Isolation and Characterization of New *Pseudomonas aeruginosa* Phages in Al- Diwanyia City

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Abstract

This study was carried out during the period from October/ 2011 to March/ 2012.It included the isolation and identification of 50 *Ps.aeruginosa* isolates from 200 samples collected from different clinical and environmental sources and detection of their susceptibility to eighteen antibiotics. The distribution of these isolates were: 21 from 50 burn samples, 4 from 40 UTI samples,10 from 50 samples of operation rooms and 15 from 60 sample of sewage water. The obtained results showed that all *Ps.aeruginosa* isolates were sensitive to imipenem and meropenem. Five types of phages were isolated from (60) samples of sewage water according to difference in shape and diameter of the plaques primarily named (ZM_1,ZM_2,ZM_3,ZM_4,ZM_5). Results showed that the highest adsorption rate of isolated phage on bacterial cells was about 3-5 minutes, phage particles have an eclipse time about 7.5 ±0.5 minutes. The maximum lysis activity of phages was documented in about two hours, while complete lysis time was about 5 hours. The calculated data showed that the burst size was about 125 ±18 pfu\cell in time period about 30 ±5 minutes. The results also showed that the *Ps.aeruginosa* isolates from sewage were more susceptibleto phage infection than isolates from clinical cases. The isolated phage arrived the maximum activity in the neutral medium between 6.5-8.5. This activity declined after pH 9. In 45Cº the phage activity was not affected but it decreased continuously when temperature increased 50Cº. In 70Cº the activity dramatically reduced and diminished in about 8-10 minutes. Scanning Electron Microscope results showed the phage particles possessing icosahederal head and diameter ranging between (51-74nm) with no tail. According to International Committee on Taxonomy of viruses, the phage (ZM_1) was assigned to the family *Corticoviridae*.

1-Introduction

*Ps.aeruginosa*is an emerging nosocomial pathogen worldwide with increasing prevalence of multi-drug resistance. *Ps.aeruginosa* exists widely in natural environment, especially in health care settings, and has been shown difficult to be eradicated(1, 2).Bacteriophages are viruses that infect bacteria. They are obligate parasites, replicating within the bacterial cell. A phage particle consists of a nucleic acid, DNA or RNA, which is enclosed in a protein or lipoprotein coat. The envelope itself protects the genetic information and is involved in the adsorption to the host (3). These viral particles can be found in the natural environment such as water, soil and air (4). During the last decades, it has become widely accepted that bacterial viruses or bacteriophages

are extremely abundant and exert enormous influences on the biosphere. Phages kill between 4–50% of the bacteria produced everyday, are a driver of global geochemical cycles and a reservoir of the greatest genetic diversity on Earth (5).The International Committee of the Taxonomy on Viruses (ICTV) used virion morphology and nucleic acid composition as a basis for the classification of phages. Recently, new phage classification schemes based upon protein similarities have been developed for complementing the traditional classification (6). Phages and phage derived products such as vectors, cosmids and promotors were important for the development of recombinant DNA techniques. Other phage derived products like integrases, DNA ligases and various other enzymes are an integral part of today's molecular biology. Phage-derived proteins are currently being used as diagnostic agents, therapeutic agents and for drug discovery (7). One of the possible replacements for antibiotics is the use of bacteriophages or simply phages as antimicrobial agents. Phage therapy involves the use of lytic phages for the treatment of bacterial infections, especially those caused by antibiotic resistant bacteria. In general, there are two major types of phages, lytic and lysogenic (4). This work aimed to isolate the lytic phages of *Ps.aeruginosa*and determine of phage kinetics (Adsorption rate, Eclipse period, Lysis time, Burst size and Latent Period, Fitness).

2- Materials and Methods:

2-1-Bacterial strain and growth media:

Ps.aeruginosa strains were isolated from 200 clinical and environmental samples, this bacterium was stored in 60% glycerol at -80^{o}C and when necessary, maintained on nutrient agar slants at 4^{o}C and all the isolates were identified according to (8).

