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# Methicillin-Resistant *Staphylococcus aureus* Phages: Isolation and Identification

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**Among 27 *Staphylococcus aureus* isolates, only 3 were methicillin-resistant *S. aureus* (MRSA) and 24 were Methicillin sensitivity *S. aureus* (MSSA). Phages specific for MRSA were isolated from collected soil samples. The spot test and single plaque technique were used for detection and biological purification of phages. Bacteriophages of MRSA found to be limited occurrence in soils from where the samples had be taken. Two single plaques (A and B) having different morphology were picked up as a single phage isolates. The titers were 6.4 x $10^8$ pfu $ml^{-1}$ and 1.7 x $10^{10}$ pfh $ml^{-1}$ for phage isolates A and B, respectively. The optimum pH and thermal inactivation point were found to be 9, 6 and 75, 65°C for A and B isolates, respectively. Biologically characterization and morphology of phage particles confirmed two phage types, that can designated as phage MRSA-A and phage MRSA-B.**

**Key words:** MRSA- phage, The spot test, Single plaque technique, Phage titer, Particle morphology.

Bacteriophages are viruses that specifically infect and lyse bacteria. A method using phages for the treatment of bacterial infectious disease is called bacteriophage therapy. Recently, phage therapy has gained an increasing attention because it has many advantages over chemotherapy. Phages have high specificity for their target bacteria, indicating that they do not harm the normal intestinal microflora. Phages are effective against multidrug resistant pathogenic bacteria because the mechanisms by which they induce bacteriolysis differ completely from those antibiotics. Moreover, phages have self limitation, meaning that the number of phages remain in very low level after killing the target bacteria[1].

Successful uses of phage therapy to control bacterial infectious diseases have been reported since 1980s. Polish and Soviet groups reported a series of successful clinical usages of phages for drug resistant bacterial infections in human[2,3]. Much of the recent research has focused on using phages to control diseases caused by a variety of human pathogenic bacteria including *Salmonella*[4,5], *Listeria*[6,7], *Campylobacter* spp[8], vancomycin-resistant *Enterococcus faecium*[9], *Pseudomonas* spp.[10], *Bacillus anthracis* [11], *Vibrio* spp.[12], *E. coli* [13], *Mycobacterium* spp.[14], *Klebsiella pneumonia* [15], *S.aureus* [16].

Phage therapy may be an alternative to antibiotics, because it has proved to be medically superior to antibiotic therapy in many ways[17,18]. Also, the developmental costs of phage therapy are expected to be much less than those involved in the development of novel antibiotics.

*S.aureus* is a pathogen of pyogenic inflammatory diseases, food poisoning, and toxic-shock syndrome; it is also a major causative agent for opportunistic and/or nosocomial infections,

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often with a high mortality rate [19]. According to one report, 150% of clinical *S. aureus* isolates in Japan today carry multidrug resistance, typically known as MRSA[20] . Moreover, certain MRSA strains also have already acquired resistance to vancomycin (vancomycin-resistant *S.aureus* (VRSA), a unique antibiotic previously considered to be effective against MRSA[21].

The purpose of this study was to isolate and identification a new lytic phage that infect MRSA and to investigate its lytic property toward its host bacterium under controlled conditions in the laboratory. Thus the study provides useful information for further use of the phage in controlling *S. aureus* infection in human.

## MATERIALS AND METHODS

### Bacterial isolates and methecillin resistance detection

During 2012, twenty nine strain of *S.aureus* were isolated from samples submitted to the microbiology Lab from patients in Assiute University Hospital, Assiut, Egypt. The identity of these isolates was confirmed using colonial morphology on blood agar plates, Gram stain and positive catalase and coagulase test[22,23].

According to The Clinical and Laboratory Standards Institute (CLSI) guidelines[24], detection of methecillin resistance was done. Briefly, a suspension equivalent to MacFarland 0.5 was prepared from each strain. Then a swab was dipped and streaked on the surface of Muller-Hinton agar supplemented with 6 $\mu g.ml^{-1}$ oxacillin and 4% NaCl. If any growth was detected on the plat incubated at 48 hrs at 35°C, the isolate was considered oxacillin or methecillin resistant. The methecillin resistance strains were confirmed by minimum inhibitory concentration test (MIC) in Mueller-Hinton broth, using a final inoculation of $7.5 \times 10^4$ CFU.

