valuation of some viral kinetics of Lytic Bacteriophages Infecting Methicillin Resistant *Staphylococcus aureus* (MRSA)

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Abstract:

Background:

Bacteriophages represent important groups of viruses that drive the bacterial populations in the ecosystems directly and control their numbers, unlike antibiotics they can kill the bacterial host without damaging or any harm to the normal microbial flora, so bacterial resistance to antibiotics is no more valuable problem if the use of phages becomes more efficient in use.

Aim:

The purpose of this study was to isolate and characterizing the phages that able to infect methicillin resistant *Staphylococcus aureus* (MRSA) and determination of phages kinetics.

Methods:

Different techniques were used to isolate and propagate the MRSA phages, then phage kinetics were determined such as adsorption rate, eclipse period, burst size , phage host range, phage sensitivity to pH and temperature and the determination of phage nucleic acids.

Results:

Two types of anti-staphylococcal lytic bacteriophages primarily named Phage 1 and Phage 2 were isolated from sewage water. Phage 1 exhibited broad host spectrum it was lytic against all *S. aureu*s isolates included for the study. Conversely, phage 2 have narrow host range as they could inhibit only 30 isolates, None of these phages inhibited a bacterium other than staphylococci species. The adsorption rate of Phage 1 was 2.1×10^{-10} ml min^{-1} while Phage 2 was 1.5×10^{-10} ml min^{-1}. Eclipse and Latent periods of Phage 1 were 6, 13 minutes and for phage 2 were 8, 16 minutes respectively, the burst size of Phage 1 and Phage 2 were 83±15 pfu\cell and 37±10 pfu\cell respectively. Complete lysis time of Phage 1 particles was 4 hours while the complete lysis time of Phage 2 particles was 5 hours. Phages passaging results showed a remarkable increment in plaque forming unit (pfu) which were reached its maximal elevation at six passage in Phage 1 and fourth passage in Phage 2. Fitness of Phage 1 and Phage 2 were 22 ± 5 pfu\cell and 20 ± 2 pfu\cell respectively through six generations. The Phage 1 and Phage 2 particles were stabled at a wide range of pH (6-10) and temperatures (30-50°C), the optimal temperature of two phages were 37°C.

Conclusion:

Overall findings suggested that MRSA phages are well distributed in the environment and represent a good and promising tool to control the infections result by this bacteria in which antibiotic resistance will be overwhelmed successfully especially in external infections such as burns and skins infections, which are safe and very specific with less side effects than antibiotic.

Key words: MRSA phages, phage kinetics

Introduction

Opportunistic pathogens represent a serious problem especially in hospitals (nosocomial infections), one of the most frequent bacterial causes is the *Staphylococcus aureus* , this bacteria can cause different infections, some of these infections are simple such as skin boils and others represent dangerous ones such as pneumonia, septicemia, meningitis and endocarditis(1). One of the most remarkable hazard of this bacteria; is the development of antibiotic resistance which resulted in the emergence of many strains which can resist wide range of antibiotics (multidrug resistance)(2,3). This phenomenon increased the risk of the infections caused by this bacterium. The hallmark point in the development of resistance is the emergence of methicillin resistant *Staphylococcus aureus* (MRSA)(4). This medical complication drag back the pharmaceutical companies in continues need to find and discover new effective drugs. Some of investigators start to looking for extra-ordinary therapies one of which is bacteriophages(5).

Bacteriophages represent important groups of viruses that drive the bacterial populations in the ecosystems directly and control their numbers(6). More than 95% of phages belong to the group *caudoviralis* which had the typical tailed shape. Estimations indicated about 10^{31} phage particle on the earth and about 10^{25} successful infection in each second (7,8). International committee on viral taxonomy (ICVT) had grouped phages in about 12 distinct group depending on the type of the nucleic acid, shape and the bacterial host(9).

Phages naturally found in aquatic and terrestrial and biological ecosystems they kill about 4-50% of bacterial population every day. They use the bacterial components to reproduce there numbers rapidly and the released to infect another bacteria specifically (9,10). From 1917 the date of the first phage documentation, large and diverse number of phages were isolated and studied, these phages were able to infect specific genera or species of bacteria on the bases of the presence of specific receptors on the host cell(9).

Due to the highly specificity of infection that used by phages, unlike antibiotics they can kill the bacterial host without damaging or any harm to the normal microbial flora, so bacterial resistance to antibiotics is no more valuable problem if the use of phages become more efficient in use(11). The use of phages to control the bacterial infections was carried due to the natural source of the phages and highly effective nontoxic action (12,13).

The purpose of this study was to isolate and characterizing the phages that able to infect methicillin resistant *Staphylococcus aureus* (MRSA) and determination of phages kinetics.

Materials and methods:

A total of 96 different clinical specimens were collected from hospitals and patients of different age groups who were attended to the general teaching and pediatrics and maternity hospitals of Al-Diwanyia city/Iraq during the period from October 2015 to march 2016.