2-2-Antibiotic Susceptibility Testing:

The antibiotic susceptibility of *Ps.aeruginosa* isolates were determined by disk diffusion method (9).Results were recorded by measuring the inhibition zone (in millimetres) and interpreted according to Clinical and Laboratory Standards Institute documents CLSI, (10).

2-3-Phage Isolation and Propagation:

Samples of sewage water were collected from different environmental sourcesin 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 *Ps.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 (11).

2-4-Plaque assay and Spot test:

To detect the presence of phages in supernatant, spot test was done as described by (11, 12), 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 ofdiluted 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(13).The numbers of plaques were counted after incubating the plates overnight at 37^{o}C.

2-5-Phage Propagation and Purification:

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 (13).

2-6- Phage kinetics:

2-6-1-Adsorption Rate:

Adsorption assay involved adding 10^6 pfu/ml from a fresh phage lysate to cells suspended in Luria-Bertani (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) (14).

2-6-2- Eclipse Period:

Eclipse time assay involved adding 10^7 pfu/ml from a fresh phage lysateto cells grown 1h. to10^8 cfu/ml; 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 modelled adsorption, eclipse, and a linear accumulation of phage after eclipse over time (14).

2-6-3- Lysis Time:

The 5×10^7 pfu/ml were added to 10-mL of cells at 10^8cfu/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 (14,15).

2-6-4-Burst Size and Latent Period:

For burst size assays,10^6pfu/ml was added to suspensions of exponentially growing cells in flasks (10^8 cfu/ml). The mixture was diluted 1000-fold after 5 min to curtail further adsorption.

At 5.5 and 6.5 min phages were titered 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. 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 (14).

2-6-5-Fitness:

Fitness was determined by passage at low phage/cell ratios (not exceeding 0.1 by the end of the transfer) across four to five consecutive transfers, the rate of increase in phage titers were measured from the end of the first or second passage to the end of the last passage (14).

2-6-6-Susceptibility of Bacterial Isolates to Phage Infection:

At this test the routine test dilution RTD (the highest dilution that just failed to give confluent lysis) the titer of the phage was 10^3pfu/ml. single colonies of test strains were inoculated into 3 ml of nutrient broth and incubated at 37°C for 4 hours. A lawn was made with this culture on a well-dried nutrient agar plate. The phage at the RTD was spotted (0.05 ml each) onto the lawn, and the plate was incubated at 37°C for 20 hours. The presence or absence of lysis was recorded (15).

2-7-pH and thermal stability testing:

Briefly, certain amount ofphage particles were treated under specified conditions. Samples were taken at different time intervals and supernatantsfrom centrifugation were used directly in theassays. Initial phage concentration was about 3.5×10^{10} PFU/ml in LB medium (15).

2-9- Electron Microscopy:

Observation of phage morphology was carried byscanning electron microscopy of and performed as described by (13), 10^8 pfu/ml were sedimented for 60 min at 25, 000 rpm. The supernatant was discarded and replaced by ammonium acetate solution, Phages were sedimented again at for 60min at 25, 000 rpm and a drop of phage suspension was deposited on a grid. After 5 min, the phage particles were stained with 2% (w/v) potassium Phosphotungstate (pH 6.8-7.2) for 10 s. The grids were allowed to dry for 20 min and examined under a scanning electron microscope at 60Kv.

3- Results:

3-1-Isolation and Identification of *Ps.aeruginosa* Isolates:

In the present study fifteenisolate of *Ps.aeruginosa* were isolated from (60) samples of sewage water that were collected from different environmental sources, these isolates were used as hostsfor primary isolation of phages; Also thirty five isolate of *Ps.aeruginosa* were isolated from 140 clinical (for both sexes of different ages who referred to Al-Diwanyia Teaching Hospital and Afak General Hospital) and hospital samples. The distributions of these isolates were: 21 from 50 burn samples, 4 from 40 UTI samples,10 from 50 samples of operation rooms.

