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Bacteriophages Mediated Biocontrol of Multidrug Resistant *Staphylococcus arlettae* on Eggshell Surface

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Abstract

Eggs are a widely consumed food product but can act as vehicle for multidrug-resistant *Staphylococcus* spp., posing significant food safety and public health challenges. Among *Staphylococcus* spp., *S. arlettae* which is often isolated from poultry environments, exhibits antibiotic resistance and virulence factors that complicate conventional decontamination efforts. This study explored the potential of bacteriophages as a biocontrol tool to mitigate *S. arlettae* contamination on eggshells. Five lytic *Staphylococcus* phages were isolated from sewage samples collected from livestock farms and characterized using Transmission Electron Microscopy. Phages were identified as tailed phages of Myovirus, Siphovirus, and Podovirus. The host range of the phages varied, with *Staphylococcus* phage BASU SA1 exhibiting the highest lytic activity, lysing 66.67% of tested strains. A cocktail of BASU SA1 and BASU PS3 phages was evaluated for efficacy in reducing *S. arlettae* contamination on eggshells at a multiplicity of infection of 1000. The treatment resulted in a significant bacterial reduction of $5.1\log_{10}$ cfu/cm² within 2 hr at room temperature. The findings of the present study demonstrate the potential of phage-based interventions in enhancing food safety in a highly specific, environmental friendly manner, with the potential of an effective alternative to antibiotics and chemical sanitizers. Further large-scale studies in natural settings need to be conducted to validate these results and support their integration into food safety protocols.

Keywords: Biocontrol, *Staphylococcus arlettae,* Eggshell, Bacteriophages

Introduction:

Staphylococcus arlettae, a coagulase-negative bacterium from the genus *Staphylococcus*, was initially discovered in the nasal passages and on the skin of poultry and goats (Schleifer et al., 1984). Since its identification, it has been recovered from various sources, including veterinary samples (Suwannarach et al., 2017; Nobrega et al., 2018; Sanz et al., 2018), human clinical samples (Dinakaran et al., 2012; Dziri et al., 2016; Andreis et al., 2017; Teeraputon et al., 2017), soil (Nanjani and Soni, 2014), mobile phone surfaces (Kurli et al., 2018), and biological safety cabinets (Lavecchia et al., 2018). Although commonly recognized as a commensal organism, *S. arlettae* has been associated with infections linked to excessive antibiotic use, such as intramammary infections in dairy goats, exudative epidermitis in pigs, bovine mastitis, and rheumatic mitral stenosis in humans (Dinakaran et al., 2012; Park et al., 2013; Gosselin et al., 2019). Recent studies reported that isolates from chicken farms harbored plasmids containing antibiotic resistance genes such as *aadD*, *aacA-aphD*, *cfr*, *ermB*, *ermC*, *ermT*, *fexB*, *fosD*, and *tetL*. These plasmids also contained elements, which promote recombination within and between plasmids, emphasizing their role in disseminating resistance genes and impacting antimicrobial treatments (Liu et al., 2017). Additional isolates from chicken farms and dairy herds affected by mastitis carried multiple multidrug efflux pump genes, such as *norA*, which confer resistance to antibiotics like chloramphenicol, tetracycline, and erythromycin (Xu et al., 2015; Liu et al., 2017; Nobrega et al., 2018). Moreover, virulence-associated genes were identified in *S. arlettae* strains, including those coding for fibronectin/fibrinogen-binding proteins, hemolysin III, programmed cell death toxin, autolysins, and regulatory factors for virulence, such as *agrA*, *agrB, agrR, agrV,* and *agrZ* (Vandenesch et al., 1993; Li et al., 2004; Dinakaran et al., 2012).

The emergence of multidrug- resistant pathogens has increased the pressure on effective control measures to combat antibiotic resistant pathogens and stop the spread of resistant bacteria are needed. Several strategies, including biological, chemical, and physical treatments have been employed to control pathogens in egg and egg products. However, these methods often compromise food quality and may lead to the formation of harmful

compounds. The lytic bacteriophages are natural alternatives for different kinds of antibiotics to combat bacterial infections. Bacteriophages are also emerging as a "Green" technology to deal with the issues in food industries (Sulakvelidze, 2016).

