

## Isolation Of Bacteriophages Active Against Methicillin-Resistant Staphylococcus Aureus Strains

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**Abstract:** In recent years, the emergence of antibiotic-resistant *S. aureus* strains, including methicillin-resistant *S. aureus* (MRSA) has complicated the treatment of *S. aureus* infections. The increasing rate of resistance of pathogenic bacteria, such as *Staphylococcus aureus*, to classical antibiotics, has directed research toward identification of other means to fight infectious disease. One particularly viable option in this regard is the use of bacteriophages. The aim of this study was the isolation of Staphylophages that lyse MRSA strains isolated from human. In this study, 13 isolates of *S. aureus* were collected from laboratories of some hospitals in Isfahan city. The Staphylophages were isolated from sewage by filtration and enrichment in *S. aureus* overnight culture. Bacteriophage isolation was performed by plaque formation test in double layer agar. Finally, detection of Staphylophage was confirmed by TEM electron microscopy. All 13 *S. aureus* isolates were resistant to methicillin. Isolated Staphylophages could lyse all the 13 MRSA isolates belonging to the Siphoviridae family. These Staphylophages could lyse all of MRSA isolates in culture media. Therefore, they can be considered as potentially effective agents in the treatment of staphylococcal infections. However, this conclusion needs to be confirmed by animal and human clinical evaluations.

**Keywords:** *Staphylococcus aureus*, Bacteriophage, methicillin, Antibiotic resistance

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### I. Introduction

*Staphylococcus aureus* is a Gram-positive bacterium opportunistic pathogen that causes nosocomial infections of surgical wounds and infections associated with indwelling medical tools like catheters. It causes skin and soft tissue infections in healthy individuals [1]. Treatment of such infections is largely based on antibiotics; however, the increasing prevalence of antibiotic resistance in clinical isolates of *Staphylococcus aureus* is a major problem.

Methicillin-resistant *Staphylococcus aureus* (MRSA) was first observed in hospitals. But, at the beginning of the year 2000, community-acquired MRSA also started to be reported (Calfee 2015). MRSA-related osteomyelitis [2], nasopharyngeal colonization [3], skin infections [4], food-chain animal infections, and infections in immunocompromised patients [5,6], among others, have been reported. Studying the resistance mechanisms presented in previous studies [1,7] reveal that MRSA strains have become endemic in many hospital environments. In addition, these MRSA strains also frequently exhibit resistance to a variety of other common antibiotics [2,8].

Novel treatment options, including bacteriophages, have been reviewed previously [3]. This bacterium causes a wide variety of human infections ranging from simple abscesses to fatal sepsis, as well as endocarditis, pneumonia, mastitis, phlebitis, meningitis, and toxinoses. Owing to high-cost requirements, withdrawal of pharmaceutical industry from the discovery of new antibiotics has made it essential to develop alternative therapeutic regimen so as to cheap, easily available, highly potent and with minimum side effects to alleviate these infections [6,7].

As a result, investigations for new and alternative antimicrobials effective against *S. aureus* have become increasingly relevant. Bacteriophages (phages) were investigated as far back as 1921 to eliminate bacteria including staphylococci in human infections. Human phage therapy studies have been a renewed interest for which evidence from a number of reviews has been recently published [9,10]. All sequenced *S. aureus* bacteriophages are grouped into three classes based on genome size: class I (< 20 kb), class II (~40 kb) and class III (> 125 kb) [11].

Previous studies and clinical trials have shown that bacteriophage therapy is effective in the treatment of many *S. aureus* related diseases, including lung infection, sepsis, and leg ulcers [11]. Therefore, in this study, we report the isolation of *S. aureus* bacteriophages from sewage and present the results of the microbiological

and molecular characterization of selected phages that can be utilized in developing biocontrol agents against staphylococcal contamination.

## **II. Materials and methods**

### **Bacteria:**

The present study included 13 *S. aureus* strains previously isolated from patients hospitalized at the hospitals of Chaharmahal and Bakhtiari province and kept in the microbiology laboratory of the Faculty of Veterinary Medicine, Shahrekord University. All strains were confirmed as *S. aureus* by the coagulase test, microscopic observation, and PCR assay for *S. aureus* detection.

