and the mortality because of this organism is very high (Falagas et al., 2008)

P. aeruginosa characterized by low antibacterial susceptibility, which is attributable to a concerted to the multidrug efflux pumps which result from genes encoding antibiotic resistance like, mexAB, mexXY and low permeability of the bacterial cellular envelopes (Cosgrove, 2006) Furthermore, *P. aeruginosa* can do acquired resistance by mutation in chromosomally encoded genes and by the horizontal gene transfers of antibiotic resistance determinants. *P.aeruginosa* development of drug resistance needs some different genetic events, this includes acquisition of different mutations and horizontal transfer of the antibiotic resistance genes.

The mechanisms of antibiotic resistance include production of antibiotic-degrading, antibiotic-inactivating enzymes, outer membrane proteins variation to evict the antibiotics and mutations to change the targets of antibiotics. The antibiotic-degrading enzymes of *P. aeruginosa* like extended-spectrum β-lactamases like PER-1, PER-2, Ampcephalosporinases, metallo-b-lactamases, OXA-type carbapenemases. It can also change the antibiotic targets (Poole, 2011).

There is an urgent need of developing novel and alternative strategy to treat the infections with *P. aeruginosa* particularly in burn patients. Using of Bacteriophages is one of the important promising alternatives to treat MDR bacterial infections (Sulakvelidze et al., 2001) Phages can bind to one or more specific receptors on the surfaces of bacterial hosts (Sulakvelidze et al.,2001), (Chan et al.,2013), (Rhoads 2009) Because of rise in antibiotic resistance, phage therapy has seen a great interest among physicians (Pirnay,2012). with the successful clinical experiments demonstrating the safety and efficacy (Rhoads 2009) and, Wright et al., Nevertheless, there are many limitations to phage therapy due to evidence that bacteria may evolve resistance to phage infection (Labrie et al., and Vale and Little 2013) While there are many mechanisms of phage resistance, phage attachment to the binding-site receptor make selection pressure for bacteria to down-regulate expression of the receptor to escape from phage infection(Labrie et al.,) Given the evolving phage-resistance, modern approaches to phage therapy must be study on this inevitability [(Turne and Chao1988) and, Goldhill & Turner). In this study we utilize phages that infect MDR bacterial *P. aeruginosa*. This approach of phage therapy should be effective; success when phage lyse the target bacterium.

MATERIALS AND METHODS

Isolation of *P.aeruginosa*

Wound swab specimens were taken from hospitalized patients with moderate to severe burn wounds showing clinical symptoms and signs of infection attending the Burn unit at deferent Iraqi Hospitals between September 2015 and April 2016. Swabs were inoculated during two hours onto Tryptic Soy Broth (TSB) after the primary isolation on blood and McConkey agar.

Wounds, was detected by standard bacteriological methods that included: colony morphology, bacterial Gram staining, pyocyanin production, growth at 44°C, catalase test, oxidase and Triple Sugar Iron (TSI) fermentation.

Antimicrobial susceptibility test:

Kirby-Bauer method was done **(**Ohmagari, 2005) to carryout antimicrobial susceptibility test for (Poole, 2011). different antimicrobial agents as shown in table 2.

Table 2 : Antimicrobial agents used in susceptibility test

Id	Antibiotics	Cod	Concentration	Group
1	**Ceftazidime**	CAZ	10	Third generation of cephalosporins
2	**Cefotaxime**	CTX	30	Third generation of cephalosporins
3	**Piperacillin**	PRL	30	Penicillin
4	**Erythromycin**	E	15	Macrolides
5	**Vancomycin**	VA	10	Glycopeptide
6	**Amikacin**	AK	10	Aminoglycoside
7	**Meropenem**	MEM	10	B-lactam
8	**Ciprofloxacin**	CIP	5	Quinolone

Using a sterile cotton swab, submerged into a bacterial suspension standardized to match the turbidity, 0.5 McFarland standards (1.5×10^8 CFU/ml). Serial dilutions of overnight Brain Heart Infusion culture was done to the tested bacteria, third dilution was used.

