

Efficacy of combined phage-gentamicin therapy for the control of antibiotic-resistant *Staphylococcus aureus*

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ABSTRACT

Introduction: Biofilm is a leading cause of antibiotic resistance and chronic infections. Studies have shown that microbial biofilms are 100–10,000 times more resilient to antimicrobial substances. Several studies have demonstrated the efficacy of bacteriophage-antibiotic combinations in planktonic cultures of *S. aureus*. This study was undertaken to evaluate the efficacy of phage-antibiotic combinations in eradicating *Staphylococcus aureus* biofilms.

Methods: The study evaluated the combined treatment of phage and antibiotic at a specific dose, order, and timing, yielding the most effective killing outcome compared with treatment with either one alone.

Results: The present study demonstrated the superior effectiveness of the phage-antibiotic combination compared to phage-only or antibiotic-only treatments. Combination therapy outperformed mono-treatments. The combined bactericidal effect of the antibiotic and phage is more pronounced in the Phage First (PF) treatment. Sequential treatment, involving phage application before eight hours of antibiotic exposure at a concentration of 10⁸ PFU/mL, proved the most efficient in eradicating both biofilms and the planktonic form of *S. aureus*.

Conclusion: The synergistic effect of phages and antibiotics on biofilm eradication was observed, and the combination proved beneficial for treating bacteria in the biofilm matrix. Phage-antibiotic synergism offers a promising avenue for developing antibiotic-phage combinations that meet therapeutic requirements for treating *S. aureus* infections. The results of this study offer fresh insights into phage therapy.

Keywords: Bacteriophage, Biofilm, *S. aureus*, Synergism & antibiotic-resistant

INTRODUCTION

The ESKAPE group, which includes *Staphylococcus aureus*, *S. epidermidis*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, and *Enterococcus faecalis*, is of significant medical importance. These nosocomial multidrug-resistant pathogens have become prevalent, underscoring the urgent need for effective control measures.^{1,2} Biofilms, a formidable adversary, are a leading cause of antibiotic resistance and chronic infections. Their resilience is staggering, with studies revealing that microbial biofilms are 100–10,000 times more resistant to antimicrobial agents, such as antibiotics, than planktonic cells. This is primarily due to the poor antibiotic penetration into the complex polysaccharide matrix (glycocalyx) of biofilms, making them a significant challenge in the field of infectious diseases.³

However, a new player has emerged from the cocktail of bacterial viruses (bacteriophages). These phages, with their unique ability to penetrate biofilms, are heralding a new era in antibacterial strategies. They

offer a safe and powerful alternative in the face of rapidly emerging antibiotic resistance, as they can effectively target the protective matrix of extracellular polymeric substances (EPS) in which biofilms are embedded, thereby providing high antibiotic tolerance and biofilm stability.⁴⁻⁶

S. aureus, a member of the ESKAPE group of organisms, is responsible for numerous community and hospital-acquired infections, such as infections of the skin and soft tissues, wound infections, endocarditis, osteomyelitis, and life-threatening bacteremia.^{7,8} The treatment of staphylococcal infections has become an international concern with the emergence of high levels of penicillin resistance, followed by the development and dissemination of strains resistant to nafcillin, oxacillin, methicillin, and now even vancomycin.^{9,10} Therefore, developing novel antibacterials to combat the staphylococcal threat is not just important but imperative and represents a significant unmet medical need. Bacteriophage (phage) therapy appears to be a promising standalone treatment alternative for staphylococcal infections resistant to conventional antibiotics.

Several studies have demonstrated the efficacy of bacteriophage-antibiotic combinations in planktonic cultures of *S. aureus*.¹¹⁻¹³ Furthermore, the distinct modes of action adopted by phages and antibiotics render them effective against pathogens with phage/antibiotic resistance.¹⁴ Therefore, there is little chance that antibiotic and phage resistance will develop simultaneously. In addition, bacteria resistant to one treatment will be taken by the other.¹⁵ Moreover, modifying the multi-drug efflux pump's activity during phage resistance development might enhance antibiotic sensitivity.¹⁶ This present in vitro study was planned to evaluate the potential of Phage-Antibiotic Synergy (PAS) on planktonic and biofilm states of *S. aureus* using different concentrations and addition timings of the phage and antibiotic, with

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special reference to complete eradication of the *S. aureus* biofilm state.