### **Sampling and preparation of sewage:**

The sampling was carried out using a sterile glass bottle and sewage samples were taken from a refinery in Isfahan province. The samples were then transferred to the laboratory and were centrifuged at 8000g for 10 minutes, then the supernatant filtered by sterile 0.2 µm Minisart filters (Sigma- Aldrich, Cat. No.: 16534K).

### **Preparation of bacteria and adding sewage:**

At first, one milliliter of overnight bacterial culture medium was added to 20 milliliters of BHI liquid culture medium and the suspension was incubated for 3 hours at 37 ° C. Then 20 milliliters of filtered sewage were added to this suspension and incubated at 37 ° C for 24 hours. Then it was centrifuged for 10 minutes at 8000 g and supernatant was filtered through a 0.45 µm sterile syringe filter (Sigma- Aldrich, Cat. No.: CLS431225).

### **Phage isolation:**

Bacteria were grown in 20 mL of BHI medium and incubated for 4h at 37°C. Then 20 mL of the filtered sewage were added to the bacteria culture and incubated for 24h at 37°C. After Centrifuge for 10 minutes at 8000 g, the supernatants were filtered through a 0.2 µm syringe filters (Sigma- Aldrich, Cat. No.: CLS431229). Phage isolation was performed using the double agar method. First, 9 mL semi-solid BHI (containing 0.7% agar) was placed into tubes and sterilized. When the temperature of the semisolid medium reached to about 45 ° C, 0.1 mL of overnight cultured and filtered bacteria added to it and spread throughout the culture medium. This culture medium was then added to a solid and sterile BHI medium (containing 1.5% agar) to form a two-layer culture. When the agar was tightened, 20 µl of the filtered sewage was placed in the center of the culture medium and incubated for 24 hours at 37 ° C.

### **Electron microscopy:**

The phage suspension was centrifuged for 90 minutes at 20000g. The supernatant was then slowly withdrawn from the tube and the pellet adhered to the tube wall was dissolved in 50 mL of Phage buffer and again centrifuged as described above. After centrifuge, the supernatant solution was removed and the pellet was dissolved in 25 mL of Phage buffer. For the coloring of the phages, 10 µL of the suspension transferred to a carbon-treated copper grid (400 Mesh) and placed 210 seconds in this mode. The grid was then placed in the room temperature for 20 seconds. Then, 20 µL of uranium acetate was poured onto the grid and after 160 seconds, the excess uranium acetate removed gently using a drying paper and the grid leaved in room temperature for 30 minutes to completely dry.

## **III. Results**

### **Plaque formation:**

After inoculation of the sewage into double layer agar, lytic bacteriophages were isolated and the phage plaques were completely formed in the plates. These plaques indicated that these phages had lytic effects on 13 isolates of *Staphylococcus aureus* (Figure 1).

### **Electron microscopy:**

In the images taken by the TEM electron microscope, the isolated bacteriophages were in the size of about 65 nm and had hexagonal and symmetrical head with a non-contractile, thin and simple tail. Considering the characteristics of morphology, it seemed that the isolated bacteriophages belonged to Siphoviridae family. Siphoviridae is a family of double-stranded DNA viruses in the order Caudovirales (Figures 2-4).

#### IV. Discussion

In the current study, phages against 13 MRSA strains belonging to the family Siphoviridae were isolated from liquid sewage samples had been taken from a refinery in Isfahan. In the previous studies, phages against MRSA have been isolated from different sources, such as hospital wastewater, cow's milk, sewage/pond water, soil samples collected from poultry, and fecal samples from livestock farms [12-16].

Treatment of staphylococcal infections with antibiotics is becoming increasingly difficult regarding the widespread presence of *S. aureus* strains resistant to multiple antibiotics. The epidemiology of *Staphylococcus aureus* has continued to change during the past few decades. Methicillin-resistant strains initially isolated in healthcare settings are now emerging in the community [17]. To control the spread of these strains, it is important to understand the epidemiological relatedness between the organisms [11].

Staphylococcal infections are the most important cause of antibiotic-resistant healthcare-associated infection, which may result in a prolonged hospital stay or the use of medical devices. In this regard, a critical hallmark of a chronic staphylococcal infection is the ability of bacteria to grow as a biofilm. Rahimzadeh et al. (2017) using the bacteriophage Phi11 prevented the formation of *S. aureus* biofilm on medical devices such as catheters [18].

