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# Control Of Methicillin Resistant Staphylococcus Aureus By Bacteriophage, Endolysin, Bacteriocin

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**ABSTRACT**

Foot ulcers are the main cause of hospitalization and mortality of diabetic patients over the world. Approximately 25 % of diabetic patients will suffer from wound infections during their lifetime. Resistance of bacterial pathogens to available antibiotics has become a great load to the human health care, resulting in a significant progression of morbidity and mortality. Therefore, the use of novel solutions with antimicrobial properties such as bacteriophages attracted the attention of many investigators. In the current study, twelve multidrug resistant (MDR) S.aures isolates were isolated from hospitalized diabetic foot patients and bacterial identification was done based on biochemical and molecular characteristics using 16srRNA. Bacteriophages are a potential tool to treat foot ulcer resulting from biofilm-associated infections. Phage cocktails were used to detect S. aureusinsusceptibility. An adaptation procedure was performed in cases of bacterial resistance. Bacteriophage with lytic activity against MDR S.aureus. bacteriophage are characterized physically through the evaluation of their lytic activities at a wide range of temperatures (40–90° C) and pH values (2–12) control the rate of adsorption. Transmission electron microscope (TEM) study revealed that phage belong to Podoviridae family. The present study opens a new window for the application of bacteriophages as promising antimicrobial agents against MDR bacteria.

**Keywords:** Diabetic foot ulcer, Bacterial profile, Antibiotic sensitivity, Biofilm production, Gene expression

## Introduction

Staphylococcus aureuscan cause a wide range of infections in humans. The most common sitesaffected are skin and soft tissue; manifestations of infectionsin these sites include folliculitis, furuncles and carbuncles, impetigo, mastitis, woundinfections, and staphylococcalscalded skin syndrome.More serious infections include bacteremia, pneumonia, endocarditis, bone and joint infections, and toxic shock syndrome. S. aureus can also be responsible for outbreaks of food poisoning.(1)

Staphylococcus aureusis afrequent inhabitant of the human skin flora colonizing up to 30%of individual at any timeprimarily through nasal carriagerequiring A suitable portal of entry into the

body.The skin normally provides such to progressive infection, however a breach in the skin as aresult scratch, cut or burn provides a suitable infection for opportunistic bacteria.Across all continents resulting both delayed wound healing and systemic infections(**2**)**.** The discovery of staphylococcal drug resistance and the subsequent global epidemic that (MRSA)hasattend agreat concentration the urgent treatment(**3**)**.**Only S.aureus and S.epidermis are significant in theirinteractions with humans. S. aureus colonizes mainly the nasal passages, but it may be found regularly in most other anatomical locales, including the skin, oral cavity and gastrointestinal tract. S epidermis's is an inhabitant of the skin.Bacterial infections are responsible for significant morbidity and mortality in clinical settings.Staphylococcus aureus has long been recognized as a major pathogen of hospital acquired infections. Over the last decade, MRSA (methicillin-resistant Staphylococcus aureus) strains have become endemic in hospitals worldwide. In addition, it is now incipient community pathogen in many geographical regions (4)**.**The isolation of such resistant variants of Staphylococcus aureusin particular and other multi drug resistant bacteria in general of certain concerns.This clearlyneed that there is anwwd to search for other effective protocol for treatment of staphylococcal infections baswd on phage therapy(5), Nanomaterial(6), Plant extract ,natural protein either singly or in combination with antibiotics (7). MRSA accounts for a high proportion of surgical siteinfections, being responsible for 64% of such infections in 2007/2008(8)**.** Fewer than 5% of S. aureus isolates are sensitive to penicillin, once the drug of choice for Staphylococcal infections. MRSA was first reported in the UK just two years after the introduction of methicillin in 1959. Horizontal transfer of the mecA gene, which encodes a penicillin-binding protein, results in resistance not only to methicillin, but also to broad spectrum β-lactams such as the third-generation cephalosporin's, cefamycins and carbapenems(9)**.** The proportion of MRSA isolates from blood cultures taken from cases of bacteremia in England has risen dramatically from less than 5% in 1990 to around 40% by the end of the 1990. As well as mortality rates of almost double those associated with MSSA (methicillin-sensitive S. aureus) infections, MRSA has a considerable financial burden on both hospitals and society in general (10)**.** Vancomycin has been the most reliable therapeutic agent against MRSA for the past 3 decades. However, despite its sustained in vitro microbiologic inhibitory activity, clinicians continue to debate its utility for MRSA infections (11).

Widespread empirical use of vancomycin to cover Gram-positive organisms, including MRSA, has likely contributed to the growing burden of less susceptible strains or completely resistance as VRSA (Vancomycin-resistant S. aureus), and many health care facilities have reported an upward trend of vancomycin MICs for MRSA isolates over the past 5 years(12)**.**

Recently the endolysins of the bacteriophages are highly evolved molecules that have been specifically developed by phages to quickly and efficiently allow their progeny to be released from the host bacterium. These enzymes damage the bacterial cell wall's integrity by hydrolyzing the four major bonds in its peptidoglycan component (13).

A diabetic foot ulcer is defined in the research system as a 'full-thickness' lesion of the skin, that is, a wound penetrating through the dermis; lesions such as blisters or skin mycosis are not included in this system. The term ulcer can be ambiguous in this context. In medicine, a skin ulcer is generally defined as a non-healing or poorly healing wound. Information on the duration of the ulcer is essential to define non-healing. Unfortunately, this temporal information is frequently missing in patients with a diabetic foot ulcer. due to loss of sensation and impaired vision, the duration is frequently not known. A foot ulcer is defined in the current system, according to the International Consensus on the Diabetic Foot, as a full-thickness wound below the ankle in a diabetic patient, irrespective of duration.

Skin necrosis and gangrene are also included in the current system as ulcers. Gangrene was defined in the International Consensus on the Diabetic Foot as a continuous necrosis of the skin and the underlying structures (muscle, tendon, joint or bone)

Therefore, our study designed tothe evaluate Efficiency of multi drug (endolysin phage dependent and bacteriocin) to control of alone or in cocktail S.aureus (MAR).

**Materials and Methods**

**1-Isolation and identification of S.aureus:**

A total of 60 patients were subjected to microbiological Analysis involving their available samples diabetic foot of swab in pus of injured foot. The isolation of pathogens were routinely carried out on nutrient agar, and Baired parker media (each sample was processed on the three media). In critical care of Zagazig University Hospital, Zagazig University, Egypt in period From June 2018 till December 2018. Almost 60 isolates were obtained and were kept as Slope cultures on Baired barker agar medium. All samples were taken from male and female adults Sixty bacterial isolates were clinically isolated from post-operative pus (60) of patients admitted to Zagazig University hospitals Zagazig Egypt .sterile cotton swabs pus samples, were immersed in the clinical samples and streaked aseptically onto baired barker agar plates (oxide) which selectively support the Growth of S.aureus bacteria .all plates were incubated 37c for 48h . greenish black colonies were showed single Colonies of them were picked up and purified onto Baired parker agar .Slope cultures of them on the same medium were made and stored 4 °C throughout the experiment then make susceptibility test for all 60 isolates. The specimens were cultured onto blood agar and Baired parker media (Oxoid), using the plate streaking technique . The plates were incubated at 37 °C for 48 h. The isolated bacterial colonies were chosen and picked up according to culture characteristics then purified by successive subculturing on the same medium as appropriate and stored at 4 °C till used. The bacterial isolates were identified according to their morphological using Gram staining technique. All isolates were tested for their susceptibility to different antimicrobial agents using Bauer disk diffusion method.**Fig(1)**

