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Research Article

Evaluation of Phage-Antibiotic Synergy (PAS) Against Biofilm Formed by Colistin-Resistant *Klebsiella pneumoniae*

Alakh Narayan Singh[1], Pooja Verma[2], Srishti Singh[3], Nandini Upadhyay[4], Kavindra Nath Tiwari[2], Gopal Nath[1*]

[1]Department of Microbiology, Institute of Medical Sciences, Banaras Hindu University, Varanasi-221005, India

[2]Department of Botany, MMV, Banaras Hindu University, Varanasi-221005, India

[3]Department of Botany, CMP Degree College, University of Allahabad, Prayagraj, India

[4]Banasthali Vidyapith, Rajasthan-304022, India

***Correspondence author:** Gopal Nath, Department of Microbiology, Institute of Medical Sciences, Banaras Hindu University, Varanasi-221005, India; Email: gopalnath@gmail.com

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Abstract

The emergence of Antibiotic-Resistant Microbes (AMR) against last-resort antibiotics, including carbapenems, colistin, etc., is a pressing global concern. The dry pipeline of antibiotics necessitates the discovery of alternatives to counter AMR. *Klebsiella pneumoniae* is known for its MDR status and ability to form biofilm, posing additional challenges to antibiotic therapy. Bacteriophage therapy emerges as the most promising alternative to treat the growing antibiotic-resistant infections associated with biofilm. It will be very interesting if the synergic effect is examined between phages and antibiotics at subinhibitory concentrations.

The novel phage ΦKpnBHU3 was isolated from the sewage water and characterized for host specificity and physiological stability (temperature and pH). The isolated phage, in combination with colistin in sub-MIC concentration, were further evaluated for eradication of planktonic and biofilm forms of *K. pneumoniae in-vitro*. Whole genome sequencing of phage ΦKpnBHU3 was performed for identification, evolutionary relationship, and bioinformatics analysis.

This study showed that combining phage ΦKpnBHU3 (1×10^9 PFU/mL) with the sub-inhibitory concentration of colistin (12.2µg/mL) produced a synergistic antibacterial effect and successfully eradicated planktonic and biofilm forms of *K. pneumoniae*. While the phage-first therapy had excellent antibacterial synergy, simultaneous and antibiotic treatment resulted in limited antibacterial synergy. Phage-Antibiotic Synergy (PAS) based therapy has been suggested to eradicate the bacteria more efficiently and thus may lead to the relevance of those antibiotics which are being reported as ineffective.

PAS may significantly reduce antibiotic usage, reducing selection pressure and thus restoring antibiotic activity. However, further research is required to comprehend the PAS's molecular mechanisms and preclinical and clinical evaluation regarding efficacy and safety.

Keywords: *Klebsiella pneumoniae;* Colistin; Biofilm; Synergy; Phage-First (PF); Antibiotic-First (AF)

Introduction

The emergence of Antimicrobial Resistance (AMR) pathogens, especially colistin-resistant *Klebsiella pneumoniae*, is an upsurge at an alarming rate and poses a severe antimicrobial crisis worldwide. Moreover, *K. pneumoniae* is a key trafficker in acquiring Antimicrobial Resistance genes (ARGs) from environmental sources, and its distribution among other bacterial genera pushes us back to the pre-antibiotic era. In 2022, Antimicrobial Resistance Collaborators reported that approximately 600,000 deaths were caused by antibiotic-resistant *K. pneumoniae* in 2019 and marked as an urgent clinical threat [1,2]. The scenario is further complicated as biofilm formation is the most significant virulence property of *K. pneumoniae* [3]. It has been reported that 60-80%

of bacterial infections in humans are associated with biofilm formation, which hinders and complicates antibiotic therapy [4-6]. The antibiotic resistance of mature biofilm somehow increases approximately 10-1000 times compared to planktonic bacteria. Furthermore, biofilm formation resists phagocytosis, antimicrobial factors and exogenous stressors [6-10]. Moreover, various environmental factors like temperature, pH, nutritional content, oxygen levels, osmolality and the presence of other bacteria also complicate the eradication of biofilm in natural settings [11,12].

The current antibiotics crisis and biofilm formation have sparked the renaissance of phage therapy as a potential therapeutic alternative against the infection caused by antibiotic-resilient bacteria [13]. Phages have proven their therapeutic potential in numerous experimental phage therapies and clinical trials where traditional antimicrobial agents have failed [14,15].

Phages disrupt biofilm with their lytic enzymes, such as depolymerases, endolysin, and peptidoglycan hydrolases and cause the death of the biofilm's basal layer, resulting in the complete eradication of biofilm [16-19]. Bacteriophages initiate their adsorption (infection) upon binding with specific receptors and secrete lytic enzymes that disrupt the bacterial biofilm matrix. The disruption of the biofilm matrix opens the pathway for deeper penetration of bacteriophages and antimicrobial agents inside the biofilm. Furthermore, antibiotics and bacteriophages have different receptors and mechanisms for bacterial killing [20,21]. However, the emergence of phage-resistant mutants during therapy is the main reason for treatment failure, limiting their therapeutic potential [22].

