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Isolation and Characterization of Lytic Bacteriophages Infecting *Pseudomonas aeruginosa* From Sewage water

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Abstract : This study was carried out during the period from April/ 2015 to January/ 2016. It included the isolation and identification of 50 *P.aeruginosa* isolates from 150 samples collected from different clinical and environmental sources. The distributions of these isolates were: 22(44%) from 50 burn samples, 8(32%) from 25 samples of urinary tract infections, 10(28.5%) from 35 samples of operative rooms and 10(25%) from 40 samples of sewage water.One type*P.aeruginosa* phage was isolated from 100 samples of sewage water according to difference in shape and diameter of the plaques primarily named Phage1.The results showed that the highest adsorption rate of isolated phage on bacterial cells was about 5-7 minutes, phage particles have an eclipse time about 6.5 minutes. The maximum lysis activity of phages was documented in about two hours, while complete lysis time was about 6 hours. The calculated data showed that the burst size was about 15±5 pfu\cell in time period about 14 minutes. The results also showed that the *P.aeruginosa* isolates from sewage water were more susceptible to phage infection than isolates from clinical cases. The isolated phage reached the maximum activity in the neutral medium between pH 6-8. This activity declined after pH 9. At temperature between 35-40C^{o} the phage activity was not affected but it decreased continuously when temperature increased to 50 C^{o}. At 55C^{o} the activity dramatically reduced and diminished in about 8-10 minutes. The phage host range was determined and the lytic phage infected all *P.aeruginosa* isolates, were isolated from the different environmental and clinical sources. While it was unable to infect other genera that used in this study (*Staphylococcus aureus*, *Escherichia coli*, *Streptococcus pyogenes*, *Klebsiella pneumoniae* and *Proteus vulgaris*).
Key words: Bacteriophages, *P.aeruginosa*, Phage kinetics, Host Range.

Introduction:

Pseudomonas is one of the most diverse genera known, with members in diverse environmental niches, such as soil, water, animals, and plants. *Pseudomonas aeruginosa* differs from other species of this genus in being pathogenic to animals[1]. This Gram-negative rodshaped bacterium is an opportunistic pathogen, causing various types of infection (e.g., skin, eyes, ears, respiratory tract, urinary tract, gut-derived sepsis). Immunocompromised patients and patients on immunosuppressive treatments, such as patients suffering from cystic fibrosis, burn wounds, AIDS, and cancer, are the most frequently infected by this organism. *Pseudomonas aeruginosa* strains are naturally resistant to several antibacterial drugs[2]. In addition, some clinical isolates have a hypermutator phenotype, facilitating the rapid evolution of resistance to drugs to which they were initially sensitive. *P. aeruginosa* strains can also acquire resistance from mobile genetic elements, such as plasmids [3]. Most *P. aeruginosa* strains are still susceptible to colistin, a polymyxin antibiotic, but increasing numbers of reports of pan-resistant strains are emerging[4,5].

Bacteriophages (or phages) are the viruses that infect bacteria, The largest group is the *Caudovirales*, or tailed phages, that make up 96% of all phages. There are an estimated 10^{31} phages on the planet with 10^{25} infections occurring every second [6]. There are at least 12 distinct groups of phages; and, each phage species is specific to its bacterial host. The exact morphology and genetic material (DNA or RNA) varies according to the phage species. The typical structure of a phage is a hollow head filled with phage DNA or RNA and a tunnel-like tail for injecting the genetic material into the bacteria[7].

The large number of virulent bacteriophages infecting *P. aeruginosa* that have been fully sequenced (more than 40 in 2010) bears witness to the high degree of interest in this field[8]. Bacteriophage genomes are much less conserved than bacterial genomes. It is therefore not possible to identify a particular subset of sequences present in all bacteriophages infecting a given bacterial species. Given the very high abundance of bacteriophages (10^{32} particles on earth) and the diversity of environments in which *P. aeruginosa* is found, the number of bacteriophages infecting *P. aeruginosa* discovered to date may represent the tip of the iceberg [9]. The phages take over the molecular machinery of the bacteria to reproduce in large numbers and are ultimately released by host cell lysis[10]. Unlike antibiotics phages kill target bacteria specifically and do not destroy host's normal micro flora thereby prevents bacterial dysbiosis, which may otherwise lead to secondary infections[11]. Thus, application of phages to inhibit bacterial growth could be natural, nontoxic and effective substitute of antibiotics. The efficacy of phages to control bacterial infections had been extensively reviewed[12,13]. The present study aimed to evaluate the antibacterial activity of isolated bacteriophages from sewage water. By the following objectives:

1. Isolation and identification of *P.aeruginosa* and determination the antibiotic resistance profile.
2. Isolation of and characterization of *P.aeruginosa*phages from sewage water.
3. Enumeration of bacteriophage by double agar overlay plaque.
4. Measurement of the attachments rate of bacteriophage to bacterial cell.

Experimental

Samples Collection:

Water Samples:

One hundred of sewage water samples were collected from different environmental sources in a sterile method in clean containers, from this samples ten *P.aeruginosa* were isolated and used as a hosts for isolation of phages. The samples were filtrated by using the Millipore filter unit size 0.45µm, in an attempt to obtain phage containing water samples[14].

Bacterial Isolates:

In this study, 40 isolates of *P.aeruginosa* were isolated from 110 clinical (for both sexes of different ages who referred to AL-hilla Teaching Hospital and mirgan Teaching Hospital, during the period from April/ 2015 to Augast/ 2015) and hospital environmental sources. These included 50 swabs from burn patients, 25 samples from patients suffering from urinary tract infections (UTI) for both sexes of different ages and 35 swab from operation rooms (Surgical operative beds, surgical instruments, tables, sink, walls and floor). All swabs and specimens were transported to the laboratory of mirgan Teaching hospital without delay. The samples were immediately inoculated in MacConkey agar, blood agar and nutrient agar and incubated for overnight at 37°C aerobically[15].

Antibiotic Susceptibility Testing:

*P.aeruginosa*isolates were tested for their sensitivity to 18 commonly used antibiotics by disk diffusion method [16]. Results were recorded by measuring the inhibition zone (in millimeters) and interpreted according to Clinical and Laboratory Standards Institute documents CLSI,[17].

Phage Isolation and Propagation:

Samples of sewage water were collected from different environmental sources in a clean containers about 500 ml from this samples. In brief, sewage samples were collected, centrifuged (10,000 rpm, 10 min, 4^{o}C)

and supernatants were filter sterilized (0.45 µm Millipore filter). 50 ml filtered sewage sample and 50 ml sterile nutrient broth was mixed with 5.0 ml overnight culture of *P.aeruginosa*and incubated at 37^{o}C overnight. The bacteria were removed by centrifugation; supernatant was filtering sterilized and checked for the presence of phages[18].

Plaque Assay and Spot Test:

To detect the presence of phages in supernatant, spot test was done as described by Garbe,[19], the phage titer was determined by plaque assay by employing double agar overlay technique. Briefly, each of the phage suspension was serially diluted. 100 µl of diluted phage and 100 µl host bacterium (10^8 CFU/ml) were mixed with 5.0 ml soft agar (0.75 % agar, w/v) and poured quickly on top of the solidified nutrient agar plate[18].The numbers of plaques were counted after incubating the plates overnight at 37^{o}C.

Phage Propagation and Purification:

Isolated phages were purified by successive single-plaque isolation until homogenous plaques were obtained by the standard procedure described by[18,20]. Briefly, one well separated phage was picked with sterile pasture pipette along with the surrounding cell mass and inoculated into 5.0 ml nutrient broth, in which 1% overnight culture of host strain was added and incubated at 37^{o}C with agitation at 240 rpm. After complete lysis, the mixture was centrifuged (10,000 rpm, 10 min), filter sterilized and treated with chloroform (1% V/V) to remove any bacterial contamination. Purified phages were stored in 60 % glycerol at -80^{o}C for long term storage. Short term stock preparations were maintained at 4^{o}C.

Phage kinetics:

Adsorption Rate:

Adsorption assay was involved adding 10^6 pfu/ml from a fresh phage lysate to *P.aeruginosa* suspended in LB in flasks (about 10^8 cfu/ml), five minutes later, spinning the sample to pellet adsorbed phage. At five minutes unspun and spun suspensions were plated to obtain total phage and free phage densities (N_{total},N_{free}) respectively. Adsorption rate was calculated from $N_{free}= N_{total}e^{-5\alpha}$[21].