3-2-Antibiotic Susceptibility of *Ps.aeruginosa* Isolates:

The obtained results shown in table (3-1) reveal a remarkable elevation in *Ps.aeruginosa* resistance to antibiotics that used in this study in general and especially for the beta-lactam antibiotics. The obtained results showed that all *Ps.aeruginosa* isolates were sensitive to imipenem and meropenem except 2 isolates were resistant to meropenem and all isolates from urine are sensitive to imipenem, meropenem, amikacin, ciprofloxacin.

Table (3-1): Results of antibiotic susceptibility of *Ps.aeruginosa*

Antibiotic disk	Resistance according to site of infection			Total resistance isolated bacterial From 35 isolated
	21 Burns	4 Urine	10 Operation rooms	
Piperacillin	20(95%)	3(75%)	10(100%)*	33(94%)
Ticarcilline	19(90.5%)	3(75%)	10(100%)*	32(91.5%)
Carbenicllline	20(95%)	3(75%)	9(90%)	30(85.5%)
Cefotaxime	20(95%)	2(50%)	9(90%)	31(88.5%)
Ceftriaxone	19(90.5%)	3(75%)	9(90%)	31(88.5%)
Ceftazidime	20(95%)	3(75%)	10(100%)*	33(94%)
Aztreonam	19(90.5%)	3(75%)	9(90%)	31(88.5%)
Cefoxitin	18(85.7%)	3(75%)	9(90%)	30(86.2%)
Cephalexin	20(95%)	3(75%)	10(100%)*	33(94%)
Cefixime	19(90.5%)	2(50%)	9(90%)	30(85.5%)
Cefepime	19(90.5%)	3(75%)	9(90%)	31(88.5%)
Amoxi-clav	20(95%)	2(50%)	10(100%)*	32(91.5%)
Nalidixic acid	20(95%)	2(50%)	9(90%)	31(88.5%)
Amikacin	8(22.8%)	0(0%)**	7(70%)	15(42.8%)
Gentamicin	12(34%)	1(0%)	8(80%)	21(60%)
Ciprofloxacin	10(47.6%)	0(0%)**	8(80%)	18(51.4%)
Imipenem	0(0%)**	0(0%)**	0(0%)**	0(0%)**
Meropenem	0(0%)**	0(0%)**	2(20%)	2(5.7%)

*: all isolates were resistant for this antibiotic;**: all isolates were sensitive for this antibiotic.

3-3-Isolation of*Ps.aeruginosa* Phages:

Five types of phages were isolated from (60) sample of sewage water primarily named (ZM_1,ZM_2,ZM_3,ZM_4,ZM_5) by using fifteen *Ps.aeruginosa* isolates that were isolated from sewage water and the phage effectively were tested against 35 clinical strains by using plaque assay method. Characteristics of these phages were determined by the plaques appearance, diameter, turbidity and the presence of a halo. Infection of *Ps.aeruginosa* by phage exhibited the clear plaques on the surface of nutrient agar. From plaque assay, all the *Ps.aeruginosa* isolates were susceptible to phage lytic infection, Plaques on agar plates revealed a big hollow zone with slightly wrinkled margin. plaque diameter of phage infection were various in range , small plaques (SP) were diameter (1-4 mm), large plaques (LP) were diameter (6-10 mm) as show in table (3-2). ZM_1 was chosen to study its different characteristics.

Table (3-2): Number and size of plaques

Phage name	Plaque diameter (mm)	Number of plaque	Turbidity	Halo
ZM_1	9-10	14	C	+
ZM_2	6-8	32	C	+
ZM_3	2-4	60	C	+
ZM_4	1-3	90	C	-
ZM_5	1-2	100	C	-