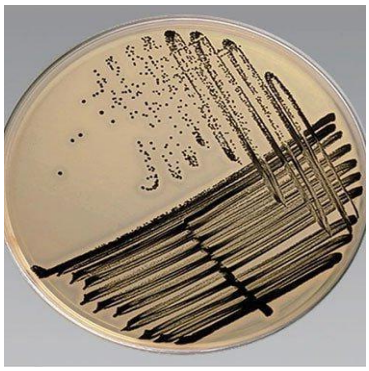


**Figure (1): Photograph of S. aureus on Baired barker media**

**Preparation and partial purification of Bacteriocin**

The bacteriocin producing bacteria P. aeruginosa TA6 was grown in Luria broth and it was incubated at 37 °C for 18 hours. The following time, the culture broth was subjected to centrifugation at 10,000 rpm for 15 min at 4 °C for separation of bacterial cells. The supernatant was filtersterilized by passing through a 0.45 µm pore sized filter membrane (Millipore, MA, USA). This CFS was further partially purified by ammonium sulfate precipitation (14)**.**To achieve the maximum saturation of bacteriocin, 70% of ammonium sulfate was added by constant agitation at 4 °C overnight. Then precipitates were recovered by centrifugation at 10,000 rpm for 30 min at 4 °C. The resulting pellets were re-suspended in 20 mM sodium phosphate buffer (pH 7.2) and were designated as `partially purified bacteriocin(15).For removal of the salt from the partially purified bacteriocin, ultra-filtration was done through a pre-treated dialysis tubing of 6-8 kDa cutoff size(14)**.**The active fractions, thus obtained, were collected and pooled for assessment of inhibitory Activity (16).The bioactivity of bacteriocin was analyzed at each point of purification by agar well diffusion assay (AWDA), in terms of AU/mL. After partial purification, protein concentration was measured (17).**Fig(2)**

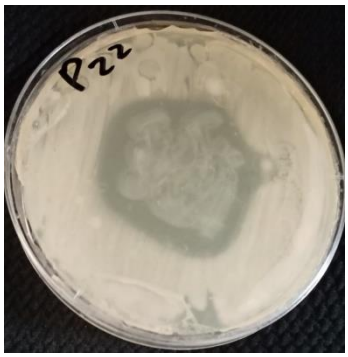



**Fig (2) morphology of bacteriocin**

**Preparation and partial purification of Endolysin**

Up to 100 ml of broth containing bacteria (S. aureus) was incubated for 18 h at 37°C. Next day, the bacteria were put in 1 L of broth for 3 h (1 × 1012). A total of 300 ml of the bacteriophage at titer 1 × 1013 pfu/ml was added for 20 min (1:10 MOI) after dividing the total volume in 50 ml tubes and then were put directly in ice. They were centrifuged at 10,000 rpm for 15 min and the sediment was taken. The sediment was put in 6 ml of 0.05 M phosphate buffer + deoxyribonuclease (5 mg). Then it was incubated for 60 min at 37°C. Ethylenediaminetetraacetic acid (EDTA) (0.005 M) was added and centrifuged at 10,000 rpm for 1 h and the supernatant was taken. Disodium tetrathionate (0.3 M) was added and mixed for 1 h at 4°C. Ammonium sulfate was added to 85% saturation and incubated for 18 h at 4°C. Next day, this was centrifuged at 10,000 rpm for 1 h. Then the mixture was resuspended in 5 ml of 0.05 M phosphate buffer (pH 6.1). Dialysis against 200 ml of the phosphate buffer saline (PBS) with (2×) conc. And pH = 6.1 at 4°C overnight was done. Then it was added to column chromatography Sephadex G100 in 0.1 M phosphate buffer (NaCl = 8 g/l, KCl = 0.2 g/l, Na2HPO4 = 1.4 g/l, KH2PO4 = 0.2 g/l) pH 6.1, in 18 × 0.5 cm column. They were collected in 0.5 ml Eppendorf tubes at 10 min intervals. From each Eppendorf tube, 10 µl was dropped by using automatic pipette to bacterial lawns of the specific bacteria to see which Eppendorf tube contain the endolysin.Fig(3)

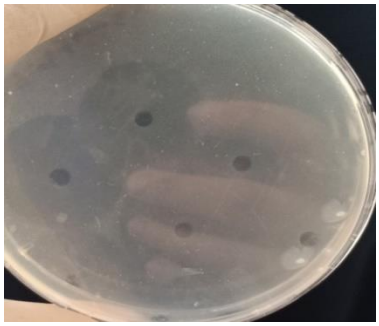


**Fig (3) morphology of endolysin**

**Antimicrobial susceptibility testing**:

Staphylococcus aureus Isolates were tested for susceptibility to Cefoxitin (30µg), Cefepime (30µg), Ceftazidime (30µg), Amoxicillin/clavulanic acid (30µg), Imipenem (10µg), Gentamicin (10µg), Sulphamethoxazole (25µg), Amikacin (30µg), Erythromycin (15µg), Trimethoprim (30µg), polymyxinB (300µg), and Ciprofloxin (5µg). From the results of Disc diffusion method, the highest multi drug resistant isolates were selected and the antibiotic susceptibility automated system as following: an aliquot of 145µL of cellular suspension, with an optical density between 0.55 and 0.63, was added in 3mL saline solution (0.45% NaCL, pH 7.0) into a clear plast (polystyrene) test tube (12mm×75 mm). Which indicated resistance or sensibility shown by the bacteria through several antibiotic concentrations in the anti-bio gram cards. Susceptibility profiles were performed with 12 antibiotics, Cefoxitin, Cefepime, Ceftazidime, Amoxicillin/clavulanic acid, Imipenem, Gentamicin, Sulphamethoxazole ,Amikacin (30µg), Erythromycin , Trimethoprim , polymyxin B, and Ciprofloxin. Were determined according to Clinical Laboratory Standard Institute (CLSI) guidelines, 2015.

**Determination of the minimum inhibitory concentration (MIC)**

MIC values of antibiotics were determined by the microdilution method following the recommendations of Papich [18]. Stock solutions of antibiotics were prepared and added to the bottom of a 96-well microtiter plate (Nunc Inc., Roskilde, Denmark). 100 mL of this solution was added to the first well of the 96-well plate and serially diluted. 100 mL of an overnight culture of S.aureus was added to each well at a final concentration of 5 × 105 CFU/mL (colony-forming units per milliliter). The microtiter plates were incubated at 35°C for 24 h and the MIC determined as the lowest concentration of antibiotics showing no visible bacterial growth.

**Molecular identification of the most resistant staphylococcus aureus isolate**

The most resistant isolate was confirmed with molecular characteristics as 16S rRNA. PCR products were sequenced using the standard Sanger method on ABI 3730XL DNA Sequencer at Macrogen sequencing services (Macrogen, Seol, South Korea). The partial nucleotide sequence of 16s rRNA region for bacterial isolate was done from the forward and reverse directions.

**Characterization of bacteriophage**

The morphological, biological and physiological properties of isolated phage were determined.

**Morphological characteristics of phage**

In order to observe phage morphology, transmission electron microscopy (TEM) of the isolatedphage was performed as described by(19). One drop of each purified phage with high titers was placed on

400 meshes carbon coated cupper grid stained with 2% (w/v) potassium phosphorrtungestic acid (pH 7.2) for 10 sec and examined using a Hitachi H600A electron microscope at 80 KV in Electron Microscopy Unit, at Faculty of Agriculture, Mansoura University, Egypt.

**Biological properties of isolated phage**

**Phage adsorption rate**

The adsorption rate of phages was determined by the method of (20). Phage suspension was added at a multiplicity of infection (MOI) of 0.1 to the host culture and incubated at 37°C. Aliquots were taken at 5 min intervals and the number of free infectious phage particles was calculated by phage titration. Adsorption rate constant was determined by the equation: K= (2.3/BT) x log (Po/P), whereas K= adsorption rate constant; B= Bacterial host concentration; T: Time (min); Po= Unabsorbed (free) phage concentration at the beginning; P= Unabsorbed (free) phage concentration at the end.