Bacteriophages are viruses that exclusively kill bacteria and often noted as the most abundant biological entity on earth, with estimates of their population being in the range of 10^{31} viral particles across the biosphere (Batinovic et al., 2019). Since their discovery, bacteriophages have been considered an important weapon to fight human and animal infections of bacterial origin due to their specific ability to attack the associated target bacteria. Most of the bacterial contamination can be reduced through treatment with commercial phage cocktails (de Melo et al., 2018). Many studies to investigate the use of bacteriophage to kill the pathogenic bacteria present on food products like *L. monocytogenes* (Iacumin et al., 2016), *Escherichia coli* (Gundogdu et al., 2016), *Salmonella* spp. (Anjay et al., 2022), *Shigella sonnei* (Soffer et al., 2017) and *Staphylococcus aureus* (El Haddad et al., 2016) are reported. The aim of this study was the isolation and characterization of lytic bacteriophages with their evaluation of biocontrol action against multidrug resistant *S. arlettae.*

Materials and Methods:

Bacterial strains

The multidrug resistant *Staphylococcus* spp. (isolated from milk samples) used in this study was obtained from the Department of Veterinary Public Health and Epidemiology, Bihar Veterinary College, Patna (Figure 4). Among these, a *Staphylococcus* strain S31 possessing resistance against most of the antibiotics was specifically utilized to enrich sewage samples for the isolation of Staphylococcus bacteriophages and to assess the bacteriophages' activity on eggshell surfaces. All *Staphylococcus* strains underwent thorough testing to confirm their purity, morphology, and biochemical and molecular characteristics. These strains were subsequently used to evaluate the lysis efficiency and determine the host range of the isolated bacteriophages.

Sample collection, isolation and purification of lytic *Staphylococcus* phages

Ten sewage samples were collected from three livestock farms associated with Bihar Animal Sciences University, Patna, India. These included five samples from cattle farms, one from a sheep and goat farm, and four from poultry farms. Approximately 45 ml of the supernatant from each sewage sample was mixed with 5 ml of 10X brain heart infusion broth and inoculated with 1 ml of an early log-phase culture of the indicator bacterial strain S31. The mixtures were incubated at 37°C for 16-18 hr and subsequently centrifuged at 7000 rpm for 10 min.

The supernatant was sequentially filtered using syringe filters with pore sizes of 0.45 µm and 0.22 µm. The presence of lytic phages in the filtered samples was confirmed through the appearance of clear zones during spot testing on soft agar layers (Figure 1A). The lytic phages were purified using the agar overlay assay with three successive passages (Anjay et al., 2022).

Propagation, titration and naming of lytic *Staphylococcus* phages

The high-titer phage stocks were prepared by inoculating 100 µl of the purified phage filtrate and 1 ml of an overnight culture of the host bacterial strain into 100 ml of New Zealand Casamino Yeast extract medium followed by incubation at 37°C for over 48 hr. The phage titer, expressed as plaque-forming units per milliliter (Pfu/ml), was determined using the agar overlay assay and stored at 4°C. The phages were named according to the system proposed by Adriaenssens and Brister (2017), which includes the complete host genus name, the word "phage," and a unique identifier.

Transmission Electron Microscopy of phages

For Transmission Electron Microscopy, phage suspensions were mixed with an equal volume of Tris Magnesium buffer, followed by the addition of 2 ml of 5M NaCl. After 15 min., 2.2 g of solid polyethylene glycol-6000 was added to the suspension, which was then incubated at 4°C for 2 hr. The mixture was centrifuged at 12,000 rpm for 30 min. under refrigeration, and the resulting pellet was resuspended in 300 µl of TM buffer. Next, the suspension was treated with 300 µl of chloroform and centrifuged at 12,000 rpm for 5 min (Anjay et al., 2022). The supernatant obtained was used as the sample for Transmission Electron Microscopy analysis. The study was conducted at the Division of Plant Pathology, ICAR-Indian Agricultural Research Institute, New Delhi, using an electron microscope (JEOL JEM-1011, Japan Electronics and Optics Laboratory, Tokyo, Japan).

