ISSN: 2588-4115; Infection Epidemiology and Microbiology. 2022;8(2):121-128
10.52547/iem.8.2.121

Tarbiat Modares University

# Isolation and in *Vitro* Characterization of Specific Bacteriophages against Methicillin-Resistant *Staphylococcus aureus*

ARTICLE INFO

*Article Type*
**Original Article**

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**How to cite this article**
Esmaeilzadeh MR., Sabzi S., Hajihossein Tabrizi A. Isolation and in *Vitro* Characterization of Specific Bacteriophages against Methicillin-Resistant *Staphylococcus aureus*. Infection Epidemiology and Microbiology. 2022;8(2): 121-128

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*Article History*
Received: December 04 ,2021
Accepted: Junuary 23 ,2023
Published: June 22 ,2022

ABSTRACT

**Backgrounds:** Bacteriophage therapy could be an alternative strategy for the treatment of antibiotic-resistant bacteria. This study aimed to evaluate the antibacterial activities of isolated bacteriophages against methicillin-resistant *Staphylococcus aureus* (MRSA) isolates.
**Materials & Methods:** A total of 16 clinical isolates of MRSA were collected from medical diagnostic laboratories in Tehran, Iran. A specific bacteriophage was isolated from hospital sewage using double-layer agar. Phage morphology was evaluated by transmission electron microscopy (TEM). Different bacteria were selected to determine the bacteriophage host range using spot test. Phage susceptibility to temperature and pH was evaluated by double-layer agar method. *In vitro* assay was carried out on human epithelial type 2 (HEp-2) cells to investigate the effect of bacteriophage on the adhesion of MRSA to human epithelial cells.
**Findings**: TEM suggested the *Myoviridae* family for the isolated phage. The effective titer of bacteriophages was $1.8\times10^7$ PFU/mL. The isolated bacteriophage was stable at 4 ˚C and pH=8. The isolated bacteriophage was specific for all clinical isolates of MRSA and had no lytic activity against other pathogenic bacteria. In evaluating the binding and invasion of MRSA to the HEp-2 cell line, as expected, the lytic activity of specific bacteriophages was observed following inoculation.
**Conclusion:** The specificity and lytic activity of this phage on MRSA and MRSA-infected HEp-2 cell line emphasized that the isolated bacteriophage may serve as an effective prophylactic and alternative therapeutic agent in hospital settings.

**Keywords:** Methicillin-resistant *Staphylococcus aureus*, Bacteriophage, Phage therapy

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[ DOI: 10.52547/iem.8.2.121 ] [ Downloaded from iem.modares.ac.ir on 2024-11-21 ]

## Introduction

*Staphylococcus aureus* is a Gram-positive bacterium that could cause minor to life-threatening infections. *S. aureus* infections were initially easily treated with penicillin, but over time and with the indiscriminate use of antibiotics, resistant strains were emerged [1]. Today, infections caused by methicillin-resistant *S. aureus* (MRSA) strains are spreading rapidly, and treatment of these infections is more difficult [2]. In a meta-analysis study in Iran, about 7500 *S. aureus* isolates were collected from patients in different places, of which about 54% of the isolates carried the *mec* gene, causing resistance to methicillin [3]. Resistant infections in various tissues and organs that could not be treated with antibiotics are considered important healthcare problems that could endanger human life [4]. In this situation, alternative treatment options and antibacterial agents must be developed. Additionally, because bacteria rapidly become resistant to new antibiotics, as well as due to the high costs of producing antibiotics, there must be a minimal motivating force to develop new antibiotics [5].

Bacteriophage (phage) is a virus that attacks bacterial cells and induces cell lysis. Phage therapy was initially presented by Felix d'Herelle (1999) over a century ago [6, 7]. The antibacterial effect of bacteriophages has been well known since their discovery [8]. Bacteriophages have surface receptors on bacterial cells for binding, including lipopolysaccharide (LPS), peptidoglycan, outer membrane proteins, oligosaccharides, capsules, and type IV fimbria [9]. Also, phage therapy could be considered as a treatment option without major side effects on microbiota and eukaryotic cells [10, 11]. Furthermore, due to detailed and extensive studies, the limitations of phage therapy have been removed, which has made them good options for use in treatment systems [12, 13].

**Objevtives:** The present study aimed to characterize and *in vitro* evaluate the isolated specific bacteriophage and its antibacterial activities against MRSA isolates.

