Isolation and Characterization of PAP a Lytic Bacteriophage Specific for *Pseudomonas aeruginosa*

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Abstract

There has been a frightening increase in drug-resistant strains of *Pseudomonas aeruginosa* as a result; there has been a repeated attention in alternative antimicrobial treatments, as bacteriophages. Current study was included isolation and characterization of a lytic phage specific to *Pseudomonas aeruginosa* (which we designed as PAP referred to a ***P****seudomonas* ***a****eruginosa* specific **p**hage) from sewage water of General Baqubah Hospital treatment plant. In this study, a phage PAP was isolated and titrated, using double layer agar technique. and determination of the latent periods and burst size of phage PAP then determines their stability to some of physical and chemical condition (temperature, UV light, Alcohol, chloroform and isopropanol exposure).The results detected a lytic phage in contradiction of an antibiotic-resistant *Pseudomonas aeruginosa*, the stock titer about 8×10^9 PFU(plaque forming units)/ml. In phage PAP growth cycle, the latent period was 20 min, a burst size was 126 PFU/ infected cell. We found that the phages PAP stable at 4°C and 37°C while they declined quickly after boiling for 10-20 min. unaffected in the presence of chloroform in different concentration, affected by 10% isopropanol, more affected by 70% alcohol.

Keywords: *Pseudomonas aeruginosa* phage ; isolation; characterization

Isolation and Characterization of PAP a Lytic Bacteriophage Specific for *Pseudomonas aeruginosa*

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عزل وتوصيف العاثي البكتيري المحلل PAP المختص ببكتريا الزوائف الزنجارية

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الملخص

هناك زيادة مخيفة في عزلات الزوائف الزنجارية المقاومة لمضادات الحياة وبالنتيجة هناك اعادة اهتمام في العلاجات الضد ميكروبية البديلة مثل العاثيات البكتيرية. تضمنت الدراسة الحالية عزل وتوصيف العاثي المحلل المختص بالزوائف الزنجارية (رمزنا له PAP التي تشير الى الحروف الاولى من *Pseudomonas aeruginosa* specific **p**hage) المعزول من مياه مجاري مستشفى بعقوبة العام. في هذه الدراسة العاثي PAP تم عزله ومعايرته باتباع تقنية الاكار ثنائي الطبقة. وتحديد فترة السكون وحجم الانفجار ثم تحديد ثباتيته لبعض الظروف الفيزيائية والكيميائية(التعرض لدرجة الحرارة، الاشعة فوق البنفسجية، الكحول، الكلوروفورم، ايزوبروبانول).كشفت النتائج عن وجود العاثي المحلل للزوائف الزنجارية المقاومة لمضادات الحياة، عيارية المخزون حوالي 8×10^9وحدة تكون البقع/مل. كانت فترة السكون في دورة نمو العاثي PAP هي 20 دقيقة وحجم الانفجار 126وحدة تكون البقع/خلية مصابة. اما بالنسبة لثباتية العاثي فوجدنا انه ثابت عند درجة الحرارة 4م° و 37 م° لكنه يتثبط بسرعة بعد الغليان لمدة 10-20دقيقة وقل تركيزه. لم يتاثر بالتراكيز المختلفة من الكلوروفورم. تاثر بتركيز 10%من الايزوبروبانول اما تاثير الكحول فكان اشده عند تركيز 70%.

الكلمات المفتاحية: عاثيات الزوائف الزنجارية، العزل ، التوصيف

Introduction

Pseudomonas aeruginosa is important opportunistic pathogen and a broad host range (vertebrates; invertebrates, plants and abiotic environments) (1) *Pseudomonas* spp. are the chief concern with concern to proteolytic degradation of milk, beside with other organisms (2,3) In humans, *P. aeruginosa* is the most public cause of many different diseases such as chronic lung infection in cystic fibrosis (CF) patients, otitis, dermatitis, urinary tract infection, bacteremia (4,5,6), and a main cause of nosocomial infections (6) In addition, *P. aeruginosa* is vastly active in the formation of biofilms on biotic and abiotic surfaces and so moderates the efficiency of numerous antibiotics (7). Multi-drug resistance (MDR) in *P. aeruginosa* is credited to a combination of resistance mechanisms, mostly including multi-drug

efflux pumps, target site modifications, β-lactamase and aminoglycoside-modifying enzymes (8) Consequently, *P. aeruginosa* infections are weakening the ability of conventional chemical antibiotic treatments and a high interest is increasing towards the lytic phages therapy(7).Phage therapy is one of numerous possible therapeutic methods and has been considered meanwhile the late 1980s (9). Bacteriophages are viruses that infect merely bacteria, cannot infect eukaryotic cells (10). The highest characteristics distinguish bacteriophage therapy from antibiotic therapy: (a) phages proliferate at the infection position; (b) they target merely specific bacteria, with no effect on normal flora; and (c) they can adjust to resistant bacteria (11). Because the little or rare bacteriophage researches in Iraq so we describe the isolation of phages for *P. aeruginosa* and further their characterization. This is perhaps the first research of the isolation of the phage from Baqubah city by using *P. aeruginosa* as a host.

