



Original Article

Specification of Bacteriophage Isolated Against Clinical Methicillin-Resistant *Staphylococcus Aureus*



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ABSTRACT

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Objectives: The emergence of resistant bacteria is being increasingly reported around the world, potentially threatening millions of lives. Amongst resistant bacteria, methicillin-resistant *Staphylococcus aureus* (MRSA) is the most challenging to treat. This is due to emergent MRSA strains and less effective traditional antibiotic therapies to Staphylococcal infections. The use of bacteriophages (phages) against MRSA is a new, potential alternate therapy. In this study, morphology, genetic and protein structure of lytic phages against MRSA have been analysed.

Methods: Isolation of livestock and sewage bacteriophages were performed using 0.4 µm membrane filters. Plaque assays were used to determine phage quantification by double layer agar method. Pure plaques were then amplified for further characterization. Sulfate-polyacrylamide gel electrophoresis and random amplification of polymorphic DNA were run for protein evaluation, and genotyping respectively. Transmission electron microscope was also used to detect the structure and taxonomic classification of phage visually.

Results: Head and tail morphology of bacteriophages against MRSA were identified by transmission electron microscopy and assigned to the *Siphoviridae* family and the *Caudovirales* order.

Conclusion: Bacteriophages are the most abundant microorganism on Earth and coexist with the bacterial population. They can destroy bacterial cells successfully and effectively. They cannot enter mammalian cells which saves the eukaryotic cells from lytic phage activity. In conclusion, phage therapy may have many potential applications in microbiology and human medicine with no side effect on eukaryotic cells.

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Introduction

Staphylococcus aureus is a gram-positive, non-motile, non-sporulating bacterium which causes hospital and community infections [1]. This bacterium is responsible for several life threatening infections including endocarditis, osteomyelitis, skin

and soft tissue infections and wound infections leading to a high mortality rate each year [2]. It has been reported that mortality of methicillin-resistant *Staphylococcus aureus* (MRSA) infections have increased amongst AIDS patients in the United States of America [3]. Based on a systematic review and meta-analysis in Iran, 7,464 samples of *S. aureus* were isolated from patients from

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different cities, with $52.7\% \pm 4.7\%$ of strains carried the *mecA* gene which is responsible for resistance to methicillin [4].

Staphylococcus can induce cellular apoptosis after invasion of host cells, and invasion mediated by Staphylococcal fibronectin-binding proteins allow the other microorganisms to bind to the hosts cells and initiate secondary infections [5]. Due to the methicillin resistance of *S. aureus* to most commonly used antibiotics such as oxacillin, nafcillin, methicillin and even vancomycin, treatment of MRSA is more challenging compared to other resistant strains [6]. In addition, indiscriminate and overuse of antibiotics has made the penicillin susceptible *S. aureus* strains, resistant to the population to β -lactam antibiotics [7].

Bacteriophages (phages) can be found in all habitats and more significantly colonization of bacterial strains. A phage is a type of virus that can attack and destroy bacterial cells [8]. Twelve distinct groups of phages have been discovered which are highly specific against bacterial species [9]. The order of *Caudovirales* bacteriophages can be categorized into 3 major families including *Siphoviridae*, *Myoviridae*, and *Podoviridae*. All members of the *Caudovirales* order, have double stranded DNA, with a morphological head and tail structure [9]. The typical structure of a phage includes a head that is filled with DNA or RNA, and a tail used for injection of the genome into the bacterial cells. Due to receptor limitations on phages, they are unable to infect mammalian cells [10].

Isolation of phages against many bacterial populations have been carried out from wastewater, sewage, retail raw chicken meat, soil, blood cultures, cerebrospinal fluid, superficial wounds, deep wound swabs, respiratory tract specimens, urine and miscellaneous specimens [8]. After the process of phage isolation specifically against MRSA, they can be used as an alternative therapy to treat MRSA infections. Due to the inability of bacteriophages to enter mammalian cells, no side effects on eukaryotic cells have been reported [11].

The main aim of this study was to isolate and characterize lytic phages against MRSA reporting their morphology, genetic material and structural protein composition.

Materials and Methods

1. Bacterial strain and growth media

Bacterial strains were isolated from clinical specimens from hospitals in the west of Iran, Kermanshah. Isolates were incubated at 37°C for 24 hours on blood agar (Oxoid Ltd, Basingstoke, UK) and then sub-cultured on TS agar (Tryptic Soya, Liofil- chem, Italy). Single colonies were tested with tube coagulase and the DNase test, and growth on mannitol salt agar (Difco, Detroit, Mich., USA).