Isolation and Identification

Swabs were transported in semiliquid medium, then specimens were streaked on Mannitol salt agar (14), the samples which can ferment the media were transported to CHROM agar then finally conformation of the identification done by using VITEK2-compact.

Antibiogram assay:

The sensitivity towards 19 type of antibiotics were carried out to the isolated bacteria *S. aureus* by using disk diffusion assay according to CLSI (16).

Phage isolation and propagation:

Sewage samples were collected from different environmental places and were centrifuged 10000rpm, then phages isolation was done by filtration through Millipore filter paper 0.45µ, filtrated phages were propagated by adding the filtrate to overnight *S.aureus* culture at 37Cº. After incubation for 24 hour the bacteria were removed by centrifugation and the filtrate was checked for the presence of the phages (17).

The plaque assay and the spot test:

The detection of the presence of phages in the supernatant was done by using spot assay which was described by Chang *et, al,* (18).

While the titer of phages was checked by the plaque assay using double layer ager (17).

Phage purification :

Purification of phage was carried out by using successive single-plaque isolation to homogenous plaque by direct picking up to the plaque and propagation of this plaque again. (19).

Phage kinetics :

Most phage kinetics were calculated such as adsorption rate, passage, fitness, eclipse period, lysis time, burst size, latent period, optimum temperature, pH effects, and host range Chang *et al.* (18).

Passage :

This test was carried to evaluate the ability of phages to multiplication through many generation of phage progeny (20).

Phage adsorption rate:

The ability of phage particles to adsorb successfully on the host cell was measured by using the equation $N_{free} = N_{total}\ e^{-5}$, were $N_{total}\ e^{-5}$ is the total phage number which added to the broth 10^6 pfu/ml and after five minutes free phage particles N_{free} represent the un adsorbed(20).

Eclipse period assay:

This test was done to calculate the time required by the phage after entry until the release of the new phage progeny (20).

Lysis time :

Lysis time of the bacterial host and the changes of phage titer over the time of the experiment was determined depending on (20,21).

Burst size and the latent period :

Burst size (the phage progeny at each replicate) is the calculated titer of phage yield at a given time periods. While latent period represent the time required by phage to be released into the environment (20).

Phage fitness:

Fitness represent the monitoring the phage densities over many generation and the time required to each generation, the equation $[\log_2(N_t/N_0)]/t$, where N_t is the number of phage at time t hours, corrected for dilutions over multiple transfers (20).

Phage stability at pH variation:

MRSA phages were tested for the stability at different pH values ranged from (4-12) by using phosphate buffer saline.

Phage stability at temperature variation:

MRSA phages were tested for the stability and ability to multiplication at different temperature degrees.

Host range :

The host range of MRSA phage represent a test for the ability of this phage to infect different bacterial species other than MRSA. Bacterial isolates include :

*Pseudomonas aeruginosa***,** *Staphylococcus saprophyticus***,** *Escherichia coli, Streptococcus pyogenes, Klebsiella pneumoniae* **,** *Staphylococcus epidermidis* and *Proteus vulgaris***,** all these isolates were idnetifed by VITEK-2 compact.this test was done according to (19).

Phage DNA extraction :

The phage DNA was extracted from 200 µl of phage lysate**,** the extraction was carried by the using of wizard genomic DNA purification kit (Geneaid).

Data statistical analysis :

All statistical analysis of data were performed in one-way ANOVA by estimation of the LSD (probability level 0.05) using Graph Pad Prism version 5.04 software (22).

Results:

***S. aureus* strain identification:**

Forty one isolate of *S. aureus* isolated from 96 clinical and hospital samples, these included 31 swab from burn patients, 28 swab from wound patients , 12 sample from patients of urinary tract infections (UTI) for both gender of different ages groups, and 25 swab from operative rooms. Biochemical and morphological characterization tests showed that 41 (42.7%) isolates were *S. aureus*, 18(58%) isolates from burns, 12(42.8%) isolates from wound, 8(32%) isolates from operative rooms and the last 3(25%) isolates from UTI infections as in figure (1) shows the percentages of *S. aureus* according to the site of infection**.**

