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Characterization of Bacteriophage Targeting S. aureus Isolated from Caprine Mastitis

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ABSTRACT
The current study was conducted to characterize phages virulent to Staphylococcus aureus isolates from caprine mastitis as well as from cattle/buffalo to assess their potential in phage therapy. Four bacteriophages were analyzed based on biological properties, namely the lytic range, multiplicity of infection (MOI), efficiency of plating (EOP), resistance to variations in temperature and pH, as well as growth kinetics. Out of four bacteriophages studied, only one isolate (isolate number 1) showed lytic activity, lysing 32 out of 121 isolates of S. aureus with lytic range of 26.44%. The optimal MOI of the phage was calculated to be 1.71, and the phage had a high EOP score of 0.88, which demonstrates high efficiency of infection. The tested phage showed stability at temperature changes between 4 °C and 55 °C and variations in pH of 5–11, showing the highest stability in the close to 37 °C and neutral pH. The latent period for this phage according to the one-step growth analysis was estimated 20 minutes with a burst size of 295 PFU. These findings show that the characterized bacteriophage was having good biological properties suitable for application in therapy but its narrow host range requires further investigations.

Keywords: Bacteriophage, Staphylococcus aureus, Lytic activity, MOI, EOP, Phage stability, One-step growth curve, Phage therapy.

Introduction

Caprine mastitis continues to be one of the important health issues affecting dairy goat production, leading to economic losses mainly due to reduced milk yield, deterioration in milk quality, treatment expenses, and in some cases, culling of animals (Bergonier et al., 2003; Contreras et al., 2007). Staphylococcus aureus (S. aureus) is commonly identified as one of the main pathogens responsible for both clinical and subclinical mastitis in goats. (Virdis et al., 2010). In the studies involving short duration therapy against established S. aureus mastitis, the bacteriological cure rate has been reported to be between 9-35% only (Mishra et al., 2011). Multiple virulence features, including the capacity to form biofilms and survive intracellularly, are known to be possessed by this organism. Such characteristics often lead to chronic infections and ineffective treatments (Melchior et al., 2006). Recently, the rise of antimicrobial resistance in bacteria, particularly the methicillin-resistant strains of Staphylococcus aureus (MRSA), and the possibility of transmission through the horizontal route have worsened the situation (Kadariya et al., 2014). Overuse of antibiotics in mastitis therapy is regarded as the main factor responsible for the spread of antimicrobial resistance and thus reduced effectiveness of conventional methods of treatment (Oliver et al., 2011). Increasing number of multidrug-resistant strains of Staphylococcus aureus associated with goat mastitis has led to a great demand for new effective methods of therapy. In this respect, bacteriophages may offer an effective solution to this problem due to their powerful antibacterial action, high specificity, and capacity to lyse antibiotic-resistant bacteria (Mishra et al., 2014). Bacteriophages are the viruses that infect only bacterial cells and possess highly specific capacity to lyse them (Abedon, 2015). Obligate lytic bacteriophages are used in the treatment of bacterial infections and as biocidal agents in the food industry as they cause lysis of bacteria (Mishra et al., 2022). They also have a unique ability to reproduce and multiply within the infected area while affecting the normal microbial flora minimally (Sulakvelidze et al., 2001). Several studies emphasize the potential utility of these viruses to treat bacterial infections including mastitis in which S. aureus is involved (Gill and Hyman, 2010). Nonetheless, very little research data exist concerning the bacteriophages that infect and lyse Staphylococcus aureus associated with goat mastitis, particularly in India. Therefore, the present study was undertaken to characterize the procured bacteriophages specific to Staphylococcus aureus from mastitis and to evaluate their biological properties for possible therapeutic use.

Material and Methods

Procurement of phage

A total of four bacteriophages (n = 4) virulent to Staphylococcus aureus were procured from ICAR–National Research Centre on Equines (NRCE), Hisar, and used in the present study.

Propagation of Bacteriophages

All procured bacteriophages were propagated to obtain high-titer lysates using both conventional liquid culture and soft agar wash methods. In the liquid culture method, an exponentially growing culture of S. aureus was inoculated with bacteriophage suspension and incubated under suitable conditions to allow phage replication and bacterial lysis. The lysate was subsequently centrifuged to remove cellular debris, followed by filtration through a 0.22 µm membrane filter to obtain a clear phage suspension. In the soft agar overlay method, an actively growing bacterial culture as well as the bacteriophage suspension was mixed with molten soft agar and overlaid onto pre-solidified nutrient agar plates. After solidification, the plates were incubated to allow plaque formation. The phages in the top agar layer was harvested using SM buffer. The harvest was further clarified by centrifugation and filtered through a 0.22 µm membrane filter to obtain purified phage lysate.