METHODS

The study plan, with the reference number Dean/2022/EC/3329, was approved by the Institutional Ethics Committee at the Institute of Medical Sciences (IMS), Banaras Hindu University (BHU), Varanasi-221005, Uttar Pradesh, India.

Thirty-five isolates of *S. aureus* were isolated from the swab/pus specimens of patients with chronic wound (wound that failed to proceed through an orderly and timely series of reparative steps to produce anatomic and functional integrity within a period of 6 weeks) infections attending the wound clinic of Sir Sundar Lal Hospital, Banaras Hindu University, Varanasi-221005, Uttar Pradesh, India, from June 2022 to August 2023.

The wound surface was gently cleaned 3 times with sterile gauze soaked in sterile saline (0.9% NaCl). Two swabs/ tissue biopsies were collected to isolate aerobic and facultative anaerobic bacteria. All these swabs were put in a tube containing 1 mL of sterile normal saline. The tube containing the swab was vortexed for 5 min to release bacteria entrapped in the swab. A hundred microliters of the above suspension were inoculated on MacConkey (MA) and Blood agar (BA) plates and incubated overnight at 37 °C. The next day, the plates were examined for bacterial colonies. If colonies did not appear, broth subcultures were plated onto fresh solid media, and the old plates were incubated for an additional 24 h. The colonies were identified as *S. aureus* by colony morphology, Gram's staining characteristics, and performing a battery of different biochemical tests (Catalase test, Coagulase test, Alkaline Phosphatase test, DNase test, Methyl Red test, Voges Proskauer test, Urease test, and Mannitol fermentation test). The isolates were then subjected to antimicrobial testing using a modified Kirby-Bauer disc diffusion method following CLSI 2019 guidelines,¹⁷ and were identified as multidrug-resistant (MDR) if they exhibited resistance to three or more antibiotic classes. Using a cefoxitin disc (30 µg), the *S. aureus* strain was screened for methicillin resistance in accordance with CLSI (2019) guidelines.¹⁷ A zone of inhibition ≤21 mm was considered a positive result for MRSA strain. The lawn culture on MHA was prepared after matching the turbidity of the test inoculum with a 0.5 MacFarland standard.¹⁸ The isolates were stored on peptone agar slants for further use.

The antibiotic susceptibility of all *S. aureus* isolates was determined for gentamicin by broth dilution method to determine MIC following the protocols of CLSI 2019 guidelines.¹⁷ The *S. aureus* (ATCC 25923) strain served as the reference strain.

S. aureus phages were recovered from raw wastewater in Varanasi's municipal drainage system. A 50 mM Tris-HCl solution (pH 7.5) was thoroughly mixed with 10 mL of sewage water. 0.5 mL of chloroform was added to the mixture. The supernatant was collected after 10 min of centrifugation at 10,000 rpm and 4°C. A 0.22 µM membrane filter was used to filter the supernatant. A mixture of 10 mL 2X BHI broth and 10 mL aliquots of the supernatants was used. Next, 1 mL of an overnight culture containing a mixture of 10 *S. aureus* strains was added to the final mixture, which was then incubated at 37°C with constant shaking. The suspension was centrifuged for ten minutes at 10,000 RPM and 4°C. It was then filtered to remove the bacterial debris. The supernatants were diluted 10-1,000-fold and mixed well with 5mL of molten (0.4%) BHI agar containing 0.1 mL of *S. aureus* (1×10^8 CFU/mL) to obtain isolated plaques. The BHI agar plate was then incubated at 37°C for overnight. Based on the morphological variations of the plaques, the plaques were propagated and characterized. Plaques with different morphologies were selected and propagated for bulk production.

