**Research Article**

Ameer Hamza, Shehla Perveen, Zaigham Abbas, Shafiq Ur Rehman*

**The Lytic SA Phage Demonstrate Bactericidal Activity against Mastitis Causing Staphylococcus aureus**

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**Abstract:** *Staphylococcus aureus* is the major causative agent of mastitis among dairy animals as it causes intra-mammary gland infection. Due to antibiotic resistance and contamination of antibiotics in the milk of diseased animals; alternative therapeutic agents are required to cure mastitis. Lytic bacteriophages and their gene products can be potential therapeutic agents against bacteria as they are host specific and less harmful than antibiotics. In this study, *Staphylococcus aureus* were isolated from milk samples of the infected animals and identified biochemically. SA phage was isolated from sewage water showing lytic activity against *Staphylococcus aureus* isolates. The highest lytic activity of bacteriophages was observed at 37°C and pH 7, and the most suitable storage condition was at 4°C. SA phage efficiently reduced bacterial growth in the bacterial reduction assay. The characterization and bacterial growth reduction activity of the bacteriophages against *Staphylococcus aureus* signifies their underlying potential of phage therapy against mastitis.

**Keywords:** Mastitis, *Staphylococcus aureus*, bacteriophage, lytic activity, phage therapy.

**1 Introduction**

Bovine mastitis is the mammary gland inflammation in dairy cattle caused by some bacterial pathogens. *Staphylococcus aureus* is the most potent pathogen for bovine mastitis around the globe [1]. It is considered as the most prevalent disease in developing countries that often affects quality as well as quantity of the milk [2]. Pakistan’s primary industry is agriculture and its economy mainly relies on the livestock and dairy products [3]. Bovine mastitis is a very expensive disease that is directly related to the decline in production rates of the dairy farms thus causing economical loss in the developing countries [4].

The emergence of multi drug resistant bacteria is the main challenge these days in veterinary medicine. Methicillin resistant *Staphylococcus aureus* (MRSA) causing mastitis is one of the main problems in cattle farming [5-7]. Although antibiotics is the first choice to fight against bacterial infections, the emergence of multidrug resistant pathogens and the presence of antibiotic residues in the milk and milk products are major concerns [8]. This scenario demands an alternative therapy to treat mastitis in cattle, caused by *Staphylococcus aureus*, and bacteriophage therapy might be a potential option for such infections [9,10].

This study was conducted to isolate and characterize potent bacteriophages that can kill *Staphylococcus aureus* causing bovine mastitis. Because of the wide diversity of *Staphylococcus aureus*, bacteriophages isolated from local sources may be a potential tool to treat complicated infections from the same environment. This study will provide a promising alternative therapy for mastitis affected cows and buffalos to prevent economic losses due to reduced milk production.

**2 Materials and Methods**

**2.1 Sample Collection for Staphylococcus aureus**

Milk samples of mastitis diagnosed cows and buffalos were collected in sterile glass containers from the fields of...
Jhang, Pakistan. The samples were brought to the Teaching Hospital, College of Veterinary and Animal Sciences and initially tested with surf field mastitis test to confirm a high count of somatic cells. Positive samples were transported immediately to the Microbiology laboratory at the Department of Microbiology and Molecular Genetics, University of the Punjab, Lahore.

2.2 Isolation and Characterization of *Staphylococcus aureus*

Milk samples were serially diluted up to $10^{-3}$ under aseptic conditions and equal volumes were spread on the surface of sterile Luria Bertani (LB) agar plates in duplicate. Plates were incubated aerobically at 37°C for 24 hours. After incubation, the bacterial colonies were selected on the basis of morphological features and further purified by re-streaking. Purified bacterial colonies were identified through morphological and biochemical characteristics such as Gram's staining, catalase test, DNase test and growth on Mannitol salt agar (MSA).

2.3 Isolation of Bacteriophage

Waste water samples were collected aseptically from the main waste water drain of Punjab University Lahore, Pakistan. Briefly, the solid debris was removed through centrifugation and the supernatant was mixed with 2X L-broth. The *Staphylococcus aureus* strain SA-08 was added as a host bacterium (100 µl, $10^5$ Cfu) followed by incubation at 37°C for 24 hours with shaking. Chloroform (1%) was added and the flask was left unshaken for 15 minutes followed by centrifugation at 11,000×g for 5 minutes. Polyethylene glycol 6000 (PEG6000) and sodium chloride (NaCl) were added to final concentrations of 10% and 1M, respectively, followed by overnight incubation at 4°C. The mixture was centrifuged at 11,000×g for 20 minutes at 4°C and the pellet was re-suspended in 1mL phosphate buffered saline (PBS) and filter (<0.45 µm) sterilized [11]. The detection of bacteriophages and their quantification was done through plaque formation using double-layer agar method [11]. Isolated bacteriophages were picked with the help of a sterilized Pasteur pipette and dropped into fresh L-broth aliquot containing a few drops of chloroform. The preparation was kept at 4°C for short term storage and kept at -20°C after adding 5% glycerol for long term storage. The bacteriophage was purified by picking the single isolated bacteriophage plaque and growing through double-layer agar technique. The plaque purification was done 2-3 times until homologous plaques were observed.

