

# Isolation and Characterization of a Novel Lytic Phage N22 and Its Effect on Drug-Resistant *Klebsiella Pneumoniae*

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**Background:** *Klebsiella pneumoniae* (KP) infections present a significant clinical challenge and are frequently associated with elevated drug resistance. The use of phage therapy has resurged in response to escalating antibiotic resistance. This study aimed to address the multidrug resistance crisis in intensive care units by exploring the use of ceftazidime/avibactam (CAZ/AVI), a widely used clinical antimicrobial agent, in conjunction with phage therapy.

**Materials and Methods:** We screened a clinical strain of KP from ICU and successfully isolated phage N22 from hospital wastewater. We conducted an in-depth analysis of the physiological and biochemical properties of phage N22 and determined its optimal multiplicity of infection with the clinical KP strain. The inhibitory effects of phage N22 in combination with CAZ/AVI on biofilm formation were investigated. Comparative efficacies of these combinations were evaluated using a *Galleria mellonella* (*G. mellonella*) model.

**Results:** Phage N22 inhibited KP biofilm formation. The impact of varying phage N22 concentrations when used alongside CAZ/AVI was examined, and the combination of phage N22 and CAZ/AVI was more effective against KP than CAZ/AVI alone.

**Conclusion:** This study provides a preliminary investigation into the effects of combining CAZ/AVI with phage therapy, highlighting its potential significance in developing novel therapeutic strategies for bacterial infections resistant to CAZ/AVI. The findings underscore the importance of advancing highly effective phage agents as alternative treatment modalities for patients with infections refractory to conventional antibiotics.

**Keywords:** phage therapy, drug-resistant, *Klebsiella pneumoniae*, ceftazidime/avibactam

## Introduction

*Klebsiella pneumoniae* (KP) is one of the most common gram-negative microorganisms identified in hospitals and can cause various clinical infections, including pneumonia, urinary tract infections, soft tissue infections, abscesses, and bloodstream infections.<sup>1,2</sup> Previous reports have documented a mortality rate exceeding 50% in patients with KP infections.<sup>3-5</sup> In addition, KP can form biofilms encapsulated in extracellular polymers, with the most clinically significant KP biofilms developing on the internal surfaces of catheters and devices.<sup>6,7</sup> During biofilm infections, planktonic bacteria released from biofilms can spread into the bloodstream, potentially causing a disseminated infection.<sup>8-10</sup> In the post-antibiotic era, increasing reports of KP strains producing extended-spectrum beta-lactamases

or carbapenemases have further exacerbated the clinical outcomes of infections. The extremely high morbidity and mortality rates associated with KP infections impose a heavy burden on the healthcare systems of many countries. Therefore, developing efficient therapeutic approaches to combat multidrug-resistant KP infections is imperative.<sup>11,12</sup>

The growing issue of antimicrobial resistance has led to phage therapy resurgence in recent years.<sup>13–15</sup> Phages are ubiquitous self-replicating viruses that infect bacteria by binding to highly host-specific bacterial receptors.<sup>16</sup> Rational phage therapies offer benefits beyond traditional antibiotics, particularly for the effective treatment of biofilms.<sup>15</sup> Notably, sub-inhibitory antibiotic concentrations have been shown to enhance phage vitality, thereby improving phage-mediated bacterial elimination. This phenomenon, known as phage–antibiotic synergy, can be particularly beneficial when antibiotics are found to be ineffective against certain pathogens.<sup>17</sup> The primary advantage of this approach is its potential to decelerate the evolution of resistance and guide it in a more favorable direction, thereby achieving a more robust suppression of bacterial populations.<sup>18–21</sup> In KP biofilms, the integration of rational phage therapy with antibiotic treatment may hold greater therapeutic promise than the application of phage or antibiotic therapy alone.<sup>22,23</sup>

Ceftazidime/avibactam (CAZ/AVI) is frequently used as an antimicrobial agent in clinical settings, particularly within intensive care units (ICUs).<sup>24,25</sup> However, the emergence of bacterial resistance to CAZ/AVI, coupled with its limitations in preventing and controlling biofilm formation, has diminished the effectiveness of clinical treatments.<sup>26</sup> In the context of the multidrug resistance crisis in ICUs and the need to reduce reliance on antibiotics, the application of bacteriophages presents a promising avenue for inducing evolutionary trade-offs in laboratory studies and potentially restoring drug susceptibility in clinical settings.<sup>27</sup> This study aimed to isolate and characterize bacteriophages from clinical KP isolates that demonstrate synergistic antibacterial activity in combination with CAZ/AVI. The isolated phage was designated phage N22, and a detailed characterization of its physiological and biochemical properties was conducted to ascertain the optimal multiplicity of infection (MOI) when used in conjunction with a clinical strain. Subsequently, we examined the inhibitory effects of phage N22 in combination with CAZ/AVI on biofilm formation, along with the impact of varying phage N22 concentrations with CAZ/AVI. The comparative efficacy of CAZ/AVI and phage N22 alone versus their combination was investigated using *G. mellonella* larvae as a model system.