C: clear;**+:** presence of halo; **-:** no visible halo

As in figure (3-1)(A,B) that showed plagues of *Ps.aeruginosa* phages

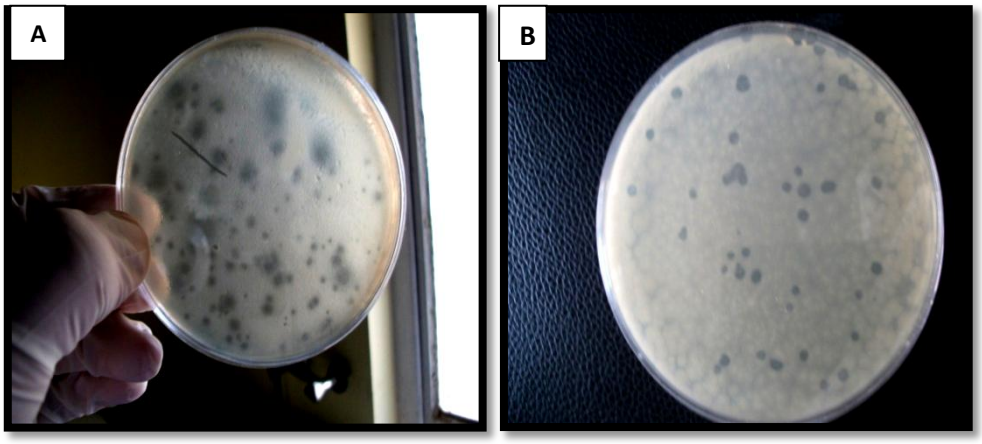


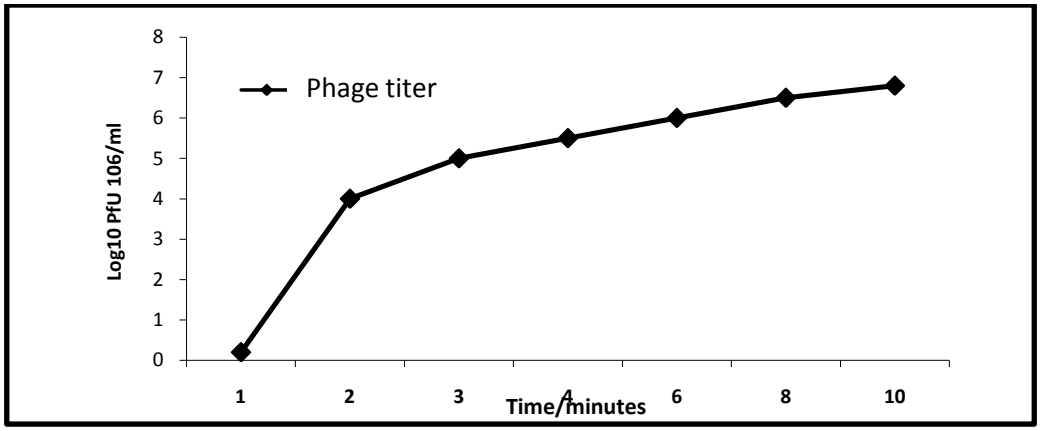
Figure (3-1)(A,B): plaques of *Ps.aeruginosa* phages on nutrient agar after 24 hr. in 37°C, LP:

3-4- Phage kinetics:

3-4-1- Adsorption Rate:

The obtained results showed that thephage particles are able to achieved the higher level of adsorption at a period of time between (3-5) minutes and the increment of time have significant

effects (P > 0.05) of increasing the number of phage particles that adsorbed on the surface of bacterial cells as in figure (3-2).



Figure(3-2): Adsorption rate of phage particles on bacterial cells

3-4-2- Eclipse Period:

The calculated data explained that thephage particles have an eclipse time about:7.5 ±0.5 minutes.

3-4-3-Lysis Time:

The present study showed that thephage particles recorded highly significant ability (P > 0.05) to cause lysis of the bacterial cells and these effects reached to the higher rate after two hours of mixing of phage particles with bacterial cells, also showed that there are dramatic decrease in number of bacterial cells that correlated with time. Complete lysis time was about 5 hours as in figure (3-3).