The disruption of bacterial biofilm matrix leads to the dispersal of bacterial cells as planktonic and subjected to easily accessible antimicrobial agents. Phage-Antibiotics Synergy (PAS), a combination therapy, has been adopted to alleviate the emergence of phage and/or antibiotic-resistant mutant bacteria during the therapy [17]. The co-administration of phage and antibiotic therapy may have comparatively higher efficacy than treating bacterial infections with bacteriophages and antibiotics alone, reducing the emergence of resistance mutations [23]. The recent application of Phage-Antibiotic Synergy in varying grades of bacterial infection has remarkable breakthrough in phage therapy across the globe [24-30]. In most of the recent studies, phage and antibiotics were administered simultaneously, while some studies have shown that administration of phage-first has better efficacy [31-34].

The arena of phage therapy advances with the application of Phage-Antibiotic Synergy and this combination therapy could exhibit synergistic, additive or antagonistic effects depending upon the interaction of phage and particular antibiotics [26,32,35,36]. In Phage-Antibiotic Synergy, the varying time and concentration of both phage and antibiotics significantly impact the successful eradication of bacterial infection [37].

In previous *in-vitro* studies, researchers executed the Phage-Antibiotic Synergy with antibiotics with respect to their MIC value; however, none of the authors claimed the complete eradication of bacterial biofilm in their study design. Therefore, the present study used colistin-resistant *K. pneumoniae* (KpnBHU109), a well-characterized lytic phage ΦKpnBHU3 (OL976437) and colistin, to evaluate the Phage-Antibiotic Synergy both on planktonic and biofilm states with sub-inhibitory MIC of colistin and also at different concentration and timing of addition of both i.e., phage (ΦKpnBHU3) and antibiotic (colistin).

Material and Method

Bacterial Strain

The *K. pneumoniae* (KpnBHU109) strain used in the study was isolated from the non-healing wound sample of a male patient admitted to the general surgery ward of the University Hospital, Banaras Hindu University, Varanasi, India. *K. pneumoniae* (KpnBHU109) strain was confirmed by conventional biochemical method and further by PCR amplification of 16S-23S rDNA internal transcribed spacer (ITS) region as described earlier [38]. The forward primer sequence was 5′ ATTTGAAGAGGTTGCAAACGAT 3′, and the reverse sequence was 5′ TTCACTCTGAAGTTTTCTTGTGTTC 3′. The PCR amplification was carried out in a thermal cycler (T100 Thermal Cycler, BIO-RAD, CA, USA). As per standard practice, an antibiotic sensitivity test was done by modified Kirby-Bauer disc diffusion method for gentamycin, amikacin, cefpirome/cefepime, levofloxacin, piperacillin+tazobactam, ampicillin+sulbactam, imipenem, ertapenem, meropenem while for polymyxin-E (colistin) by broth dilution method to determine Minimum Inhibitory Concentration (MIC) of colistin according to the recommendation of Clinical and Laboratory Standard Institute (CLSI, 2020) against *K. pneumoniae* KpnBHU109. All the antibiotics and polymyxin-E (colistin) were brought from Hi-Media, Mumbai, India. The *K. pneumoniae* (ATCC 700603) was used

as the reference strain. The Phage-Antibiotic Synergy study plan was approved by the Institutional Ethical Committee at the Institute of Medical Sciences, Banaras Hindu University, Varanasi, India (Reference no. Dean/2022/IAEC/3254).

Selection of Bacterial strain and K. pneumoniae-specific bacteriophage

The well-characterised phage ΦKpnBHU3 (GenBank accession number: OL976437), which exhibits broad-spectrum activity against *K. pneumoniae* isolates, was available to us for this study. It had already been characterised morphologically and genetically [39].

Time-kinetics assay of PAS on the planktonic form of K. pneumoniae

The study examined three different conditions, i.e., administration of phages 8 h prior to antibiotics (PF), administration of bacteriophage 8 h prior to antibiotics (AF), and simultaneous administration of phage and antibiotics (SIM). In brief, 180µL of logarithmic growth phase (0.6 OD ≈ 1×10^8 CFU/mL) of *K. pneumoniae* (KpnBHU109) was inoculated into a 96-well ELISA plate. Subsequently, 20µL of phage composition (1×10^9 PFU/mL), antibiotics 12.2 µg/mL, or a combination of both (phage and antibiotics) was inoculated into the respected well in a specified sequence. The inoculated plates were incubated at 37°C for 72 h. The bacterial load from the inoculated plate were counted at different time intervals (0 h, 2 h, 4 h, 8 h, 16 h, 24 h and 48 h), respectively, according to the standards Colony Forming Unit (CFU/mL) method described by Miles and Mishra with slight modification [40].

Administration of bacteriophage: before, after and simultaneously with antibiotic

K. pneumoniae (KpnBHU109) in the logarithmic growth phase (0.6 OD ≈ 1×10^8 CFU/mL) was inoculated into a 96-well ELISA plate, and furthermore, the bacterial culture was subjected to the three treatment strategies; phage first (1×10^9 PFU/well), antibiotic first (12.2 µg/well) and finally phage + antibiotic (1×10^9 PFU/well + 12.2 µg/well) simultaneously in triplicates set and incubated for 16-18 h at 37°C. After the incubation period, the growth was assessed per the standard CFU counting protocols [41].

Assessment of timing of addition of bacteriophages on the lysis of planktonic form of K. pneumoniae

In this study, to optimize the timing of the addition of colistin after phage introduction, we added the colistin at different time intervals, i.e., 0, 2, 4, 6, 8, 12, 24, and 48 hr after the phages at the concentration of 10^6, 10^7, 10^8 and 10^9 PFU/mL in the LB broth containing 1×10^8 CFU/ mL of the *K. pneumoniae*. Subsequent subculture was done for the bacterial cell counting after 24hr of adding the antibiotics.