Material and Methods

1. *Pseudomonas aeruginosa* isolation and characterization

Pseudomonas aeruginosa was isolated from wound samples of patients in General Baqubah Hospital in Baqubah, Iraq, by using nutrient agar (Oxoid, UK) and pseudomonas agar (HiMedia, India). Isolate gave positive results for catalase, oxidase, citrate utilization, urease and growth in 40°C, while negative for indol, methyl red, Vogas-Praskaur and H_2S production test but had ability to produce pyocyanin when growing on Pseudomonas P medium. The cells were single cells, Gram-negative and rod shapes (12). *P. aeruginosa* isolate was multiple-resistant to most of the antibiotics, It possessed high resistance against penicillin; tetracycline, erythromycin, piperacillin, oxacillin, cephalothin, tobramycin and gentamicin. The bacterial strains (*P. fluorescens, E. coli, Proteus, Salmonella, Klebsiella, Enterobacter cloaeca, Acenetobacter, Streptococcus pyogenes and Staphylococcus aureus*) were previously isolated in Almoqdadia Laboratory and were obtained from there. All bacteria were grown at 37°C in nutrient broth and nutrient agar. All bacteria were subcultured once and glycerol stocks were done and stored frozen at -80°C until further use.

2. demonstration of *pseudomonas aeruginosa* phage (PAP)

2.1 Preparation of phage suspension

PAP phage suspension was prepared from sewage water of General Baqubah Hospital treatment plant (2.5L), this wastewater treatment plant was selected because it receives effluents from hospital i.e. contain pathogenic bacteria (host for bacteriophage). then aseptically filtered 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 0.45 µm pore sized filter to remove bacterial cells and cellular debris (13).

2.2 Phage enrichment and isolation

Briefly, five ml of logarithmic phase suspension of *Pseudomonas* were inoculated in nutrient broth and mixed with (45ml) bacteriophages suspension (as prepared in above step) 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 (14).

2.3 Testing the suspension of expected phage

By the spotting technique, 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 on bacterial law. The plates were inverted and incubated at 37^0C overnight, and then examined for the presence of clear zones of lysis (15). One of the cultured nutrient agar plates not processed with suspension of expected phage stock solution as a negative control.

2.4 Purification of phage

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 (NaCl, 5.8 g; $MgSO_4$_$7H_2O$, 2 g; 1 M Tris_Cl pH 7.5, 50 ml; 2 % gelatin, 5 ml; the volume was completed to 1,000 ml by ddH_2O). This mixture was centrifuged at 5000rpm for 25min at 4^0C and filtered through Millipore Membrane Filter (0.22 µm). 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 (16).

2.5 Large scale amplification of phage

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 (17) with some modifications. Briefly, Materials from the centre of the plaques were scraped off using a sterile inoculation loop and were transferred to sterile nutrient broth(100ml) containing the specified organism(*pseudomonas*) 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 (17).

2.6 Titration by plaque assay

Phage titer was determined by plaque-forming units (PFU) per ml using the double-layer agar plate method as described by Adams. Briefly, 100 µl of diluted phage solution in SM buffer, 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 (18).

2.7 Host range

The ability of Pseudophage to infect more than one bacterial species and genus was determined. The bacterial strains *P. aeruginosa, P. fluorescens, E. coli, Proteus, Salmonella, Klebsiella, Enterobacter cloaeca,Acenetobacter, Streptococcus pyogenes and Staphylococcus aureus* were obtained from hospitals in Diyala, Iraq. By spotting technique (15) was determined the host range of Pseudophage, in brief 1 ml of log phase suspension of host

strains was spreader separately on sterile nutrient agar then a 0.2 ml aliquot of phage lysate (8×10^9pfu/ml) was spot inoculated at the center of each plate. Then the plates were incubated at 37°C and examined after 24 h. A clear zone in the bacterial lawn was recorded as host susceptible.