## Materials and Methods

### Bacterial Strains

The *Klebsiella pneumoniae* strain ASM41783V1 (KP-ASM) employed in this study was isolated from an adolescent male patient in the intensive care unit (ICU). The patient had been admitted to the ICU due to multiple infections resulting from injuries sustained in a traffic accident. The isolation of bacterial strains from patients is a standard procedure in hospital settings. The use of these samples was ethically approved by the Clinical Research Ethics Committee of Shenzhen People's Hospital. Informed consent was obtained from the patient in accordance with the Declaration of Helsinki.

### Isolation and Purification of KP Phages of N22

The KP-ASM was utilized as a host. Phage N22 was successfully isolated from the sewage system of Shenzhen People's Hospital using a double-layer agar method (Sangon Biotech, 9002–18-0). In brief, 10 mL of sewage sample was combined with 10 mL of Luria-Bertani (LB) medium (Solarbio) and a resistant strain KP-ASM. This mixture was incubated in a conical flask at 37 °C with shaking at 180 rpm for 48 hours. Following incubation, the mixture was filtered through a 0.22 µm membrane filter (Millex-GP), and the filtrate was subjected to serial dilution. The presence of phage activity was then assessed using double-layer agar plates.

### Plaque Assay and Plaque Purification of N22

In summary, following the isolation of individual plaques in the preceding step, they were cultured overnight in 4 mL of LB medium containing 2% bacteria. The subsequent day, the phage mixture was filtered through a 0.22 µm filter membrane, yielding a filtrate with potential phages, which was stored at 4 °C for future use. Concurrently, 100 µL of the phage solution was combined with 350 µL of host KP-ASM (OD<sub>600</sub> = 0.4–0.6). Subsequently, 3 mL of LB-agar (0.6%) at

40 °C was poured onto pre-prepared LB-agar plates. After the surface layer solidified, the plates were inverted and incubated at 37 °C for 6 hours. The process is repeated five to seven times to obtain a purified phage filtrate. Each resulting phage filtrate from these repetitions is stored at 4 °C for subsequent experimental use. The most recent phage filtrate was expanded through culturing by combining 4 mL of the filtrate with 100 mL of a KP-ASM bacterial solution in the logarithmic growth phase. This mixture was incubated at a constant temperature of 37 °C and agitated at 200 rpm for 1 to 3 hours. Following the erosion of the bacterial solution by the phage, the mixture was centrifuged at 8000 ×g and 4 °C for 10 minutes. The supernatant was filtered through a 0.22 µm membrane in order to isolate a pure phage filtrate, which was then preserved at 4 °C for the purpose of replicating the experiment.

## Transmission Electron Microscopy

Using a straw, take 10–20 µL of the sample and place it on a wax disc or sealing membrane using the drop-adding or suspension method. Use a copper mesh to ensure the supporting membrane contacts the sample surface, and let it stand for 10–30 minutes. Remove the copper mesh and excess droplets with filter paper strips, then let it partially dry. Stain with 1–2% phosphotungstic acid by applying a small drop to the wax disc or sealing film. Position the copper mesh with the sample on top of the dye solution. The sample was allowed to remain in contact with the solution for a duration of 1–10 minutes. Subsequently, the sample was dried either under an incandescent lamp or through natural air drying. The mesh was then removed, and any excess droplets were carefully wiped away using filter paper strips. Following the drying process, the grids were examined using a transmission electron microscope, specifically the Zoom-1 hC-1, operated at 80 kV.

## Host-Range Determination of N22

Thirty clinical gram-negative bacterial strains were mixed with phage N22, and the mixture was added to LB plates containing semisolid medium. After the medium solidified, the plates were incubated at 37 °C for 4–6 hours. The presence of plaques was then assessed to determine the host spectrum of the phage.