After identification of *S. aureus* positive specimens, they were stored in 20% glycerol at -20°C. The Oxacillin susceptibility test was performed to identify MRSA strains (0.5 McFarland suspension of *S. aureus* was spotted onto Mueller-Hinton agar supplemented with 4% NaCl and 6 ug of oxacillin per mL). To initiate the analysis, subculture bacterial strains were added to the nutrient agar. In addition, the presence of the methicillin resistance gene (*mecA*) was determined by multiplex polymerase chain reaction (PCR; Eppendorf epGradient S thermocycler).

2. Isolation of phages against MRSA

Phages against clinical MRSA in sewage and stool samples were isolated based on the enrichment method described by Cerveny, et al with some modifications [12]. Briefly, sewage and stool samples were collected from the main sewage pipe and livestock farming in Ilam University of Medical Science and Ilam University, respectively. Samples were centrifuged and supernatants were filtered through 0.45 and 0.2 μm sterile filters. Twenty mL of filtered sewage sample and 20 mL sterile nutrient broth were mixed and spiked with 5.0 mL of overnight culture clinical MRSA, and then incubated at 37°C for 24 hours. To deplete bacterial strains, the mixture had undergone centrifugation and the supernatant was then sterilized by 0.45 and 0.2 μm filters. Moreover, to check for the presence of phages, a double layer agar method was used as described by Adams [13].

2.1. Plaque assay

Double agar overlay technique was performed to determine plaque formation. Each of the phage suspensions was serially diluted. This was performed by adding 1 mL of diluted phage and 1 mL of host bacterium that was mixed with 2 mL of molten soft-agar (0.75 % agar, w/v). This was poured quickly onto the surface of a solidified nutrient agar plate (1/5 % agar, w/v; Difco Laboratories) [13].

2.2. Phage purification and concentration

All of the isolated phages were purified by single-plaque isolation, and mix plaques with CaCl_2 were obtained by the standard procedure presented by Sambrook et al [14]. One separated phage was picked with a sterile pipette tip along with the surrounding cell mass, and then inoculated into 100 μL CaCl_2 with 200 μL of host strain that had been cultured overnight. One mL of top agar was added and quickly poured onto a solidified nutrient agar plate.

Phages were concentrated based on the published methods described by Yamamoto et al [15] with some modifications. MRSA isolated from host cells was added to the isolated phages and then incubated for 24-48 hours at 37°C to complete the

lysis of host cells. In the next step, culture fluid was centrifuged and filtered through 0.4 µm filters. Polyethylene glycol 8,000 was added to make the final concentration of 20%. Finally, the pellet was collected following centrifugation (12,000 rpm for 20 minutes in 4°C).

3. Random amplified polymorphic DNA-polymerase chain reaction (RAPD-PCR) analysis

Phage DNA was extracted by DNA extraction kit (NOREGEN®). RAPD-PCR was performed based on the Johansson et al [16] study with some modifications.

Four different primers used in this study which included: primer 1: (5'-ACGCAGGCAC-3'), primer 2: (5'-AACGCGCAAC-3'), primer 3: (5'-CCG CAGCCAA-3') and primer 4: (5'-AACGGGCAGA-3') that were selected based on a relatively similar study [17]. RAPD-PCR cycles are described in Table 1.

The PCR mixture consisted of 1 µL Taq DNA polymerase, 1 µL primer, 1 µL dNTPs, 2 µL PCR buffer, 1 µL phage DNA, and 6.5 µL distilled water (for 12.5 µL reaction). DNA band patterns of each primer were obtained after gel electrophoresis (0.8% agarose gel) of the RAPD-PCR (12.5 µL). DNA molecular weight marker (1,000 bp) was used as a standard. Four types of MRSA phage were isolated from the stool samples.

4. Sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

Phage sedimentation (with 20% PEG and 12,000 rpm, 20 minutes in 4°C) was used for sodium dodecyl SDS-PAGE separation. Formaldehyde was used to prevent the effects of PEG on SDS-PAGE.

MRSA-specific phages were mixed with an equal volume of sample buffer (0.0625 M Tris-HCl; pH 6.8, 15% glycerol, 1% SDS, 1% Beta-mercaptoethanol and bromophenol blue), and heated

in a boiling water bath for 10-15 minutes [18].