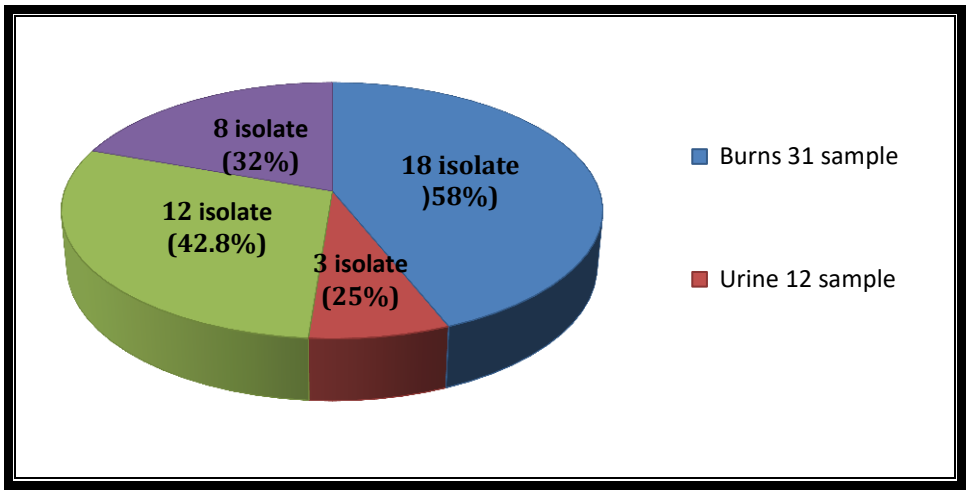
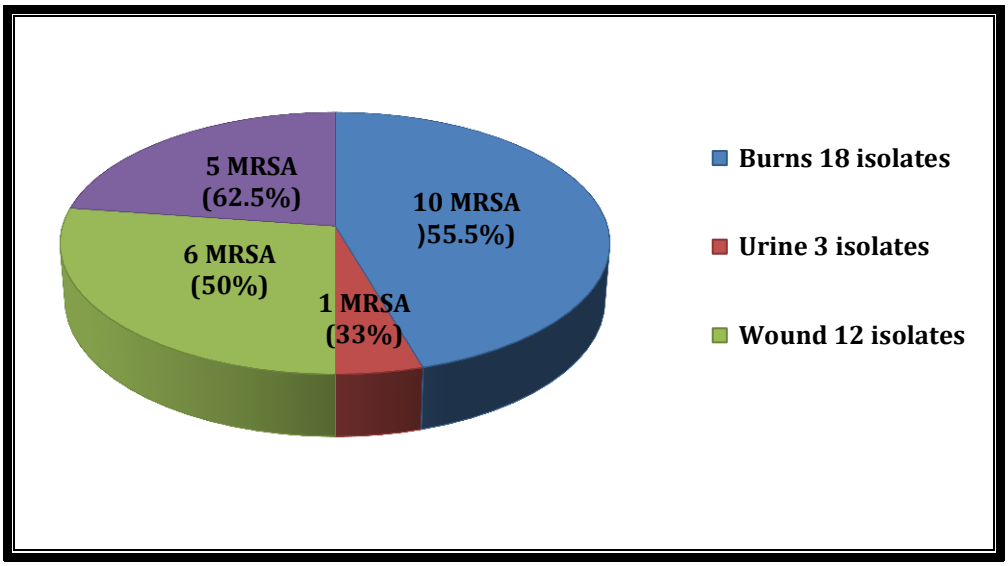


Figure (1): Isolation percentages of *S. aureus* according to the site of infection.

Detection of methicillin resistant *S. aureus* (MRSA):

Results revealed that out of 41 *S. aureus* isolates, 22(53.6 %) isolates were resistant to both of these antibiotics and 19(46.4) were sensitive to both of these antibiotics in disc diffusion test (Figure 2 a,b).



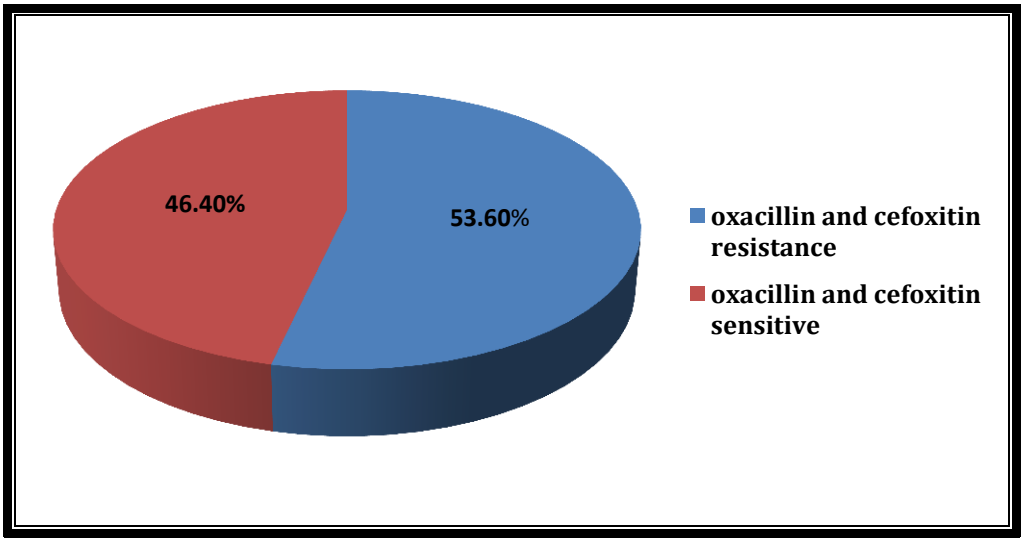


Figure (2 a,b): Percentage of Oxacillin resistance isolates of *S. aureus*

Antibiotic Susceptibility:

In this study 19 antibiotics performed to all *S. aureus* isolates for testing their susceptibility to antibiotics and identify the most effective one against *S. aureus* particularly MRSA. The results revealed that all bacterial isolates showed high resistance (100%), to Penicillin G, Ampicillin and Amoxi-clav, also revealed that all *S. aureus* isolates (100%) were sensitive to Vancomycin (Figure 3).