Host range of Bacteriophages

The host range of each phage was assessed against 21 *Staphylococcus* strains using a spot test. In this method, 10 µl of phage lysate was spotted onto a bacterial lawn. After allowing the spot to fully absorb, the plates were incubated overnight at 37°C and examined for clear zones of lysis, indicating phage activity. The lysis profile of each phage was generated using Gene Cluster 3.0 and Java Tree View software, following the protocol described by Anjay et al. (2022).

Temperature and pH stability of Bacteriophages

The stability of bacteriophages was evaluated at temperatures of 42°C, 65°C, and 80°C. One milliliter of bacteriophage lysate, with concentrations of 10^7 pfu/ml was placed in a water bath set to the respective test temperatures for 60 min. Following the incubation, the samples were immediately cooled on ice for 10 min. Serial 10-fold dilutions were prepared, and plaque-forming units were determined using the agar overlay method (Manohar et al., 2019).

Phage stability under different pH conditions (pH 3, 6, 9, and 12) was also assessed. A 100 µl aliquot of bacteriophage lysate, with concentrations of 10^7 pfu/ml was added to 900 µl of NZCYM broth adjusted to the respective pH values. The mixtures were incubated at 37°C for 24 hr. The phage titers at various pH levels were then determined using the agar overlay method (Camens et al., 2021).

Biocontrol of *Staphylococcus* spp. in raw egg:

Fifteen eggs were purchased from Poultry Research and Teaching Centre, BASU, Patna, and their equatorial radius, short polar radius, and long polar radius were measured using a digital calliper (Aerospace). The surface area of the eggs was calculated using the formula provided by Makalatia et al. (2018). The eggs were cleaned with cotton gauze soaked in 70% ethanol before the experiment. To test the efficacy of the bacteriophage cocktail, the eggs were contaminated by immersing them for 5 min at room temperature in a suspension containing *Staphylococcus* strain S31 at a concentration of 10^5 cfu/ml (200 ml per egg). Ten of the contaminated eggs were sprayed with approximately 2.5 ml of a bacteriophage cocktail containing *Staphylococcus* phage BASUSA1 and *Staphylococcus* phage BASUPS3 at a concentration of 1×10^8 pfu/ml (MOI of 1000), using a syringe. The remaining five eggs were sprayed with SM buffer as controls. All eggs were incubated for 2 hr at room temperature. After incubation, bacterial counting was performed by breaking the eggs and discarding their contents (Makalatia et al., 2018). The eggshells were collected in 50 ml centrifuge tubes containing 10 ml of BHI broth and vortexed. *Staphylococcus* counts from treated and control eggs were determined by preparing 10-fold serial dilutions and inoculating them onto Mannitol Salt Agar plates, following the methods described by Miles and Mishra (1938). The bacterial concentrations were expressed in cfu/cm². The bacterial reduction was calculated by subtracting the average bacterial concentration, expressed in $\log_{10}$ units, on eggshells treated with the bacteriophages cocktail from that on untreated eggs.

Results and Discussion:

Bacteriophages are the most abundant organisms on Earth and play a critical role in regulating the diversity, abundance, evolution, and physiology of microbial communities within specific habitats (Lehti et al., 2017). In the present study, five lytic *Staphylococcus* phages were isolated, purified, and propagated using a multiple antibiotic-resistant *Staphylococcus arlettae* strain (S31) through an enrichment method. Of these, one phage was isolated from poultry farm sewage and four from cattle farm sewage at BASU, Patna. Previous research has similarly identified phages targeting methicillin-resistant *Staphylococcus aureus* (MRSA) from sources such as hospital wastewater, cow's milk, sewage, pond water, soil samples from poultry farms, and livestock fecal samples (Kahankova et al., 2010; Gupta and Prasad, 2011; Deghorain and Van Melderen, 2012; Jun et al., 2013; Chang et al., 2015).