## The Multiplicity of Infection (MOI) of Phage N22

The optimal multiplicity of infection (MOI) was determined by co-incubating log-phase KP strain ASM (KP-ASM) with serial dilutions of bacteriophage N22 in 1.5 mL polypropylene tubes. The phage-to-bacteria ratios were adjusted to 10, 1, 0.1, 0.01, 0.001, 0.0001, and 0.00001, respectively. The mixtures were subsequently incubated in a shaking incubator at 37 °C for 4 hours. Following incubation, the samples were centrifuged at 10,000 rpm for 1 minute and filtered to remove the host bacteria. Phage titers were quantified using a double-layer agar assay. The MOI was determined based on the ratio of N22 that yielded the highest phage titer relative to KP-ASM. This experiment was conducted in triplicate.

## N22 Growth Curve

KP-ASM was infected with phage N22 at a multiplicity of infection (MOI) of 0.01 and incubated at 37 °C for 10 minutes. Subsequently, unabsorbed phage particles were eliminated by centrifugation at 10,000 revolutions per minute (rpm) for 1 minute. The resulting cell pellet was promptly re-suspended in 1 mL of fresh Luria-Bertani (LB) medium. This suspension was then added to 5 mL of LB broth and incubated in a shaking incubator at 30°C. Samples were collected at 5-minute intervals over a 60-minute period. Phage titers were quantified using a double-layer agar plate assay. The experiment was conducted in triplicate.

## Stabilization Analysis of N22

To assess the thermal stability of phage N22, we exposed the phages to a series of environmental temperatures ranging from ambient room temperature to 80 °C. Samples were collected at 10-minute intervals over a one-hour incubation period. Subsequently, phage N22 was diluted, and titers were determined using the double-layer agar method. For the evaluation of pH stability, the pH of the phage buffer was adjusted to values between 4 and 10. Following a one-hour incubation at 30 °C, phage N22 was diluted, and titers were quantified using the double-layer agar technique.

## The Minimal Inhibitory Concentration (MIC) of the Host Bacterial ASM41783VI

MICs were determined via broth microdilution. KP clinical strains with OD<sub>600</sub> 0.6–0.8 were diluted to OD<sub>600</sub> 0.001 in LB broth. 200 µL of bacterial solution was added to the first well, followed by 100 µL in each subsequent well. Polymyxin B (1024 µg/mL) was added to the first well, mixed, and 100 µL was transferred to the next well for serial dilution. The eleventh well's 100 µL was discarded, and the twelfth well served as a positive control. After 18 hours of incubation at 37 °C, 10 µL of thiazolyl blue (5 mg/mL) was added to each well, and absorbance at 595 nm was measured after a further 30 minutes at 37 °C. The MIC was defined as the lowest drug concentration inhibiting 90% of bacterial growth.

## Sequencing of the Phage N22 Genome

The phage DNA was extracted utilizing the E.Z.N.A Viral DNA Kit from Omega following the specified protocol. The specimen was subsequently transferred to Shanghai Personalbio Biological Technology Co., Ltd. for genomic analysis.

## Combined Experiments with Phage N22 and Ceftazidime/Avibactam (CAZ/AVI)

To evaluate the impact on bacterial growth when exposed to both phage and antibiotics, we employed an optical microtiter plate assay system. This assessment included varying concentrations of antibiotics, encompassing the minimum inhibitory concentration (MIC), across multiple orders of magnitude of phage titer over time. This study aims to ascertain the efficacy of antibiotics and bacteriophages across various stoichiometries in clinically pertinent scenarios.

Bacterial growth was monitored over a 24-hour period, both in the presence and absence of the bacteriophage and antibiotic. The effectiveness of CAZ/AVI and bacteriophage, individually and in combination, against KP-ASM was assessed. Growth curves are presented as means ± standard deviation (SDs). The absorbance of the culture was monitored at 37°C for 24 hours. The resultant absorbance data were transformed into a heat map illustrating the percentage reduction in the bacterial population.

## G. *Mellonella* Experiment

To assess the therapeutic efficacy of phage treatment in vivo, *G. mellonella* larvae were utilized as the experimental model. Specifically, three-month-old *G. mellonella* larvae were injected with 50 µL of various solutions, including a phosphate-buffered saline (PBS) control group, a bacterial ( $3.15 \times 10^5$  CFU/mL) infection group, a bacterial ( $3.15 \times 10^5$  CFU/mL) and phage ( $1.705 \times 10^5$ ) mixture group, a bacterial ( $3.15 \times 10^5$  CFU/mL), phage ( $1.705 \times 10^5$ ), and 64 µg/mL CAZ/AVI treatment group, and a bacterial, phage, and 128 µg/mL CAZ/AVI treatment group. Each group n = 24 and the experiment was repeated three times.