**Physical characteristics of phage**

**Thermal stability**

Thermal inactivation point of phages in vitro was carried out by exposure the phage to different degrees of temperature, 40, 50, 60, 60, 70, 80 and 90°C for 10min using water bath and then directly cooled under tap water treated phage was diluted and assayed by the plaque assay.

**pH stability**

The activity of phage to survive at different pH levels was evaluated by exposing the phages suspension to different pH values from 2 to 12. One hundred µl of each phage suspension was added to 900 µl of different buffer solution at different pH degrees ( glycin-HCl buffer at pH values 2, 3 and 4; phosphate buffer solution at pH values 5,6,7 and 8; glycin- NaoH solution at pH values 9 ,10 and 11 and KOH or HCL to other extreme pH values for 24h at 4°C then, each phage suspension was checked for survival quantitatively by using plaque assay **.**

**Effect of storage on the phage infectivity**

The infectivity of isolated phagewas examined by plaque assay after incubation at different temperatures (4°C, room temperature and 20°C) for eight months (20).

**Phenotypic characterization of biofilm production**

1. **Congo red agar test**

Have described a simple qualitative method to detect biofilm production by using Congo Red Agar (CRA) medium.CRA medium was prepared with brain heart infusion agar (Oxoid, UK) 37 g/L, sucrose 50 g/L, and Congo red indicator (Oxoid, UK) 8 g/L. First Congo red dye was prepared as a concentrated aqueous solution and autoclaved (121° C for 15 minutes) separately from the other medium constituents(21). Then, it was added to the autoclaved brain heart infusion agar with sucrose at 55° C. In this test, Congo red dye was used as a pH indicator, showing black coloration at pH ranges between 3.0 and 5.2. Plates with the Congo red agar medium were seeded and incubated in an aerobic environment for 24 to 48 hours at 37ºC. Isolates were interpreted according to their colony phenotypes. Black colonies with dry consistency and rough surface and edges were considered a positive indica-

tion of slime production, while both black colonies with smooth, round and shiny surface and red colonies of dry consistency and rough edges and surface were considered as intermediate slime producers. Red colonies with smooth, round, and shiny surface were indicative of negative slime production **Fig(4).**

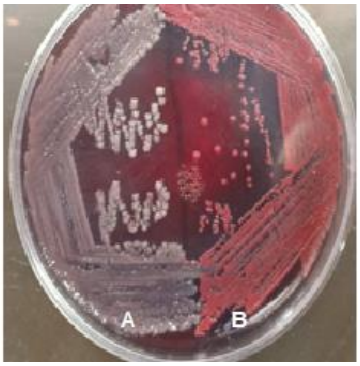



**Fig (4)** Investigation of biofilm producer S. aureus using CRA method: (A) dry black colonies representing strong producer, (B) smooth red non-biofilm producing colonies.

**2. Quantitative detection of biofilm by microtiter plate method**

The biofilm assay is performed by using flat-bottom micro titer plates (Techno Plastic Products, Switzerland) as described by O'Toole (22). P. aeruginosa isolates were grown at 37°C in tryptic soy broth (TSB; Oxoid, UK). The bacterial cells were then pelleted at 6000 g for 10 min, and the cell pellets were in 5 mL of fresh medium. The optical densities (ODs) of the bacterial suspensions were measured using a spectrophotometer (Model 6305, Jenway Ltd., Essex, UK) and normalized to an absorbance of 1:00 at 600 nm. The cultures were diluted 1:40 in fresh TSB, and 200 µL of cells were aliquoted into a 96-well polystyrene microtiter plate and inoculated for 24 h at 37°C without agitation. After incubation at 37°C for 24 h, the planktonic cells were aspirated, and the wells were washed three times with sterile phosphate-buffered saline (PBS). The plates were inverted and allowed to dry for an hour at room temperature. For biofilm quantification, 200 µL of 0.1% aqueous crystal violet solution was added to each well, and the plates were allowed to stand for 15 min. The wells were subsequently washed three times with sterile PBS to wash off the excess crystal violet. Crystal violet bound to the biofilm was extracted with 200 µL of an 80:20 (v/v) mixture of ethyl alcohol and acetone, and the absorbance of the extracted crystal violet was measured at 545 nm on ELISA reader (stat fax 2100, USA). A negative control, crystal violet binding to wells was measured for wells exposed only to the medium with no bacteria. All biofilm assays were performed in triplicate. The interpretation of biofilm production was according to the criteria described by Stepanović et al. (23). Based on these criteria, optical density cutoff value (ODc) is defined as an average OD of negative control +3 × SD (standard deviation) of the negative control. The ability to produce biofilm of each P. aeruginosa isolate was classified according to the following criteria: OD ≤ ODc = not a biofilm producer, ODc < OD ≤ 2x ODc = weak biofilm producer, 2x ODc < OD ≤ 4x ODc = moderate biofilm producer, and 4x ODc < OD = strong biofilm producer **Fig (5) .**

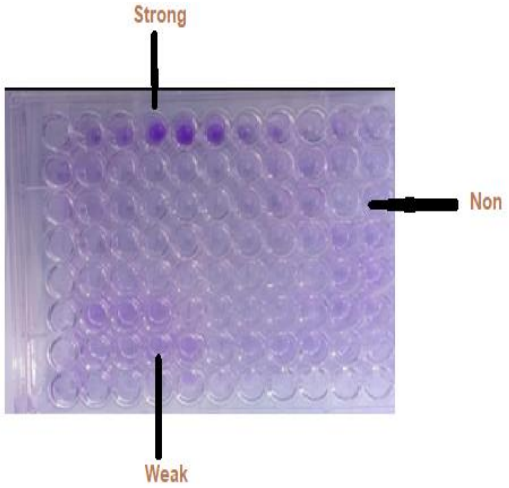
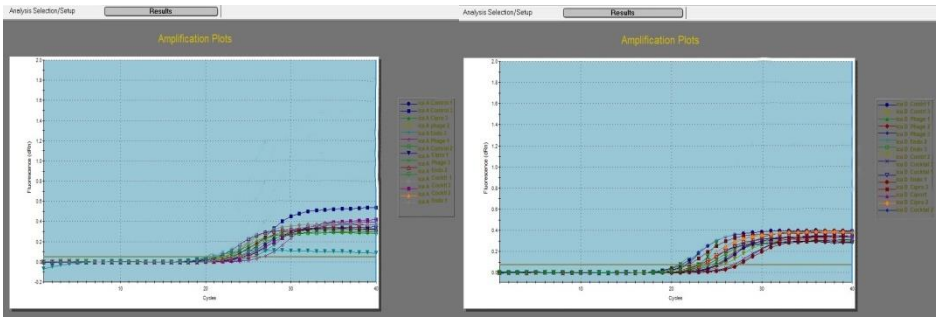



**Fig (5)** Microtiter plate method showing none, strong, and weak biofilm producers differentiated by crystal violet stain in 96-well tissue culture plate.

**DNA extraction and PCR amplification**

DNA extraction of samples was performed usingheQIAamp DNA Mini kit (Qiagen, Germany, GmbH).Then, isolates were screened for the presence of the major biofilm genes, including intercellular adhesion factors (icaA) and (icaD) **.Fig(6)**




**Fig(6)** DNA extraction adhesion factors (icaA) and (icaD).

**PCR products visualization and analysis**

The products of PCR were separated by electrophoresis on 1% agarose gel (Applichem, Germany,GmbH) by running 20µl of the PCR products.Thegelwas photographed by a gel documentation system (Alpha Innotech, Biometra) and the data were analyzed bycomputer software.