The isolated phages were named *Staphylococcus* phage BASU SA1, BASU SA3, BASU CS2, BASU CS1, and BASU PS3. The plaque sizes formed by these phages ranged from pinpoint to 1 mm (Figure 1B-1F), providing insights into their lytic efficiency and interaction with host bacteria. Plaque morphology varies among phages, reflecting differences in replication cycles and lytic efficiency. Clear plaques are typically associated with virulent phages that efficiently lyse host cells, while smaller or turbid plaques may indicate temperate phages or slower lytic cycles.

Recent changes in phage taxonomy by the International Committee on Taxonomy of Viruses replaced the morphology-based families *Myoviridae*, *Podoviridae*, and *Siphoviridae* with the class *Caudoviricetes*, encompassing all tailed bacterial and archaeal viruses with icosahedral capsids and double-stranded DNA genomes (Barylski et al., 2020). In this study, morphological analysis using TEM identified two phages as myoviruses, two as siphoviruses, and one as a podovirus (Figure 2A-2E). The findings demonstrate that morphological analysis complements the new taxonomic framework by providing tangible evidence of structural diversity among tailed bacteriophages.

Phages rely on prokaryotic hosts to reproduce, attaching to specific host cells, injecting their genetic material, and exploiting host resources to replicate, ultimately leading to host cell lysis and death. The lytic profiles of the isolated phages demonstrated varying effectiveness, with *Staphylococcus* phage BASU SA1 lysing 66.67% of tested isolates, followed by BASU PS3 (57.14%), BASU SA3 (47.62%), BASU CS2 (42.86%), and BASU CS1 (33.33%) (Figure 3). The differences in the lytic profiles of the isolated phages are likely due to variations in their host range and the efficiency with which they infect and

lyse bacterial isolates. Several factors such as receptor specificity, phage-host interactions, environmental and physiological conditions, and phage characteristics may contribute to the differences in lytic ability of phages. The observed variability in lytic profiles highlights the diversity in phage-host interactions and underscores the importance of selecting phages with complementary lytic activities for therapeutic or biocontrol applications. Combining phages with different host ranges could enhance the overall effectiveness of phage-based biocontrol by targeting a broader spectrum of bacterial pathogens.

The comparative lysis profile of *Staphylococcus* phages developed against antibiotics resistance pattern of *Staphylococcus* strains showed that phages were able to lyse 81.82% of the resistant strains (Figure 4). The finding underscores the efficacy of *Staphylococcus* phages in targeting antibiotic-resistant strains, demonstrating their potential as a valuable tool in combating resistant bacterial infections. Phages are highly specific to their bacterial hosts, targeting particular surface receptors. The high lysis percentage (81.82%) suggests that the phages were well-suited to the surface receptors commonly found on the antibiotic-resistant *Staphylococcus* strains tested. This may be due to the evolutionary adaptation of these phages to infect such strains.

The temperature stability study of bacteriophages revealed complete inactivation of all phages at 80°C. However, two phages, BASU SA3 and BASU CS2, demonstrated survival at 65°C, with an approximate reduction of 5 $\log_{10}$ pfu/ml. Moderate differences in phage stability were observed when incubated at 37°C and 42°C (Figure 5). Similarly, the pH stability study showed complete inactivation of all phages at pH 3 and pH 12. However, at pH 6 and pH 9, a moderate reduction in phage concentrations was observed (Figure 6). The findings align with the known sensitivity of bacteriophages to extreme environmental conditions, such as high temperatures and extreme pH values. Variations in stability among phages reflect differences in structural robustness, highlighting the need to consider environmental conditions when utilizing phages in therapeutic or biocontrol applications.