Assessment of different concentrations of bacteriophages on the lysis of *K. pneumoniae* when colistin was added after 8hr of bacteriophage administration

The study's optimization of bacteriophage concentration when colistin was added at 8hr was based on the results obtained in the section 2.2 study.

Assessment of different concentrations of phages when added simultaneously with colistin

In this group, we simultaneously administered phage at different concentrations and colistin at a subinhibitory concentration to the KpnBHU109 culture (1×108 CFU/mL) to determine the most effective phage concentration.

Assessment of PAS in different combinations on biofilm

Biofilm formation by K. pneumoniae (KpnBHU109)

K. pneumoniae biofilm was grown in a 96-well microtiter plate and evaluated according to the method described earlier by [42,43]. The fresh colonies of *K. pneumoniae* KpnBHU109 were inoculated into 10 mL of LB broth. The culture was incubated for 18 h at 37 oC with shaking at 150 rpm. The bacterial culture was quantified and adjusted to 0.5 MacFarland (1.5×10^8 CFU/mL), and 200µL of bacterial suspension was distributed into eight wells of a flat bottom 96-well microtiter plate containing LB broth. The inoculated plate was incubated on a shaking platform at 37 oC for 24, 48 and 72 hr, permitting mature biofilms to be established at each well's bottom. Subsequently, the inoculated wells were gently washed three times with PBS to remove free-floating bacteria. The biofilm biomass was fixed with methanol (200µL/well), followed by a 20 min incubation period and stained with 1% crystal violet (200µL/well) for 20 min. The plate was washed thoroughly with distilled water, and the plate was air-dried. The stained biofilm was decolourized by adding 200µL of ethanol (95%) to each well for 30 min. The optical density (OD) of stain

from biofilm was measured using a micro-ELISA auto-reader (Thermo Scientific Multiskan Fc) at a wavelength of 570 nm. The experiment was performed in triplicate.

In-vitro activity PAS on K. pneumoniae (KpnBHU109) biofilm

In this study, after 72 h of incubation, the *K. pneumoniae* (KpnBHU109) biofilm was gently washed with PBS to remove the free-floating bacterial cells. The adherent biofilm was treated with three different conditions, i.e., administration of phage first (1×10^9 PFU/well), administration of antibiotic first (12.2 μg/mL) and simultaneous administration of phage+antibiotic in triplicates. The treated biofilm was further incubated at 37°C for 24 h. Subsequently, the wells were gently washed with PBS, and the biofilm biomass was measured using crystal violet staining according to the earlier protocol.

Assessment of the efficacy of the phage and antibiotic (synergistic) treatment on the K. pneumoniae (KpnBHU109) biofilm

The bacterial suspension (supernatant) was removed from the biofilm-containing wells after 72 hr of incubation and gently rinsed thrice with PBS to remove any planktonic cells. The *K. pneumoniae* biofilm was administrated to three treatments, i.e., phage (1×10^9 PFU/well), colistin (12.2μg/mL), and phage + colistin (simultaneous) in the triplicate set. The treated adherent biofilm was incubated at 37°C for 18 h. The adherent biofilm was rinsed with PBS and dried. The adherent biofilm was inoculated with 100μL of normal saline and scraped with sharp needles from each well. The biofilm samples were inoculated in the LB broth and incubated at 37°C for 18 h.

Result

Antibiotic sensitivity test and phage efficacy against clinical strains

The antibiotics sensitivity testing of clinical isolates of *K. pneumoniae* revealed that KpnBHU109 was resistant to all the antibiotics tested, including imipenem, ertapenem, meropenem and colistin having MIC value ≥24.4 μg/mL (supplementary Table S1). Therefore, KpnBHU109 was used for further experiments. The PCR amplification of 16S-23S rDNA ITS region PCR product of *K. pneumoniae* is shown in supplementary Table 1.

Effect of PAS on the planktonic form of K. pneumoniae (KpnBHU109)

Time-kill assays of the planktonic form of K. pneumoniae (KpnBHU109)

The synergistic antibacterial efficacy of colistin (sub-MIC concentration) and the fixed concentration of phages was determined in the three different studies, i.e., PF, AF and SIM. The time interval between the administration of phage and antibiotics was 8 hr, as depicted in (Fig. 1).

The synergistic efficacy of phage and antibiotic was superior to either the phage or antibiotic alone in reducing the viable planktonic bacterial count. However, in monotherapies, phage treatment has achieved the highest level of bacterial killing (about 4 log reduction) in 24-hour intervals. However, colistin reduced planktonic bacterial count by 1 log in 48 h intervals (Fig. 1). Interestingly, the phage treatment followed by colistin administration after 8 hr significantly reduced the planktonic bacterial count from 1×10^8 CFU/mL to 0 CFU/mL. Additionally, the experiment in all three settings yielded similar results.

Intriguingly, when the phage was added alone, it increased phage count after 24 hr of the treatment. However, when phage and colistin were administered simultaneously, the PFU count was found to be the lowest. Similarly, the overall killing efficacy of the combination therapy had the following descending order: Phage First (P-F) 8 log reduction > Simultaneous administration (SIM) 6 log reduction > Antibiotic first (A-F) 3 log reduction respectively.