## Materials and Methods

**Bacterial preparation and susceptibility testing:** In this study, 16 clinical isolates of MRSA from different sources were collected from medical diagnostic laboratories in Tehran, Iran. The standard strain of *S. aureus* (ATCC 33591) was selected as a positive control, and all isolates were confirmed by catalase, coagulase, oxidase, and DNase production as well as mannitol fermentation. E-test (Liofilchem s.r.l, Roseto Degli Abruzzi, Italy) was carried out to verify resistance to methicillin, suggested by the Clinical and Laboratory Standards Institute (CLSI) guidelines.

**Bacteriophage isolation and purification:** To isolate specific bacteriophages, a wastewater sample was collected from the hospital and sent to the laboratory immediately. Then the sample was placed in a refrigerator overnight to settle the sediments. The sample was centrifuged (Sigma, Germany) at 4˚C and ×15,000 g and then passed through a 0.22-micrometer filter (Jet-Biofiltration, China). Then 15 mL of the centrifuged solution and bacterial suspension (containing $10^6$ CFU/mL of MRSA) from a 12-hour culture were added to the brain-heart infusion (BHI) medium (Merck, Germany) with 0.7% agar and then to a plate containing BHI broth with 1.5% agar [14]. After 24 hours, plaques were observed and removed using a sterile pipette. These plaques were added to BHI broth for enrichment, and enriched phage was then used to assess plaque formation. For phage purification, all steps were repeated five times, and the phage solution was stored at 4°C until used for further studies [15].



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**Morphological assessment of the phage:** The isolated bacteriophage was examined for size and morphology using a transmission electron microscope (TEM; 150 kV Philips-CM 30, Germany). For this purpose, a drop of phage was poured on a carbon-coated copper gride and then painted with 2% uranyl acetate.

**Bacteriophage titration**: Bacteriophage titration was carried out in three steps. First, *S. aureus* was inoculated on LB agar and plated at 37°C for 24 hrs. Then a single colony of the bacterium was inoculated in 25 mL of tryptone broth at 37°C. Finally, 1 mL of SM buffer was pipetted in a tube, and a dilution of $10^{-10}$ was prepared using serial dilution. Besides, 300 μL of bacterial culture was poured in 9 tubes, and 100 μL of each dilution was serially added to each tube. The tubes were then vortexed and placed at room temperature for 20 minutes.

The tubes were labeled, and 4 mL of molten agar (40-50 °C) was poured in tube No. 1 and vortexed. Then the suspension was transferred to LB agar plate using a sterile pipette. This procedure was carried out for each dilution, and the plates were incubated at 37°C. Finally, a plaque assay was carried out to determine plaque formation.

**Phage susceptibility to heat**: A certain concentration (aliquot) of phage suspension ($1\times10^8$ CFU/mL) was incubated at 4, 25, 37, 50, 60, and 70°C and pH=7 for 60 min. Bacteriophage titration was carried out using the double-layer agar method. All experiments were performed in triplicate.

**Phage susceptibility to PH**: A certain concentration (aliquot) of phage suspension ($1\times10^8$ CFU/ml) was incubated at 4°C in the pH range of 3 to 12 for 60 min. Bacteriophage titration was carried out using double-layer agar. All experiments were performed in triplicate.

**Determining the host range of bacteriophage:** Different bacterial standard strains were obtained from the Pasteur Institute of Iran, and the spot test was performed to determine the host range of bacteriophage. For this purpose, *Salmonella enterica* serovar Typhimurium (ATCC14028), *Shigella dysenteriae* (ATCC13313), *Citrobacter freundii* (ATCC43864), Enteropathogenic *Escherichia coli* (ATCC35401), *Enterococcus faecalis* (ATCC29212), *Listeria monocytogenes* (ATCC19114), *Cronobacter sakazakii* (ATCC29544), *S. aureus* (ATCC 33591), and 16 clinical isolates of MRSA were used. Each bacterial species was inoculated in BHI broth for 24 hrs. Then 100 μL of bacterial culture was added to 4 mL of BHI agar, and double-layer agar was prepared after pouring into the plate with BHI agar. Then 10 μL of bacteriophage suspension was spotted, and the plates were incubated at 37°C for 24 hrs. In addition, sensitivity to bacteriophages was determined based on the formation of the inhibition zone. All experiments were carried out in triplicate.