The phage cocktail composed of *Staphylococcus* phages BASU SA1 and BASU PS3 was tested for its biocontrol efficacy against *S. arlettae* on chicken eggshells. At an MOI of 1000 and after 2 hr of incubation, the bacterial count on treated eggs was significantly reduced ($P < 0.0001$) compared to the control group, as determined by two-way ANOVA at a 95% confidence interval. The *S. arlettae* count on untreated eggshells was 5.3 ± 0.2 $\log_{10}$ cfu/cm², whereas the treated group showed a count of 0.2 ± 0.1 $\log_{10}$ cfu/cm², representing a 5.1 $\log_{10}$ cfu/cm² reduction. These results align with findings by Makalatia et al. (2018), who reported a 90% *Escherichia coli* and *Salmonella* reduction within 15 min of phage application, with further decreases over the following 18 hr. Similar outcomes have been observed by Poojari et al. (2022) demonstrated ~90 % reductions in *E. coli* and *Salmonella* levels on chicken meat using a phage cocktail at MOI 0.01–0.1 over several hr. Additionally, El-Shibiny et al. (2017) applied phages ZCSE1 against *Salmonella* and ZCEC1 against *E. coli* to egg surfaces, achieving ~2 $\log_{10}$ reductions to undetectable levels within 1 hr. These consistent observations highlight the rapid and substantial biocontrol potential of phage cocktails against foodborne bacteria on eggshells and poultry products.

Based on the findings, phages appear to be a promising alternative to antibiotics for bacterial control and as a prophylactic measure in the food industry. Multiple studies have shown that applying phages to contaminated food surfaces can significantly reduce bacterial counts, enhancing food safety. However, further large-scale experiments in natural farm environments are necessary to validate these conclusions and support the practical application of phages in food decontamination.

Conclusions:

Eggs are a significant food source worldwide but can serve as vehicles for multidrug-resistant *Staphylococcus* species, such as *Staphylococcus arlettae*. These pathogens, often acquired from poultry farming environments, present serious public health risks due to their antibiotic resistance and virulence factors. Phage biocontrol is a promising, effective alternative to chemical treatments, with potential for integration into food safety protocols. The specific phage preparation, especially composed with multiple phage clones with overlapping host ranges, significantly reduces external contamination of eggs and, therefore, may get an effective practical application in farm production via eliminating contamination and increasing the product quality and safety.

Conflict of Interest:

The authors declare that there is no conflict of interest.

Data Availability:

All the data in relation to present study is available with corresponding author.

Author's Contribution:

All authors have made significant contribution in the designing of the manuscript, data analysis and interpretation of result.

Ethical Statement:

In this study, all procedures were performed in compliance with relevant laws and institutional guidelines and approval of the Institutional Animal Ethical Committee is not required.

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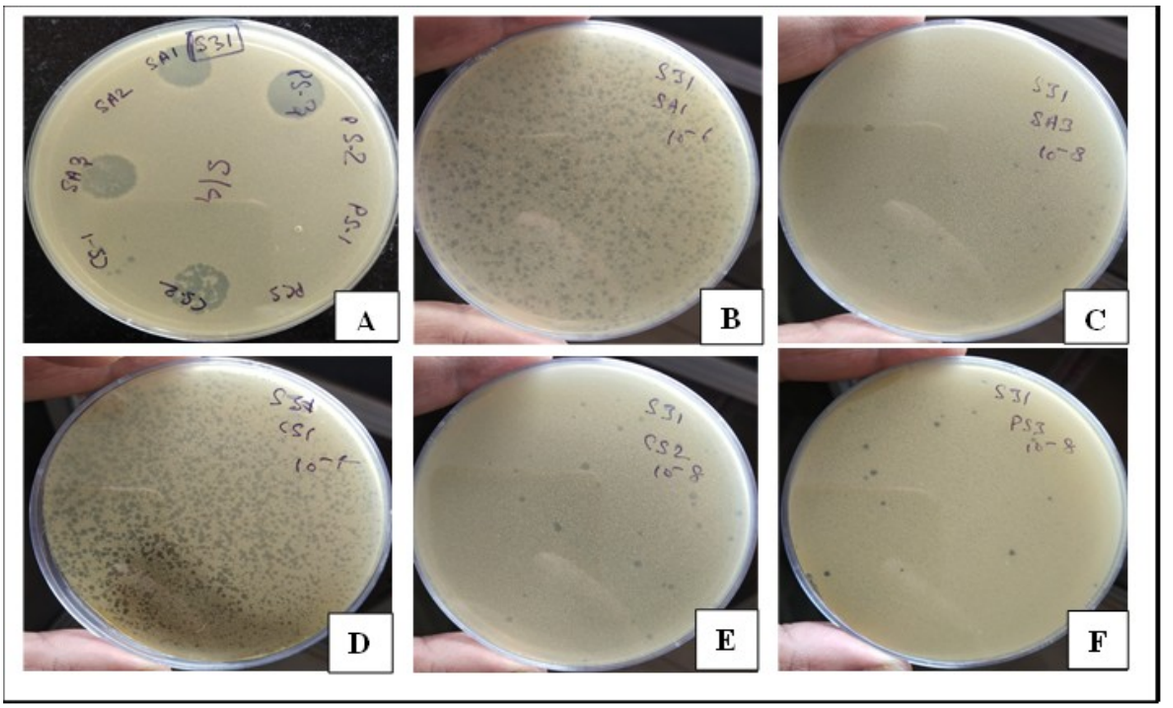