Eclipse Period:

Eclipse time assay were involved adding 10^7 pfu/ml of phage lysate to 10^8 cfu/ml of *P.aeruginosa*; after 5.5 minutes, samples were taken over chloroform every 30 second, until approximately 1.5 min before the average lysis time of the phage. Titers were taken of the treated samples, yielding a combined estimate of free phages and intracellular phages. Eclipse time was then estimated by fitting the data numerically to a simulation that modeled adsorption, eclipse, and a linear accumulation of phage after eclipse over time. Parameter values for phage density, eclipse, and the slope of linear phage increase were fit by empirical least squares in which the difference log(observed phage density) – log(model phage density) was normalized by log(observed phage density) to enforce equal weighting of the squared deviations at all time points[21].

3-6-3- Lysis Time:

The 10^7 pfu/ml of phage lysate were added to 10-ml of *P.aeruginosa* at 10^8 cfu/ml, grown for five minutes, and then diluted 10^5-fold and 10^3-fold in separate flasks to stop adsorption. Infective centers (a mix of untreated free phage plus infected cells) were plated at various time points to determine changes in titer [21].

Burst Size and Latent Period:

For burst size assays 10^6 pfu/ml of phage lysate were added to suspensions of exponentially growing cells of *P.aeruginosa* in flasks (10^8 cfu/ml). The mixture was diluted 10^3-fold after 5 min to curtail further adsorption. At 5.5 and 6.5 min phages were tittered both before and after treatment with chloroform. Treatment with chloroform kills cells, and because 6.5 min precedes the end of eclipse, all infections were failed to leave progeny; the only plaques in the chloroform treatment were derived from free phage. The initial density of infected cells can be determined by comparison of these titers. At 15.5, 16.5, and 17.5 minutes, chloroform-treated samples were plated to estimate phage density. Burst sizes for each replicate were calculated as the titer

of phage produced at late time points/the number of initially infected cells, calculated from initial time points. The latent period is defined as the time at which virus progeny are released into the environment[21].

Effect of pH on Stability of Phage:

The stability of *P.aeruginosa* phage was studied using phosphate buffer saline at different pH (4, 5, 6, 7, 8, 9, 10 , 11 and 12). An aliquot (1 ml) of lysate (1×10^9pfu/ml) was suspended in 9 ml of the respective buffer in sterile test tubes. Tubes were incubated at room temperature for 1 h. Contents of the tubes were serially diluted and pfu in each was determined by plaque assay using host bacterium *P.aeruginosa* as mentioned above[22].

Optimum Temperature for Phage Stability:

The stability of *P.aeruginosa* phage was evaluated at different temperatures (10, 20, 30, 37, 40 and 50^{o}C). A 1 ml aliquot of phage lysate (1×10^9pfu/ml) was dispensed in sterile screw capped tubes. Tubes were incubated at the respective temperatures for 1 h. Contents of the tubes were serially diluted in sterile phosphate buffer saline (pH 7) and pfu values were determined on sterile nutrient agar medium[22].

Phage Host Range:

The ability of *P.aeruginosa* Phage 1 to infect more than one bacterial genus was determined. The bacterial isolates *Staphylococcus aureus, Escherichia coli, Streptococcus pyogenes, Klebsiella pneumoniae* and *Proteus vulgaris* were obtained from the clinical cases and identification according to Forbes *et al*., [15] and finally identified via VITEK 2 – Compact. To this, 0.5 ml of the mid-log phase suspension of host bacterium was mixed separately with 4 ml of soft agar (0.6% w/v) and then poured onto a sterile nutrient agar plate. Once the overlay was gelled and dried, a 100µl aliquot of phage lysate (6×10^9pfu/ml) was spot-inoculated at the centre of each plate. Then the plates were incubated at 37^{o}C and examined after 24 h. A clear zone in the bacterial lawn was recorded as complete lysis[18].