Assessment of the Lytic Range of Staphylococcus aureus Bacteriophages

The lytic activity of the procured bacteriophages was evaluated against a panel of 121 Staphylococcus aureus isolates obtained from milk samples taken from goats, cattle and buffaloes. The host range of the phages was determined using the spot test method as used by Mishra et al., 2011. and Mishra et al., 2012. The appearance of lytic zones at the spot sites was considered indicative of susceptibility of the bacterial isolate to the respective bacteriophage.

Determination of Multiplicity of Infection (MOI)

The multiplicity of infection (MOI) of the procured bacteriophage was determined as the ratio of infectious phage particles to host bacterial cells. Exponentially growing cultures of Staphylococcus aureus were prepared in broth, and the viable bacterial count was determined by the standard plate count method and expressed as colony-forming units per milliliter (CFU/mL). The bacteriophage stock was titrated using the double agar overlay technique to determine phage concentration in terms of plaque-forming units per milliliter (PFU/mL). For MOI determination, a fixed volume (0.1 mL) of host bacterial culture was mixed with an equal volume (0.1 mL) of appropriately diluted phage suspension. The total number of bacterial cells and phage particles in the reaction mixture were calculated based on their respective CFU/mL and PFU/mL values. The MOI for each bacteriophage was calculated as the ratio of total PFU to total CFU.

Efficiency of Plating (EOP) Assay

The efficiency of plating (EOP) of the procured bacteriophages was determined using the double agar overlay method. Exponentially growing cultures of Staphylococcus aureus were prepared in broth at 37 °C. Serial dilutions of the phage stock was prepared in SM buffer, and aliquots of each dilution were mixed with the host bacterial culture and allowed to adsorb for 7–10 min. The mixture was then mixed with molten soft agar (0.7%), and overlaid onto nutrient agar plates. After incubation at 37 °C for 18–24 h, plaques were counted to determine phage titers (PFU/mL). The EOP was calculated as the ratio of phage titer on the test bacterial strain to that on the propagation host strain.

Temperature Stability of Bacteriophages

The thermal stability of the isolated bacteriophages was evaluated by incubating phage suspensions at different temperatures (4 °C, 25 °C, 37 °C, 45 °C, 55 °C, 65 °C, and 80 °C) for 1–2 h. Following temperature exposures, the residual lytic activity of the phages was assessed using the spot assay method. Briefly, exponentially growing cultures of Staphylococcus aureus were mixed with molten soft agar and overlaid onto nutrient agar plates to form a uniform bacterial lawn. Aliquots of temperature-treated phage suspensions were then spotted onto the lawn and allowed to adsorb. The plates were incubated at 37 °C for 18–24 h, and lytic activity was determined based on the presence of clear zones of lysis.

pH Stability of Bacteriophage

The pH stability of the procured bacteriophage was evaluated by incubating phage suspensions in buffers of varying pH (3–12). Sodium acetate buffer (pH 3–6) and phosphate-buffered saline (pH 7–12) were used, and the mixtures were incubated for 1–2 h. Following incubation, residual lytic activity was initially screened using the spot assay method. Briefly, exponentially growing cultures of Staphylococcus aureus were mixed with molten soft agar and overlaid onto nutrient agar plates to form a uniform bacterial lawn. Aliquots of pH treated phage suspensions were spotted onto the lawn and allowed to adsorb. Plates were incubated at 37 °C for 18–24 h, and lytic activity was assessed based on the presence of clear zones. For quantitative analysis, serial dilutions of pH treated phages were prepared and plated using the double agar overlay method. Plaques were counted, and phage titers (PFU/mL) were calculated to determine the pH activity profile.