2.8 Effect of media on plaque shape

The morphology of plaques due to PAP phage was studied on three different media: pseudomonas agar, nutrient agar and MacConkey agar media by double layer agar technique (18). An aliquot of 0.1 ml phage lysate [8×10^9 pfu/ml] was mixed with 0.1 ml of the host culture in 3ml of semi-solid media this mixture was poured onto the surface of the respective medium. The plates were incubated at 37°C for 24h then observed the development of plaques and their morphology.

2.9 One-Step Growth Curve

It was determined as defined by Hyman and Abedon (19) with few variations. Briefly, 9 ml of the log phase culture of *P. aeruginosa* was mixed with 1 ml of phage lysate (8×10^9 pfu/ml) in a test tube. Phages were permissible to adsorb at 37°C for 10 min. then centrifuged (10,000g, 4°C, 20 min) and the pellet was suspended in 10 ml of SM buffer. 0.1 ml was pipetted from the suspension at an interval of 5 min for 1 h. and chloroform (1% v/v) was added to the supernatant to kill any unlysed bacteria. Just before the culture was removed at each time interval, 0.lml of *P. aeruginosa* culture was added to a slopy agar tube to which the culture from the water bath was added. This was then plated immediately on Nutrient agar plates and incubated overnight, the number of PFU was then determined for each time.

2.10 Stability to some of physical condition

Five temperatures (4, 37 and 100 °C) used to study the thermal tolerance of phage in nutrient broth. An aliquot of phage was taken every 10 min for 1 h, and tittered by double-layer method. In addition, phage was also exposed to ultraviolet light (30 W, 35 cm wavelength) treatment in tube and petri dish for 5,15, 25, 35, 45,55 and 65 min, and then tittered immediately by double-layer agar plate method (20).the results were stated as the percentages of existence phages from original particles in each treatment.

2.11 stability to some of chemical agents

To analyze phage chemical stability, phage was subjected to treatment with chloroform (10, 30, 60 and 100 %, v/v) for 72 h at 4 °C , isopropanol (10, 30, 60 and 100 %, v/v) for 72 hr. In addition, phage was also exposed to alcohol (10, 30, 70 and 100) for 72hr and then tittered immediately by double-layer agar plate method (20). The results were stated as the percentages of existence phages from original particles in each treatment

Results and discussion

1. Phage isolation

After preparation of mixed bacteriophages suspension from General Baqubah Hospital sewage water as in paragraph (2.2.1), enrichment and propagation of *pseudomonas aeruginosa* phage, which we designed as PAP referred to a ***P****seudomonas* ***a****eruginosa* specific **p**hage, if present in sewage water as in paragraph (2.2.2). Then we tested the final suspension for present of PAP by spotting method as mentioned in paragraph (2.2.3). From results of this study, clear zones appeared in spotted locations indicated the presence and isolation of PAP, Figure (1). Then the clear zone by sterile suspended with SM buffer then filtered through 0.22µm filter. The spotting method repeated for three times to ensure that these clear zone for bacteriophage activity not bacteriocin. In each time, the filtered suspension store at 4°C for further work.

Figure (1) Clear Zone

2. Phage Titration

Titration of PAP was determined from large scale filtered suspension by double layer agar after diluted of sample. The results showed the stock titer about 8×10^9 PFU/ml, table (1) and

from this result indicated that the dilution (10^{-8}) gave the best countable number of plaques per plate, so (10^{-8}) dilution would use for all further experiments.

Table (1) PAP Titration

Plate no.	Dilution	Plaque no.	Stock titer per ml (plaque no. × invert dilution)/ 0.1*	Dilution titer (Plaque no.×DF**)/0.1*
1	10^{-1}	Clear	-	-
2	10^{-2}	Semi Clear	-	-
3	10^{-3}	TMTC***	-	-
4	10^{-4}	250	2.5×10^{7}	2.5×10^{4}
5	10^{-5}	167	1.6×10^{8}	1.6×10^{4}
6	10^{-6}	66	6.6×10^{8}	6.6×10^{3}
7	10^{-7}	49	4.9×10^{9}	4.9×10^{3}
8	10^{-8}	8	8×10^{9}	8×10^{2}
9	10^{-9}	2	2×10^{9}	2×10^{2}
10	10^{-10}	0	-	-

*Volume of phage plated (ml) is 0.1ml

**Dilution Factor (DF)=10

***Too much to count

Other researchers as Li and Zhang in 2014 (21) found the titer of phage SPW Staph aureus from large scale preparation was more than 10^{9} pfu/ml while Ahiwale and co-worker (2012), found the titer of Pseudophage (BVPaP-3)was $6.6*10^{9}$PFU/ml. These differences may be from the sample, counting of bacteria, conditions of work and characters of each phage.