Following bulk production, the liquid was collected and dialyzed for 24 hours against a hypertonic solution containing 30% polyethylene glycol (PEG 6000) in 2.5 M NaCl, using a 20-nm membrane. After that, the dialyzed fluid was concentrated and washed three times at 4°C using phosphate-buffered saline (PBS).¹⁹

With a few modifications, the previously reported protocol by Parasion et al. (2012 for one-step growth curve experiments) was followed. The *S. aureus* strains were recovered in the early log phase (OD₆₀₀ = 0.2) after centrifugation at 10,000 ×g for 10 min at 40°C. The pellet was then

suspended in freshly prepared BHI broth at a concentration of 1×10^8 CFU/mL. The bacterial cultures were then treated with bacteriophage at a multiplicity of infection (MOI) of 0.01 and left to adsorb at room temperature for ten minutes. After removing the unbound phages from the supernatant by centrifugation and discarding the supernatant, the pellets were reconstituted in 10 milliliters of fresh BHI medium and incubated for 90 minutes at 37°C with shaking. Samples were taken at 5-minute intervals, diluted by serial tenfold dilution, and phage counts were determined using the soft agar overlay method. By dividing the average phage number at the plateau by the number at the latent phase, the burst sizes of phages were calculated as described by Sváb et al. (2018).²⁰

The phage's thermal stability was evaluated at a range of temperatures (37, 40, 50, 60, 70, and 80°C) for a duration of 180 minutes. The pH stability of the bacteriophage samples was assessed by mixing them with BHI in multiple tubes at varying pH levels. The mixture was subsequently incubated for 180 minutes at 37°C. The double-layer agar plate method was used to estimate bacteriophage titers.

The study examined three distinct setups: the first involved administering the phage 8 hours before the antibiotics (PF); the second involved giving the antibiotic and the phage simultaneously (SIM); and the third involved giving the antibiotic 8 hours before the phage (A-F). In a 96-well plate, 180 µL of bacteria in the logarithmic growth phase was added, representing a concentration of 1×10^8 CFU/mL. Following that, 20 µL of either an antibiotic (16 µg/mL) or phage suspension (1×10^6 PFU/mL) or a mixture of both (10 µL of each antibacterial agent) was added to the designated wells in the specified sequence. A temperature of 37°C was maintained for 48 hours for the 96-well plates. At various times, the number of live bacteria was counted (0 h, 4h, 8h, 16h, 24h and 48h) using standardized CFU counting protocols (spread plating).

A flat-bottom 96-well microtiter plate was used to inoculate *S. aureus* with 200 µL of BHI broth per well. After that, the plate was incubated at 37 °C for 4 days to allow biofilm to grow along the edges of each well. After washing the wells with phosphate-buffered saline to remove any remaining free-floating bacteria, the biofilm biomass was quantified by adding 200 µL of methanol to each well and incubating for 20 minutes. The methanol was then aspirated, and the plate was allowed to air-dry. Wells were filled with 200 µL of 1% crystal violet solution and left at room temperature for 20 minutes. After drying the plates, the excess stain was removed using deionized water. The following day, 200 µL of ethanol was added to each well, and the wells were left for 30 minutes to extract the stain from the bacteria. Using a micro-ELISA auto-reader set to 570 nm, the optical density (OD) of the stained adhering biofilm was determined.

Following a two-week incubation period, the bacterial suspension was extracted from the wells and washed with PBS to exclude any planktonic cells. Next, the wells were treated in triplicate with phage first (PF; 1×10^6 PFU/well), antibiotic first (AF; 16 µg/mL), and phage + antibiotic (SIM; simultaneous). The microtiter plates were incubated at 37°C for 24 hours. Following the previously described procedure, the wells were washed with PBS, and the amount of biomass was quantified using crystal violet staining.