**Quantitative analysis of virulence gene expression**

The virulence gene expression was analyzed byquantitative real-time PCR (qRT-PCR) and the 16SrRNA housekeeping gene served as an internal controlto normalize the expressional levels between samples.Primers were utilized in a 25- µl reaction containing12.5 µl of the 2x

Quanti Tect SYBRGreen PCR Master Mix (Qiagen, Germany, GmbH), 0.25 µl of Revert Aid Reverse Transcriptase (200 U/µL) (Thermo Fisher), 0.5µl of each primer of 20 pmol concentration, 8.25 µl ofwater, and 3 µl of RNA template. The reaction was performed in a Stratagene MX3005P real-time PCR withspecific conditions mentioned in the **Table (1)**. Amplification curves and Ct values were determined by the Stratagene MX3005P software. To estimate the variation ofgene expression on the

RNA of the different samples,the Ct of each sample was compared with that of thepositive control group according to the "ΔΔCt" methodstated by (24) .

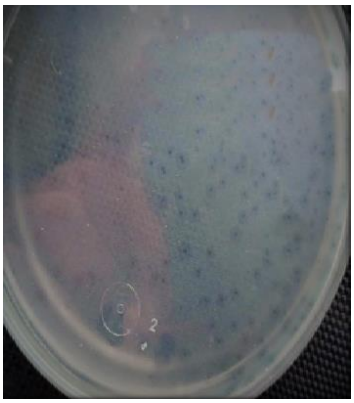
**Table (1): Primers sequences, target genes and cycling conditions for SYBR green rt-PCR.**

| Target gene | Primers sequences | Reverse transcrip-tion | Primary dena-turation | Amplification (40 cycles) | | | Reference |
|---|---|---|---|---|---|---|---|
| | | | | Secondary denaturation | Annealing (Optics on) | Extension | |
| 16S rRNA | CCTATAAGACTGGGATAACTTCGGG | 50˚C 30 min. | 94˚C 15 min. | 94˚C 15 sec. | 55˚C 30 sec. | 72˚C 30 sec. | **Mason et al., 2001** |
| | CTTTGAGTTTCAACCTTGCGGTCG | | | | | | |
| icaA | CCT AAC TAA CGA AAG GTA G | | | | 49˚C 30 sec. | | **Rohde et al., 2001** |
| | AAG ATA TAG CGATAA GTG C | | | | | | |
| icaD | ATGGTCAAGCCCAGACAGAG | | | | 60˚C 30 sec. | | **Rohde et al., 2001** |
| | AGTATTTTCAATGTTTAAAGCAA | | | | | | |

**Results**

**Isolation of Bacteriophages**

Atotal of 60 bacterial isolates were obtained from 60 patients with diabetic foot ulcers. The age group of these patients ranged from 30 to 75 years and the maximum number of patients was in the age group of 61 to 75 years were isolated from that sample collected Sewage from elberka (Ain shams) **Table (2)**and **Fig (7)**



**Fig(7)** plaque morphology of staphylococcus aureus

**Table (2) Isolation of phages active against staphylococcus aureus**

| No. of Sources | Phage sources | Formation of Lytic area |
|---|---|---|
| **A** | Sewage from the university hospital 1 (zagazig) | – |
| **B** | Sewage from the university hospital 2 (zagazig) | – |

| | | |
|---|---|---|
| **C** | Sewage from kfr-elhamam | – |
| **D** | Sewage from kfr-elhamam 2 (zagazig) | – |
| **E** | Sewage from country eltyba (zagazig) | – |
| **F** | Sewage from station water (Ain shams) | – |
| **G** | Sewage from elberka (Ain shams) | + |

**(-) : Negative (+): positive**

**Antibiotic susceptibility testing**

Antimicrobial susceptibility was tested by the agar disk diffusion method on Mueller-Hinton agar (Becton Dickonson, Germany), according to the guidelines of the National Committee for Clinical Laboratory Standards (NCCLS) (18). The following antibiotics were tested **Table(1):**Cefoxitin (30µg), Cefepime (30µg), Ceftazidime (30µg), Amoxicillin/clavulanic acid (30µg), Imipenem (10µg), Gentamicin (10µg), Sulphamethoxazole (25µg), Amikacin (30µg), Erythromycin (15µg), Trimethoprim (30µg), polymyxinB (300µg), and Ciprofloxin (5µg) (Becton DickonsonSensi-disc; Becton Dickonson From the results of Disc diffusion method, the highest multi drug resistant isolates were selected and the antibiotic susceptibility automated system as following: an aliquot of 145µL of cellular suspension, with an optical density between 0.55 and 0.63, was added in 3mL saline solution (0.45% NaCL, pH 7.0) into a clear plast (polystyrene) test tube (12mm×75 mm). Which indicated resistance or sensibility shown by the bacteria through several antibiotic concentrations in the anti-bio gram cards. Susceptibility profiles were performed with 12 antibiotics, Cefoxitin, Cefepime, Ceftazidime, Amoxicillin/clavulanic acid, Imipenem, Gentamicin, Sulphamethoxazole ,Amikacin (30µg), Erythromycin , Trimethoprim, polymyxin B, and Ciprofloxin.**Table(3)**

**Table (3):** Antibiotic susceptility profile of tested isolates

| Isolation no | Cephalosporin | | | B.lactam | | Aminoglycosides | | Sulfamide | | polypep-tide | Quino-lone | Macro-lides | index |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Cefoxitin (FOX) | Cefepime (FEP) | Ceftazidime (CAZ30) | Amoxicil-lin clavu-linic acid (AMC) | Im-penem (IPM) | Amika-cin (AK) | Gen-tamicin (CN) | Sulpha-methox-azole (SXT) | Trime-thoprim (SRO) | polymyxinB (PB) | Ciproflox-in (CIP) | Erythro-mycin (E) | |
| 1 | 20 (s) | 18 (S) | 19 (S) | 19 (S) | 14 (s) | 27 (S) | 17 (S) | 22 (S) | 26 (S) | 12 (R) | 22 (S) | 20 (S) | **8.3%** |
| 2 | 20 (s) | 15 (S) | 12 (R) | 12 (R) | 20 (S) | 13 (S) | 8 (R) | 20 (S) | 10 (R) | 0 (I) | 20 (S) | 20 (S) | **33.3%** |
| 5 | **25 (s)** | **10 (R)** | **10 (R)** | **22 (S)** | **10 (R)** | **22 (S)** | **17 (S)** | **15 (s)** | **20 (S)** | **14 (s)** | **8 (R)** | **16 (s)** | **33.3%** |
| 6 | **23 (s)** | **25 (S)** | **28 (s)** | **13 (s)** | **13 (s)** | **21 (S)** | **16 (S)** | **19 (S)** | **25 (S)** | **13 (s)** | **20 (S)** | **18 (s)** | **0** |
| 7 | **26 (s)** | **12 (R)** | **30 (s)** | **15 (s)** | **15 (s)** | **17 (S)** | **10 (R)** | **20 (S)** | **20 (S)** | **0 (I)** | **15 (s)** | **12 (R)** | **25%** |
| 8 | **18 (s)** | **20 (S)** | **30 (s)** | **23 (S)** | **17 (S)** | **15 (R)** | **20 (S)** | **0 (I)** | **18 (S)** | **0 (I)** | **5 (S)** | **15 (R)** | **16,6%** |
| 9 | **15 (s)** | **10 (R)** | **25 (S)** | **12 (R)** | **15 (S)** | **10 (R)** | **8 (R)** | **0 (I)** | **20 (s)** | **0 (I)** | **0 (I)** | **18 (S)** | **11.1** |
| 10 | **15 (S)** | **14 (S)** | **26 (S)** | **14 (S)** | **15 (S)** | **18 (S)** | **10 (R)** | **0 (I)** | **18 (S)** | **15 (R)** | **12 (R)** | **15 (S)** | **33.3%** |
| 11 | **22 (s)** | **10 (R)** | **0 (I)** | **12 (R)** | **13 (S)** | **15 (S)** | **18 (S)** | **0 (I)** | **0 (I)** | **0 (I)** | **0 (I)** | **0 (I)** | **16.6%** |
| 12 | **12 (R)** | **15 (S)** | **12 (S)** | **28 (S)** | **20 (S)** | **25 (S)** | **10 (R)** | **0 (I)** | **30 (S)** | **18 (S)** | **0 (I)** | **20 (S)** | **16.6%** |

| | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 14 | **0 (I)** | **0 (I)** | **0 (S)** | **18 (S)** | **20 (S)** | **25 (S)** | **11 (R)** | **0 (I)** | **12 (R)** | **12 (R)** | **0 (I)** | **20 (S)** | **25%** |
| 15 | **19 (s)** | **0 (I)** | **8 (R)** | **29 (S)** | **0 (I)** | **18 (S)** | **0 (I)** | **0 (I)** | **8 (R)** | **0 (I)** | **7 (R)** | **13 (S)** | **25%** |
| 16 | **10 (R)** | **0 (I)** | **12 (R)** | **0 (I)** | **16 (S)** | **12 (R)** | **0 (I)** | **0 (I)** | **12 (R)** | **0 (I)** | **17 (S)** | **0 (I)** | **33.3%** |
| 17 | **22 (S)** | **30 (S)** | **21 (S)** | **30 (S)** | **17 (S)** | **15 (S)** | **9 (R)** | **7 (R)** | **0 (I)** | **0 (I)** | **20 (S)** | **8 (R)** | **25%** |
| 19 | **25 (S)** | **29 (S)** | **30 (S)** | **40 (S)** | **25 (S)** | **27 (S)** | **12 (R)** | **18 (R)** | **28 (S)** | **11 (R)** | **0 (I)** | **17 (S)** | **25%** |
| 22 | **18 (S)** | **35 (S)** | **38 (S)** | **15 (S)** | **21 (S)** | **18 (S)** | **17 (S)** | **0 (I)** | **31 (S)** | **12 (R)** | **18 (S)** | **0 (I)** | **8.3%** |
| 23 | **14 (S)** | **35 (S)** | **40 (S)** | **14 (S)** | **12 (R)** | **10 (R)** | **10 (R)** | **0 (I)** | **25 (S)** | **0 (I)** | **20 (S)** | **0 (I)** | **25%** |
| 25 | **20 (S)** | **10 (R)** | **10 (R)** | **26 (S)** | **20 (S)** | **20 (S)** | **16 (S)** | **25 (S)** | **15 (S)** | **18 (S)** | **20 (S)** | **12 (R)** | **25%** |
| 26 | **15 (S)** | **0 (I)** | **0 (I)** | **16 (S)** | **11 (R)** | **13 (S)** | **12 (R)** | **0 (I)** | **12 (R)** | **15 (S)** | **0 (I)** | **15 (S)** | **25%** |
| 27 | **15 (S)** | **16 (S)** | **15 (S)** | **11 (R)** | **17 (S)** | **16 (S)** | **0 (I)** | **0 (I)** | **12 (R)** | **10 (R)** | **18 (S)** | **0 (I)** | **25%** |
| 28 | **15 (S)** | **15 (S)** | **10 (R)** | **20 (S)** | **15 (R)** | **15 (S)** | **15 (S)** | **25 (S)** | **15 (S)** | **10 (R)** | **17 (S)** | **0 (I)** | **25%** |
| 29 | **15 (R)** | **15 (S)** | **13 (R)** | **10 (R)** | **12 (R)** | **12 (R)** | **0 (I)** | **0 (I)** | **15 (S)** | **0 (I)** | **17 (S)** | **0 (I)** | **41.6%** |
| 31 | **26 (S)** | **13 (S)** | **15 (S)** | **17 (R)** | **18 (S)** | **12 (R)** | **12 (R)** | **22 (S)** | **11 (R)** | **15 (S)** | **26 (S)** | **0 (I)** | **33.3** |
| 32 | **25 (S)** | **0 (I)** | **15 (S)** | **15 (S)** | **20 (S)** | **0 (I)** | **0 (I)** | **22 (S)** | **0 (I)** | **0 (I)** | **22 (S)** | **25 (S)** | **0** |

| | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 33 | **26 (S)** | **11 (R)** | **12 (R)** | **15 (S)** | **22 (S)** | **0 (I)** | **0 (I)** | **22 (S)** | **0 (I)** | **0 (I)** | **22 (S)** | **22 (S)** | **16.6%** |
| 35 | **15 (S)** | **18 (S)** | **14 (S)** | **15 (S)** | **18 (S)** | **18 (S)** | **11 (R)** | **0 (I)** | **15 (S)** | **0 (I)** | **28 (S)** | **0 (I)** | **8.3%** |
| 36 | **25 (S)** | **22 (S)** | **17 (S)** | **25 (S)** | **32 (S)** | **22 (S)** | **20 (S)** | **0 (I)** | **20 (S)** | **14 (S)** | **25 (S)** | **0 (I)** | **0** |

**Determining the MIC of Phage**

Confirmation the identification of multidrug resistant bacterial isolates by Vitek 2 the isolate which recorded highly resistant to antibiotics were selected tightly and represented all isolates for bio-chemical identification and for testing the susceptibility to MIC (µg /ml) with Vitek 2 as shown in **Table (4)**. The results of susceptibility pattern of selected isolates to different MIC using Vitek 2 showed that isolates were multidrug resistant (MDR) which gave MRI reached to 0.46.

**Table (4): Susceptibility and MIC (µg /ml) for selected gram positive bacteria using different groups of antibiotics**

| Susceptibility Information | MIC Interpretation for isolates | |
|---|---|---|
| **Antibiotic** | **Antibiotic group** | **SP15a** |
| **Cepahlosporins** | **Cefoxitin (FOX)** | **-** |
| **B- lactams** | **Benzylpenicil-lin(penicillin G)** | **≥ 0.5(R)** |
| | **Ampicillin(AMP)** | **≥ 32 (R)** |
| | **Oxacillin(OXA)** | **2 (R)** |
| **Aminoglycosides** | **Gentamicin High Level (synergy)** | **ND** |
| | **Streptomycin High Level (synergy)** | **ND** |
| | **Gentamicin (CN)** | **≤0.5 (S)** |
| **Quinolones** | **Ciprofloxacin (CIP)** | **4(R)** |
| | **Levofloxacin(LVX)** | **0.25 (S)** |
| **Macrolides** | **Erythromycin(E)** | **2 (R)** |
| **Lincosamides** | **Clindamycin(CM)** | **(-)** |
| **Streptogarmins** | **Quinupristin/ Dalfopristin(QDP)** | **2(*R)** |
| **Oxazolidnone** | **Linezolid(lzd)** | **2(S)** |
| **Glycopeptides** | **Vancomycin(VAN)** | **≤ 0.5 (S)** |
| **Teteracycline** | **Tetracycline(TET)** | **≥ 16 (R)** |
| **Teteracycline** | **Tigecycline(TGC)** | **≤ 0.12 (S)** |
| **Nitrofurantoin** | **Nitofuration(NIT)** | **≤ 16 (S)** |
| **MRI** | | **0.46** |

**Effects of Bacteriophages, endolysin,bacteriocin and gene expression on staphylococcus aureus**

Results in **Table (5)**showed the effect in the first hour was not strong with the effect of bacteriocin,endolysin, bacteriophage and gene expression **Table(6)**, but it seemed to show an effect with the cocktail. In the second hour, the effect of the cocktail seemed to show its strength, with the effect of the cocktail being stronger on staph. At the third, fourth and fifth hours, the effect of the cocktail with phage, bacteriocin, endolysin and bacteriophage appeared to have a stronger, as for the sixth hour, the strength of the cocktail appeared, then came Bacteriophage, Bacterosin and endolysin.