Assessment of phage administration before, after and simultaneously with colistin

The phage alone significantly reduces the bacterial load in the planktonic stage by 4 logs, while the sub-inhibitory concentration of colistin (12.2μg/mL) reduces the planktonic cell by 1 log. Intriguingly, when combination therapy was given with Phage First (PF), it reduced the bacterial count by 8 logs, followed by the simultaneous administration by 6 logs, and the least when colistin was added first, i.e., by 3 logs, respectively (Fig. 2).

Assessment of the timing of the addition of colistin after phage on the lysis on the planktonic form of K. pneumoniae

The planktonic bacterial cell was completely eradicated in this experiment when colistin was added with 1×10^9 PFU/mL, 8 h after the phage administration. Moreover, a similar observation was made with the MOI of 0.1 and 0.01; even at 0.001, there was

a notable reduction in the viable cell count, but complete eradication could not be achieved. In addition, a similar pattern of decrease in planktonic cell count was observed with the phage concentrations of 10^7, 10^8 and 10^9 PFU/mL when the colistin was added 6 hr after the phage administration. Ironically, 10^8 PFU/mL did not show a similar pattern of rational reduction in planktonic cell count. Bacterial reduction was insignificant when the colistin was added 12hr, 24hr and 48hr after the phage administration. Intergunigally, when colistin was added to the bacterial suspension after 0hr, 2hr and 4hr of phage concentrations (10^7, 10^8 and 10^9 PFU/mL) administration, there was no significant reduction in the bacterial count observed after 24hr of colistin administration (Fig. 3).

Assessment of different phage concentrations on the lysis of K. pneumoniae when the colistin was added 8h after phage administration
In this experiment, the optimum concentration of phage dose was optimised when the colistin was added 8hr after the phage administration. The phage concentration of 1×10^9 PFU/mL resulted in the complete clearing of the planktonic form of the bacteria after 12 hr. However, a decrease in planktonic form with 10^7 could be seen. However, 10^8 PFU/mL showed an inferior result than 10^9 PFU/mL but complete eradication could not be achieved (Fig. 4).

Assessment of different phage concentrations on the lysis of K.pneumoniae when the colistin was added simultaneously
The simultaneous administration of phage concentration of 1×10^9 PFU/mL and colistin resulted in the most effective clearing of the planktonic form of bacteria when the subculture was done after 48 h. Notably, the phage concentration of 10^7 and 10^8 PFU/mL resulted in a higher bacterial count when the subculture was done after 48h of incubation (Fig. 5).

Effect of PAS in different combinations on the K. pneumoniae biofilm
Biofilm formation by *K. pneumoniae*
The *K. pneumoniae* isolate used in the present study was a strong biofilm former.

In-vitro efficacy of PAS on K. pneumoniae (KpnBHU109) biofilm
The *in-vitro* efficacy of the phage at a concentration of 1×10^9 PFU/mL and colistin (12.2µg/mL) at sub-inhibitory were evaluated separately for their effectiveness in biofilm eradication; the colistin had minimal impact, while the phages disrupted the biofilm by 69%. Intergunigially combining phage and colistin in different orders resulted in the complete eradication of biofilm. The greatest biofilm disruption was observed with phage treatment first, followed by simultaneous treatment, and colistin first treatment in descending order.

Evaluation of the combined treatment of K. pneumoniae (KpnBHU109) biofilm
The complete eradication of *K. pneumoniae* biofilm was observed with the Phage-First (PF) treatment after 24 h of administration. Simultaneous administration of phage and colistin (SIM) resulted in complete eradication after 48 h of incubation; however, viable bacterial count was observed in the colistin-first (AF) treatment.

Evaluation of colistin susceptibility in the presence of phage in different treatment orders
The combination therapy of phage ΦKpnBHU3 and colistin has synergistic antibacterial efficacy in different treatment sequences: PF, SIM, and AF, respectively. Intriguingly, after 24 h of incubation, the MIC value of colistin was determined to be 6.1 µg/mL for SIM treatment (a 4-fold reduction). In contrast, no reduction in the MIC value was observed in the case of AF treatment. Interestingly, no viable bacterial counts were observed in PF treatment after 24 h of incubation (Fig. 6).