Statistical Analysis:

Data analyses were performed with Graph Pad Prism version 5.04 Software (Graph Pad Software Inc., San Diego, CA, USA). All of the descriptive variables were expressed as the mean ± standard error (SE).The group analyses were performed using one-way ANOVA and Turkey's post-hoc analyses by determination of LSD on probability level 0.05 [23].

Result and Discussion

Forty isolate of *P.aeruginosa* isolated from 110 clinical and hospital samples, these included 50 swab from burn patients, 25 sample from patients suffering from urinary tract infections (UTI) for both sexes of different ages groups, and 35 swab from operative rooms. Biochemical and morphological characterization tests showed that 40 (36.6%) isolates were identified as *P.aeruginosa*, 22(44%) isolates from burns, 10(28.5%) isolates from operative rooms and the last 8(32%) isolates from UTI infections. Also, ten isolate of *P.aeruginosa* isolated from 40 samples of sewage water that were collected from different environmental sources, these isolates were used as hosts for primary isolation of phages, as in figure (1) shows the percentagesof *P.aeruginosa* according to the site of isolation**.***P. aeruginosa* is a nosocomial pathogen and leading cause of health care associated infections these infections are difficult to eradicate. It is an emerging multidrug resistant pathogen around the globe and also in Asia[24].The high frequency of *P.aeruginosa* may be explained by the fact that it is regarded as an opportunistic pathogen [25,34,35].Prolonged antimicrobial use as treatment for pseudomonic infections is seemed to be risk factor for infection with *Ps.aeruginosa* especially multidrugs–resistant *P.aeruginosa* (MDRPA) [26,33]. Forty isolate of *P.aeruginosa* were tested for their antibiotics susceptibility, toward 18 antibiotics using Kirby-Bauer disc diffusion methods. Screening for antibiotic susceptibility was done by agar screen methods on Muller-Hinton agar. The antimicrobial potency of selected antibiotics against the *Pseudomonas* is summarized in table(1). The obtained results from this study reveal a remarkable elevation in pseudomonal resistance to the beta-lactam antibiotics represented by penicillins. One types of *P.aeruginosa* phage was isolated from (100) samples of sewage water primarily named Phage 1 using ten *P.aeruginosa* isolates that were used as hosts for primary isolation of phages, the phages effectively were

tested against 40 *P.aeruginosa* isolates by using plaque assay method. Characteristics of these phages were determined by the plaques appearance, diameter, turbidity and the presence of a halo in the culture media as in table (1). Infection of *P.aeruginosa* by phage exhibited the clear plaques on the surface of nutrient agar. From plaque assay, all the *P.aeruginosa* isolates were susceptible to phages lytic infection, plaques on agar plates revealed a big hollow zone with slightly wrinkled margin as in figure (3) that showed plaques of *P.aeruginosa* phages. Sewage water was chosen as the phage source because it has yielded a wide diversity of phages in the past [14]. Isolation of of one type of lytic phages against *P.aeruginosa* host bacterium from sewage illustrates that human extra is a rich repertoire of this bacterium as well as antipseudomonas phages. However, the renowned specificity of the interaction of a phage with its bacterial host means that the chances of successful treatment can be greatly increased by having