5. Transmission Electron Microscopy

After transferring the samples containing CaCl₂ to the laboratory, samples were prepared with uranyl acetate (2%) for transmission electron microscopy (TEM) performed at the TEM Lab, Institute of Biochemistry and Biophysics, University of Tehran, Tehran, Iran. Firstly, the same amount of sample and glutaraldehyde (2.5%) was placed on a carbonic grid. After 1.5 minutes, the grid was left to dry on filter paper then washed with deionized water. One drop of uranyl acetate (2%) was added to the sample, and the grid was dried out on filter papers; samples were then ready for TEM. Particles were examined using a ZEISS EM 900 electron microscope (80 kV accelerating voltage; TEM Lab, Nano Kefa Institute, Iran).

6. Host range

The host range of each phage was determined by inoculation of bacterial species into a double-layer agar, and the phages were tested against all the clinical MRSA.

Results

1. Detection of *mecA* gene in clinical MRSA

To determine the presence of the methicillin resistance gene (*mecA*) in clinical MRSA samples, multiplex PCR was used (Figure 1).

2. RAPD-PCR

RAPD-PCR was performed on the phage-extracted DNA from

Table 1. Random amplified polymorphic DNA-polymerase chain reaction cycle programs.

Time	Temperature
45 s	94°C
120 s	30°C
60 s	72°C
4 cycles	
5 s	94°C
30 s	30°C
30 s	72°C
25 cycles	
10 min	72°C

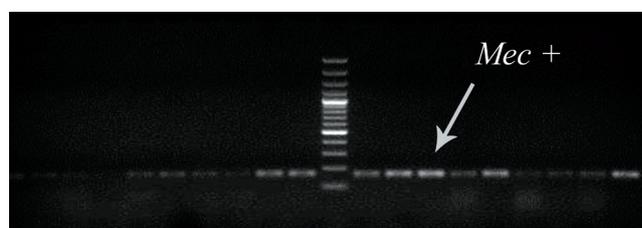


Figure 1. Multiplex PCR for detection of *mec+* gene in *Staphylococcus*. The presence of the methicillin resistance gene (*mecA*) was evaluated in clinical MRSA. DNA molecular weight marker (1,000 bp) was used in this figure. MRSA = methicillin-resistant *Staphylococcus aureus*.

stool samples. In this study, 4 different primers were used which differentiated between all MRSA specific phages (Figure 2).

3. Structural protein analysis

The structure of MRSA specific phage proteins shown in Figure 3 revealed 2 major protein bands approximately 30 and 48 kDa in weight, and a minor structure protein band, of approximately 80 kDa (Figure 3).

4. Phage morphology

Based on the morphological features of the bacteriophages observed by electron microscopy, all the phages examined showed a long icosahedral capsid and non-contractile tails. According to the International Committee on Taxonomy of viruses, these phages were belonged to the *Siphoviridae* family [19] of the *Caudovirales* order [20]. The morphology of MRSA-specific bacteriophage has been illustrated in Figure 4.

Discussion

S. aureus is a source of many human and animal infections such as osteomyelitis, endocarditis, skin abscesses and many more [2]. Overuse of antibiotics has led to the emergence of MRSA. The emergence of MRSA strains highlights the necessity for therapeutic improvements in the future [21]. It has been suggested that the combination of bacteriophages and antibiotic therapy maybe more effective than unilateral treatment [21]. Due to the failure of treatment of Staphylococcal infections by routinely used antibiotics, this study highlights the role of phage therapy as a possible solution [22]. Phage therapy has many potential applications in human medicine and dentistry as well as in veterinary science and agriculture, and it can be an appropriate alternative therapy especially for multi drug resistant bacteria [23,24].

The first report of phage therapy in clinical medicine was described by Felix d'Herelle [25]. Many researchers think bacteriophages can play an important role to defeat resistant bacteria such as *S. aureus*. There has been a remarkable increase in the number of Staphylococcus phages with marvellous progress in targeting resistant *S. aureus* [22].

In a similar study, different MRSA-specific phages belonging to the *Podoviridae* family, have been isolated from farm animals, and visualized using electron microscopy which showed the possibility of using lytic bacteriophages against MRSA strains [26]. In another study, 2 new phages isolated from a farmyard were classified into the *Siphoviridae* family which had lytic activity against MRSA [27].

There are a wide variety of phages on the planet capable in destroying a large number of different bacterial populations [23].