2.4 Host Range Determination of Bacteriophage

The host range of isolated phage SA was checked against different *Staphylococcus aureus* strains isolated from mastitis infection in buffalos. The sensitivity of some bacterial strains other than *Staphylococcus aureus* from phage SA were also tested.

2.5 Characterization of Bacteriophage

Purified bacteriophages were characterized for their potential as therapeutic agents by parameters such as morphological, physiological i.e., the effect of pH and temperature on stability of bacteriophages, the bacterial growth reduction ability and one step growth curve [12].

2.6 One Step Growth Curve of Bacteriophage

One step growth of SA phage was executed in duplicates as reported by Capra et al. (2006) with a few modifications. The host bacterial strain was incubated at 37°C to reach mid-exponential stage (O.D 600 as 0.4-0.6, approximately $10^7$ Cfu/mL) and collected by centrifugation. Filtered (0.45 µm) bacteriophages ($10^8$ Pfu/mL) were supplemented with the bacterial re-suspension and incubated at 37°C for one minute. The mixture was centrifuged at 13000 rpm for 30 seconds to separate unbound phages, pellet was re-suspended in 100 mL LB broth followed by incubation at 37°C. Samples were collected at 5 minute interval for up to 60 minutes, centrifuged, and the phage titer was assessed by double layer agar method.

2.7 Bacterial Growth Reduction Assay

Overnight grown bacterial culture ($10^8$ Cfu/mL) was added to two LB broth flasks. One flask was inoculated with respective phage ($10^8$ Pfu/mL), the other was used as a control (no phases). Flasks were incubated on a shaking incubator at 120 rpm at 37°C. Optical density (O.D 600 nm) was measured at an interval of 2 hours for up to 24 hours and plotted against the incubation time for comparison of growth reduction with control.
2.8 Storage Stability of SA Phage

Purified bacteriophages were kept at different storage temperatures (-20, 4, 25°C) for 2 months to determine the most suitable storage conditions. The PFU/mL was calculated by double layer agar technique after two months incubation.

2.9 Examining Bacteriophage stability at wide range of pH and Temperature

The activity of SA phage at different pH and temperatures was checked by incubating it at different pH and temperature values. Briefly, known concentrations of purified bacteriophages were mixed in L-broth having different pH levels (5-10) and incubated for one hour at 37°C. Similarly, to study the effect of different temperatures on viability of bacteriophages, a known virus concentration was incubated at different temperatures (28, 37, 50, 68, 80 and 105°C) for 24 hours in L-broth (pH 7.0). The incubated phages were independently mixed with exponential growth culture of the host bacteria and the number of plaques was counted by the double layer agar method [13].

2.10 Morphology Study by Transmission Electron Microscopy

Samples for electron microscopy were prepared by negatively staining the SA phage with 5% uranyl acetate after washing it three times with 0.1 M ammonium acetate solution (pH 7.0). The morphology of the purified phage SA was examined by JEOL JEM 1010 transmission electron microscope, operating at 100 KV.

3 Results

Nine different strains of Staphylococcus aureus were isolated from milk samples of the infected animals. The bacterial strains were identified through cellular morphology on Gram’s stain reaction as Gram positive cocci and through colonial morphology that displayed yellow colored colonies on LB agar with smooth surface and entire margins. However, biochemical characteristics of the purified bacterial colonies confirmed Staphylococcus aureus as they produced catalase, DNase enzymes and production of yellow colored colonies on the surface of mannitol salt agar (MSA). The antibiotic resistance pattern of the isolated strains is mentioned in Table 1.

Table 1. The staphylococcus aureus strains isolated from mastitis infection, presented here are their antibiotic resistance pattern along with their susceptibility to the SA phage.