**Table (5) Effects of Bacteriophages, endolysin and bacteriocin on staphylococcus aureus:**

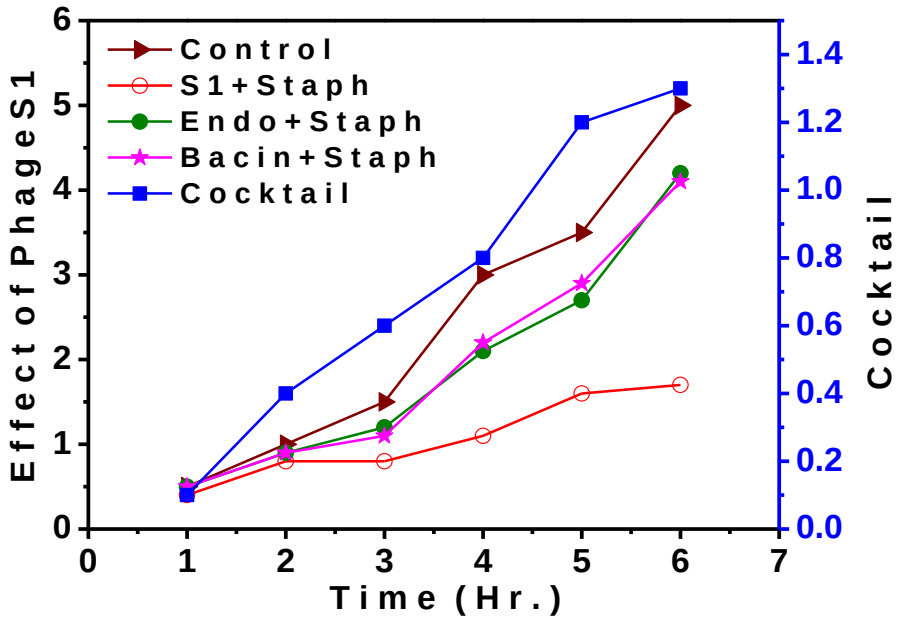


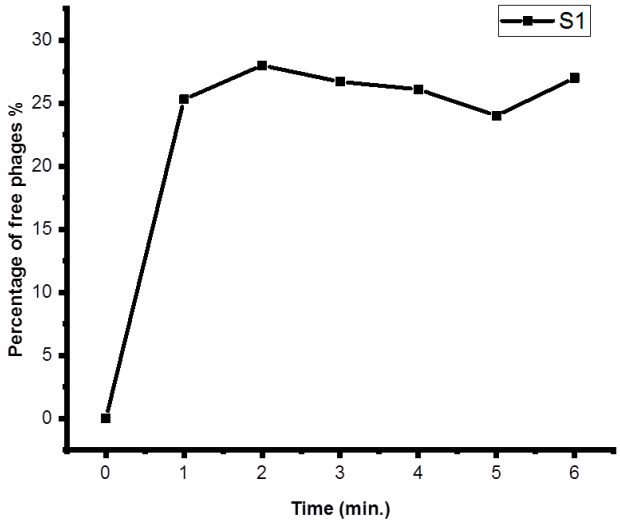

Table (6):Compression**16S rRNA** and gene expression

| Group | Sample No. | 16S rRNA | icaA | | icaD | |
|---|---|---|---|---|---|---|
| | | CT | CT | Fold change | CT | Fold change |
| Control | 1 | 19.23 | 20.92 | - | 20.82 | - |
| | 2 | 22.13 | 22.67 | - | 23.12 | - |
| | 3 | 19.12 | 20.73 | - | 20.21 | - |
| Ciprofloxacin | 1 | 19.39 | 24.01 | 0.1312 | 24.11 | 0.1232 |
| | 2 | 21.76 | 26.5 | 0.0544 | 26.72 | 0.0727 |
| | 3 | 19.02 | 21.10 | 0.7219 | 23.28 | 0.125 |
| Phage | 1 | 19.76 | 23.2 | 0.2973 | 21.7 | 0.7845 |
| | 2 | 21.34 | 21.9 | 0.9862 | 22.72 | 0.7631 |
| | 3 | 20.38 | 23.78 | 0.2891 | 22.5 | 0.4897 |
| Endolysin | 1 | 20.41 | 25.46 | 0.0973 | 25.12 | 0.1150 |
| | 2 | 18.02 | 23.51 | 0.0323 | 22.1 | 0.1174 |
| | 3 | 19.89 | 22.21 | 0.6113 | 22.59 | 0.3275 |
| Cocktail | 1 | 19.65 | 24.98 | 0.0802 | 24.82 | 0.0836 |
| | 2 | 20.88 | 26.34 | 0.0330 | 26.29 | 0.0467 |
| | 3 | 19.34 | 24.82 | 0.0683 | 24.11 | 0.0780 |

**Effect time on number of phage**

Results in **Table (7)**as shownwhen the phage stops working at any minute In the first minute, a coin appears naturally, but in the second minute it starts to decrease, and when we reached the third minute, it stopped completely As for the fourth minute, it reached the highest activity in it, then the fifth minute. Decrease a coin for the fourth minute, and then increased again in the sixth minute.

**Table (7) Profound effect of phage was observed as minute**




**Molecular identification of S.aureus isolates using 16S rRNAgene**

16s rRNA region of multidrug bacterial isolate was amplified from isolated DNA of cells mixed with PCR reaction mixture and specific primer sets. The size of PCR product was estimated by comparing its electrophoresis mobility with those of standard DNA lader (100 bps). The partial nucleotide sequence of 16s rRNA region for bacterial isolate was done from the forward and reverse direction and represented as 684bps for forward direction **(Fig.8)** and 677 bps for reverse direction **(Fig.9)**. showed that Egyptian isolate were be found to be highly homologous with Staphylococcus aureus strain SaEg01 16S ribosomal RNA gene that recorded on Gene-Bank and significant similarities for 16sRNA gene of S.aureus isolate with some other related genera recorded in gene bank was done using Blast research alignment . The Gene-Bank nucleotide sequence accession numbers for partial sequences of 16S rRNA gene generated in this study using DDBJ Center and recorded as Staphylococcus aureus SaEg01 LC596095.