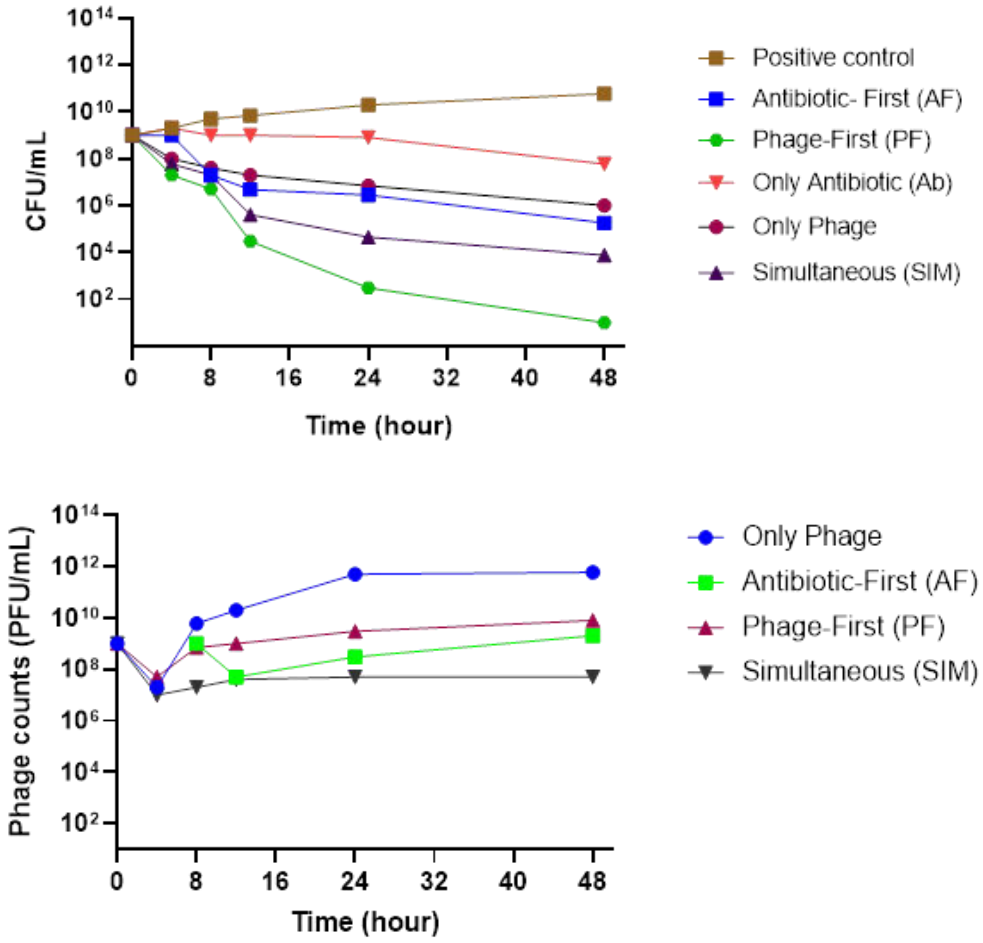

Figure 1: a: The cell count (CFU/mL) in the time-kill kinetics of the planktonic form of *K. pneumoniae* KpnBHU10[9] (1×10^8 CFU/mL) and phage (1×10^9 PFU/mL); b: The phage counting (PFU/mL) in the time-kill kinetics of the planktonic form of *K. pneumoniae* KpnBHU10[9] (1×10^8 CFU/mL) and phage (1×10^9 PFU/mL).

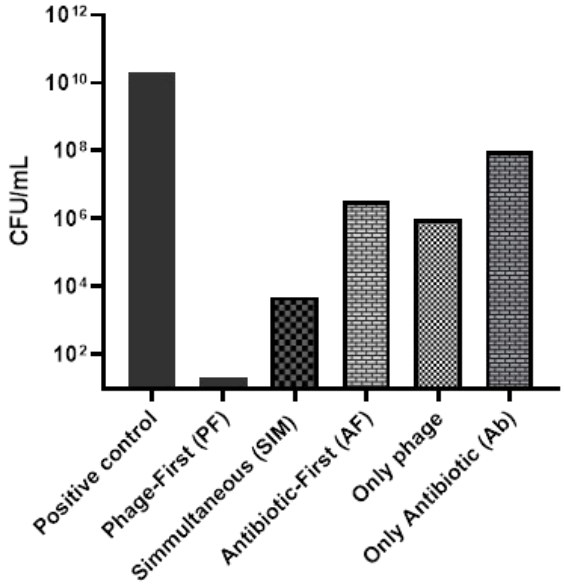

Figure 2: The cell counts of planktonic *K. pneumoniae* KpnBHU109 (1×10^8 CFU/mL) following a sequential treatment of phage (1×10^9 PFU/mL) and colistin (12.2 μg/ mL).

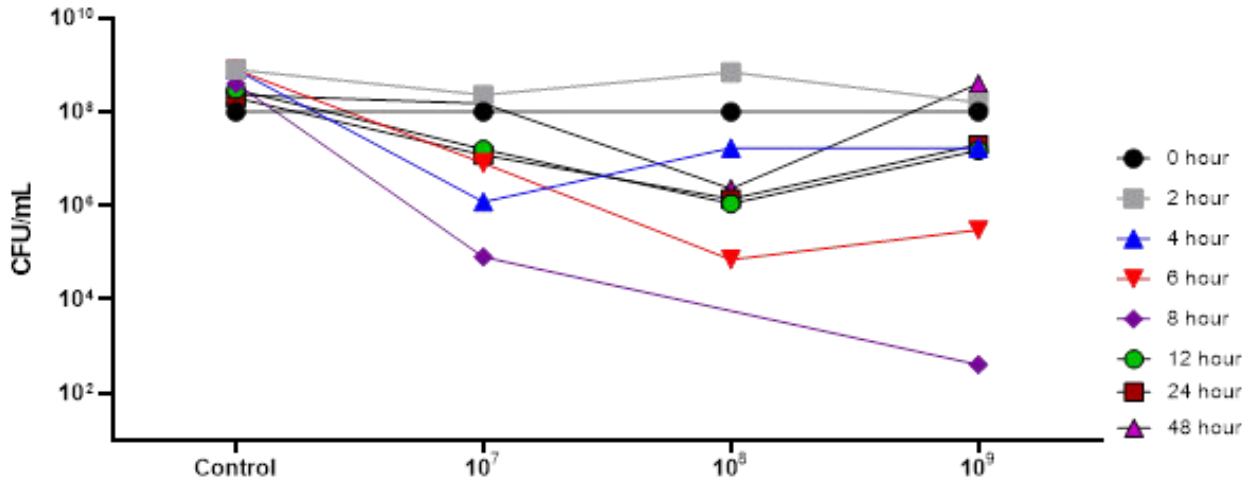

Figure 3: Assessment of timing of addition of phage on the lysis on the planktonic form of *K. pneumoniae.*