3. Influence of Growth Medium on the properties of Plaques

The plaques formed by phage PAP were clear and transparent round zones on all three different media and uniformed per each plate of pseudomonas agar, nutrient agar but multiformed on MacConkey agar. The average plaque diameter (mm) was 3, 2, multisized on pseudomonas agar, nutrient agar and MacConkey agar media respectively. the edge of plague was confluent on nutrient agar while irregular on both of pseudomonas agar and MacConkey agar media. Number of plague per plate was 38, 60 and 24 in pseudomonas agar, nutrient agar and MacConkey agar media respectively. Thus, from the results, it can be concluded that

phage PAP responded differently to its host on different growth media and the best media was nutrient agar. In contrast, Ahiwale and coworkers (2012) found that BVPaP-3 did not respond differently to its host on different culture media.

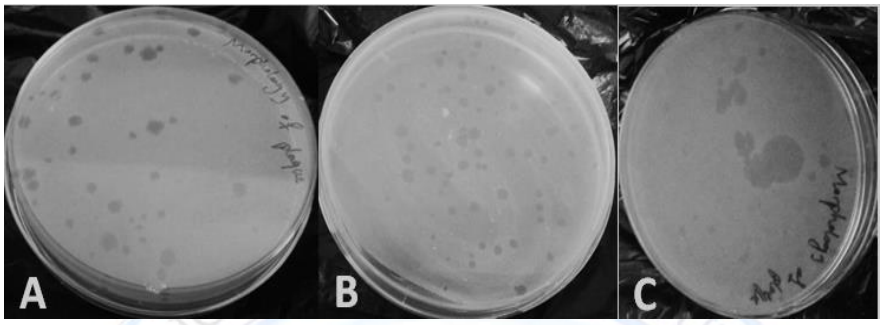

Figure (2) Morphology of PAP ´s Plaques on Different Media

A- Pseudomonas agar B- Nutrient agar C-MacConkey agar

4. Host Range

The cross infectivity of phage PAP was estimated to check its range of activity against other genera of bacteria. *P. aeruginosa, P. fluorescens, E. coli, Proteus, Salmonella, Klebsiella, Enterobacter cloaeca, Acenetobacter, Streptococcus pyogenes and Staphylococcus aureus* were tested to check the spectrum of activity of phage PAP. PAP could lyse *P. aeruginosa* only but was unable to lyse *P. fluorescens* and the above genera of bacteria. Indicated that PAP highly specific for *P. aeruginosa* so they called broad host range bacteriophage. These results are similar to previous study as Ahiwale and coworkers (2012) but contrarily to those in the literature of Bielke and coworkers in 2007(22) found Salmonella phages infected more one genera (wide host range phage). These differences because the bacteriophage attached to specific receptor on surface of bacteria and some of bacteria have the same receptor.

5. One step growth curve of PAP

The PAP growth parameters, latent period, rise period and burst size, were determined from the change of the number phages. The latent periods and rise period of phage PAP was 20 and 25min respectively. The burst size of the phage was 126 PFU per infected cell after 45

min at 37°C which Calculate by dividing the concentration of bacteriophage at time=45min by the concentration of bacteriophage at time=25min) as shown in figure (3). While Ahiwale and coworkers (2012) found the latent period and burst size of BVPaP-3 was 15min and 44PFU/infected cell after 35min at 37°C respectively, while Li and Zhang (2014) found the latent period, rise period and burst size of Staph aureus was 15min, 50min and 44PFU/infected cell respectively. These significant characteristics vary among viruses but are often analogous for related ones. Thus, they can assist taxonomic criteria, commonly for high-level taxonomy. The number of burst size, and the time which take to make them or latent period, are chiefly determined by the complexity and dimension of the virion so it is estimated that these principles are conserved between phages with analogous morphology (23).

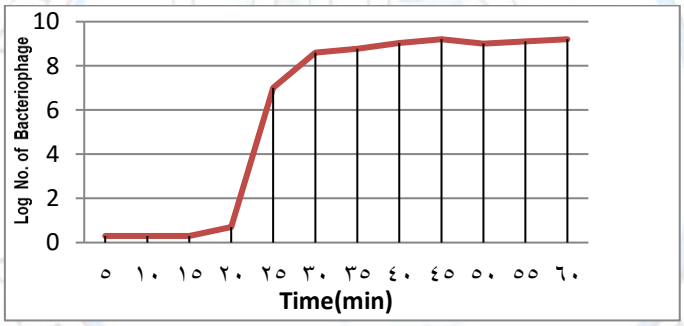

Figure (3) curve of PAP growth

6. Stability to some of physical condition and chemical agents

To evaluate the suitability of phage PAP for potential therapeutic use in the further, its physical and chemical stability were examined. We found that the phage PAP stable at 4°C and 37°C for about 60 min while towards extreme conditions, it declined quickly after boiling for 10-20 min and was completely inactivated after 30 min as in figure (4).Because, the high temperature denatured the chemical composition of bacteriophage. Previous study had shown analogous results (7, 21)

