Bacteriolytic Activity study of Aerophage on Pseudomonas aeruginosa

Zahraa Jaafar Jameel(PhD)[1]

Abstract

Background: The increasing incidence of antibiotic resistance in bacterial pathogens has justified a reassessment of the value of phages as antibacterial agents for medical applications.

Objective: To assess the effect of bacteriolytic activity against P. aeruginosa by use multi methods for isolation of bacteriophage.

Patients and Methods: This study was performed from from November, 2016 till April, 2017. A total of 60 samples collected from urine, stool, diabetes leg, ear, burns and wounds infections in General Baqubah Hospital to isolate Pseudomonas aeruginosa and tested for 6 groups of antibiotics. Aerophage was isolated using three methods: spotting; double-layer agar plate and liquid broth method. After enrichment of aerophage, the bacteriolytic activity of aerophage was done by spotting method.

Results: *Pseudomonas aeruginosa* isolates resistance were 10(43.5%) for Aztreonem and 8(34.8 %) to Ticarcillin-Claculanic acid followed by Imipenem 4(17.4%), Gentamicin and when the study done Levofloxacin 3(13%) for each finally Cefepime2(8.7%). high significant differences of aztreonem with both of cefepim and gentamycin ($P<0.01$) while significant differences of aztreonem with both imipenim and levofloxacillin ($P<0.05\%$). while the results of bacteriolytic activity showed all ear and leg diabetes isolates were sensitive to aerophage (100%) followed by urine isolates(75%), stool isolates(50%) and burns isolates(25%) while all wound isolates were resist to aerophage(100%). The result of relation between Antibiotic resistance phenotype profile and Aerophage sensitivity that no detect to susceptibility of isolates to aerophage but the source of isolates may be detected to infection with aerophage.

Conclusion: Aztreonem antibiotic was widely used in Baqubah city recently. All three methods to isolate aerophage were effective. The result of relation between Antibiotic resistance phenotype profile and Aerophage sensitivity that no detect to susceptibility of isolates to aerophage but the source of isolates may be detected to infection with aerophage.

Key words: *Pseudomonas aeruginosa*, phage, antibiotics.

Corresponding Author: dr.zahraa@sciences.uodiyala.edu.iq

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[1] Department of Biology - College of Science - Diyala University- Iraq.

agar then subculture on Macconkey agar and blood agar. Cultural, Microscopical and Biochemical characterization was done to identification *P. aeruginosa* [13].

Antibiotic Susceptibility Testing

The antimicrobial susceptibility assay was performed on Mueller-Hinton agar by the disc-diffusion method (Kirby–Bauer)[14]. Selection of antibiotics and growth inhibition zones were interpreted according to the Clinical Laboratory Standards Institute [15]. The antimicrobial disks: Ticarcillin-Claculanic acid(10µg), Cefepime(30µg), Imipenem(10µg), Aztreonem(30µg), Gentamicin(10µg) and Levofloxacin(5µg) were of commercial grade (Mast Group, UK).

Preparation of aerophage suspension

PAP phage suspension was prepared from sewage water of General Baqubah Hospital treatment plant (2.5L), this waste water treatment plant was selected because it receives effluents from hospital i.e. contain pathogenic bacteria (host for bacteriophage); then aseptically filtered it through 0.8 µm pore sized cellulose filter to eliminate particles debris followed centrifugation at 2500rpm for 10min.Finally the supernatant was aseptically filtered through 0.45mm pore sized filter to remove bacterial cells and cellular debris [16].

Enrichment and isolation

Five ml of overnight suspension of Pseudomonas aeroginosa mixed with (45ml) bacteriophages suspension and 10 x nutrient broth (5 ml). The mixture was incubated at 37℃ with shaking at 180 rpm for 24 hr. At the end of incubation period, the suspension was screened by centrifugation at 3000rpm for 10 minutes and the supernatant was filtered through 0.22µm filters to remove bacteria. The suspension of expected phages was kept at 4°C [17].

Testing the suspension of aerophage by spotting method

Bacteriophage lysis assay was done. An overnight culture of bacteria was spread on nutrient agar and then a single drop of suspension of expected phage stock solution was added by micropipette (100µl) on bacterial law. The plates were inverted after dried and incubated at 37^0C overnight, and then examined for the presence of clear zones (plaques) [18].