Figure 1 A: Isolation of bacteriophages using spot test against *Staphylococcus arlettae*
B-F: Plaques of different size produced by the *Staphylococcus* phages.

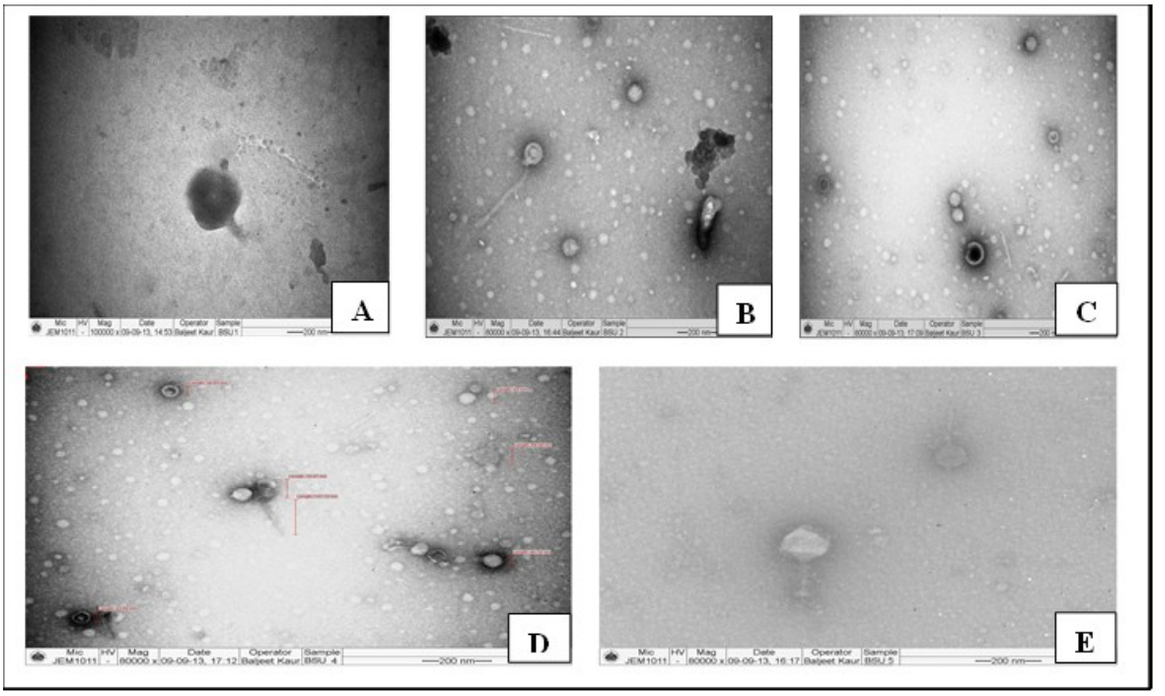

Figure 2: Transmission Electron Microscopy of *Staphylococcus* phages
A: BASU SA1 (Siphovirus), B: BASU CS2 (Siphovirus), C: BASU SA3 (Podovirus), D: BASU CS1 (Myovirus) and E: BASU PS3 (Myovirus)