In brain heart infusion broth, bacterial seed cultures were grown to a mid-logarithmic phase as inocula. The concentrated pellet was diluted to 1×10^8 CFU/mL after centrifugation at 1912 g for 15 min. After that, the standardized bacterial culture was treated in triplicate for 16 hours at 37°C using three distinct protocols: phage first (1×10^6 PFU/well), antibiotic first (16 µg/mL/well), and phage + antibiotic (simultaneous/well). Standard CFU counting procedures were used to evaluate the growth following the incubation period, according to Boswell M et al. (2019).²¹

RESULTS

A total of 35 strains of *S. aureus* were isolated from patients with chronic wound infections. Twenty-nine isolates (7.2%) were found to have gentamicin resistance (MIC ≥32 µg/ml) by broth dilution.

A total of four broad-spectrum bacteriophages against gentamicin-resistant *S. aureus* were isolated and labeled as φSA01 to φSA04. Phages

showed varied lysing efficacy when tested against 29 gentamicin-resistant *S. aureus* isolates, i.e., 74.28.2%, 57.1%, 51.4%, and 40% efficacy for ϕ SA01, ϕ SA02, ϕ SA03, and ϕ SA04, respectively. Nevertheless, these phages showed no lytic activity against *P. aeruginosa*, *E. coli*, *K. pneumoniae*, and *A. baumannii*.

Since ϕ SA01 exhibited higher lytic activity (74.3%), we selected this phage for further characterization and to test its antibiofilm efficacy in combination with gentamicin. The phage ϕ SA01 retained its lytic activity within the pH range of 4.0–10 (Figure 1) and temperature at 37°C to 60°C; however, at 70°C, it lost its activity. The latent period was 22 min, and the burst size was 78 PFU/bacterial cells for the virus.

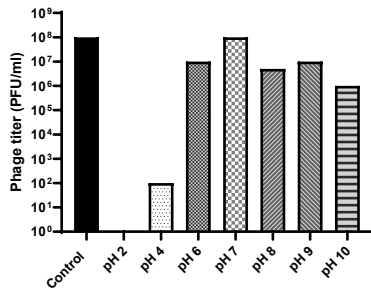


Figure 1: pH sensitivity of phages

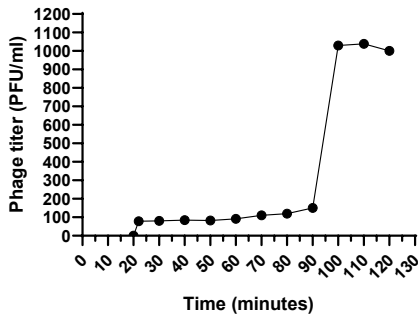


Figure 2: One-step growth curve

A time-kill assay showed that a combination of phage and gentamicin resulted in synergistic antibacterial activity. An in vitro study was conducted to investigate the synergistic antibacterial impact of gentamicin (sub-MIC level) in combination with a fixed dose of bacteriophage in three sequential treatments that is PF, AF, and SIM. As depicted in (Figure 3), the combined bactericidal effect of the antibiotic and phage was more pronounced in PF treatment. The phage replicated in each of the three treatment settings; however, when gentamicin was added to SIM and AF, the phage replication was decreased. In the case of monotherapies, the treatment with phage only achieved the highest level of killing (about 3 log reduction) over 24 h. On the other hand, the treatment with gentamicin only reached the maximum level of killing (1 log) in 48 hours. When comparing three sequential combinations, the PAS combination effectively reduced bacterial count. The overall killing efficiency of the combinations followed this order: P-F approx. 7 log reduction) > SIM (approximately 5 log reduction) > A-F (approximately 2 log reduction).

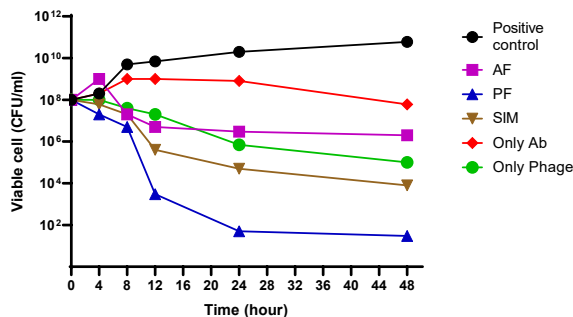


Figure 3: Bacterial colony counts in the time-kill assays

The percentage of biofilm inhibition was determined by assessing the ability of the phage and the antibiotic (gentamicin) to reduce the optical density (OD) relative to the negative control. Percentage biofilm inhibition = ((optical density (OD) of control - OD of treatment) / (optical density (OD) control) x 100).