Testing the suspension of aerophage by double-layer agar plate method

As described by Adams. Briefly, 100 µl of diluted phage solution in SM buffer (NaCl, 5.8 g; MgSO4_7H2O, 2 g; 1 M Tris_Cl pH 7.5, 50 ml; 2 % gelatin, 5 ml; add ddH2O to 1,000 ml), 100 µl of a bacterial overnight culture, and 3 ml of molten soft agar (which had been pre-warmed at 45 ^{0}C in a water bath) mixed in a tube and immediately poured into a 1.5 % nutrient agar containing petri dish. Plates were incubated for 24 h after which plaque forming units (PFU) were counted on each plate [19].

Testing the suspension of aerophage by liquid broth method

As described by Fortier and Moineau, 2009. P. aeruginosa cultured into 5ml nutrient broth until an OD600nm~ 0.1then inoculated with 50µl of bacteriophage suspension. Incubation at 37^0C for 24hr [20].

Purification of aerophage

By mass multiplication: Materials from the centre of the clear zone were scraped off using a sterile inoculation loop and were transferred to sterile SM buffer. This mixture was centrifuged at 5000rpm for 25min at 4^0C and filtered through Millipore Membrane Filter (0.22µ). The filtrate was collected in sterile amber bottles. The spotting assay was again carried out as mentioned earlier. This cycle step was repeated for a minimum of three times to ensure the purity of the phage. All the phage lysate were stored at 4^0C [21].

Large scale amplification of aerophage

Small-scale concentration of phages was performed by spreading phages on the top-agar layer containing the respective host bacterium by plaque picking. Briefly, clear zone was cut by sterile loop and eluted with SM buffer finally purified with chloroform and stored at 4°C. These solutions were then used for preparing concentrated phage solutions in larger scale using broth media as described by Sambrook and Russel with some modifications. Briefly, Materials from the center of the plaques were scraped off using a sterile inoculation loop and were transferred to sterile nutrient broth(100ml) containing the specified organism(P. aeruginosa) and incubated overnight for about 24 h at 37^0C. This mixture was centrifuged at 5000rpm for 25min at 4^0C and filtered through Millipore Membrane Filter (0.22µ). The filtrate was collected in sterile amber bottles and then purified with chloroform and stored at 4°C until further use [22].

Bacteriolytic activity of aerophages

By spotting technique [18]was determined the sensetivity of bacteria to aerophage, in brief 1 ml of overnight suspension of each isolates of P. aeruginosa was spreader on sterile nutrient agar separately then a 100µl aliquot of phage lysate was spot inoculated at each plate. Then the plates were incubated at 37°C and examined after 18 h. A clear zone in the bacterial lawn was recorded as sensitive.

Statistical Analysis

Statistical significance comparison of antibiotics was calculated by the Kruskal-Wallis test, which is a non-parametric test to compare samples from two or more groups of independent observations [23]. P-values <0.05 were considered to be significant. This test was selected because it does not require the groups to be normally distributed and is more stable to outliers. All statistical analyses were performed using the SPSS Version 16.0 and excel program 2010.

Results

Isolation bacteria

From a whole of 60 samples (10 samples from each source) collected from diabetes leg, stool, urine, ear, wounds and burns infections in General Baqubah Hospital, 23

isolates were able to grow on Pseudomonas agar. Colonies were picked up on Macconkey agar, blood agar. Finally purified on nutrient slant. Purified colonies were Gram negative stained, positive to catalase and oxidase test, and all the isolates were able to cultivate at 42ºC, pyocyanin pigment which is a diagnostic character [1] so identified as P. aeruginosa. Distribution the isolates according to sample source of infection are shown in Figure (1).