**Bacterial adherence and the effect of a specific bacteriophage using HEp-2 cell line:** Bacterial adhesion assay was carried out *in vitro* on human epithelial type 2 (HEp-2) cells to assess the effect of bacteriophage on MRSA adherence to human epithelial cells. HEp-2 cells were obtained from the cell bank of the Pathobiology Department, School of Public Health of Tehran University of Medical Sciences (Tehran, Iran). In the next step, the cells were added to the culture flasks containing minimum essential medium (MEM; Sigma-Aldrich, USA), 10% fetal bovine serum (Sigma-Aldrich, USA), penicillin (100U/mL) (Sigma-Aldrich, USA), and streptomycin (100 μg/mL) (Sigma-Aldrich, USA); then they were protected at 37°C with %5 $CO_2$. After the cell line accomplishment, the cells were trypsinized and counted. Then $2\times10^5$ cells were added into each vial of 12-cell plates and stored at 37°C with %5 $CO_2$. Afterward the culture medium was removed from the

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cells, and the vials were washed twice with phosphate-buffered saline (PBS) solution. The vial without the bacteria and phage was selected as a negative control. Moreover, 150 μL of MRSA suspension ($1–2×10^8$ CFU/mL) was added to well A, and 150 μL of MRSA suspension ($1–2×10^8$ CFU/mL) plus 150 μL of the phage solution ($10^6$ pfu/mL) was added to well B. The plate was stored at 37 °C for 4-3 hrs. After incubation, the cells were washed with PBS to remove unattached bacteria; after fixation with methyl alcohol, they were finally stained with crystal violet staining (Merck, Germany).

## Results

**Antibiotic susceptibility testing**: MRSA isolates were confirmed by cefoxitin E-test according to the CLSI guideline (Fig. 1). MIC of ≥8 μg/mL was considered as MRSA.

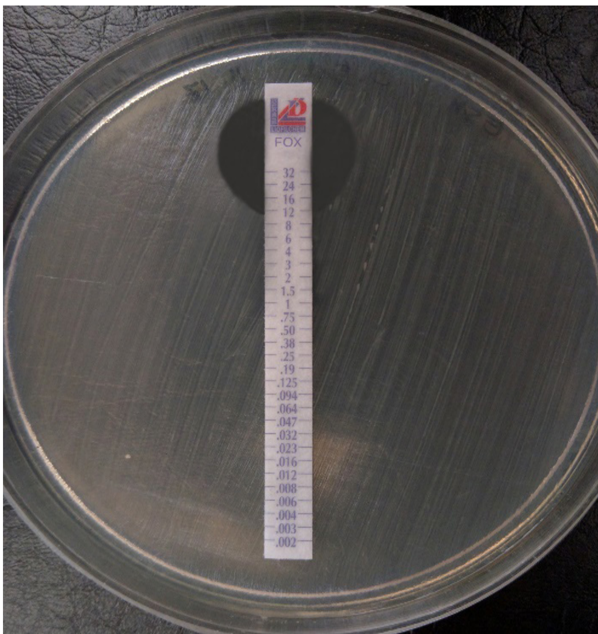


**Fig. 1)** MIC test strip of cefoxitin (FOX strip, 0.002-32 μg/mL). MRSA isolates had MIC of >8 μg/mL

**Determination of bacteriophage morphology**: Using microscopy, bacteriophages were observed with an icosahedral head of approximately 46 and 90 nm along with a very short tail. Transmission electron microscopy showed that isolated bacteriophages belonged to the *Myoviridae* family (Fig. 2).

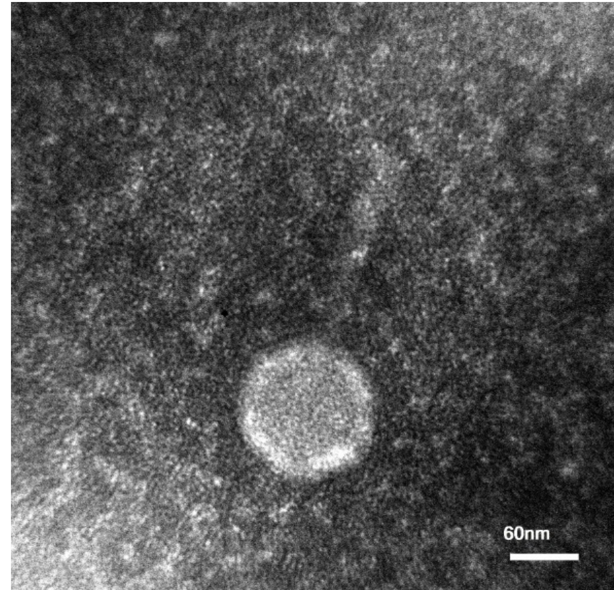


**Fig. 2)** Morphology of phage (*Myoviridae* family) using TEM

**Bacteriophage titration:** Based on the double-layer agar assay, the titer of bacteriophages was $1.8×10^7$ PFU/mL. To be able to report the number of plaques, 30-300 plaques should be formed on the media (Table 1). Bacterial dilution of $10^4$ was used to report the number of phages according to the following formula:

Number of Viruses (phages)in 1mL suspention

$$= \frac{\text{number of plaques}}{\text{dilution factor} \times \text{phage solution volume}} = \frac{180}{0.0001 \times 0.1}$$

The number of phages in 1 mL of suspension= $1.8×10^7$ PFU/mL

**Table 1**) Number of bacteriophage plaques in each sample

| | Number of plaques in different dilutions of bacteriophage suspensions | | |
|---|---|---|---|
| Dilution of specific bacteriophage | $10^4$ | $10^5$ | $10^6$ |

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| *Staphylococcus aureus* | 180 | 98 | 52 |
|---|---|---|---|

**Susceptibility of bacteriophage to heat:** As indicated in Line Chart 1, the highest level of stability was reported at 4°C.

Line Chart 1) Effect of temperature on bacteriophage

**Susceptibility of bacteriophage to pH**: The results of bacteriophage susceptibility to different pH values are shown in Line Chart 2. Each quantification was performed three times. According to the results, the optimum stability of phage was at pH 6 to 8.

**Line Chart 2)** Effect of pH on bacteriophage

**Bacteriophage specificity**: The specificity of this bacteriophage against MRSA isolates was confirmed using the spot test (Fig. 3, Table 4). Also, MRSA susceptibility to isolated bacteriophages was determined based on the formation of the inhibition zone (Fig. 4).

**Fig. 3)** Specificity of isolated bacteriophages for *S. aureus* compared to other pathogens

**Fig. 4)** Effect of isolated bacteriophages on MRSA isolates and observation of lysis zone using the spot test

**The effect of isolated bacteriophages on *S. aureus* using HEp-2 cell-line:** The isolated bacteriophages were inoculated on pre-prepared infectious cell lines under sterile conditions and then examined under a microscope. A tube with healthy cells was considered as a control. According to the results, in well A, bacterial adhesion to the surface of HEp-2 cells was very well evident. However, in well B, there was no sign of bacteria or their adherence to the cells, indicating the antibacterial activity of the phage *in vitro*. According to the images related

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to the effect of isolated bacteriophages on MRSA-infected HEP-2 cell line, it was found that bacteriophages showed a good ability to reduce and eliminate the studied bacteria (Fig. 5). The bactericidal effects of isolated bacteriophages against MRSA were evaluated as completely positive, and infected HEp-2 cell lines lost their original state (deformation), and intercellular connections were largely lost.

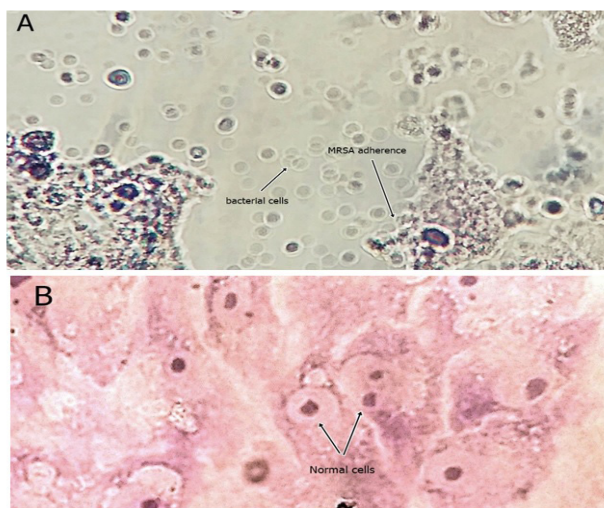


**Fig. 5)** Effect of isolated bacteriophages on bacterial adhesion to HEp-2 cells. **A**: MRSA adhesion to HEp-2 cells. **B**: HEp-2 cells after inoculation of MRSA and phage (no bacterial adhesion to cells in the exposure of phage).