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One-Step Growth Curve of Bacteriophage

The one-step growth curve was performed to determine the growth kinetics of the procured bacteriophage, including latent period, and burst size. An exponentially growing culture of Staphylococcus aureus was mixed with bacteriophage suspension at the previously determined optimal multiplicity of infection (MOI) and incubated briefly at 4 °C to facilitate phage adsorption. Following adsorption, the mixture was centrifuged to remove unadsorbed phages, and the pellet containing infected bacterial cells was resuspended in fresh broth medium. The suspension was then incubated at 37 °C, and samples were collected at regular intervals (every 5 min). Each sample was immediately processed and plated using the double agar overlay method to determine phage titer (PFU/mL). After overnight incubation at 37 °C, plaques were counted, and phage titers were plotted against time to generate the one-step growth curve. The latent period and burst size were determined from the resulting curve.

Result and Discussion

Propagation of Bacteriophages

All four Staphylococcus aureus bacteriophages were successfully propagated using both liquid culture and soft agar overlay methods, resulting in the production of stable and high-titer phage lysates. The clarity of lysates and consistent recovery of phages following filtration confirmed efficient phage amplification and absence of significant bacterial debris. These findings indicate that the selected propagation methods were suitable for large-scale phage production. Efficient propagation of bacteriophages is essential for downstream applications, including host range determination and therapeutic evaluation. The successful amplification observed in the present study is in agreement with previous reports, where conventional liquid culture and soft agar methods have been widely used for obtaining high-titer phage preparations (Kropinski et al., 2009; Hyman and Abedon, 2009). The use of soft agar overlay technique ensures uniform bacterial growth and facilitates efficient phage replication, thereby enhancing phage recovery. Overall, the consistent propagation of all four bacteriophages suggests their adaptability to laboratory conditions and suitability for further characterization studies.

Assessment of the Lytic Range of Staphylococcus aureus Bacteriophages

The lytic range of the procured bacteriophages was evaluated against 121 Staphylococcus aureus isolates obtained from milk samples taken from goats, cattle and buffaloes using the spot assay method. Among the four bacteriophages tested, only bacteriophage isolate 1 exhibited detectable lytic activity, infecting 32 out of 121 isolates, corresponding to a lytic range of 26.44%. In contrast, the remaining three bacteriophages did not show any observable lytic activity against the tested bacterial isolates (0% lysis). The observed lytic activity of bacteriophage isolate 1 indicates a moderate host range, while the absence of lysis by the other phages suggests a high degree of host specificity. Bacteriophages are generally known for their narrow host range, often infecting only specific strains within a bacterial species due to receptor specificity and adsorption mechanisms. The variation in lytic activity observed in the present study may be attributed to differences in surface receptors of S. aureus isolates, which influence phage attachment and subsequent infection. Similar findings have been reported in previous studies, where S. aureus bacteriophages demonstrated limited host range against diverse clinical isolates (Hyman & Abedon, 2010). The narrow host specificity, although limiting in terms of broad-spectrum application, is advantageous for targeted therapy as it minimizes disruption of beneficial microbiota (Hyman & Abedon, 2010). The lack of lytic activity in three bacteriophage isolates may be due to incompatibility with the tested bacterial strains, possible loss of infectivity during storage, or requirement of specific host receptors not present in the evaluated isolates. These findings highlight the importance of selecting bacteriophages with broad host range or developing phage cocktails for effective therapeutic applications.

Determination of Multiplicity of Infection (MOI)

The multiplicity of infection (MOI) for the selected Staphylococcus aureus bacteriophage host system was determined based on the ratio of total phage particles (PFU) to bacterial cells (CFU). The calculated MOI value was found to be 1.71, obtained from a phage count of 1.53×10^{10} PFU and a bacterial count of 8.92×10^{9} CFU . An MOI value close to unity indicates a balanced interaction between bacteriophages and host bacterial cells, ensuring efficient adsorption and infection without excessive phage wastage. Such conditions are considered optimal for phage propagation, as they maximize phage yield while maintaining effective host cell infection. Previous studies have reported that MOI values ranging from 0.1 to 10 are generally suitable for efficient phage amplification, depending on the phage host system (Hyman & Abedon, 2009). The MOI value obtained in the present study falls within this optimal range, suggesting favorable conditions for phage replication. The observed MOI also reflects efficient infectivity and compatibility of the selected bacteriophage with its host strain. Maintaining an optimal MOI is critical for downstream applications such as one-step growth curve analysis and large-scale phage production. Therefore, the MOI of 1.71 was considered appropriate for subsequent experimental studies. The details of the MOI calculations are provided below in the tabulated forms:

Host/Phage	Concentration (cfu/ml or pfu/ml)	Volume (ml)	Quantity (cfu or pfu)
S. aureus Host	8.92 × 1010	0.1	8.92 × 109
S. aureus phage	1.53 × 1011	0.1	1.53 × 1010

MOI Calculation (Total PFU ÷ Total CFU)

Phage	Total CFU	Total PFU	MOI (Actual)
S. aureus phage	8.92 × 109	1.53 × 1010	1.71

Efficiency of Plating (EOP) Assay

The efficiency of plating (EOP) of the selected Staphylococcus aureus bacteriophage was determined by comparing phage titers on the target host and the original propagation host. The bacteriophage exhibited an EOP value of 0.88, indicating high productivity and efficient replication on the target bacterial strain. An EOP value greater than 0.5 is generally indicative of high phage infectivity and strong host adaptability. The high EOP observed in the present study suggests that the bacteriophage retains its lytic efficiency across different S. aureus isolates, demonstrating its potential for broader application. This finding is particularly important in the context of phage therapy, where effective replication on target strains is essential for therapeutic success. Similar observations have been reported in previous studies, where bacteriophages with high EOP values showed enhanced infectivity and lytic performance against target bacterial hosts (Viazis et al., 2011). High EOP values are also indicative of efficient adsorption, replication, and release of progeny phages within host cells. Overall, the high EOP value obtained in this study confirms the strong lytic potential and adaptability of the selected bacteriophage, supporting its suitability for further characterization and possible therapeutic application. The details regarding EOP calculations are given below in the tabulated form:

EOP Calculation

Phage	Host (pfu/ml)	Target (pfu/ml)	EOP*	Efficiency*
S. aureus	3.18 × 109	2.8 × 109	0.88	High

**Based on the EOP values, phage productivity (Viazis et al., 2011, https://doi.org/10.1111/j.1365-2672.2011.04989.x) is classified as:*

High Productivity *>0.5*
Medium Productivity *0.5 - 0.2*
Low Productivity *0.001 – 0.2*
No Productivity *<0.001*

Temperature Stability of Staphylococcus aureus Bacteriophage

The thermal stability of the selected Staphylococcus aureus bacteriophage was assessed over a wide range of temperatures under both short-term and long-term exposure conditions. The phage remained viable across a temperature range of 4 °C to 55 °C after 1 h of exposure, whereas complete inactivation was observed at 65 °C and 80 °C [Fig: 1 (a)]. Long-term stability analysis revealed that the phage exhibited maximum stability at lower temperatures, remaining viable for more than 10 months at 4 °C. At moderate temperatures (25 °C and 37 °C), the phage retained viability for up to 33 days, while higher temperatures (45 °C and 55 °C) significantly reduced stability, with survival limited to 12 h and 7 h, respectively. Rapid inactivation was observed at 65 °C and above, where the phage lost viability within 15 min. The observed temperature-dependent stability pattern is consistent with previous studies, which report that most bacteriophages exhibit optimal stability at lower temperatures and are progressively inactivated at elevated temperatures due to protein denaturation and structural damage to the phage capsid (Jończyk et al., 2011). The stability of the phage at refrigeration temperature (4 °C) suggests its suitability for long-term storage, while its ability to remain active at physiological temperature (37 °C) supports its potential application under in-vivo conditions. The rapid loss of viability at temperatures ≥65 °C indicates thermal sensitivity, which is typical for most lytic bacteriophages and may be attributed to irreversible damage to viral proteins and nucleic acid structures. These findings highlight the importance of maintaining appropriate storage and handling conditions for preserving phage activity.

pH Stability of Staphylococcus aureus Bacteriophage

The pH stability of the selected Staphylococcus aureus bacteriophage was evaluated over a broad pH range (3–12) using both spot assay and plaque assay methods. The bacteriophage exhibited complete loss of activity at highly acidic (pH 3 and 4) and highly alkaline (pH 12) conditions [Fig: 1 (b)], showing no detectable lytic activity. The phage remained viable within a pH range of 5 to 11, although with varying levels of stability. Maximum lytic activity was observed at neutral pH (pH 7), which served as the control, with a phage titer of 1.3×10^{10} PFU/mL (100% survival). Slightly reduced activity was observed at pH 6 (69.2%) and pH 8 (84.6%), indicating good stability under near-neutral conditions. However, a gradual decline in phage activity was observed at more extreme pH levels, with survival