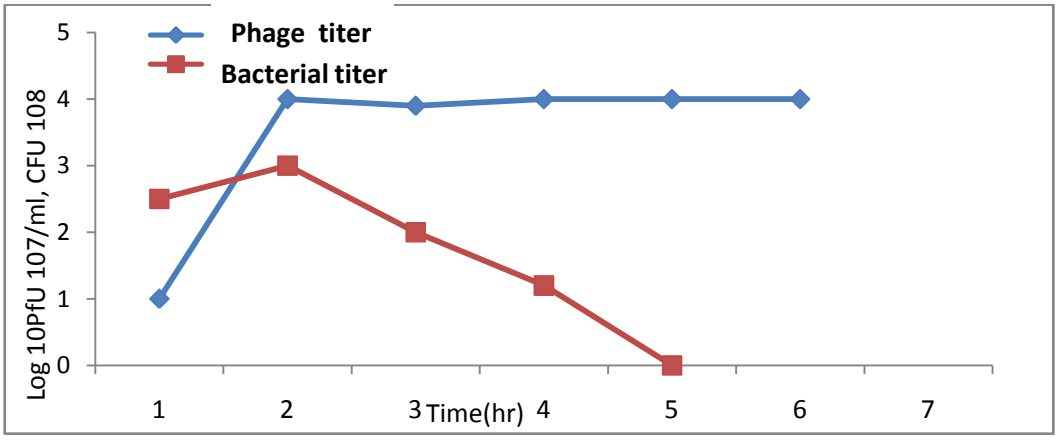


Figure (3-3): Lysis time of bacterial cells

3-4-4-Burst Size and Latent Period:

The calculated data showed the burst size about:125 ±18 pfu\cell. In time period about:30 ±5 minutes.

3-4-5-Phage Fitness:

The calculated data showed that the phage fitness(the rate of increase in phage titers) about: 20 ±4 pfu\cellthrough three generations.

3-4-6-Susceptibility of Bacterial Isolates to Phage Infection:

The obtained results showed that the *Ps.aeruginosa* isolates which were isolated from sewage water were more susceptibleto phage infection than isolates from clinical cases at (P > 0.05) as in figure (3-4).

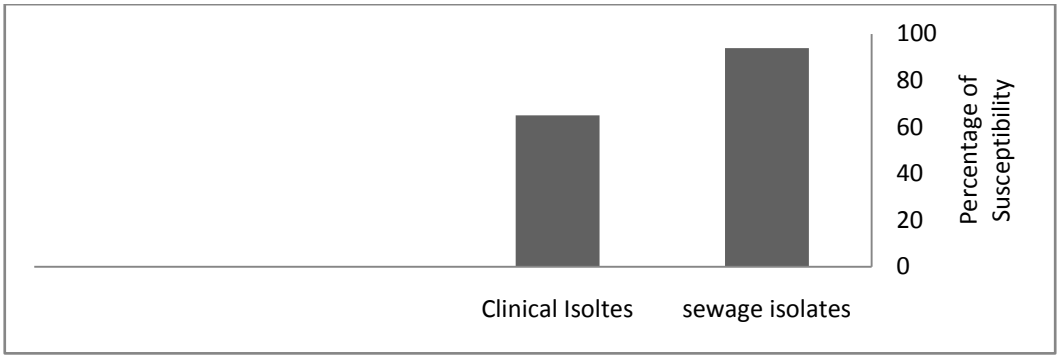


Figure (3-4):Susceptibility of bacterial isolates to phage infection

3-4-7- pH Stability:

The obtained data showed that the pH values have a significant effect (P > 0.05) on the effectiveness of phage particles and these effectively reached the highest level at pH values between 6.5-8.5 and at pH > 9 the effectively start in decline dramatically , in about pH < 4.5 the effectively diminishedas in figure (3-5).