## Pipeline Microbial Membrane Experiment

In summary, the bacterial cultures were incubated overnight and subsequently inoculated into a fresh medium the following day. The cultures were grown until they reached an optical density at OD<sub>595</sub> of 0.6. At this point, they were connected to a circulation system comprising a pipeline, an adapter tube, and a Shengnuo SN-1600VR infusion pump, which was utilized to regulate the flow rate. The flow rate was set at 60 mL per hour and maintained for a duration of 48 hours. Following this period, the adapter tube was detached, and the bacterial cells were fixed using formaldehyde and stained with a 1% crystal violet staining solution. The specimens were stained and subsequently eluted with anhydrous ethanol. Absorbance measurements were conducted at OD<sub>595</sub> utilizing a microtiter plate system. The experiment was replicated a minimum of three times, and the data presented represent the mean values from three representative experiments.

## Well Plate Biofilm Assay

Bacterial cultures with an OD<sub>595</sub> of 0.6 were inoculated with 10 µL of the original 90 µL LB medium per well in a 96-well plate. The cultures were then incubated for 24 hours and fixed with formaldehyde for 15 minutes. Following fixation, a 1% crystal violet staining solution was applied for 15 minutes, and the wells were subsequently washed three times with PBS.

Cells were eluted with absolute ethanol for 1 hour, and absorbance at OD595 nm was measured using a micro-UV visible spectrophotometer. The assay was repeated at least three times, and the data represent the mean of three experiments.

## Results

### Genomic Characteristics of Phage N22

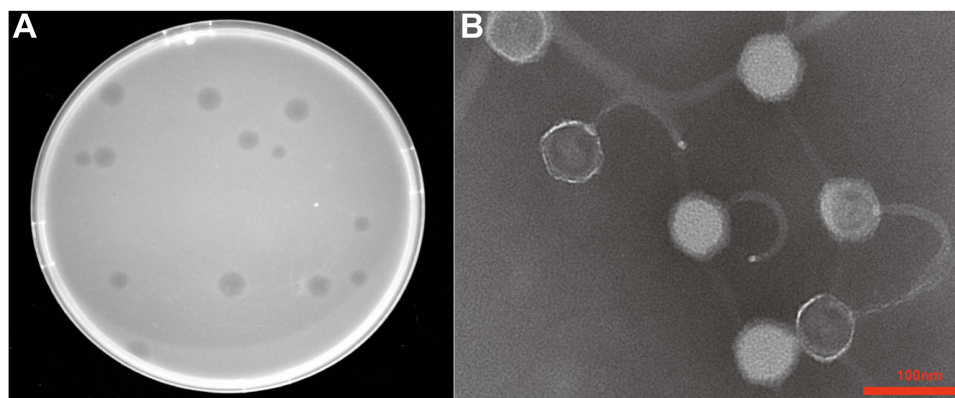
A pathogenic bacteriophage, designated N22, was isolated from untreated hospital sewage using KP-ASM as the host. Phage N22 was effectively propagated and visualized using the double-layer agar plate method (Figure 1A). Transmission electron microscopy analysis revealed that phage N22 possesses a head with a diameter of approximately  $70 \pm 1.3$  nm and a tail of approximately  $170 \pm 1.6$  nm in length, with the tail observed to be in a non-contracted state (Figure 1B).

The N22 genome is 51,626 base pairs in length, with a G + C content of 51.67% and 81 putative open reading frames (ORFs) ( $\geq 30$  amino acids). No ORFs associated with phage lysogeny, virulence, antimicrobial resistance, or integrases were identified, indicating the potential utility of N22. BLASTn analysis against the GenBank database revealed that the top 20 hits for N22 ( $\geq 90\%$  coverage) were all KP phages (Figure S1). N22 exhibited the highest similarity to KP phage UGKSKpnP2 (accession number: OV877339.1; 98% coverage; 95.93% nucleotide identity) and KP phage vB\_KpnS\_Domnhall (accession number: NC\_049835; 97% coverage; 96.72% nucleotide identity); the virus in question is classified within the genus *Webervirus*, and species *Webervirus domnhall*. Consequently, N22 was identified as a species of the *Webervirus* domain within the genus *Webervirus*, based on species delimitation criteria that require a minimum of 95% overall DNA sequence homology for bacterial and archaeal viruses.