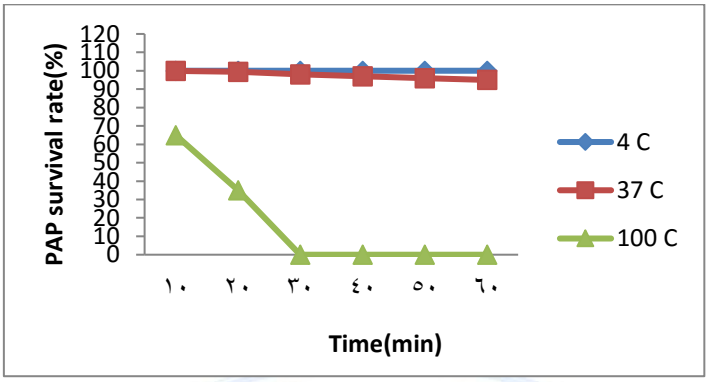

Figure (4) Stability of PAP to Temperature

Direct UV light inactivated the phage PAP which placed in petri dish rapidly while phage PAP in tube survived for 5min only. While the results of Li and Zhang (2014) showed that phage SPW of Staph aureus survived from ultraviolet light treatment for up to 40 min. Furthermore, the viability of phage PAP was largely unaffected in the presence of chloroform in different concentration (10, 30, 60 and 100%) this result compatible to most previous studies (21)while the viability of phage PAP was slightly affected by 30%and 60% isopropanol, moderate affected by 100% isopropanol while more affected by 10% isopropanol as shown in figure (5). In contrast Li and Zhang (2014) found the viability of phage SPW was little affected by 5%isopropanol.

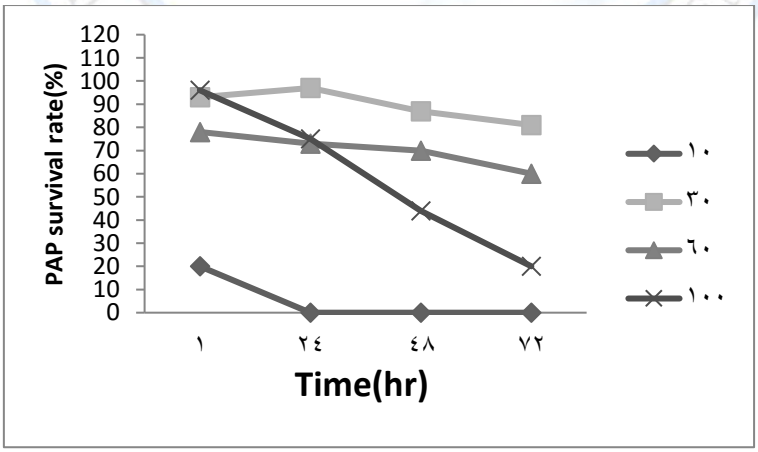

Figure (5) Stability of PAP to Isopropanol

10% alcohol unaffected on viability of phage PAP for 3 days while PAP moderate affected by 30% and 100% alcohol, but more affected by 70% alcohol as shown in figure (6).This is due to 10% alcohol more diluted and 100% alcohol volatilization quickly.

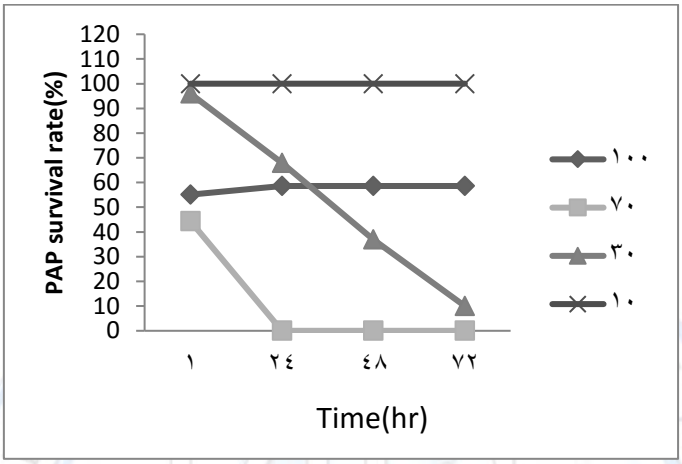

Figure (6) Stability of PAP to Alcohol

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