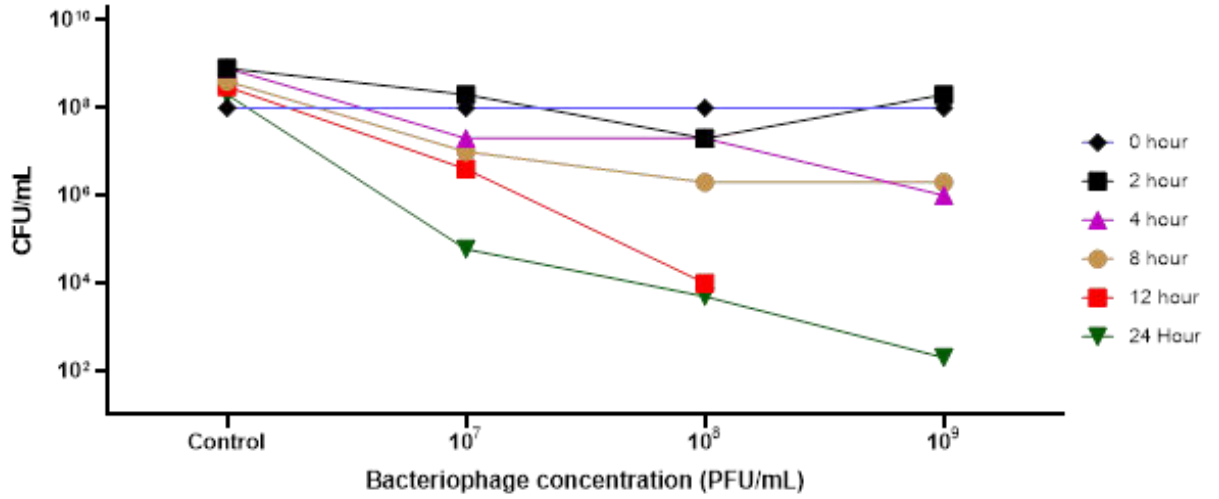

Figure 4: Assessment of different phage concentrations on the lysis of *K. pneumoniae* when the colistin was added after 8 h of phage administration.

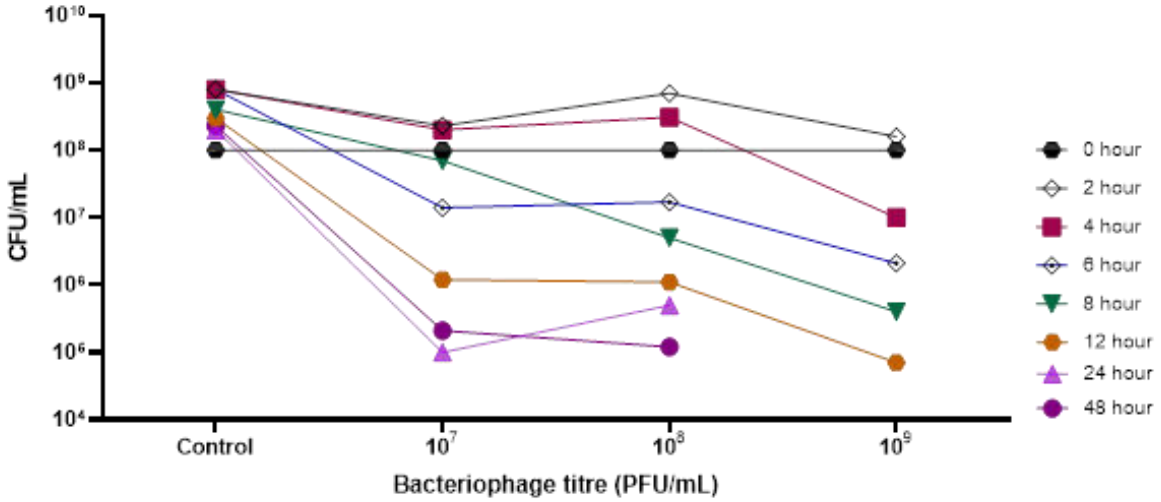

Figure 5: Assessment of different phage concentrations on the lysis of *K.pneumoniae* when the colistin was added simultaneously.