### Isolation of MRSA bacteriophages

According to the liquid enrichment technique[25] with minor modification[26], bacteriophages specific MRSA were isolated from soil samples collected from Sohag Government in April 2012. Briefly, 20g of soil were incubated overnight with 40 ml of nutrient broth at 30-33°C. Five ml of chloroform were added to samples and shaken for 10 min to kill bacteria then samples were centrifuged at 4000 rpm for 10 mints to remove soil and bacteria. The supernatant was added to 3 ml of 36 hrs old liquid culture of MRSA. After multiplication of phages (24-30 hrs at 30-33°C), bacterial cells were killed by shaking with 5 ml chloroform for 10 mints, then the samples were clarified by centrifugation at 4000 rpm for 10 mints. The supernatants (phage lysate) were subjected to phage detection.

### Phage detection

Plates were prepared by pouring a base layer of 20 ml of nutrient agar (NA) containing 1.5% agar and allowed to solidify. A mixture of 3 ml melted NA (0.7% agar) and 300 µl of tested bacteria (MRSA) was poured over a base layer. The phage lysates which prepared for each soil sample was spotted by sterile micropipette on the solidified upper layer. After incubated at 30-33°C for 36-48 hrs, plates were examined for lyses of bacterial lawn at the sites where drops had been applied[25] . The lyses clear zones were picked and transferred into eppendorf tubes containing 1ml selective medium (SM) medium and 200 µl chloroform then maintained at 4°C[27].

### Biological purification of phage isolates

The single plaque isolation technique was used to obtain pure single isolate of phages. The phage suspensions prepared from clear zones in the spot test were diluted ($10^{-4}$ to $10^{-6}$ as appropriate) in SM medium. Double layer plates were prepared as described above but the top layer contained a mixture of 300 µl MRSA culture and 5 µl diluted phage suspension. The plates were incubated at 30-33°C for 24-30 hrs, or until satisfactory plaque formation was observed. Two single plaques with different morphological characteristics were picked using sterile Pasteur pipettes. Each plaque was transferred into eppendorf tube containing 500 µl SM medium. These single isolates of phages were maintained over 200 µl of saline solution contained 20 mM $MgCl_2$ and 20 mM $CaCl_2$ at 4°C.

### Preparation of high titer phages suspension

The phage suspensions which prepared from the formed single plaques in the purification procedure were diluted ($10^{-4}$ pfu $ml^{-1}$) in SM medium. Double layer plates were prepared as described above but the top layer contained a mixture of 300 µl MRSA culture and 50 µl of diluted phage suspension. After incubation at 30-33°C for 24-30 hrs five ml of SM

medium were added to the surface of each plate showed almost complete lyses. The top agar layer of each plate was scraped off and combined in a flask together with the added SM medium. After centrifugation at 4000 rpm for 30 mints, agar and bacterial debris were sediment and the supernatant containing the phages was stored at 4°C over 3 ml of chloroform.

Titer of the prepared phage suspensions was estimated using the following method. A series dilutions of phage suspension were prepared in ninety µl of SM medium. Then, 300 µl of MRSA liquid culture were placed in each dilutions and shaken. The contents of each dilutions were transferred to a sterile test tube containing 3 ml of nutrient agar (NA) (0.7 agar) and kept at 50-55°C. Each tube was shaken separately, and the contents were poured onto previously prepared solid media plates. Plates were then incubated at 30-33°C for 24-30 hrs. The formed plaques were counted and the titer was calculated and expressed as plaque forming unit (pfu $ml^{-1}$).

**Characterization of the isolated phages**

**Optimum pH**

Eppendorf tubes containing 1 ml SM medium with various pHs (2-12) were prepared. Individual plaques for each single phage isolate were transferred to the prepared tubes (plaque/ tube). Tubes were incubated at 30°C for 60 mints, then 5 µl from each tube was spotted over double agar layer plate (four times per plate was used as a replicates). Diameter of lysed spots was measured. The average values of the replicates were calculated.