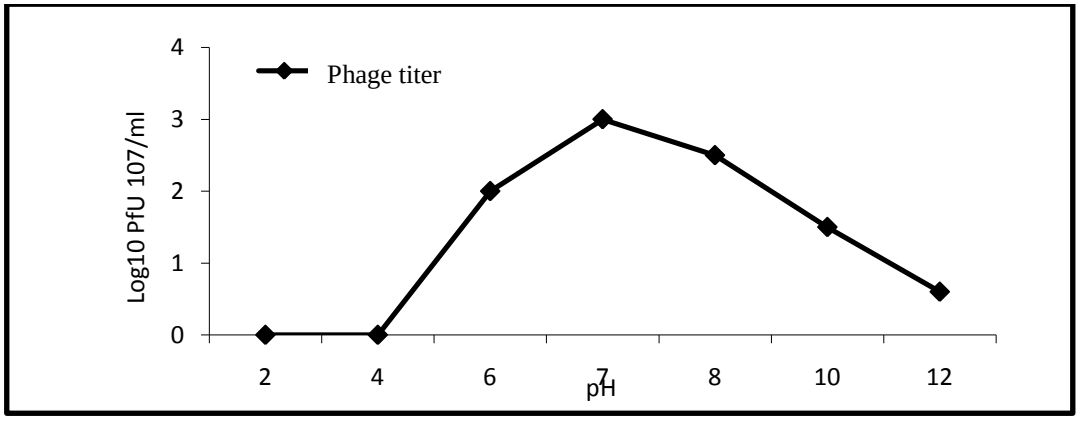
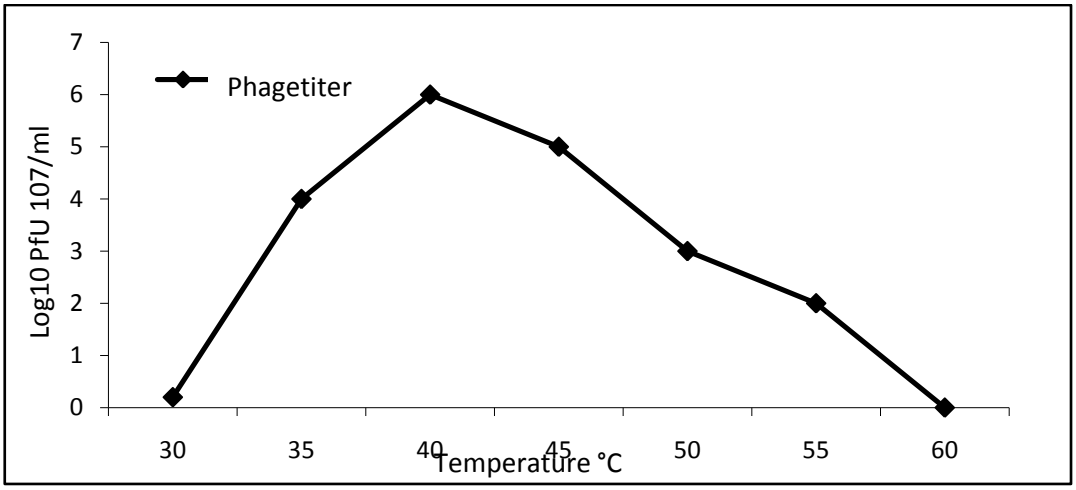


Figure (3-5): The effect pH values on phage effectiveness

3-4-8- Thermal Stability:

The obtained results showed that the effectiveness of phage particles reach their highest level in the temperature between (35-45°C). The temperature values have significant effect (P > 0.05)

on the effectiveness of phage particles which decreased in high temperature. The phage particles effectively inhibitedat 60°C after (8-10) minutesas in figure (3-6).



Figure(3-6): The effect of the temperature on phage effectiveness

3-6- Electron Micrograph:

Purified phage particles (ZM_1) possessed icosahederal head and diameter ranged between (51-74nm) as in figure (3-7). According to International Committee on Taxonomy of viruses, this phage was assigned to family *Corticoviridae*.