<>H201106-033_I11_1492R.ab1 684

AAAATGACAGCATGACGTCTACGACGCGATCTACTGCCAGTATCAGAGCT
TCCTTCCACCGGCTTCGGGTGTTACAAACTCTCGTGGTGTGACGGGCGGT
GTGTACAAGACCCGGGAACGTATTCACCGTAGCATGCTGATCTACGATTA
CTAGCGATTCCAGCTTCATGTAGTCGAGTTGCAGACTACAATCCGAACTG
AGAATAATTTTATGGGATTTGCTTGACCTCGCGGGTTCGCTGCCCTTTGT
ATTATCCATTGTAGCACGTGTGTAGCCCAAATCATAAGGGGCATGATGAT
TTGACGTCATCCCCACCTTCCTCCGGTTTGTCACCGGCAGTCAACCTAGA
GTGCCCAACTTAATGATGGCAACTAAGCTTAAGGGTTGCGCTCGTTGCGG
GACTTAACCCAACATCTCACGACACGAGCTGACGACAACCATGCACCACC
TGTCACTTTGTCCCCCGAAGGGGAAAACTCTATCTCTAGAGCGATCAAAG
GATGTCAAGATTTGGTAAGGTTCTTCGCGTTGCTTCGAATTAAACCACAT
GCTCCACCGCTTGTGCGGGTCCCCGTCAATTCCTTTGAGTTTCAACCTTG
CGGTCGTACTCCCCAGGCGGAGTGCTTAATGCGTTAGCTGCAGCACTAAG

GGGCGGAAACCCCCTAACACTTAGCACTCATCGT

**Figure (8):** Partial nucleotide sequence of 16s rRNA gene for S.aureus (forward direction)

<>H201106-033_K11_27F.ab1 677

CCCGGGGCTGTGGCTTACAATGCAGTCGTGACGAGCAGATCGCAGTAGCT
TGCTTCTCTGATGTTAGCGGCGGACGGGTGAGTAACACGTGGGTAACCTA
CCTATAAGACTGGGATAACTCCGGGAAACCGGGGCTAATACCGGATAATA
TATTGAACCGCATGGTTCAATGTTGAAAGACGGTTTCGGCTGTCACTTAT
AGATGGACCCGCGCCGTATTAGCTAGTTGGTAAGGTAACGGCTTACCAAG
GCAACGATACGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGAACTGA
GACACGGTCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAAT
GGGCGAAAGCCTGACGGAGCAACGCCGCGTGAGTGATGAAGGTCTTAGGA
TCGTAAAACTCTGTTGTTAGGGAAGAACAAATTTGTTAGTAACTGAACAA
GTCTTGACGGTACCTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGC
CGCGGTAATACGTAGGTGGCAAGCGTTATCCGGAATTATTGGGCGTAAAG
CGCGCGTAGGCGGTTTCTTAAGTCTGATGTGAAAGCCCACGGCTCAACCG
TGGAGGGTCATTGGAAACTGGGGAACTTGAGTGCAGAAGAGGAGAGTGGA
ATTCCATGTGTAGCGGTGAAATGCGCA

**Figure (9):** Partial nucleotide sequence of 16s rRNA gene for S.aurus (reverse direction)

**Morphological properties of phages (TEM)**

Transmission Electron microscopy (TEM) revealed that phage particle had head and short tail. This phage belong to podoviridae family that contain isometric head with Diameter 56.4nm and short tail with length about 22.6 nm with diameter about 32.2 nm .**Fig (10)**

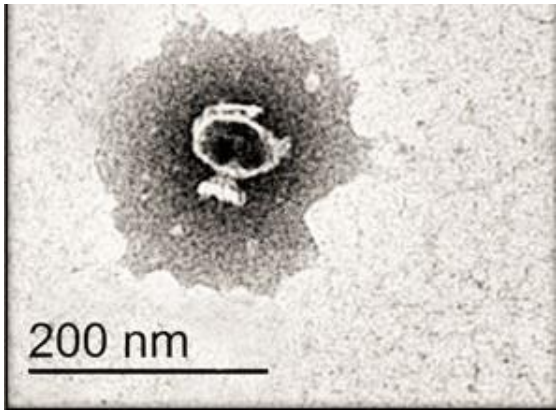



**Figure (10):** Electron micrograph illustrates bacteriophages for S.auerus

**Characterization of the isolated bacteriophage**

From the obtained results, it was found that phage particle remain infective at 40°C and decreased with temperature the read. This phage lost about 75% of infectivity at temperature 70°C and completely disappear at 80°C **(Fig. 11)**. The results also revealed that the viral infectivity increased by increasing the pH value until it reach the maximum infectivity then decreased gradually and lost their ability to lyse host at pH12. The maximum infectivity of both phages was in neutral medium at (pH=7) .phage lost their infectivity at pH 2and 12. C1 phage still infective at pH ranged from 3 to 11,.The result indicate that phage were stable at pH 4-11, but inactivated completely at pH 2 and 12 **(Fig.12)**.

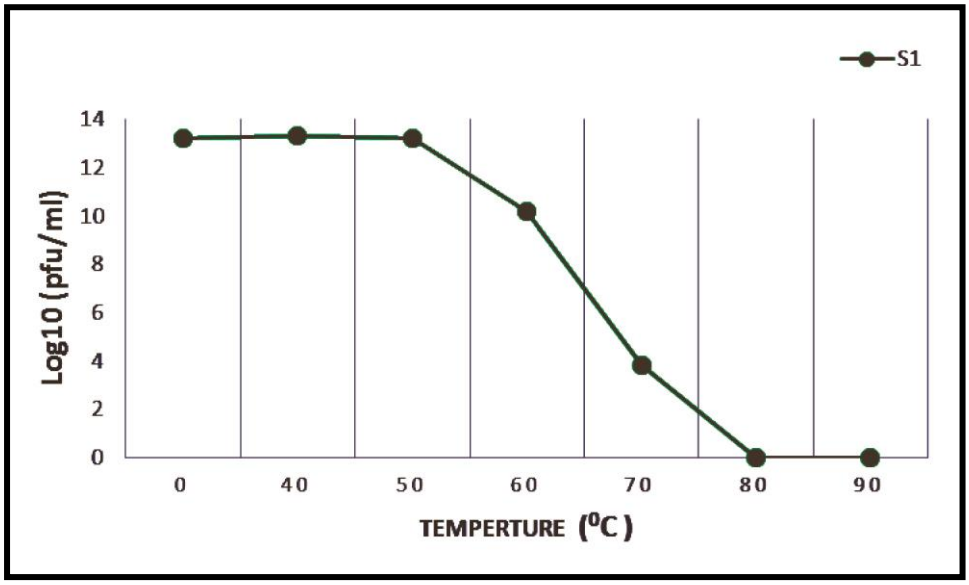



**Figure (11):** Effect of different temperature on the infectivity of phage

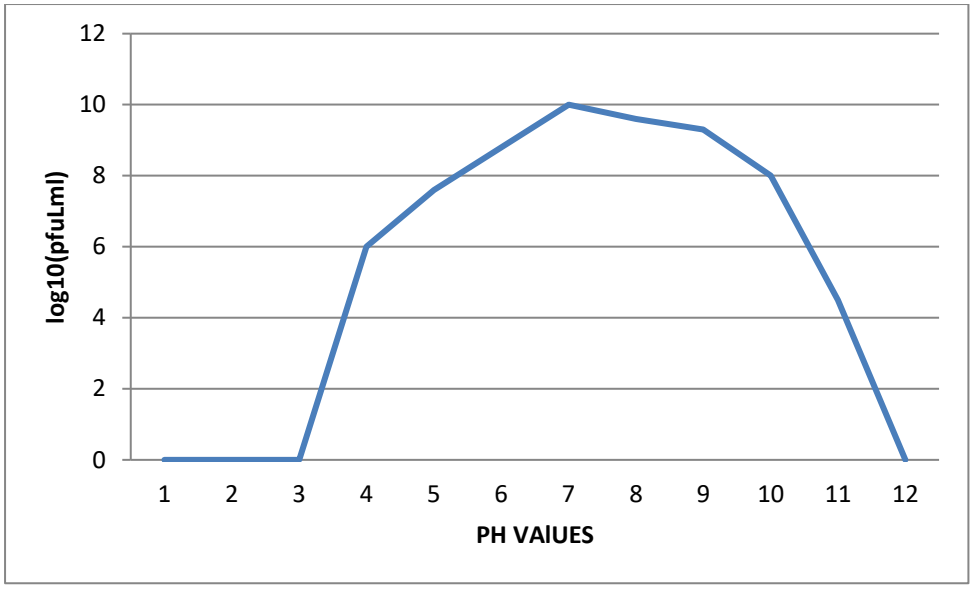



**Figure (12):** Effect of different PH values on the infectivity of phage

**Phage longevity and rate of adsorption**

The infectivity of S1 phage was determined at month intervals. The results **(Fig.13)** revealed that S1 phage were remain survive for five months at 4°C with titer 0.1x $10^6$a respectively while, at room temperature S1 phage remain survive for 5 months with low titer of about 0.1x $10^3$**(Fig.14)**. On the other hand, S1phage after stored at freezing (-20°C) was survived for five months with low titer of about 0.9 x $10^3$ PFU/ml .