multiple phages with strong lytic capabilities and wide host ranges that complement those of other phages, other investigators have also described the isolation of pseudomonas specific phages from sewage [14,18,27].The bacteria and viruses in this experiment were mixed in a ratio that only one phage is likely to adsorb to a bacterium. Moreover, it is best to infect bacteria in a exponential growth phase to have metabolic active.The phage particles are able to achieved the higher level of adsorption at a period of time between (3-5) minutes. The calculated data explained that the isolated phage particles have an eclipse time about (6.5 minutes).The phage particles recorded highly significant ($P > 0.05$) to cause lysis of the bacterial cells by casing complete lyses (clearing) of bacteria in brothalso showed that there is dramatic decrease in number of bacterial cells that correlated with time. Complete lysis time was about 6. Rate of adsorption and lysistime were determined by a series of divers of nonspecific physical- chemical factors (pH, temperature, bacterial physiological status, phage concentration, presence in the media certain substance and ions) and depends on host physiological state and cultural condition[28]. The calculated data showed the burst size (numbers of phages were produced by an infected cell) was about: Phage 1= 15±5 pfu\cell in time period about: 14 minutes. Latent period length and burst size are complex related; longer latent periods provide more time for phage reproduction within a cell, thus increasing burst size [19]. The obtained results also showed that the *P.aeruginosa* isolates which were isolated from sewage water more susceptible to phage infection of isolates from clinical cases at ($P > 0.05$).These results were agreed with those results being obtained by other studies of [14,21,29].The pH stability of phages it is important for microbiological interest, for example, their survival in the variable pH environment of gut and also for practical purposes related to the possible therapeutic uses**,**the isolated phage particles were stable and survived at pH values between 6 and 8. These results are consistent with the previous observations made by Yang *et al*.,[29]. Thermal inactivation point of phages is important parameter to be determined for their identification and classification. For example, phages with a high degree of thermo stability have better chance of survival in organic composts, in which temperature may exceed 70°C[29]. At physiological conditions, Phage 1 has the potential to infect its host within a wide temperature range, with 100% infectivity at 37^{o}C. Therefore, the isolated two phage particles have a potential to be used as a biological disinfectant to control *P.aeruginosa* in the environment. These results were agreed with results being reported by [27,29,30]. The wide host spectrum of Phage 1 suggests its polyvalent nature and signifies its importance to be used as universal anti-pseudomonas agent. The host range of a phage depends on its own genetic information as well as that of the host, including phage receptors, various cytosolic proteins and defense mechanisms[31]. These results may indicate to the cell surface variation amongst the different clinical bacterial isolates tested in this study. Some bacterial isolates may not make the receptor, or may make a receptor that is occluded. Alternatively, some strains may have post-infection restriction or abortive infection systems [32].