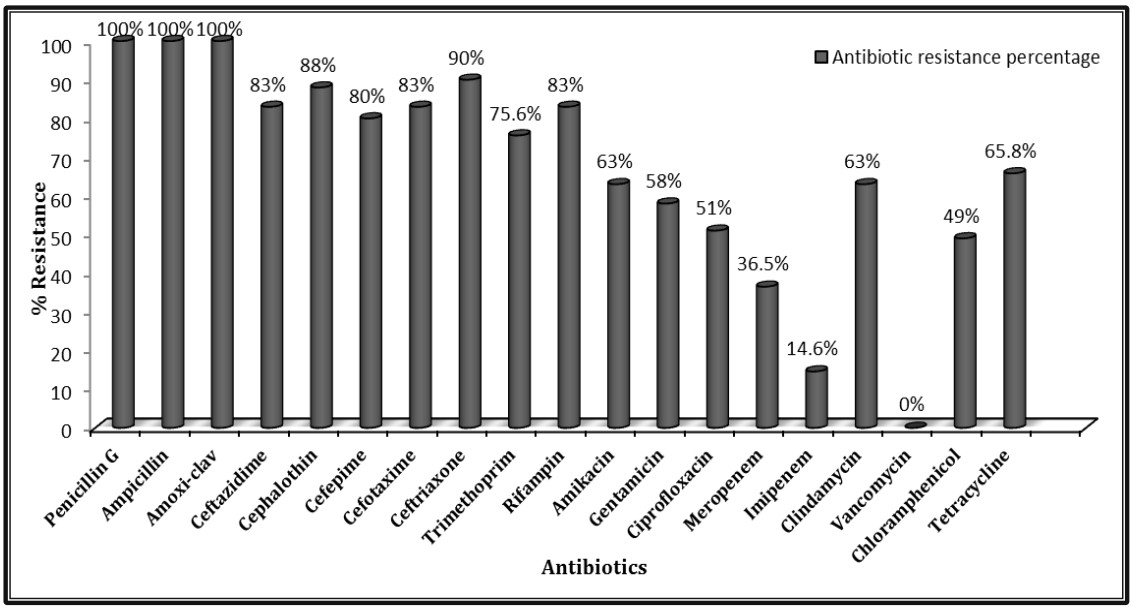


Figure (3): Antibiotic resistance profile of *S. aureus*

Isolation of MRSA Phages:

Fortunately two types of MRSA phages were obtained from 25 sample of sewage water primarily named phage 1 and phage 2 these phages were tested against 22 MRSA isolates. Plaques appeared as big hollow zone on nutrient agar with wrinkled margin as shown in figure (4) and table (1).

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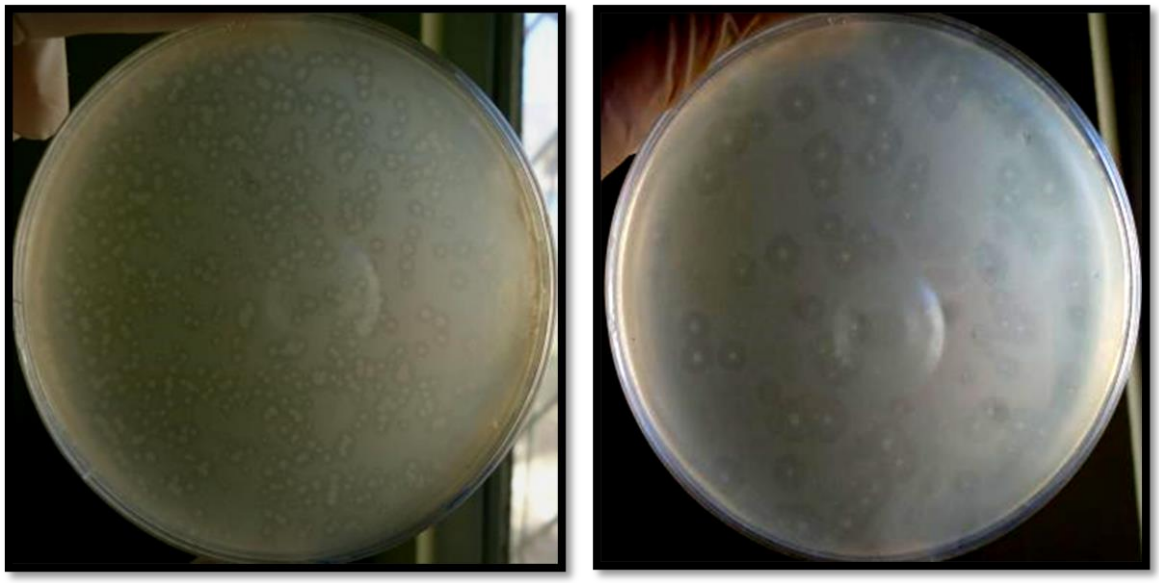
Table (1): Characteristics of MRSA phages