<table>
<thead>
<tr>
<th>Serial no.</th>
<th>Strain name</th>
<th>Multi-Drug Resistance</th>
<th>Cefoxitin (mm)</th>
<th>Oxacillin (mm)</th>
<th>Vancomycin (mm)</th>
<th>Susceptibility from SA Phage</th>
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<tbody>
<tr>
<td>1</td>
<td>SA- 07A</td>
<td>S</td>
<td>29</td>
<td>18</td>
<td>19</td>
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</tr>
<tr>
<td>2</td>
<td>SA- 07 B</td>
<td>R</td>
<td>25</td>
<td>09</td>
<td>19</td>
<td>+++</td>
</tr>
<tr>
<td>3</td>
<td>SA- 08</td>
<td>R</td>
<td>04</td>
<td>-</td>
<td>18</td>
<td>+++</td>
</tr>
<tr>
<td>4</td>
<td>SA- 09 A</td>
<td>S</td>
<td>25</td>
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<tr>
<td>5</td>
<td>SA- 09 B</td>
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<td>6</td>
<td>SA- 09 C</td>
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<td>21</td>
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<td>7</td>
<td>SA- 09 D</td>
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<td>20</td>
<td>14</td>
<td>17</td>
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<tr>
<td>8</td>
<td>SA- 09 E</td>
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<td>9</td>
<td>SA- 17 A</td>
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<td>+++</td>
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<tr>
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<td>KP-13</td>
<td>R</td>
<td>04</td>
<td>07</td>
<td>16</td>
<td>+++</td>
</tr>
<tr>
<td>11</td>
<td>PA-27</td>
<td>R</td>
<td>03</td>
<td>04</td>
<td>16</td>
<td>_</td>
</tr>
<tr>
<td>12</td>
<td>EC-15</td>
<td>R</td>
<td>05</td>
<td>13</td>
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</tr>
</tbody>
</table>

SA= staphylococcus aureus, KP= klebsiella pneumonae, PA= pseudomonas aeruginosa, EC= Enterobacter cloacae, R= resistant from drugs tested, S= sensitive from drugs tested, +++ = sensitive from SA phage and _ = non sensitive from SA phage
Bacteriophage SA was isolated and purified from the enriched waste water sample against targeted host *Staphylococcus aureus*. The plaque morphology of SA phage was done using the double layer agar method and the plaques of the SA phage were small (1-2 mm) and pinpoint in their morphology. The host range of SA phage was checked for different bacterial isolates of *Staphylococcus aureus* isolated from mastitis infections, along with environmental isolates of *Klebsiella pneumonia* (accession no: KF975429), *Pseudomonas aeruginosa* (accession no: DQ455691) and *Enterobacter cloacae* (accession no: KF975427). The Phage SA showed activity against 6 out of 12 tested strains of *S. aureus*, while no activity against the bacterial strains other than *Staphylococcus aureus* was observed. The sensitivity of different tested bacterial strains along with their sensitivity from SA phage is presented in Table 1.

The effect of different pH values on the viability of SA is important to check their maximum, minimum and optimal pH value which facilitate their proper handling during phage therapy. It was observed by treating the bacteriophages at different pH for 1 hour followed by phage titer determination. SA phage showed a great stability at a wider range of pH (4-11 pH) evidenced by no significant decrease in the Phage titer (Figure 1).

The thermal stability of phages is helpful to standardize the phage therapy techniques and keep phages working even through harsh conditions. Thermal stability of isolated SA phage was checked after incubating at the corresponding temperature and then determining phage titer by phage titer determination method. SA Phage was so resistant to extreme temperature even at 105°C for overnight incubation in oven; however, the phage titer was greatest when incubated at 37°C and 28°C (Figure 2).

One step growth curve helps in verifying the latent period and burst size of the bacteriophage. The shorter latent period with higher burst size are ideal parameters for a phage candidate for phage therapy. The one step growth curve of the SA phage is presented in Figure 3. Our results revealed that the latent period of SA phage was 30 minutes, while its burst size was 1000 phage particles per infected bacterial cell.

Bacterial growth reduction assay holds a tremendous relation towards phage therapy as it shows the potential of bacteriophages to reduce the bacterial growth during incubation. Maintained bacterial reduction is a major interest in the phage therapy. Reduction in growth of the host bacterium was observed for SA phage, compared with non-infected host culture which served as control. Phage SA phage suppressed the bacterial growth for initial 8 hours and then phage resistant bacteria emerged.
However, even after 24 hours of infection, the bacterial growth was still less in phage treated mixture as compared to the control (Figure 4).