The bacterial suspensions were spreaded at the surface of Mueller Hinton agar, left for 10 minutes to dry. The antimicrobial disks were put using sterile forceps on the agar. The plates were incubated at 37°C for 18 hrs. After incubation, Inhibition zones were measured according to (CLSI, 2011). The bacterial isolates were interpreted as susceptible, intermediate or resistant to each antimicrobial agent.

Bacteriophage isolation and purification

Wastewater samples were collected from hospitals in Baghdad proven, Iraq in sterile cups and delivered to the laboratory and characterized as the standard method (Masuda,2000) The samples were stored at4°C for 18 hours to let larger suspended precipitate. The crude samples were centrifuged at 4,500 xg for 10 minutes to remove the bacterial cells and debris. Then the supernatants were passed through a 0.22-µm-pore-size membrane filter (Sartorius AG, Gottingen, Germany) to harvest bacteriophage. The filtrate was added to an equal volume of double strength Brain Heart Infusion broth of log phase *P. aeruginosa* in concentration of 10^6 (CFU/ml) for bacteriophage enrichment. Then incubated at 37°C for 18 hours. The cultures were centrifuged at 4,500 xg for 10 minutes, the supernatant were filtered through a 0.22-µm-pore-size membrane filter. Double layer method was used with some modifications (Douglas et al., 1969) to examine the presence of a lytic phage in the filtrated suspensions. One hundred microletters of the filtrates was mixed with 400 microliters of a log phase of *P. aeruginosa* and then incubated at 37°Cfor 30 minutes. The mixtures were added to 4.5 ml of molten BHI agar (0.7% agar) which cooled down to 50°C, mixed and poured onto a BHI agar (1.5%agar). The plates were left at room temperature for 30 minutes to let the top agar to solidify. The lytic bacteriophages in the form of plaques were detected after incubation at 37°C overnight.

Purification of bacteriophage was done by taking a single plaque with a sterile glass Pasteur pipette and put into a log phase of *P. aeruginosa* culture. After incubation at 37°C for 18 hours, the bacteriophage-host mixtures were centrifuged at 4,500 xg for 10 minutes and then filtered through a 0.22-µm-pore-size membrane filter. The filtrates were subjected to the double layer method for three repeated rounds of single plaque isolation and re-inoculation was done. The elution of bacteriophage was performed by taking the plaque from the final resulting plate and adding 5 ml of SM buffer which contains 50 mM Tris-HCl, pH 7.5, 99 mM NaCl, 8 mM MgSO4 and 0.01% gelatin on top of the plate, then incubated at room temperature for 4 hours with shaking. Then centrifuged at 4,500 xg for 10 minutes and filtered through a 0.22-µm pore-size membrane filter to get bacteriophage suspension.

Determination of Bacteriophage titer

The bacteriophage containing suspensions were serially diluted using SM buffer. Each dilution was subjected to plaque assay as mentioned double layer. The plaques we recounted in the plates that containing 50-300 plaques, expressed as plaque forming unit per milliliter (PFU/ml).

RESULTS AND DISCUSSION

*P. aeruginosa*is usually involved in causing health care acquired infections with high rates of mortality (Douglas et al., 1969). The major reason of the high pathogenicity of *P. aeruginosa,* is the highly resistance to antibiotics, furthermore, its ability to develop multidrug resistance in the hospital environment (Lambert et al., 2011) *P. aeruginosa* was isolated from 23% of the swabbed burn wounds from patients having moderate to severe burned wound. The frequency of *P.aeruginosa*in this study is (18%) which is nearly the same to that of other studies previously done on burn wounds in Egypt (20%), and to that study carried in Tunisia (27%) (Kallen et al.,2010)

and South Africa (14.5%) (Chahed et al., 2014) also a previous study in the same governorate, in which (19.5%) *P. aeruginosa* prevalence rate was reported, but in that study, *P. aeruginosa* was isolated from different sources in addition to burn wounds (Coetzee et al., 2013)
While, high frequency rates were reported in other similar studies on burn patients in Pakistan and Egypt (Gad et al., 2007)