In the in vitro biofilm model, gentamicin (16 μ g/mL) was less effective at inhibiting the biofilm than phage (1×10^6 pfu/mL) alone. Additionally, the combination of phage and antibiotic (PF) had a better inhibition of the biofilm formation to the AF, SIM, and individual treatments.

The impact of the sequence of phage and antibiotic treatments on targeting biofilm was evaluated. There was a considerable reduction in biomass for all treated biofilms compared to the non-treated biofilms (Figure 4). When comparing the treatment with phage, only (59.3% residual biomass) showed stronger antibiofilm activity than the antibiotic alone (with 95.1% residual biomass). Of the three combination treatment methods, P-F and SIM exhibited greater efficacy in combating biofilms, with residual biomass levels of 15.7% and 27.1%, respectively, compared with the single treatment. In the A-F treatment, the amount of leftover biomass was (about 44.7%).

The combination of phage and gentamycin (PF) resulted in a significant reduction of the planktonic bacterial growth (8 log) than AF (3 log reduction) and SIM (6 log). In contrast, The reduction of bacterial by treatment with phage only (four logs) and gentamicin only (2 log) had less impact on viable cell count (Figure 4).

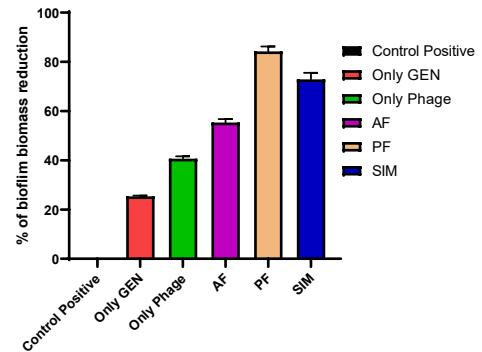


Figure 4: The percentage of biofilm biomass reduction

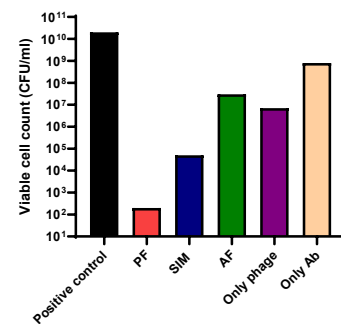


Figure 5: Viable counts of planktonic *S. aureus* (CFU/mL) following a sequential treatment of phage (1×10^6 PFU/mL) and gentamicin (16 μ g/ mL)

DISCUSSION

Since antibiotics and bacteriophages have different mechanisms for killing bacteria in both planktonic and biofilm forms, a combination of the two might be more effective. To examine this aspect, the present study was conducted.

The early observations by other authors on the synergy between phages and antibiotics have received little attention. Even though Himmelweit et al.²² used penicillin and phages to combat *Staphylococcus* effectively, his work was not well-known until recently. More than fifty years later, Huff et al.²³ saw a similar phenomenon with ciprofloxacin and *E. coli*-

specific phages, but they were unable to explain it.

There are reports of *S. aureus* being eradicated by phages. Phage-derived lysin has been used to eradicate MRSA,²⁴ and phages have been examined *in vivo* to treat *S. aureus* infections.²⁵ Nevertheless, few studies have examined the use of phages, and combinations of phages and antibiotics, to treat *S. aureus* biofilms.

This study addresses two major issues in healthcare: the scarcity of biofilm-eradication therapies and the problem of drug-resistant pathogens. In particular, we investigated whether phage-antibiotic synergy (PAS) can occur in the presence of antibiotic-resistant bacteria. The use of a combination of gentamicin and a phage specific to *S. aureus* resulted in the elimination of the biofilm of antibiotic-resistant *S. aureus*.