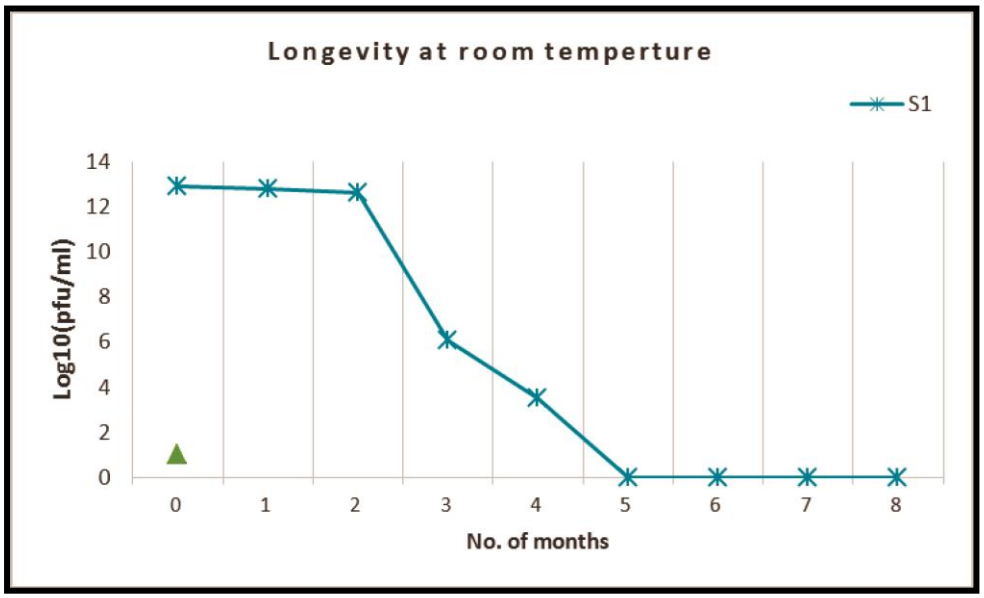



**Figure (13):**Determination the long viability of phage at 4°C

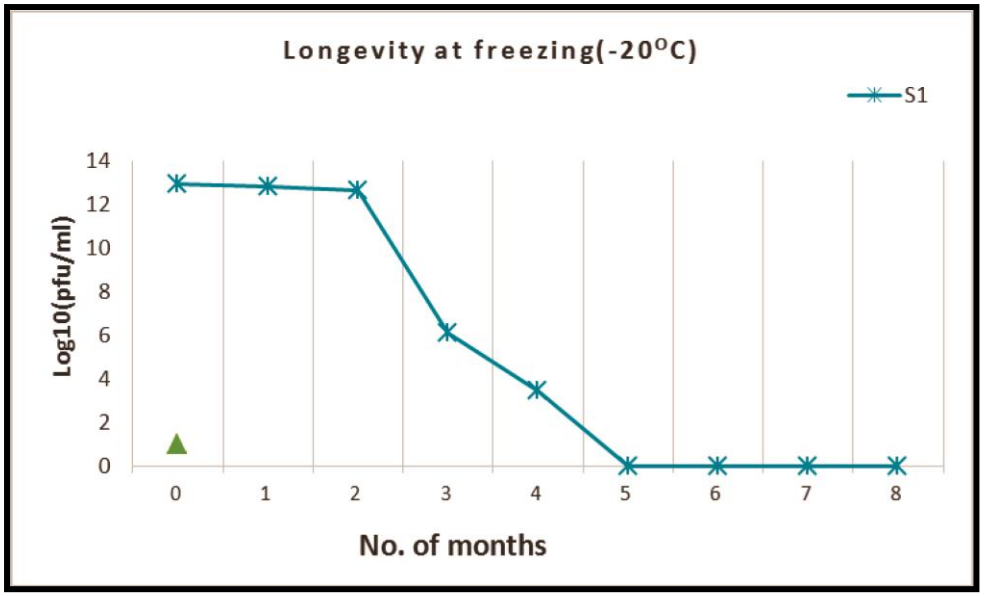



**Figure (14):**Determination the long viability of phage at room temperature

**Discussion**

Foot ulcers are considered the main cause of hospitalization and mortality of diabetic patients across the world. In recent years, the development of antibiotic resistant bacteria has made it increasingly difficult to select appropriate antibiotics for the treatment of DFI resulting in a significant upturn of morbidity and mortality . In the present study, Total of 64 bacterial isolates were obtained from 32 patients with diabetic foot ulcers. The age group of these patients ranged from 30 to 75 years and the maximum number of patients was in the age group of 61 to 75 years. The antibiotic sensitivity profiles of the bacteria were also studied. This study showed a preponderance of Gram positive cocci among the isolates from the diabetic foot ulcers. Moreover, biofilm production has been considered to be an important determinant of pathogenicity in S.aureus infections. The formation of biofilms facilitates chronic bacterial infections and reduces the efficacy of antimicrobial therapy. The situation is getting very concerning, the World Health Organization has declared it to be a “critical priority pathogen,” on which research and development of novel antibiotics should be focused.

For this reason, this work designed to find repositionable candidate’s antibiotics against P. aeruginosa biofilms, which are disreputable for their intensified drug resistance. Here we isolated 64 S.aureusfrom 32 patient suffering from foot ulcer. These findings were close to that reported earlier in UK . Many studies showed different prevalence rates of P. aeruginosa isolates in broilers worldwide: in Iraq, a low rate of 6% was reported. The proportion of MRSA isolates from blood cultures taken from cases of bacteremia in England has risen dramatically from less than 5% in 1990 to around 40% by the end of the 1990. In the current investigation, Resistance to antibiotics reached to 75% of these isolates. The present study revealed that S. aureus showed the highest resistance to Cefoxitin ,Cefepime , ceftazidime , Amoxicillin/ clavulanic acid , Imipenem , Polymixin B , Gentamicin , Amikacin , Erythromycin , Ciprofloxin and Trimethoprim/ sulphamethoxazole. isolate was multi-drug resistant with percentage of 92%. In the present study, the partial genome sequence of S. aureus was about 684bps for forward direction and 677 bps for reverse direction. Among patients with multiple isolated S. aureus strains, their biofilm production phenotype was statistically different. Based on the study of 64 S. aureus isolates, Piechota M. et al.reported, that their biofilm production differed according to their isolation site, and strong biofilm producers were found mainly from tracheostomy

tubes, sputum, throat, and nose. We detected strong biofilm production in 56–73% of S.aureus among all three isolation sites. The accessory gene regulator (agr) quorum-sensing system and intercellular adhesion (ica) group genes play an important role in S. aureus biofilm formation, but they are environmental factor-dependent. Our results about biofilm production and phage susceptibility support S. aureus phenotypic variability, even within one patient; however, such phenomena did not interfere with the bacteriophage positive lytic effect that was detected in the majority of strains. The results revealed phage were remain survive for five months at 4 oC , at room temperature S1 phage remain survive for 5 months, phage after stored at freezing (-20 oC) was survived for five months.

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