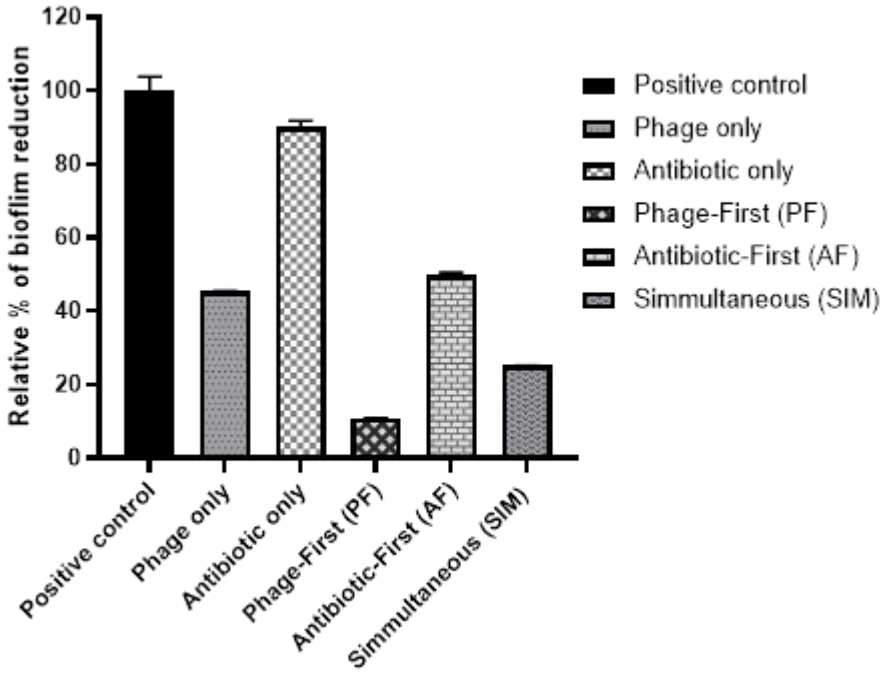

Figure 6: The relative percentage of *K. pneumoniae* biofilm reduction with different treatments.

Discussion

Before the innovation of the first antibiotic, penicillin, in 1928 and its application in clinical practice in the 1940s, bacteriophage therapy remained the primary therapeutic weapon against the war with pathogenic microorganisms. However, within 40 years, antibiotic molecules are losing their shine due to massive and often indiscriminate use. Since the antibiotic molecules are mostly broad-spectrum in action, they can kill various bacterial species. In contrast, phages are host-specific, and prior testing is mandatory.

The enthusiastic researchers explored the efficacy of phages alone and phages in combination with antibiotics (synergy) in treating bacterial infection in the 1940s. In the era of the menace of antibiotic resistance, we have restarted exploring the potential of synergy, additive or antagonistic effects of phage and antibiotics in combination treating bacterial infections. The principle of synergy (phage and antibiotics) seems logical because both phage and antibiotic have a different receptor and unique mechanisms of bacterial lysis/killing. Many encouraging studies have explored synergy through the simultaneous administration of phage and antibiotics with variable outcomes [44-46]. Furthermore, there are a few studies that have reported that administering Phages First (PF) provides better eradication of the bacteria in both states (planktonic and biofilm) [34].

However, none of the studies has explored the optimal phage dose and the time interval between administering the phage and antibiotics to have an end result with complete eradication of infection bacteria irrespective of their planktonic or biofilm state. Interstingly, we are exploring the effect of subinhibitory doses of an antibiotic for the synergy studies, which might bring the relevance of those antibiotics we have accepted as the lost ones. Therefore, the present study was planned to address the above-unresolved issue in *in-vitro* experiments. We selected the colistin-resistant *K. pneumoniae* KpnBHU109 (MIC=24.4 μg/mL) and a purified lytic phage ΦKpnBHU3 (GenBank accession number OL976437) at the various concentrations (10^7, 10^8 and 10^9 PFU/mL) was selected for the combination studies.

The first objective of this study was to examine the order of addition of phage and antibiotic (colistin) as PF, AF and SIM. It was intriguing to see that the Phage First (PF) give the best results in tackling the bacterial load in planktonic and biofilm states. However, the complete eradication/killing of *K. pneumoniae* could not be achieved at the concentration of the phage and antibiotic suspension used alone. The finding encouraged us to look into the most effective concentration of phage to completely eradicate *K. pneumoniae* in both states (planktonic and biofilm).

The second objective of this study was to optimize the optimum concentration of the phage. A phage concentration of 1 $\times 10^9$ PFU/mL was added 8 hr before the colistin, resulting in a complete bacterial eradication. Although a phage concentration of 1 $\times 10^7$ PFU/mL could decrease the bacterial count, it could not eradicate them. Surprisingly, doses other than 10^8 PFU/mL could not show a visible reduction in bacterial count. Even 10^8 PFU/mL gave an inferior result to 10^7 PFU/mL. We do not have an explanation for this observation.

The third objective was to decide the timing of adding an antibiotic (colistin) after phage administration. The 8-hour interval only when the antibiotic was added after phage administration yielded the best result. The time interval of 0, 2, 4, 12 and 24 hr between the phage and antibiotic did not result in satisfactory killing of bacterial count in both states (planktonic and biofilm). In agreement with our finding, a recently published study also showed a similar optimum timing of adding antibiotics after the phage administration was 8 hr [34]. This time interval may be required for the highest activity of phages on the bacterial cells in both states (planktonic and biofilm), making them susceptible to antibiotics even at sub-inhibitory concentrations. Interestingly, it is worth mentioning that mono phages applied at 1 $\times 10^8$ PFU/mL could not eradicate the bacteria. However, during therapy, few mutants developed with decreased MIC due to mono-phage administration, which might explain this failure. Further, the phage count did not increase in any of the three different settings of PAS studies. It is worth to mentioned that the highest phage count was observed when phage alone was administered, and counting was done after 24 h incubation.