The results of susceptibility of ten isolate, (Ps1, Ps2, Ps3, Ps4, Ps5, Ps6, Ps7, Ps8, Ps9 and Ps10) of *P.aeruginosa* to 8 antibiotics (Ceftazidime, Cefotaxime, Piperacillin, Erythromycin, Vancomycin Amikacin, Ciprofloxacin and Meropenem) was (10%, 0%, 10%, 0%, 0%, 100%, 90% and 80%) respectively as shown in the figure (1).

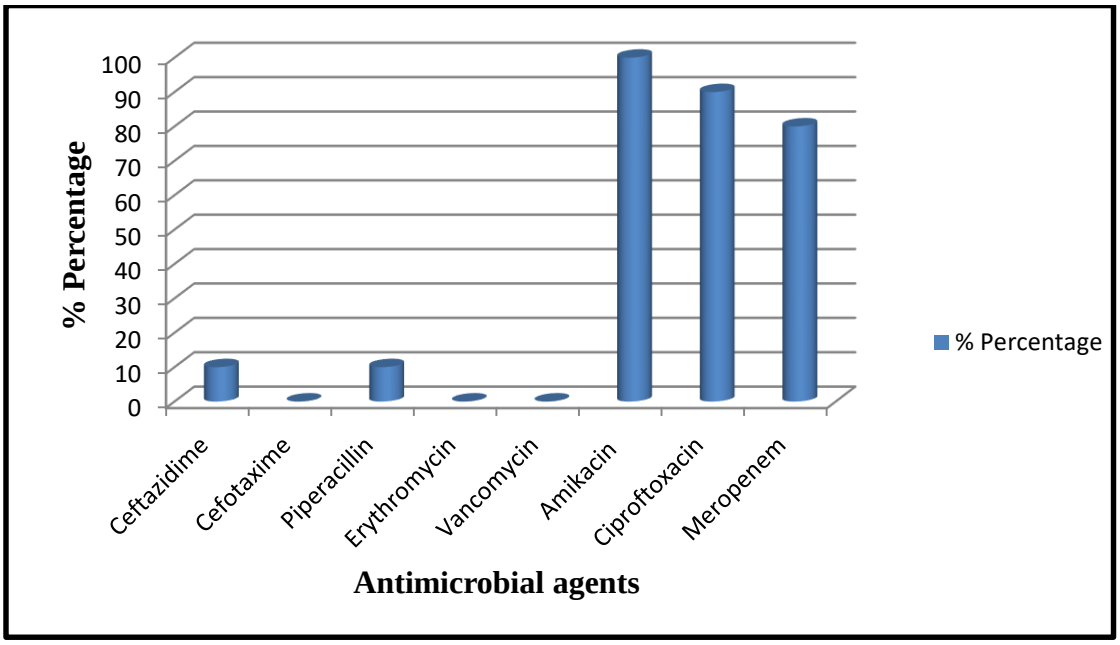

Figure (1): susceptibility of *P.aeruginosa* isolates to antimicrobial agents.

The resistance to Amikacin and Ceftazidime was relatively high (100%,90%) respectively, these results were the same as the results of study done in Egypt by Mahmoud et al., (91%) (Shahzad et al., 2012) and to another one done by Zafer et al., 2014 (60%) (Mahmoud et al., 2013)

Moreover, other different studies revealed high resistance rates of ceftazidime and other third generation cephalosporins as in Benin (67.5%) (Zafer et al., 2014) In some countries there is a high sensitivity to ceftazidime as mentioned in studies carried in some developed countries as in Brazil (28%) (Ahoyo et al., 2014)