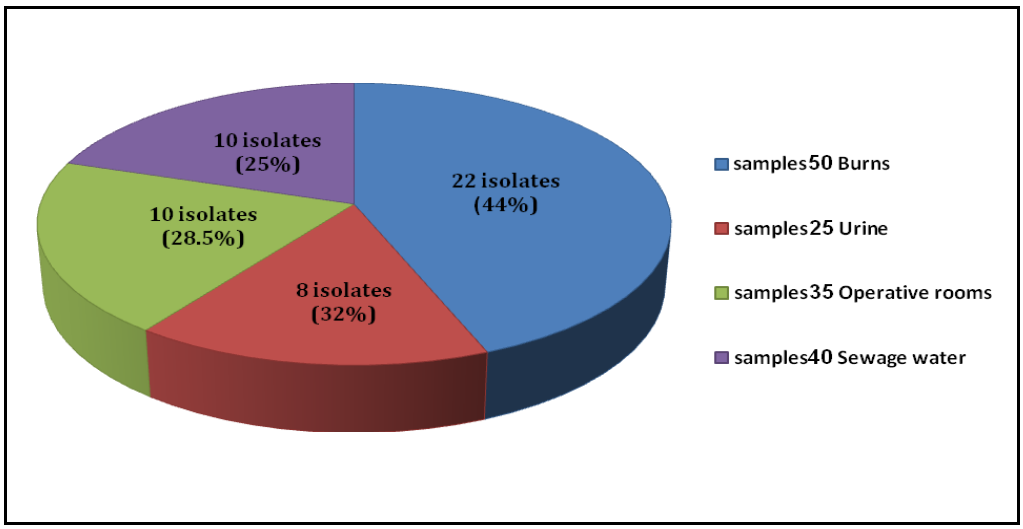

Figure (1): Isolation percentages of *P.aeruginosa*

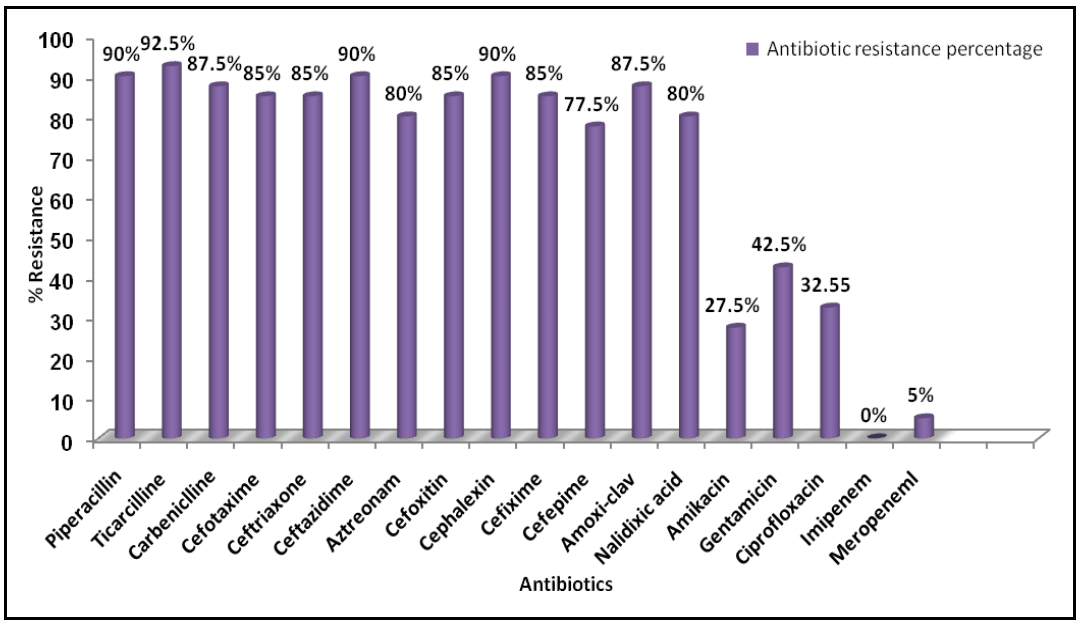

Figure (2): Antibiotic resistance profile of *P.aeruginosa*

Table (1): Characteristics of *P.aeruginosa* phages

Phage name	Plaque diameter (mm)	Number of plaques	Turbidity	Halo
Phage 1	2-5	5-20	C	+

C**:** clear ; **+:** presence of halo

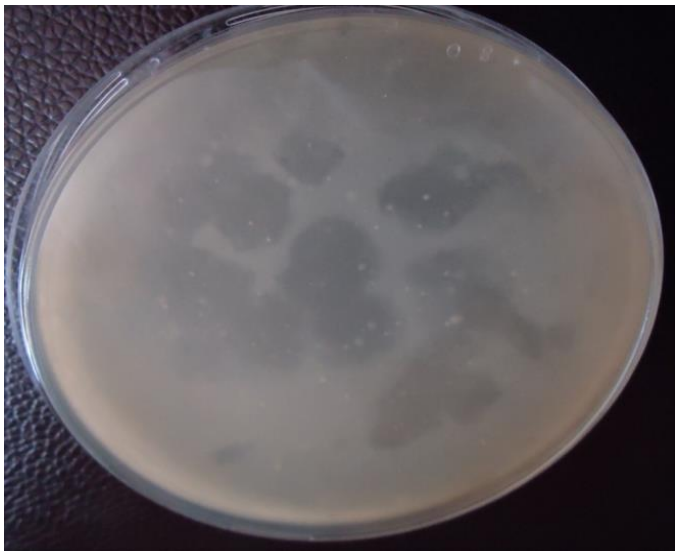

Figure (3): Plaques of *P.aeruginosa* phages on nutrient agar after 24 hr. in 37°C