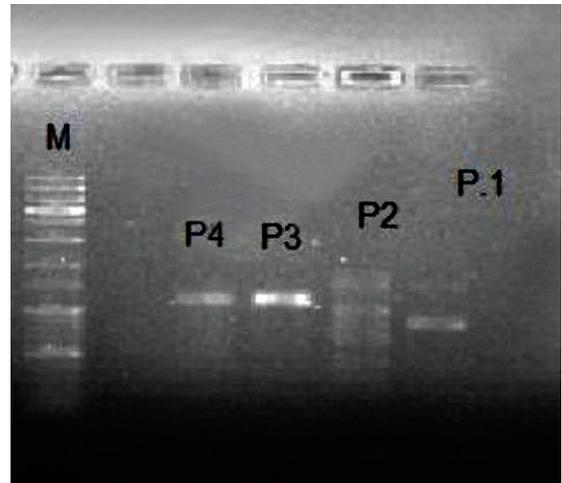


Figure 2. Agarose gel electrophoresis showing RAPD-PCR amplification of DNA from isolated MRSA specific phage. P1 = Primer number one etc; M = molecular weight marker (1,000 bp); MRSA = methicillin-resistant *Staphylococcus aureus*; RAPD-PCR = random amplified polymorphic DNA-polymerase chain reaction.

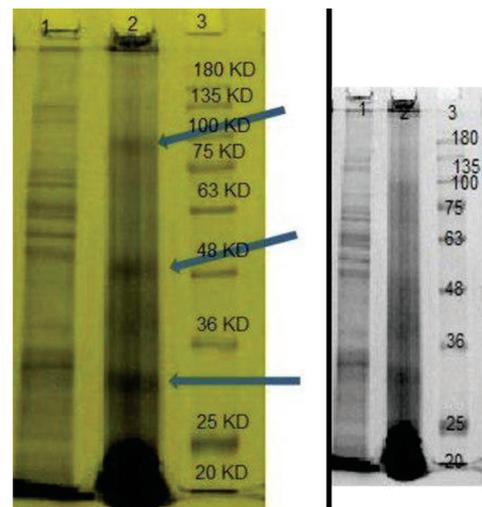


Figure 3. SDS-PAGE patterns of structural proteins isolated from MRSA-specific phage. Line-1: bacterial protein, Line-2: phage protein and line-3: molecular weight marker (1,000 bp). MRSA = methicillin-resistant *Staphylococcus aureus*; SDS-PAGE = sulfate-polyacrylamide gel electrophoresis.

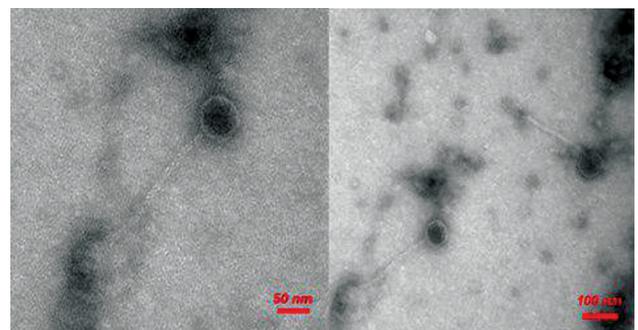


Figure 4. Morphology of MRSA bacteriophage determined by TEM. MRSA = methicillin-resistant *Staphylococcus aureus*; TEM = transmission electron microscopy.

Compared to the classic antibiotics, phage therapy is more cost-effective and without serious side effects on eukaryotic cells. Phages have complementary receptors on bacterial cell membranes [28]. The specific affinity of bacteriophages to the particular bacterial strains can prevent common side effects reported from routine antibiotics therapy. In addition, phage gene modification can improve the specificity and sensitivity of bacteriophages against bacterial strains [29]. Phenotype variation can help bacteria to escape from immune system and anti-bacterial treatments [29]. This problem can also be addressed by using phage mutants [30].

In this study, isolation and characterization of effective bacteriophages with lytic activity against MRSA has been evaluated. Based on our finding, phage therapy could be a good candidate to combine with traditional therapies.

However, to gain the benefits from the natural action of bacteriophages against the bacterial population, more clinical and in vivo studies should be conducted to show efficacy of phage therapy.

In conclusion, the phages reported in this study exhibited good bacterial lysis activity. However, these isolated phages need to be further characterized and if found to have efficacy, they can be used in commercial lysate preparations in the near future, where therapeutic potential against a wider range of bacterial strains can be further investigated. Research using animal models of phage-bacterial interactions and ultimately clinical trials will highlight this topic and may provide a powerful alternative treatment against the dangers of multi-resistant pathogens.

Conflicts of Interest

The authors have declared no conflicts of interest.

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