Phage name	Plaque diameter (mm)	Number of plaques	Turbidity	Halo
Phage 1	1-2	135	Clear	-
Phage 2	2-4	70	Clear	+



Phage kinetics:

Passages:

Phage passages were done to propagate and evaluate the effect of sub culturing on phage effectively and fitness, passaging results showed a remarkable increment in PFU which were reached its maximal elevation at six passage in Phage 1 and fourth passage in Phage 2 while absorbency of tested samples showed a dramatic decreased in optical density at two phages as in table (2).

Table (32): Passages and PFU of MRSA phages

*Passages	Phage 1		Phage 2	
	Optical density (650 nm)	PFU	Optical density (650 nm)	PFU
Passage 1	0.820	30	0.760	20
Passage 2	0.550	50	0.602	48
Passage 3	0.320	78	0.480	61
Passage 4	0.167	108	0.221	80

Passage 5	0.96	126	0.215	80
Passage 6	0.10	144	0.215	80

* Each passage represents 5 replicate

Adsorption Rate:

The two isolated phages particles showed higher level of adsorption at a period of time (4-6) minutes and which is positively correlated with time ($P > 0.05$) while dilution and shaking reduce adsorption as in figure (5). The adsorption rate of Phage 1 was 2.1×10^{-10} ml min^{-1} while Phage 2 was 1.5×10^{-10} ml min^{-1}.

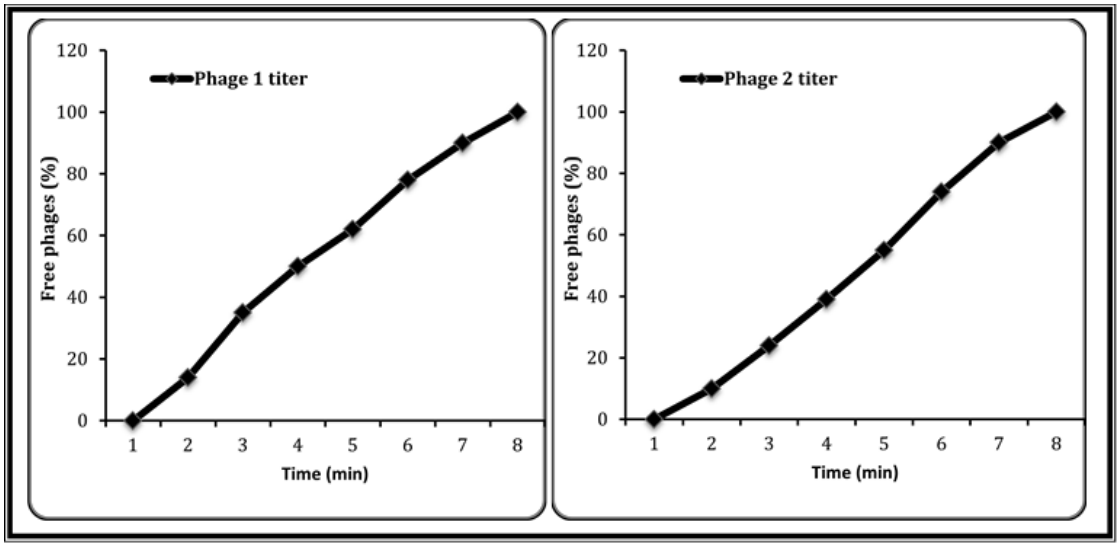


Figure (5): Adsorption rate of phage particles on bacterial cells

Eclipse Period:

Eclipse period of the obtained phages were :

Phage 1= 6 minutes

Phage 2= 8 minutes

Complete lysis Time:

The higher value of bacterial lysis recorded after about one hour for the isolated phages which was highly significant ($P > 0.05$) and correlated positively with time, Complete lysis time of Phage 1 particles was 4 hours while the complete lysis time of Phage 2 particles was 5 hours as in figure (6).