And united States of America (4.5%)[(Picao et al., 2009) may be due to the less use of antibiotics. The isolates of *P. aeruginosa* of our study were sensitive to Meropenem in a ratio of 80% , this result is nearly as the results of studies carried in Egypt as the one of(Badr et al.,2008) who found that sensitivity rate of 95.7% (Lockhart et al., 2007)

]. Other studies done by Zafer et al.,2014 and by Diab *et a*l., found that there is a higher frequency of imipenem resistance, 39.34% and 72%respectively [(Badr et al., 2008) On the other hand, 90% of *P. aeruginosa* isolates were sensitive to Ceproftoxacin and all were sensitive to Amicacin.

In this study the bacteriophage was isolated from the Iraqi hospital wastewater using the double layer method and contributed *P.aeruginosa*as a bacterial host in a concentration of 2.5×10^6pfu/ml. The results showed that the lysis of bacteria by bacteriophage which revealed as clear plaques, which indicates that it was a virulent bacteriophage. Then the plaques were determined, the average diameter was1 mm as shown in (Fig. 1).

Recently, antibiotics are widely used to control the infection with *P.aeruginosa*; but, the antibiotic resistant of *P.aeruginosa* reducing the drug efficacy and led to the need of an alternative to antibiotics in order to control bacterial infections. Bacteriophage therapy is an important and promising alternative therapy to control infections caused by multidrug resistant bacteria. That is may be because that bacteriophages and antibiotics actions are different in mechanisms (Diab et al., 2013) Bacteriophages possess an ability to inhibit the growth of bacteria. Beside, the ability of bacteriophages of infection their specific bacterial hosts only that make them preferred than antibiotics in therapeutic purposes. As a result they don't cause any harm to the normal microflora, also bacteriophages multiplication depends on their host which lead to elvate their counts at just the sites of infection, which increase their therapeutic efficacy. When their hosts are damaged, bacteriophages then rapidly cleared from the bodies of treated one (Al-Agamy et al.,

2011 and Umadevi et al., 2011) by innate immune system mechanisms. Due to all of these reasons, bacteriophage therapy may be considered as a very effective and also safe therapeutic approach. Moreover, bacteriophages normally reside in the environments of their host bacteria habitats .(Al-Marjani et al.,2013)

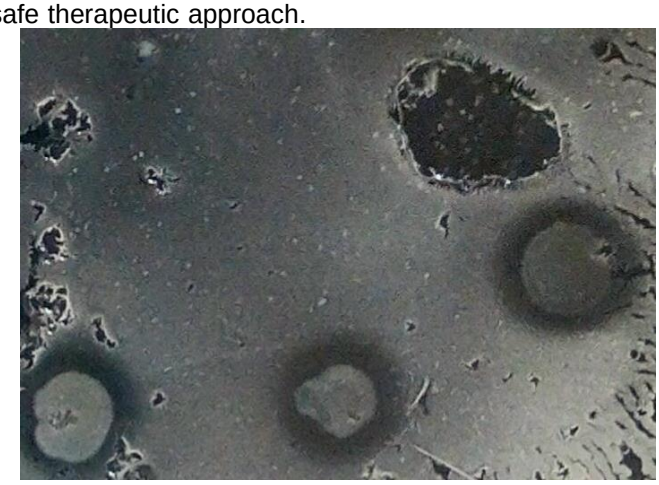

Figure 1. Plaques on *P.aeruginosa* culture

CONCLUSION

Bateriopages are important and promising tool which is safe effetciant and low cost treatment against antibiotic resistant bacteria.

CONFLICT OF INTEREST

The present study was performed in absence of any conflict of interest.

ACKNOWLEGEMENT

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AUTHOR CONTRIBUTIONS

Author contributions: NSA designed and performed isolation and also the antimicrobial resistance of the isolates. LFA designed the experiment of isolation; propagation and plaque assay of bacteriophage. Both authors wrote the manuscript, read and approved the final version.

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