The morphology of the bacteriophage SA was examined through Transmission Electron Microscopy (TEM), and found that it has a long contractile tail and icosahedral head (Figure 5) which indicates that it belongs to the family Myoviridae. The estimated head size of the SA phage was 108 nm while the tail size was about 130 nm. The genome of SA phage was isolated and observed under UV after running the DNA in 0.7% agarose gel for one hour and a half. Phage SA genome appeared to have double stranded DNA as it was not digested by endonuclease I enzyme, which digests single stranded DNA. Length of the genome is higher than 40 kb in size (Figure 6).

4 Discussion

Pakistan is an agricultural country and its economy greatly depends on agriculture and livestock. Mastitis is the bacterial infection of bovine mammary glands that has an effect on animal’s health, rate of milk production and its quality, which ultimately lead to loss of economy. An increased trend of multiple drug resistance among bacterial pathogens leads to the failure of antibiotic treatments among animals that leads to prolonged and complicated infections. Therefore, there is an urgent need for an alternative and significantly effective therapy against multi drug resistant bacterial pathogens. *Staphylococcus aureus* is one of the organisms responsible for causing mastitis [14]. This study was an effort to isolate, characterize and assess the potential of lytic SA phage, isolated from indigenous environment to kill *Staphylococcus aureus*. This is the first work from Pakistan explaining the isolation and characterization of bacteriophages against the mastitis causing *Staphylococcus aureus*. The isolated phage showed the lytic activity as well as showed marked stability at different pH and temperatures which are hallmark characteristics of this phage to be used in phage therapy.
In this study nine different strains of *Staphylococcus aureus* were isolated from milk of mastitis infected cows and buffaloes and were characterized. Isolated strains showed marked resistances from commonly used antibiotics. Antibiotic resistance in *Staphylococcus aureus* from commonly used antibiotic is reported in different studies [14]. Phage SA, isolated against target bacteria showed a narrow host range which is a hallmark characteristic of bacteriophages as reported in different studies [15,16]. A phage cocktail containing a combination of different phages is a possible solution where multiple types of bacteria can cause an infection.

The stability of SA phage over a wide range of pH is beneficial for administering it to infected cattle through oral route and for udder application. It could also be useful during food processing because the phages can face different pH environments, so it can be applied in the milk to reduce *Staphylococcus aureus* contamination during cheese and yogurt production [17]. The extreme resistance to temperature is advantageous for SA Phage in phage therapy and also for other purposes like their use to control *Staphylococcus aureus* growth in raw milk as it can easily survive in the pasteurization temperature [18]. Heat resistant phages are also important in food industry and in pasteurized food products. Bacteriophages are more resistant to high temperatures as compared to bacteria (Breitbart and others 2004). Likewise, after two months of storage at room temperature, at 4°C and -20°C, SA produced the highest titer at 4°C as compared to -20°C and room temperature (Table 2). Phage SA showed high burst size with a low burst time, favoring its use in the phage therapy. Burst size presented by phage SA is relatively higher than that reported from phage SPW [9] and Phage K [19]. Ability of the SA phage to control the growth of bacteria for first 8 hours is a hallmark potential to be used for phage therapy. Appearance of resistance after 8 hours is being observed in different bacteria, but, considering the in vivo system of a functional immune system, resistance will rarely emerge. If resistance is produced, it will reduce the virulence of bacteria as showed by different studies [20]. Morphology of the phage SA under electron microscopy placed it in the family Myoviridae, like another phage SPW, isolated against *Staphylococcus aureus* causing bovine mastitis [9]. The genome of phage SA come out as double stranded DNA and more than 40 kb in size that is in accordance with the genome size of other phages [9,19] belonging to family Myoviridae.

Here the isolation and characterization of a bacteriophage SA isolated against *Staphylococcus aureus* causing bovine mastitis is described. Phage has showed a narrow host range with high stability at differing pHs and temperatures. High burst size along with a tendency to control the bacterial growth for the initial 8 hours make this phage suitable for phage therapy. Combinations of different phages in a cocktail along with suitable multiplicity of infection might overcome bacterial phage resistance.

### Table 2. Storage stability of SA phage at different environmental conditions.

<table>
<thead>
<tr>
<th>Storage condition</th>
<th>Phage titer of SA (Pfu/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4°C</td>
<td>4.7×10⁹</td>
</tr>
<tr>
<td>Room temperature</td>
<td>5.6×10⁸</td>
</tr>
<tr>
<td>-20°C</td>
<td>2×10³</td>
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</tbody>
</table>

**Conflict of Interest:** There is no conflict of interest for this article.

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**References**


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aureus (MRSA) ST398 associated with clinical and subclinical mastitis in Belgian cows, Vet. Microbiol., 2010, 144, 166-171