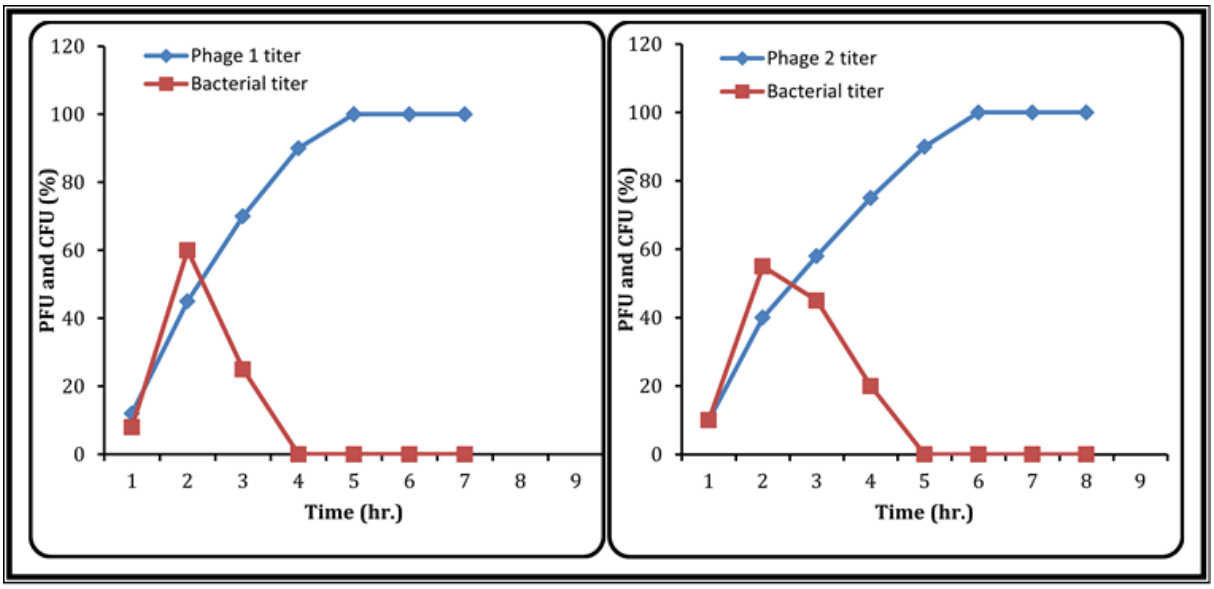


Figure (6): Complete lysis time of MRSA

Latent period and the burst size of MRSA phage

The numbers of phages were produced by an infected cell (burst size) was about:

Phage 1= 83±15 pfu\cell with time period about 13 minutes while in the

Phage 2= 37±10 pfu\cell in16 minutes.

Fitness the isolated phages:

Observations of this work indicated the fitness of the phages as follow;

Phage 1= 22 ± 5 pfu\cell through six generations

Phage 2= 20 ± 2 pfu\cell through six generations

pH Stability:

Maximum phage activity appeared near the neutral area of pH in about 7±1 and the dramatic decline in about 9 and higher also 4 and lower pH diminish the replication of the phages figure (7).

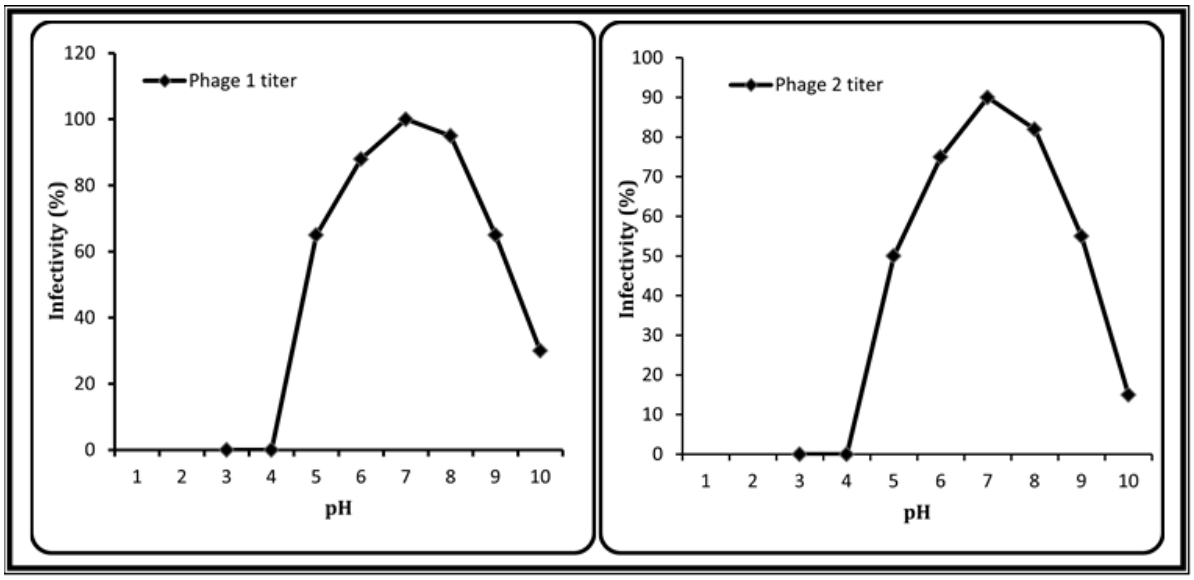


Figure (7):pH effects on MRSA phages

Thermal Stability:

Temperature degrees between (35-40°C) showed the highest effectiveness of MRSA phages (P > 0.05) this effectiveness was reduced at 50°C after (8-10) minutes as in figure (8).