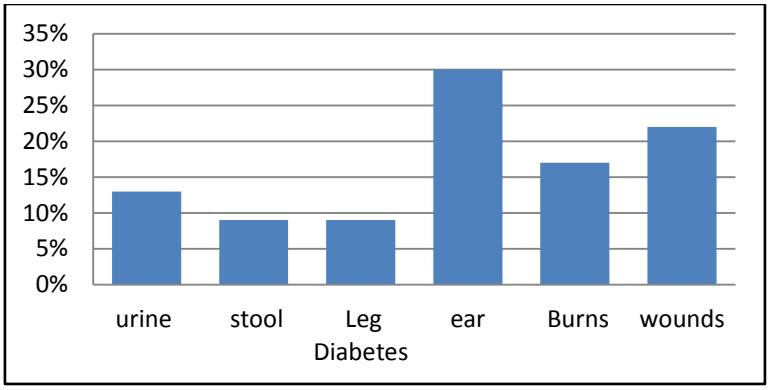


Figure (1): Distribution of P. aeruginosa isolates were studied

Sensitivity to antibiotics

The result of antibiotics sensetivity test of 23 P. aeruginosa showed high resistance associated with aztreonem 10(43.5%) followed by Ticarcillin-Claculanic acid 8(34.8%) while the sensitivity recorded with cefepime and gentamicin were 19(82.6%) as shown in table (1).

Table (1): Antimicrobials susceptibility patterns of P. aeruginosa Isolates.

Antibiotics	Symbols	S no. (%)	I no. (%)	R no. (%)
Ticarcillin-Claculanic acid	TIM	15(65.2%)	0(0%)	8(34.8 %)
Cefepime	CPM	19(82.6%)	2(8.7%)	2(8.7%)
Imipenem	IMI	18(78.3%)	1(4.3%)	4(17.4%)
Aztreonem	ATM	10(43.5%)	3(13%)	10(43.5%)
Gentamicin	GM	19(82.6%)	1(4.3%)	3(13%)
Levofloxacin	LEV	18(78.3%)	2(8.7%)	3(13%)
S: sensitive; I:intermediate; R:resistance				

Correlation between antibiotics reported, the Multiple Comparisons of antibiotics by using Kruskal-Wallis Test was showed high significant differences of aztreonem with both of cefepim and gentamycin ($P<0.01$) while significant differences of

aztreonem with both imipenim and levofloxacillin (P<0.05) as in table (2), this indicated that aztreonem in Baqubah more using than the rest antibiotics mentioned above.

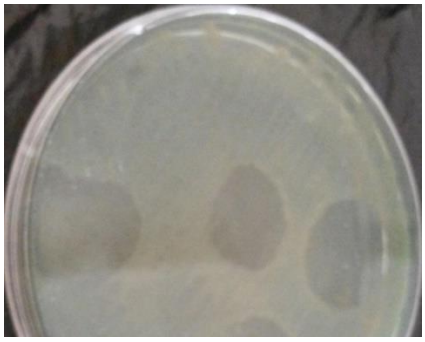
Table (2): Multiple Comparisons of significant levels for antibiotics according to senetivity degree(resistance, intermediate resistance and susceptibility).

Antibiotics	CPM	TIM	ATM	GM	IMI	LEV
CPM	-	0.121 NS	0.005 HS	0.947 NS	0.645 NS	0.691 NS
TIM		-	0.258 NS	0.150 NS	0.280 NS	0.235 NS
ATM			-	0.007 HS	0.021 S	0.013 S
GM				-	0.702 NS	0.750 NS
IMI					-	0.939 NS
LEV						-
	Kruskal-Wallis Test; (K-W = 13.718); P=0.018; (S)					

* HS:high significant P<0.01; S:significant P<0.05; NS:non significant P>0.05

Isolation of aerophage

The results of this study appeared clear zones (plagues) in spotted locations indicated the presence and isolation of aerophage, Figure (2). Separated plagues in double layer method that indicated the positive result as in figure (3). the plague under light microscope showed the clear zone surrounded by transparent cell which indicated that deed cell and opaque cell indicated that living cell as figure(4) . The result in test tube method showed clearance media which indicated the success isolation of aerophage as in figure (5). Finally the purified aerophage stored at 4°C as stock.



Figure(2): plague of aerophage by spot method.

Figure(3): isolation of phage by double layer.