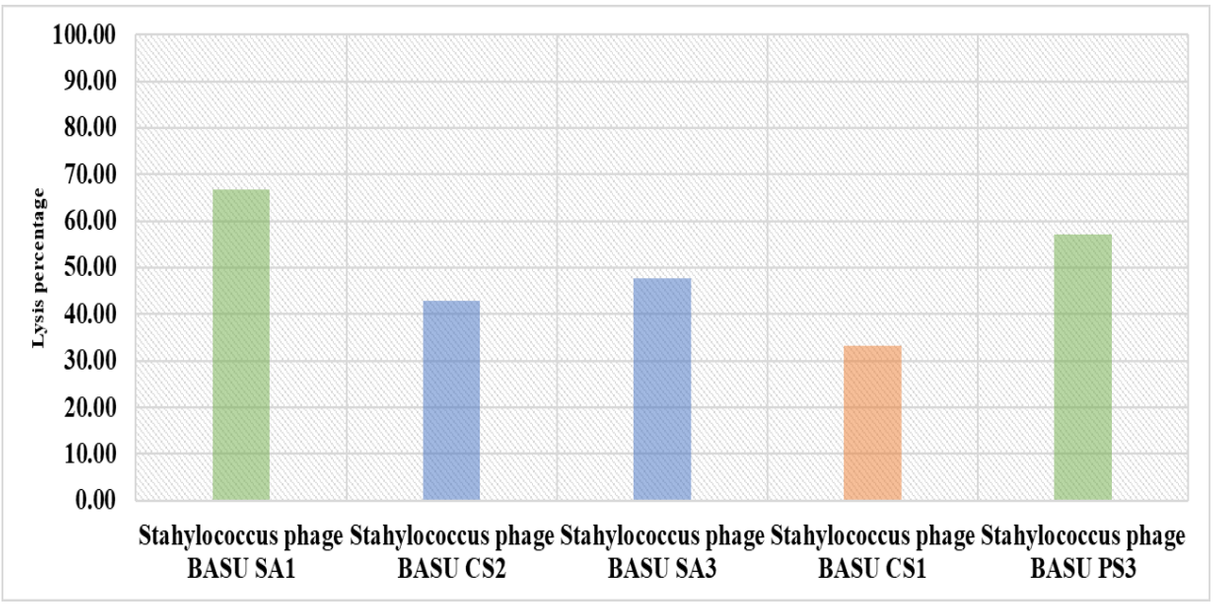

Figure 3: Lysis percentage of phages against multidrug resistant *Staphylococcus* strains.