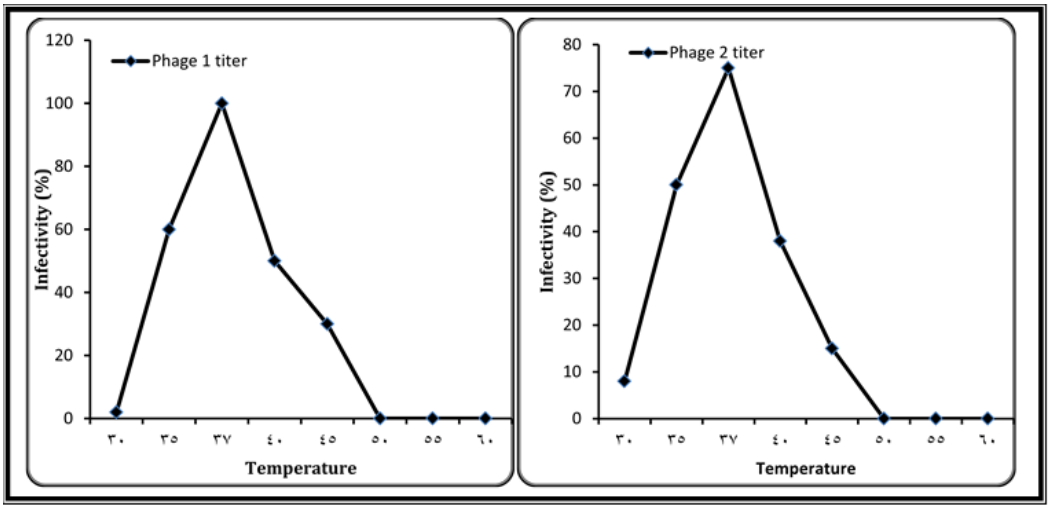


Figure (8): Temperature effects on MRSA phages

Host Range of MRSA Phages:

Both isolated MRSA phages showed remarkable activity in infecting and lysis of staphylococci species, on the other hand, none of phages can infect other species as in table (3).

Table (3): Host rang of MRSA phages with bacterial isolates

Bacterial isolates	Phage infection	
	Phage 1	Phage 2
Staphylococcus epidermidis	+	+
Staphylococcus saprophyticus	+	+
Escherichia coli	-	-
Streptococcus pyogenes	-	-
Klebsiella pneumoniae	-	-
Pseudomonas aeruginosa	-	-
Proteus vulgaris	-	-

(+) complete lysis, (-) no lysis

Phage DNA Extraction:

The results showed that the isolated two phages particles Phage 1 and Phage 2 exhibited DNA as a genetic material in single chromosome as shown in figure (9).