**Thermal stability**

Eppendorf tubes containing 1 ml of high titer phage suspension of each phage isolate were prepared. Tubes were heated in water bath at 50, 55, 60, ….. up to 95 °C for 10 mints, then cooled in ice bath. After heat treatment 10 µl from each tube was spotted over double agar layer plates. Plates were inspected for lysed spots after 24-30 hrs at 30-33°C.

**Phages morphology**

Five µl of high titer phages suspension were fixed in 5% formalin and then placed onto a sheet of parafilm. A formavar coated grids were placed (formavar side down) on the drop of phage suspension and allowed to absorb for 10 mints. The excess liquid was removed with a filter paper wick. The grids were placed above drop of filtered 2 % uranyl acetate pH 4.0 for 40 seconds[28].The grids were air dried, then examined at 50 kv in transmission electron microscopy (Joil, model GEM 1010). Standard deviation (SD) was calculated for phage dimensions as average of four particles as the following:

$$SD=\sqrt{\frac{\sum x^2-\frac{(\sum x)^2}{n}}{n-1}}$$ where x= replication values and n= number of replicates.

## RESULTS

**Bacterial isolates and methecillin resistance detection**

Based on our identification methods for 29 bacterial isolates, 27 was re-identified as *S. aureus* and 2 were coagulase negative Staphylococci. Of the 27 *S.aureus* strains, 3 were methecillin resistance *S.aureus* (MRSA). MRSA-7 was detected as more resistance strain (MIC, 32µg $ml^{-1}$) so choice for further tests.

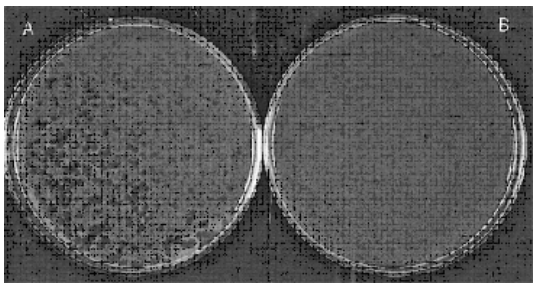



**Fig. 1.** Double layer agar plates prepared with lysates of MRSA-phage : (isolate A) big clear plaques and (isolate B) pinpoint or tiny plaques

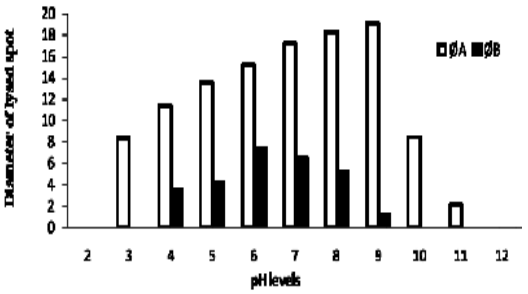



**Fig. 2.** Stability of MRSA-phage isolates to different levels of pH.

**Phages isolation and purification**

Phages specific for MRSA-7 were enriched from soil samples collected from different locations in Sohag Governorate. The spot test indicates that phages of MRSA were not common in the soils from where the samples had been taken. Two phages only were isolated from collected soils. As showen in Fig. (1), Plaque morphology (shape, size and outline) between two isolated phages was clearly different. Phage B developed small, faint and regular outline plaques compared with big, clear, and irregular outline plaques were induced by phage A one isolate of MRSA-7. The titers were 6.4 x $10^8$ pfu $ml^{-1}$ and 1.7 x $10^{10}$ pfh $ml^{-1}$ for phages isolate A and B, respectively.

**pH and temperature durability**

pH and thermal stability were studied to characterize two phage isolates which infected MRSA-7. The infectivity of the 2 phage isolates was studied at various pH (2-12). As shown in Fig. 2, isolate A and B formed lysed spots within pH range 3-11 and 4-9, respectively. Such results indicated that phage A are more tolerant to alkaline and acidic reactions, while isolate B was less tolerant. At pH 9 and 6, phage isolate A and B formed lysed spots wider than formed by any other pH tested, respectively.