The present study demonstrated the superior effectiveness of the phage-antibiotic combination compared to phage-only or antibiotic-only treatments.

Because of the complementary nature of their mechanisms of action, phages and antibiotics used to treat biofilms are more effective. Through pores and channels in the biofilm layers, phages can attach themselves to specific receptors and destroy the biofilm matrix.²⁶ Phages and antibiotics can infiltrate and adhere to cells across different biofilm layers, including the basal layer, resulting in cell death. After the biofilm matrix is destroyed, the bacterial cells are released as planktonic cells and are attacked by phages and antibiotics.²⁷ This could be why using PAS in conjunction with other methods is more effective than using them alone in killing bacterial cells producing biofilms.

In the present study, when phage and gentamicin were applied at subinhibitory doses, they produced a synergistic effect; however, this did not occur when the phage and the antibiotic were applied simultaneously. In contrast, the pre-exposure to phage and gentamicin combination eliminated the biofilm at lower antibiotic concentrations. These results suggest that the most efficient method of eliminating biofilms is to expose them to phages first, then antibiotics. A study by Tkhalishvili et al. demonstrated the advantages of applying antibiotics and phages in a staggered manner.²⁸

Phage and antibiotic treatment sequences may significantly affect film removal. A study by Kumaran et al. on the biofilm produced by the *S. aureus* strain ATCC 3556, which was treated with the antibiotics dicloxacillin, tetracycline, vancomycin, linezolid, and cefazolin using different treatment techniques, as well as the phage SATA-8505, confirms this phenomenon.²⁹ The addition of the phage before antibiotic therapy significantly decreased the bacterial load, particularly for lower dosages of vancomycin and cefazolin.

A study by Dicky J et al. on the efficacy of combining phages and antibiotics has also demonstrated that the synergistic antibacterial effects between them depend on the order of administration.³⁰

Host specificity has been reported to affect phage efficacy, along with several other parameters.³¹ According to Bauer et al., who evaluated antibiotic activity on young and mature MSSA and MRSA biofilms and found that, in addition to biofilm maturity, the bacterial strain clearly influenced antibiotic activity, strain differences in the biofilm matrix can ultimately affect the bioavailability and/or function of antimicrobial.³²

CONCLUSION

The results validate previous research on the limited efficacy of antibiotics alone and demonstrate the potential of phage therapy as a biofilm target. As demonstrated in our study, optimizing treatment regimens for biofilm-associated infections is critical, as evidenced by the superior efficacy of phage-antibiotic combination therapies. The synergistic effect of phages and antibiotics in biofilm eradication was observed, and the combination proved beneficial in treating bacteria within the biofilm matrix. Phage-antibiotic synergism provides a potential avenue for future development of antibiotic and phage combinations that fulfil therapeutic application requirements in order to treat *S. aureus* infections. Additionally, this strategy has the benefit of using subinhibitory antibiotic concentrations, which prevents the side effects of high-dosage antibiotic administration. These results add to the increasing amount of evidence that suggests

phage therapy may be used as an adjuvant strategy to address the problems caused by biofilms and ultimately improve patient outcomes.

DECLARATION

Author Contributions

SLK, and GN conceived the idea, designed the study, and critically analyzed the article. SLK reviewed the literature investigated the data's integrity. SLK, and MS executed the laboratory and clinical work. SLK wrote a preliminary manuscript, which was further by GN, and all authors reviewed the manuscript and approved the final version of the manuscript. All authors agreed to be accountable for all aspects of the research work. Note: SLK, MS, and GN are abbreviated name of authors.

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Ethical Approval

The study plan was approved by the Institutional Ethics Committee at the Institute of Medical Sciences (IMS) Banaras Hindu University (BHU), Varanasi-221005, Uttar Pradesh, India with the reference number Dean/2022/EC/3329 on 6th June 2022.

Consent/Assent

Informed written consent was obtained from all the participants before data collection.

Name of Registry and Registration number

Clinical Trial Registry of India (CTRI/2021/12/038527)

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request

Conflicts of Interest

Author(s) declare no conflict of interest

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