In the recent years, many studies have been conducted in regard to Staphylophages. (2013) isolated *Staphylococcus aureus* bacteriophages from poultry/livestock farms [19].

Isolation of phages is based on different sources, for example, in a study by Wommack et al. (2000) [20], phages were isolated from water samples from the TFF filtration apparatus used for the concentration of viruses from water samples. In a study by Dane et al. (2002), phages were extracted from soil samples by centrifuging, sonication, and filtering the supernatant [6].

Phage isolation may not be an accessible or feasible method in the reference laboratory because such testing is sensitive and many variables can affect the results, for example, in our study, sewage samples were taken from different locations and phages were not isolated from some of the locations. Therefore, the isolation process took considerable time [14].

Synnott et al. (2009) isolated phages against MRSA in liquefied sewage samples [21]. Moreover, in a study by Chang et al. (2015), phages against MRSA from skin were isolated, the results of electron microscopy showed that isolated phages belonged to the family Siphoviridae, serogroup B, and had a latent period time of 20 minutes [16]. Kwiatek et al. (2011) isolated phages that lysed 17 *S. aureus* and coagulase-negative *Staphylococcus* (CNS) isolates [14]. In our study, phages isolated from sewage belonged to the family and phages lytic activity against 13 MRSA strains.

The sufficient therapeutic effect of phages has been established in many studies. For example, Kutter et al. (2005) applied phages to treat radiation burns for treating three people infected with *Staphylococcus aureus* following a burn. After 7 days of using the phage, *Staphylococcus aureus* infection was eliminated in the wound site [22].

Gu et al. (2011) showed that the phage lysin LysGH15 might be an alternative treatment strategy for the isolation of phages that are active against *S. aureus* [23]. However, Kwiatek et al. (2012) and Sahin et al. (2013) obtained phages active against *S. aureus* clinical MRSA strains infections caused by MRSA [24].

Chhibber et al. (2013) found that co-therapy using lytic bacteriophage and linezolid is an effective treatment in eliminating MRSA from diabetic foot infections [25]. Mishra et al. (2014) isolated and characterized the therapeutic potential of bacteriophages virulent to *Staphylococcus aureus* associated with goat mastitis. The results of their study provided insight into the use of lytic bacteriophages for therapeutic interventions against multi-drug-resistant *S. aureus* inducing mastitis in goats [26].

In a study performed by Jun et al. (2013), it was exhibited a rapid and effective bactericidal activity of SAL200 against encapsulated and biofilm-forming *S. aureus* as well as against planktonic *S. aureus* cells [15].

Schuch et al. (2016) CF-301 with antibiotics for the ability to eradicate MRSA1 biofilms grown for 24 hours in polystyrene dishes. The results showed that CF-301 removed all visual biomass by 2 hours, whereas the antibiotics failed to remove biomass after 4 hours of treatment [27]. Son et al. (2010) compared the staphylococcal biofilms removal activity of a bacteriophage SAP-2 and a derivative of it, i.e., endolysin. According to their observations, endolysin SAL-2 showed lytic activity against all strains of the *Staphylococcus* genus, whereas bacteriophage SAP-2 had antibacterial activity against only some *S. aureus* strains [1].

Overall, due to the increasing threat imposed by multidrug resistance, searching for novel antimicrobials has been the subject of interest for many researchers. Some studies have described isolation and characterization of new endolysins. The potential applications of these enzymes in the fields of medicine, food safety, agriculture, and biotechnology will be intensified in the near future [19,26].

Furthermore, antibiotic resistance, virulence factors, and toxins in bacteria strains are encoded by mobile genetic elements, such as prophages. These elements can be horizontally transferred by transduction. The presence of phages in sewage may be one of the reasons for antibiotic resistance or virulence factors, as transduction may result in a nonvirulent strain of *staphylococcus* turning into a virulent one [28]. Overall, in the

present study, phages against MRSA belonging to the family Siphoviridae (order Caudovirales) were isolated from liquid sewage samples. Air-born phages can be transferred to the wards, thus, creating negative pressure in the sewage pipe can prevent phages from entering wards via aerosols. Other investigations are still required for the full molecular characterization of bacteriophages. A limitation of the present study was lack of a decent financial support [1].

## V. Conclusion

In the current study, phages against 13 MRSA strains belonging to the family Siphoviridae were isolated from liquid sewage successfully.

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