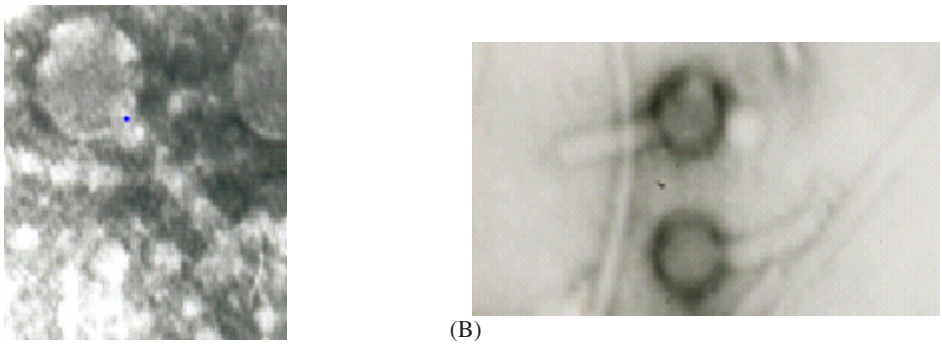
As shown in Table (1), phage isolate B have the less thermal stability, since it inactivated after incubation at 65°C for 10 mints. Whereas, isolate A exhibited the highest thermal stability, since it inactivated after incubation for 10 mints at 70°C.

**Table 1.** Thermal stability of MRSA-phage isolates exposed to 50-95 °C for 10 min

| Phage | Temperature (°C) | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| isolate | 50 | 55 | 60 | 65 | 70 | 75 | 80 | 85 | 90 | 95 |
| Phage- A | + | + | + | + | + | - | - | - | - | - |
| Phage- B | + | + | + | - | - | - | - | - | - | - |

**Phages morphology**

Two Phage isolates were negatively stained with uranyle acetate and examined with electron microscopy. As shown in Fig. 3, two phage isolates belonged to the head and tail types. phage A exhibit icosahedral head and rigid tail, typical member of the Myoviridae family. Phage B exhibit icosahedral head and short flexible tail, typical of members of Siphoviridae family. After measurements of phage particles, some variations in dimension between two isolates were noticed. In isolate A, the diameter of head was 64±2 nm, moreover, the tail length 132±2 nm and tail width was 14±2 nm. In isolate B, head diameter (44±2 nm), tail length (83±2 nm) and tail width (12± nm). Fig. 3.



(A) (B)

**Fig. 3.** Electron micrograph of the *Staphylococcus aureus* – derived phages. Purified phage particles were negatively stained with 2% uranyl acetate

## DISCUSSION

The emergence of multidrug-resistant bacteria will have a serious impact on various aspects of medical practice. Our results indicated that, 3 MRSA strains were detected from *S.aureus* which isolated from patients. MIC test for this isolates show that MRSA-7 most resistance one (MIC 32 µg/ml).

*S. aureus* is often resistant to all β-lactam antibiotic[29,30]. Several studies found MRSA in as many as 15–30% of diabetic wounds[3o,31] and at least 50% of all deaths caused by diabetic foot are the result of MRSA infection[16].

Phage therapy presents an alternative approach against the emerging MRSA threat. Our results shown, two phages were isolated from collected soil samples. Lysis spot test explained that, phage-A was more effective than phage-B against MRA-7 *in vitro*. There are many literatures available illustrating the utility of bacteriophage as therapeutic agents in many bacterial infections[32,33]. There are many literatures reported, many phages infected *S. aureus* and may be used in treatment in bacterial infection. In early study, authors used experimental phage therapy for *S. aureus* infection[34]. Furthermore, efficacy of phage cocktail BBFC-1 was evaluated against *P. aureginosa* and *S.aureus*[35]. also, The isolated phage MR-5 formed tiny plaques against its host *S. aureus* ATCC 43300 (MRSA), making its detection and enumeration difficult[36].

On the basis of the obtained results, it can be concluded that, the two phage isolates of MRSA may be belonging to two phage types. As a tailed virus of bacteria, MRSA A and B phages fell into the order Caudovirales that contains three families of tailed viruses that infect Bacteria and Archaea[37]. Possession of an icosahedral head and a long, non contractile tail would tentatively place it in the family Siphoviridae and Myoviridae[37].

Phage-A is promising agent for suppress MRSA reproduction *in vitro*. So that further studies must be undertaken such as practical phage therapy using experimental animals and phage characterization (structural proteins and phage genome).

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