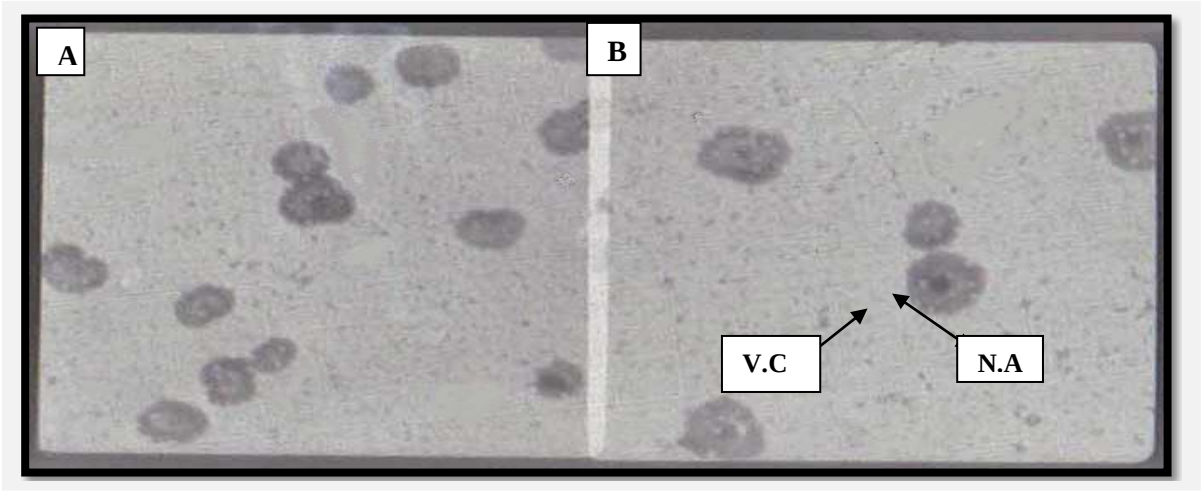


Figure (3-7): Electron microscope image of negative stained phage particles: A: 10.500 X , B: 13.500 X , V.C: viral capsid , N.A: viral nucleic acid.

4-Discussion

Ps.aeruginosa causes a broad spectrum of infections which are associated with urinary, respiratory and gastrointestinal tract, burn, wound, eyes, ears, as well as with other sites (16). Prolonged antimicrobial use as treatment for pseudomonic infections is seemed to be risk factor for infection with *Ps.aeruginosa* especially multidrugs–resistant *Ps.aeruginosa* (MDRPA) (17). The obtained results from this study shown in table (3-2) reveal a remarkable elevation in pseudomonal resistance to the beta-lactam antibiotics represented by penicillins.Bacteriophages are ubiquitous in nature and likely to be present in environments with high densities of

metabolically active bacteria (12). Phages are generally isolated from environments that are habitats for the respective host bacteria e.g. sewage, soil, water (18). In the present study, five bacteriophages specific to *Ps.aeruginosa* were isolated from (60) samples of sewage water from different sources and locations. The ability of phage to increase in number during the infectious process makes phages excellent potential diagnostic and therapeutic agents for fighting bacterial diseases (19). According to (20) a big clear plaque is a characteristic of virulent (lytic) phages (12).Thephage particles are able to achieved the higher level of adsorption at a period of time between (3-5) minutes**.** Thephage 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 5.Rate of adsorption and lysis timewere 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 (21). The calculated data showed the burst size wasabout 125±18 pfu\cell, in a time period about30 ±5 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 (22,23). The obtained results also showed that the *Ps.aeruginosa* isolateswhichwere 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 (14,15,25,26). 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.Obtained data showed that the pH values have a significant effect ($P > 0.05$) on the effectiveness of phage particles and these effectively reached the highest level at pH values between 6.5-8.5 and at $pH > 9$ the effectively start in decline dramatically , in about $pH < 4.5$ the effectively diminishedThis results was accordant with results being pointed by (25,26,27).Thermal inactivation points of phages are important parameters 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 (27).Morphological characteristics seen under an electron microscope are considered important in phage taxonomy and 96% of all phages investigated in the last 45 years have turned out to be members of the *Siphoviridae*, the *Myoviridae*, or the *Podoviridae* (24).Purified phage particles possessed icosahederal head and diameter ranged between (51-74nm)According to International Committee on Taxonomy of viruses, the phage (ZM_1) was assigned to family *Corticoviridae*(24). Kumari*et al*., (12) were isolated and examined five phages by electron microscopy of negatively stained preparations. All these phages possessed icosahederal heads and very short non contractile tails.