**Table 4**) Effect of isolated bacteriophages on the studied bacteria

| Bacteria | Lysis |
|---|---|
| *Salmonella enterica serovar* Typhimurium | - |
| *Staphylococcus aureus* (ATCC 33591) | + |
| *Shigella dysenteriae* | - |
| *Enteropathogenic E. coli(EPEC)* | - |
| *Citrobacter freundii* | - |
| *Enterococcus faecalis* | - |
| *Listeria monocytogenes* | |
| MRSA isolates | + |
| *Cronobacter sakazakii* | - |

## Discussion

*S. aureus* is typically the cause of many human and animal infections ranging from modest to more important situations such as bacteremia, endocarditis, pneumonia, and bone and joint infections [16]. The emergence of resistant bacterial strains such as MRSA highlights the need for improving treatment methods in the future [17]. Bacteriophages are a type of viruses that use and attack bacteria as hosts. Thus, phage therapy could be a good candidate to combine with traditional therapies [18, 19]. Smith et al. (1987) showed that the use of a single intramuscular dose of anti-K phage was more effective in treating muscle infection in mice than treatment with multiple doses of tetracycline, chloramphenicol, trimethoprim, as well as sulfafurazole [20]. In this study, a specific phage was isolated and tested against MRSA strains. This bacteriophage morphologically belonged to the *Myoviridae* family. The high ability of the *Myoviridae* family to lyse bacteria makes them the best candidates for phage therapy purposes [21, 22]. In a similar study, an anti-MRSA specific phage was isolated from animals and observed using an electron microscope, and its lytic properties against MRSA were evaluated, but it belonged to the *Podoviridae* family [23]. In another investigation, two novel phages belonging to the *Siphoviridae* family were isolated from the farm and showed lytic properties against MRSA [24]. Like all viruses, bacteriophages are species-specific and usually infect a single species of bacteria [25]. In this study, phage specificity test was performed using spot test based on the formation of an inhibition zone. The pathogenic bacteria examined in this study for this purpose were as follows: *S. enterica* serovar Typhimurium, *S. dysenteriae, C. freundii*, Enteropathogenic *E. coli*, *E. faecalis*, *L. monocytogenes*, *C. sakazakii*, *S. aureus* ATCC 33591, as well as 16 clinical isolates of MRSA. It was shown that all MRSA isolates were susceptible to this bacteriophage, while other bacteria showed no sensitivity. These results are in line with the findings of a

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study by Lubowska et al. (2019) [26], but they are inconsistent with the results of another study by Paolozzi and Ghelardini (2006) [27]. External factors such as pH and temperature are very crucial for bacteriophage function and survival. At temperatures below and pHs above the optimal values, less phage genetic material penetrates bacterial host cells [28]. Another purpose of this investigation was to assess the phage function at various temperatures and pH values. According to the results, the isolated phage showed the highest stability at refrigerator temperature. However, it could survive at 25 and 37°C, but its count decreased at a temperature of 50-70°C, and it was destroyed at 80-90 °C. Furthermore, the highest level of lytic activity of bacteriophages was at pH 6 to 8. Therefore, it has an optimal function at physiological pH. Bacteriophages could potentially be applied to decrease infection in various tissue microenvironments. Many different challenges, including cytotoxicity and administrative issues, remain confusing before bacteriophage therapy could be credited among new medications [29]. In this study, the efficacy of the isolated phage was investigated *in vitro* using HEp-2 cells line. Phage-treated cells showed similar results and cell morphology to untreated cells after 2 and 24 hrs of incubation. The isolated bacteriophage capacity to cover mammalian cells against MRSA infection was studied using HEp-2 cells. The results showed a decrement in MRSA-infected HEp-2 cells undergoing phage pre-treatment. Based on the results, the isolated phage could potentially diminish the bacterial burden compared to the phage-undressed HEp-2 cells. This study was assessed with regards to the treatment and control of infections. However, more investigation and *in vivo* studies are required to accurately identify bacteriophage benefits.

## Conclusion

This study showed that isolated bacteriophages could lyse MRSA strains *in vitro*. The emergence of antibiotic resistant *S. aureus* strains suggests that bacteriophages may serve as an alternative solution to this medical dilemma. However, these isolated phages need to be further characterized, and if found to be effective, they could be widely used commercially as an effective antibacterial agent.

## Acknowledgment

We sincerely thank the effort of Pedram Foroughi Shad, head of the Research Department of the Central Organization of Community-Wide Science Ambassadors, Department of Medical Biology Research, Tehran, Iran.

**Ethical Permissions:** This article does not contain any studies with human participants and animals.

**Conflicts of Interest**: The authors declare no conflict of interest.

**Authors' Contribution:** Conceptualization: SS ; data curation: AHH; formal analysis: AHH; funding acquisition: SS; investigation: SS; methodology: MRE ; project administration: SS; writing of the original draft: MRE; writing-review and editing: MRE.

**Fundings**: his study was supported by Research Department of the Central Organization of Community-Wide Science Ambassadors, Department of Medical Biology Research,Tehran, Iran.

**Consent to participate:** This article does not contain any individual person's data in any form.

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