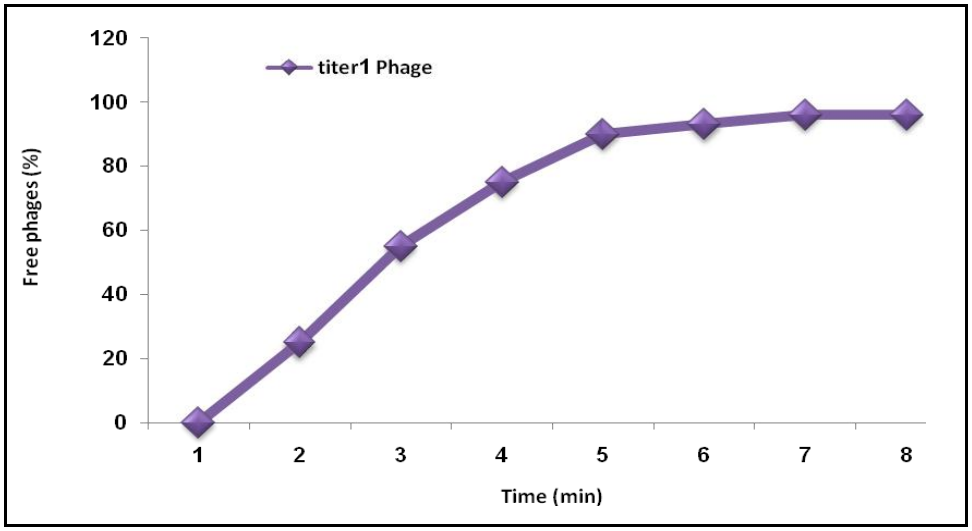

Figure (4): Adsorption rate of phage particles on bacterial cells

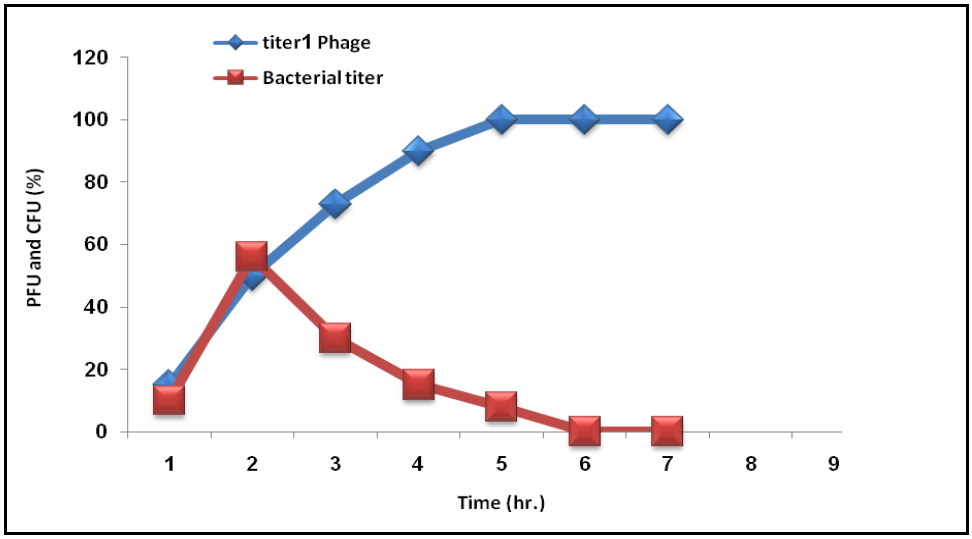

Figure (5): Lysis time of *P.aeruginosa*

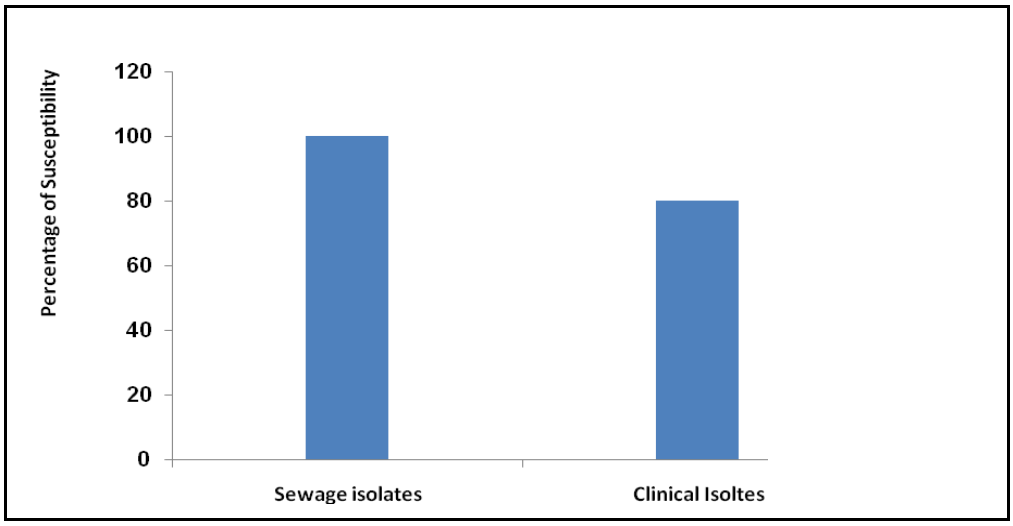

Figure (6): Susceptibility of *P.aeruginosa* isolates to phage infection

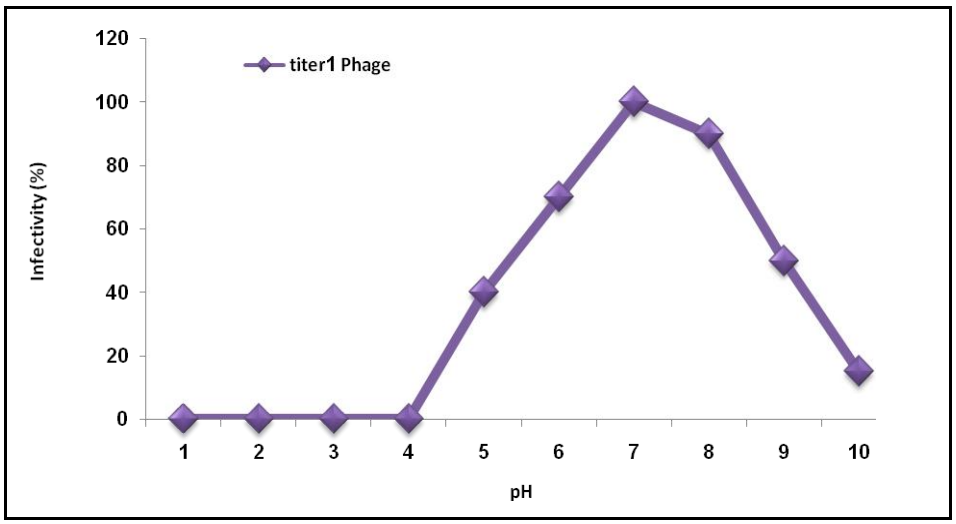

Figure (7): The effect pH on stabilityof *P.aeruginosa* phages