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عزل ودراسة عاثيات الزوائف الزنجاريه في مدينه الديوانية

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الخلاصة:

أجريت هذه الدراسة في الفترة من تشرين الأول 2011 ولغاية آذار 2012 والتي تضمنت عزل وتشخيص 50 عزله لبكتريا الزوائف الزنجاريه من مجموع 200 عينه والتي جمعت من حالات سريريه ومصادر بيئيه مختلفة بواقع (21) من أصل 50 مسحه حالات الحروق، (4) من أصل 40 عينه إدرار، 10 من أصل 50 مسحه من صالات العمليات، 15 من أصل 60 عينه ماء مجاري. تم اختبار فعالية (18) مضادا حيويا اتجاه العزلات البكتيرية لتحديد مدى مقاومتها لها.أظهرت النتائج أن جميع عزلات الزوائف الزنجاريه كانت مقاومه اتجاه Imipenem و Meropenem باستثناء عزلتين كانت مقاومه Meropenem.

خلال هذه الدراسة تم عزل خمسه أنواع مختلفة من العاثيات من مجموع 60 عينه من مياه المجاري وبالاعتماد على الاختلاف في شكل وقطر ألثغره سميت بصوره أوليه (ZM_1,ZM_2,ZM_3,ZM_4,ZM_5)، وتم اختيار ZM_1لدراسة خصائصه المختلفة.

بينت النتائج التي تم الحصول عليها بان جزيئات العاثي البكتيري استطاعت تحقيق أعلى مستوى للامتزاز في وقت بلغ أقصاه بين 3-5 دقيقة وتمتلك فتره سكون حوالي 7.5 ± 0.5 دقيقه، أن جزيئات العاثي المدروس كانت ذات فعالية عالية عند اختبارها في حل الخلايا البكتيرية، وان الفعالية بلغت أقصاها بحدود الساعتين من مزج جزيئات العاثي مع المزروع البكتيري بينما كان زمن التحلل الكلي 5 ساعات. بينت البيانات المحسوبة أن حجم التفجر كان حوالي 125 ± 18 (جزيئه عاثي / خليه) في زمن يقدر 30 ± 5 دقيقه .كما أظهرت النتائج بان عزلات الزوائف الزنجاريه المعزولة من مياه المجاري كانت أكثر حساسية للاصابه بالعاثي من العزلات المعزولة من الحالات ألسريريه. أن فعالية جزيئات العاثي تبلغ أعلى مستوى للفعالية في الوسط المتعادل بين 6.5 و8.5 وبعد 9 تبدأ هذه الفعالية بالانخفاض. كما أن فعاليه جزيئات العاثي لم تتغير كثيراً عندما تكون درجات الحرارة بحدود 45مº في حين لوحظ انخفاض طرديمتزايد في هذه الفعالية عندما تم حضن العاثيات بدرجات حرارة 50 مº و60 مº وبلغ اكبر تأثير على فعالية العاثيات في 70 مº وكانت نقطة نهاية فعالية العاثي في درجة حرارة 70 مº بعد 8-10 دقائق.

أظهرت نتائج التصوير بالمجهر الالكتروني الماسح أن جزيئه العاثي ZM_1هو من العاثيات الصغيرة ألمكعبه الشكل وغير حاوي على ذنب إذ تراوح قطره بين (51-74) نانوميتر، وحسب المنظمة العالمية لتصنيف الفيروسات وضع ضمن عائله *Corticoviridae*.