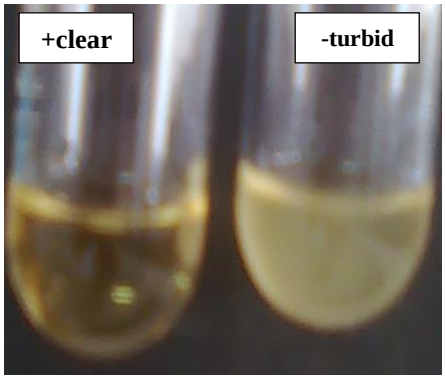


Figure (5) :Liquid broth method.

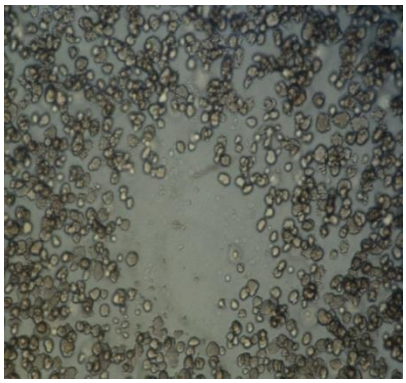


Figure (4): Plague under light microscope.

Sensitivity of P. aeruginosa to aerophage

The results showed that all ear isolates and all leg diabetes isolates were sensitive to aerophage (100%) followed by urine isolates(75%) stool isolates(50%) burns isolates(25%) while all wound isolates were resist to aerophage(100%) table(3).

Table (3) :Sensitivity of P. aeruginosa to aerophage by spotting method.

No.	Bacteria Source	No. of bacteria isolates	Sensitive to aerophage	Resistance to aerophage
			No.(%)	No.(%)
1	Wound	5	0(0)	5(100)
2	Burns	4	1(25)	3(75)
3	Urine	3	2(75)	1(25)
4	Stool	2	1(50)	1(50)
5	Ear	7	7(100)	0(0)
6	Leg Diabetes	2	2(100)	0(0)
Total		23	13	10

Results showed that ear and leg diabetes isolates high sensitive to aerophage with different antibiotic resistance phenotype table (4).

Table (4): the relation between Antibiotic resistance phenotype and Aerophage sensitivity.

No.	Source	Antibiotic resistance phenotype	Aerophage sensitivity
1	Ear	AIM;CPM;IMI;ATM;GM;LEV	+
2		-	+
3		-	+
4		-	+
5		AIM;IMI;ATM	+
6		ATM;GM	+
7		ATM	+
8	Leg diabetes	TIM;LEV	+
9		-	+
10	Urine	AIM;IMI;ATM	+
11		IMI;GM	+
12		ATM	_
13	Stool	ATM;LEV	+
14		AIM	_
15	Burns	ATM;GM	+
16		-	_
17		-	_
18		_	_
19	Wounds	AIM;CPM;ATM	_
20		AIM;ATM	_
21		ATM	_
22		-	_
23		-	_

Discussion

Pseudomonas aeruginosa was most common in ear infection followed by wounds, burns, urine, stool and diabetes. Many studies were conducted with distribution of P. aeruginosa in different sources such as which done by by Abbas (2016) In Baqubah revealed that *P.aeruginosa* from burns (18.18%), ear (11.6%)[24]. In Egypt, El fouly and coworker found that P. aeruginosa was the most common in urine (12.5%), followed by burns (10%) [25]. The reasons of variations may be due to geographical, virulence factors, environmental, nutritional reasons as well as age and immune response of participant [2].

In this study P. aeruginosa isolates resistance was 10(43.5%) for Aztreonem,. Several reports such as in Baqubah, Iraq [24] mentioned a high resistance rate in P. aeruginosa to cephalexin and cephalothin but low resistance rate to aztreonem (5%) so the rate of resistance was increased as compared with current results. One explanation for this could be its widespread use in the treatment of diseases associated

with P. aeruginosa . Also because they are inexpensive and can be obtained easily without a medical prescription, resistance is probably due to indiscriminate antibiotics usage (drug abuse) which could result in plasmid-mediated antibiotic resistance found to be common in P. aeruginosa. According to this result, the above antibiotics should be wise used for treatment disease caused by P. aeruginosa.in Al- Diwanyia, Iraq [9] found high resistance rate in P. aeruginosa to aztreonem(88.5%).