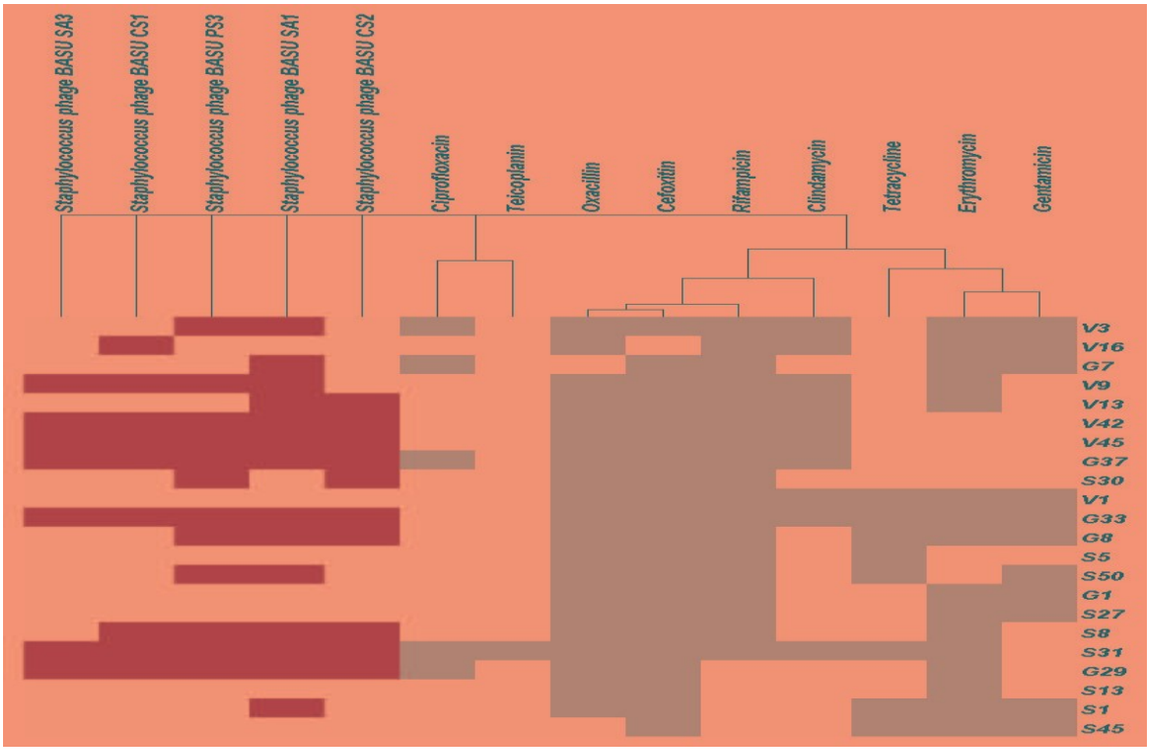

Figure 4: Comparison of lysis pattern of *Staphylococcus* phages with resistance pattern of *Staphylococcus* strains with antibiotics. Dark red colour box below the name of the phages represents strains lysis with phages and black colour box below antibiotics represents resistance.

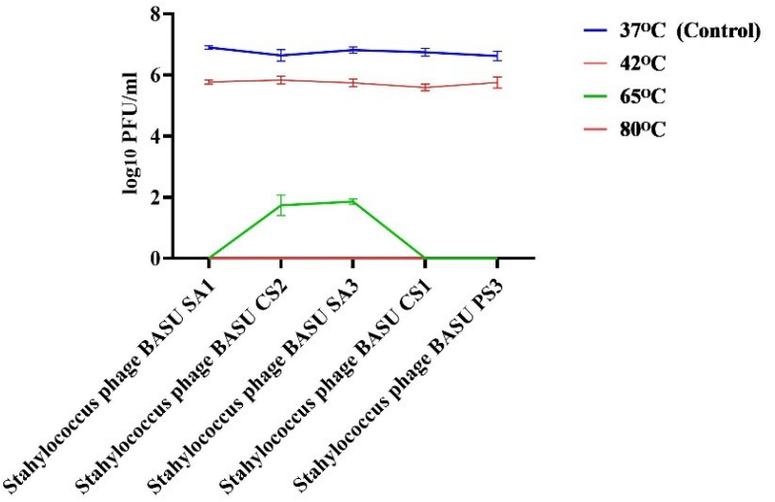

Figure 5: Temperature stability of *Staphylococcus* phages

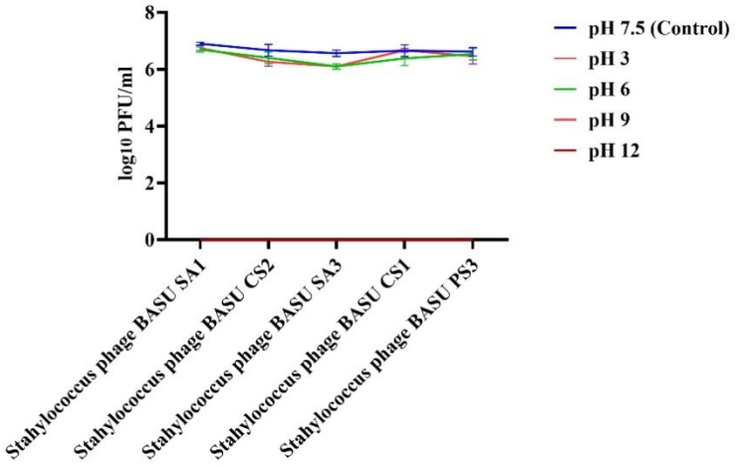

Figure 6: pH stability of *Staphylococcus* phages

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