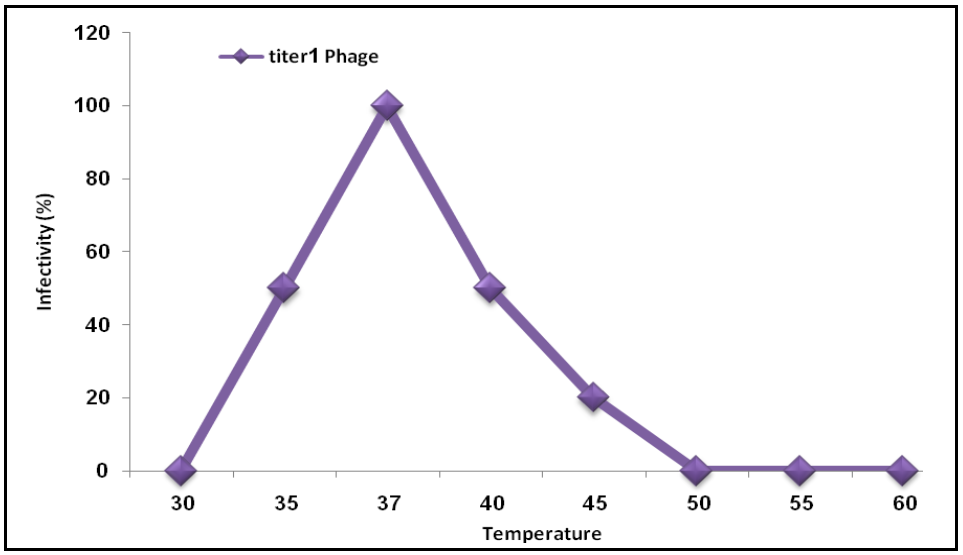

Figure (8): The effect of the temperature on stability *P.aeruginosa* phages

Table (2): Host rang of Phage1 with bacterial isolates

Bacterial isolates	Phage infection
Pseudomonas aeruginosa	+
Staphylococcus aureus	-
Escherichia coli	-
Streptococcus pyogenes	-
Klebsiellapneumoniae	-
Proteus vulgaris	-

(+) complete lysis, (-) no lysis

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