Pseudomonas aeruginosa isolates resistance were 3(13%) to Levofloxacin. Two major mechanisms lead to fluoroquinolone resistance in P. aeruginosa: structural changes in target enzymes and active efflux [26].

Pseudomonas aeruginosa isolates resistance were 3(13%) to Gentamicin. Unlike to this result, in Al- Diwanyia, Iraq[9] found high resistance rate in P. aeruginosa to gentamicin(60%).Several groups of aminoglycoside resistance mechanisms are known: enzyme modification (major), low outer membrane permeability, active efflux and, rarely, target modification [27].

Pseudomonas aeruginosa isolates resistance were 4(17.4%) to Imipenem. in Al- Diwanyia, Iraq[9] found no resistance rate in P. aeruginosa to imipenem(0%). Loss of OprD determines resistance to carbapenems only in cases of expressed chromosomal AmpC b-lactamase, and this demonstrates the close cooperation between these two mechanisms [28].

Yayan and coworker (2015) in Germany reported the resistance to cefepime, imipenem, gentamycin and levofloxacin were 23%, 27.3%, 18% and 24.5% respectively in nosocomial patients[29]. While in Pakistan, Ameen and coworkers(2015) found the P. aeruginosa resistant to imipenem was 49.5%[30].

Other resistance phenotypes are determined mainly by the production of plasmid- or integron-encoded extendedspectrum b-lactamases (ESBLs) from different molecular classes. In P. aeruginosa all possible mechanisms determining resistance to b-lactam antibiotics [enzymic inactivation, active efflux, changes in outer membrane permeability and synthesis of penicillin-binding proteins (PBPs) with lower affinity to b-lactams] may exist simultaneously or in various combinations [27].

The result of multiple comparisons of antibiotics by using Kruskal-Wallis Test indicated that aztreonem in Baqubah more using than the rest antibiotics.

Multi drug resist in P. aeruginosa makes treatment of infections caused by this organism both difficult and expensive. Improved methods for antimicrobial susceptibility testing are needed [27].

Many in vitro studies have been conducted in recent years to evaluate the prospective of phages against clinical isolates of P. aeruginosa, including MDR strains, in planktonic cultures or

biofilms[3].Local and international studies interested with isolation of aerophage.Zaman(2010) isolated P. aeruginosa phage and isolates with green and yellow coloring on Nutrient agar were lytic by isolated phages in Kirkuk[8]. Alkhozai and Alkabei (2011) isolated Five types of phages specific to P. aeruginosa from (60) sample of sewage water primarily named (ZM1,ZM2,ZM3,ZM4,ZM5) in Al-Diwanyia City[9]. Jameel (2016) isolated a lytic phage of an antibiotic-resistant P. aeruginosa in Baqubah[10].

In animals, Hawkins and coworkers (2010) in London used six bacteriophages(have in vitro activity against 90% of P. aeruginosa strains from dog ear infections) to treatment of Pseudomonas aeruginosa otitis of dogs in vivo, As showed lysis of specific bacteria in the ear without apparent toxicity[12].

In human, Wright and coworkers (2009) reported the safety of Biophage-PA, drug bacteriophage preparation to patients with chronic otitis have antibiotic resistant P. aeruginosa and showed significant clinical improvements compared to 12 placebo group individuals. Furthermore, no related side effects or local systemic toxicities were observed, which highlights the safety of phage therapy [11].

The result of relation between Antibiotic resistance phenotype profile and Aerophage sensitivity that no detect to susceptibility of isolates to aerophage but the source of isolates may be detected to infection with aerophage.

Distribution studies of P. aeruginosa lytic bacteriophages isolation by country studied by Pires et al (2015) who reported the most research in France 13.64% followed by Russia and Belgium 10.61% for each then South Korea 9.09% and others[3].

In phage therapy after encounter with specific pathogenic bacteria, can infect and kill them. As typically practiced phages then lyse bacteria, releasing new virion progeny that can again the cycle, may wandering to other places of infection anyplace in the body. Phages are sole among antibacterial agents in their ability to increase their quantities when in the occurrence of bacterial targets [31]. Finally, phage therapy more effective to treatment pathogenic bacteria but, Phage therapy in Iraq need for further researches deal with isolation, identification and safety used for human.

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