The optimum combination of phage (1 $\times 10^9$ PFU/mL) was given first, followed by the sub-inhibitory concentration of antibiotic (colistin, 12.2μg/mL) after 8 h is the most effective for eradication/killing of bacteria in both states (planktonic and biofilm). The phage first treatment leads to massive replication of phage particles that might eradicate all the susceptible bacterial hosts and also lead to the emergence of a few mutant bacteria which might have become susceptible to the sub-inhibitory concentrations of antibiotics (colistin). The other possible suggested mechanism of synergy by previous workers is that antibiotic treatments might cause the elongation of bacterial host cells, which results in the production of increased copies of phage progeny [24,36,47]. Secondly, phage treatment causes activation of bacterial SOS response due to DNA damage which results in mutagenesis and DNA repair and finally, bacterial cell cycle arrest making the bacteria susceptible to sub-inhibitory concentration of antibiotic (colistin). Similarly, phage attacks on the host bacteria resulted in elevated transcription of the recA gene, resulting in enhanced bacterial DNA repair and recombination due to stress generated by phage attack. In turn, activating the recA gene may result in modifying the target site responsible for antibiotic resistance [48]. Thirdly, the breaking down of the bacterial cell wall and EPS layer of the biofilm through the action of phage enzymes (glycan depolymerase) allows the diffusion of the sub-inhibitory concentration of antibiotics and penetrates the bacterial cell [20,49]. Fourthly, the decrease in the MIC value of the antibiotic (colistin) resulted from the phage activity resulted in a storm-like situation for the host bacteria cells and disturbing the normal physiological process, making them susceptible to the sub-inhibitory concentration of antibiotic (colistin). Finally, the fifth mechanism may be that both phage and antibiotic have different receptors and modes of action, which enhances the synergistic effect. However, this proposition may be conjectural because antibiotic-first or simultaneous addition does not give the same efficacy as the phage-first treatment.

The proposition that antibiotics may increase phage production seems unlikely because none of the 3 combinations, PF, AF, and SIM, caused higher production of phage particles than the phage-alone. However, the suggestion that phage enzymes degrade the bacterial biofilm matrix is a natural and well-established fact [50].

Our findings agree with those of other studies by Mukhopadhyay, et al., and Manohar, et al. Recently, Mukhopadhyay, et al., in their study reported that the optimum time interval of 8 hr for adding antibiotics after bacteriophage intervention for a satisfactory reduction in bacterial count; nevertheless, they could not look for a complete eradication. However, they used the antibiotic (>MIC value of colistin) on the Acinetobacter baumannii for the PAS study [34]. Similarly, Manohar, et al., reported that using specific phage and sub-inhibitory concentrations of different classes of antibiotics showed overall good Phage-Antibiotic Synergy on *Citrobacter amalonaticus* [51]. However, the lacunae of the study were simultaneously added both phage and antibiotic, and their endpoint was the reduction in bacterial load rather than complete eradication. Similarly, Wang, et al., also reported the synergy of phage and gentamicin against the *K. pneumoniae* biofilm. However, they administrated the antibiotic only after 30 min of the phage intervention [33]. To the best of our knowledge, the present study is unique in using phage-first with the optimized concentration of phage (1 × 10^9 PFU/mL) with antibiotics (colistin) 8 h later, eradicating both the forms of bacteria, i.e., planktonic and biofilm.

The present observation seems promising because antibiotics with sub-inhibitory doses become bactericidal after phage therapy. In clinical practice, PAS therapy may lead to a significant reduction in antibiotic usage and also may help reverse the so-called obsolete antibiotics due to the emergence of MDR/PDR/XDR bacteria. Finally, PAS therapy will result in the loss of resistance genes from the bacterial genome in the absence of selection pressure. Therefore, neither the phage therapy nor the antibiotic will be obsolete.

It is important to mention that before making any specific generalized statement on PAS therapy, more extensive studies are needed on different bacteria and antibiotics. However, the possibility of the antagonism effect should also be considered in further studies. Moreover, the study findings should be validated in different conditions of *ex-vivo* and *in-vivo* systems. Furthermore, it will be interesting to see that apart from the administration of mono phage, a cocktail of lytic phages should be considered to avoid any mutant development during the synergy therapy.

Conclusion

This study showed that combining phage ΦKpnBHU3 (1×10^9 PFU/mL) with the sub-inhibitory concentration of colistin (12.2µg/mL) produced synergistic antibacterial results and successfully eradicated *K. pneumoniae* in both forms, i.e., free-floating and biofilm forms. While the phage-first therapy had excellent antibacterial synergy and was effective in inhibiting the development of biofilm formation, simultaneous treatment has shown limited antibacterial synergy. This provides a potentially effective method for treating bacterial infections caused by K. pneumonia. It is very encouraging to see that PAS may significantly reduce the usage of antibiotics, reducing the selection pressure and thus restoring antibiotic relevance. However, further research is required to comprehend the molecular mechanisms of the PAS.

Conflict of Interest

The authors have declared no conflict of interest.

Data Availability Statement

The original contributions in the present study are included in the article/supplementary material; further inquiries can be directed to the corresponding author.

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Authors' Contribution

ANS: Conceptualization, Data curation, Formal analysis, Investigation, Validation, Visualization, Writing - original draft, Writing - review and editing. PV: Methodology, Investigation, Data curation, writing-original draft. SS: Methodology, Investigation. NU: Methodology, Investigation. KNT: Resources. GN: Conceptualization, Data curation, Formal analysis, Investigation, Validation, Visualization, Writing - review and editing, Supervision, Project administration.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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