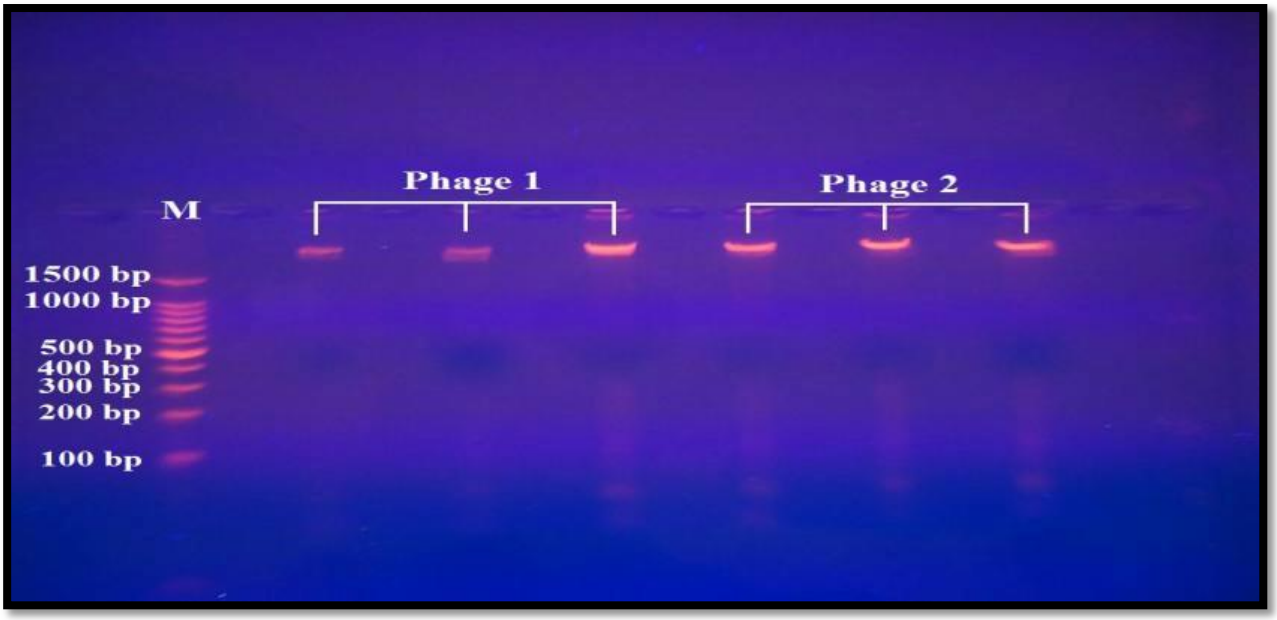


Figure (9): Phages DNA Extraction. Lane M showing 100 b.p DNA ladder; lanes 1- 6 show the DNA genome of Phage 1 and Phage 2.

Discussion:

S. aureus widely abounded in the environments that surrounds the human especially the medical centers and hospitals which indicate the clinical importance as pathogen, modern public health and medical workers have taken the minimizing this bacteria effects as priority, this priority due to the vast mechanisms that used by this bacteria to adhere many biomedical materials and infect the human and resist the most known antibiotics(23,24).

This bacteria developed many survival mechanisms against most known and used antiseptic and disinfectant preparations in addition to the most antibiotics nearly, biofilm formation of this bacteria seemed that is one of the active means in resistance and survival strategies (24,25).

All the antibiotics that used in this study (19 antibiotics) subjected to all *S. aureus* isolates to identify the most effective one against *S. aureus* particularly MRSA, because indiscriminate using of antibiotics, which may correlated with increased risk of MRSA infections (26). However, the overwhelmed *S. aureus* isolates that recovered from clinical samples were highly resistant to most antibiotics that used in the present study. This may be due to the vast and sometimes nonspecific and not necessary use of antibiotics in our daily life also the horizontal gene exchange by transformation or conjugation and sometimes by phage transduction (27,28,29).

The existence of abundant varieties and numbers of MRSA phages in the sewage water which result from human population activity and hospitals, reflect that this waste water is a rich environment to the MRSA which enter to this ecosystems through human extra activities waste (31). From another angle the high specifity of phages to infect their host and the potential lytic activity represents good hopeful start step in nonconventional and useful control technology on pathogenic bacteria, this concepts is very encouraging especially when we know that bacterial difficultly develop a successful resistance against phages. For MRSA, many researches isolated and described staphylococcus phages (32,33).

Phage adsorption represent the most important initial step in virus infection, for the phages in this study Phage 1 was 2.1×10^{-10} ml min^{-1} while Phage 2 was 1.5×10^{-10} ml min^{-1}. These values of adsorption rate are efficient in comparison to many other phages isolated previously, adsorption usually occur specifically due to presence of special receptors on the host cell or the suggesting the presence of BVPaP-3 on the tail fibers of phage which have high affinity to cellular receptors of bacteria. Such mechanism was reported in T7 phages with the adsorption rate ranging between 4.5 $\times10^{-10}$ and 8.9 $\times10^{-10}$ ml min^{-1} (33,34).

Other documented phage parameters of this work comes in conformity with the normal documented data by other investigators to most phages mainly with the value of the optimal pH which was 7±1 and the temperature of the highest lytic activity around 37Cº(35,36,37) .This findings support the hypothesis of the potential and possible usage of these phages as biotherapy which will not interfere with the normal and environmental conditions especially externally.

The vast host range of the phages that isolated in this study toward Staphylococcus species suggesting the existence of common receptors that they bind to, also the polyvalent nature of these phages indicated the genetic content that make them good and hardly resist able by bacteria also promising cloning tools.

Also the high affinity of the isolated phages as anti- Staphylococcus and the remarkable lytic activity against MRSA isolates that used in this work could be used as a safe antibacterial agents, this applications is not harmful to the normal microbial flora and its usage can reduce the undesired side effects of the traditional drugs these suggestions were supported by the finding of many investigators who found that (Phage K) is capable to control and inhibit the growth of *S. aureus* isolates also the antibacterial finding of Al-kabie to control the growth and complete healing of rats infected with *Pseudomonas aerogenosa* (34,39,40,41).

Conclusion

Bacteriophages considered a potential environmental toll that drive bacterial populations into specific behavior, mainly drive these bacteria to manage their genetic pool towards evolution. from another hand *S. aureus* phages are well distributed in the environment and represent a good and promising tool to control the infections resulted by this bacteria in which antibiotic resistance will be overwhelmed successfully especially in external infections such as burns and skins infections, which are safe and very specific with less side effects than antibiotic.

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