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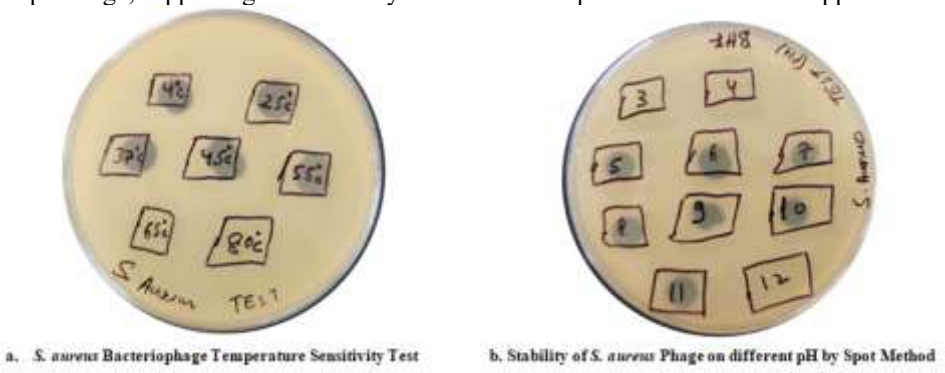
decreasing to 53.8% at pH 9, 23.1% at pH 10, and 15.4% at pH 5 and 11 [Fig: 2]. Long-term stability analysis further demonstrated that the bacteriophage retained viability for up to 31 days at pH 6 and 29 days at pH 7–10, whereas stability decreased significantly at pH 5 and 11, with inactivation occurring within 9–10 days. No long-term survival was observed at pH 3, 4, and 12, confirming extreme pH sensitivity. The observed pH-dependent stability pattern is consistent with previous studies, which report that most bacteriophages exhibit optimal stability near neutral pH and reduced viability under highly acidic or alkaline conditions due to denaturation of capsid proteins and disruption of viral structure (Jończyk et al., 2011). The ability of the phage to remain active over a relatively broad pH range (5–11) suggests good environmental adaptability and enhances its potential applicability under varying physiological and field conditions. Overall, the bacteriophage demonstrated maximum stability under near-neutral conditions and moderate tolerance to a wide pH range, supporting its suitability for further therapeutic and biocontrol applications.



a. *S. aureus* Bacteriophage Temperature Sensitivity Test b. Stability of *S. aureus* Phage on different pH by Spot Method

Fig 1: Thermal and pH stability of S. aureus bacteriophage determined by the spot test method.

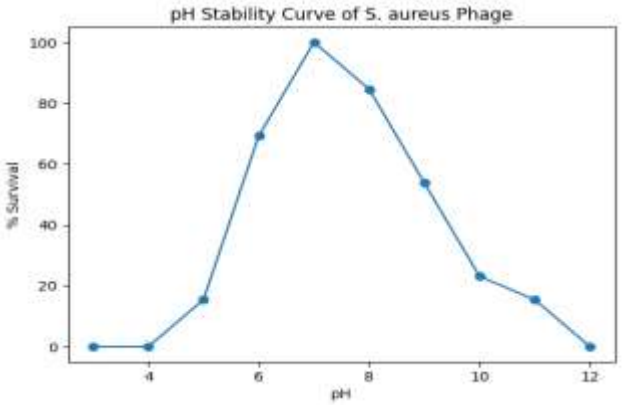


Fig 2: pH Stability curve of S. aureus bacteriophage

One-Step Growth Curve of Staphylococcus aureus Bacteriophage

The one-step growth curve of the selected Staphylococcus aureus bacteriophage was analyzed to determine its replication dynamics, including latent period and burst size. The phage exhibited a clear latent period of approximately 20 minutes, during which no significant increase in extracellular phage count was observed. Following this phase, a sharp rise in phage titer was recorded between 20 and 45 minutes [Fig: 3], indicating the active release of progeny phage particles (rise period). The phage titer increased from 1×10^5 PFU/mL at the end of the latent period to 2.95×10^7 PFU/mL during the plateau phase (≥45 min). Based on these values, the burst size of the bacteriophage was calculated to be approximately 295 phage particles per infected bacterial cell. This high burst size reflects efficient intracellular replication and effective lysis of host bacterial cells. The relatively short latent period observed in the present study suggests rapid adsorption and replication of the bacteriophage within the host cells. Such characteristics are desirable for therapeutic applications, as they enable quick bacterial clearance. The burst size obtained in this study is comparatively high and indicates strong lytic potential, which is consistent with previous reports where S. aureus bacteriophages exhibited burst sizes ranging from 50 to 300 PFU per cell (Hyman & Abedon, 2009). The well-defined growth phases, including a distinct latent period, rise period, and plateau phase, further confirm the lytic nature of the bacteriophage. The rapid increase in phage population during the rise phase indicates synchronized infection and efficient phage production. The detailed results are presented in form of tables below:

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Time Interval (min)	pfu/ml Produced by S. aureus Phage at 10-4 Dilution
0	0
5	0
10	0
15	0
20	1×10^5
25	4.0×10^6
30	6.9×10^6
35	1.11×10^7
40	1.92×10^7
45	2.95×10^7
50	Not Countable
55	Not Countable
60	Not Countable
65	Not Countable
70	Not Countable
75	Not Countable
80	Not Countable
85	Not Countable
90	Not Countable

Calculation of Latent Period & Burst Size

Phage	Latent Period*	pfu/ml before burst	pfu/ml at Plateau (≥45 min)	Estimated Burst Size**
S. aureus	20 min	1×10^5	2.95×10^7	295

**Latent period is the time interval between a phage infecting a bacterial cell and the release of new phage particles after cell lysis*

***Burst size= pfu per ml at plateau/pfu per ml before burst*

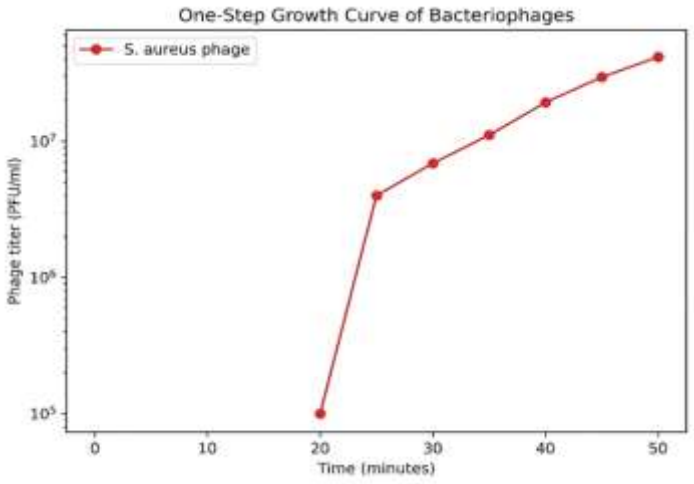

Fig 3: The growth kinetics of S. aureus phage was determined via one-step growth curve analysis. The Y-axis represents the phage titer (pfu/ml) on a logarithmic scale, while the X-axis denotes time in minutes. Phage exhibited a synchronized latent period of 20 minutes. The subsequent rise period (20–50 min) shows a sharp increase in extracellular phage particles, leading to a plateau phase where the burst size was calculated.

Conclusion

The present study successfully characterized bacteriophages targeting Staphylococcus aureus isolated from milk samples obtained from goats, cattle and buffaloes. Among the four bacteriophages studied, only one isolate exhibited lytic activity with a host range of 26.44%, indicating moderate infectivity and high host specificity. The selected bacteriophage demonstrated an optimal multiplicity of infection (MOI) of 1.71 and a high efficiency of plating (EOP) value of 0.88, reflecting efficient adsorption, replication, and adaptability to the host strain. The bacteriophage showed considerable stability over a broad range of environmental conditions, remaining viable between 4 °C and 55 °C and across a pH range of 5 to 11, with maximum stability at near-neutral pH. The one-step growth curve analysis revealed a short latent period of 20 minutes and a high burst size of approximately 295 PFU per infected cell, indicating strong lytic potential and rapid replication dynamics. Overall, the characterized bacteriophage exhibited desirable properties, including high lytic efficiency, stability under physiological conditions, and rapid replication, suggesting its potential as a promising alternative to antibiotics for the control of Staphylococcus aureus-associated mastitis. However, the relatively narrow host range highlights the need for further studies focusing on phage cocktails or broad-host-range phages to enhance therapeutic efficacy.

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