### Growth and Environmental Characteristics of Phage N22

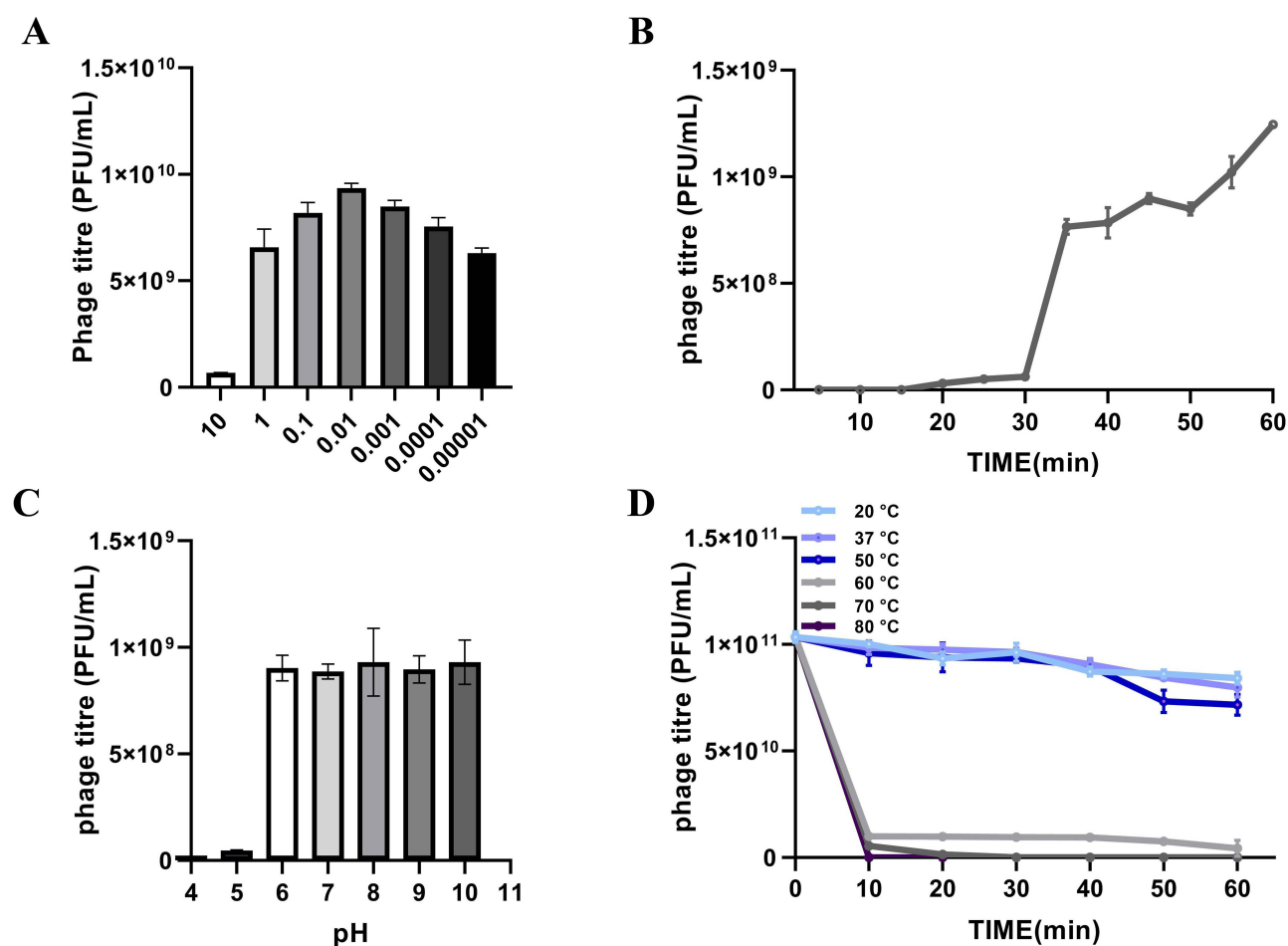
The phages and host bacteria were co-cultured at various MOIs for 6 h. The highest phage titer ( $9.35 \times 10^9$  PFU/mL) was observed at an MOI of 0.01 (Figure 2A), suggesting that this was the optimal MOI. The one-step growth curve indicated an incubation period of approximately 30 min (Figure 2B). During the subsequent burst period, which lasted for 15 min, the phage titer increased rapidly. After approximately 45 min, phage growth entered a plateau phase. Calculations indicated that phage N22 exhibited a substantial burst size of  $8.98 \times 10^8$  PFU per cell.

Phage N22 demonstrated robust tolerance to pH values ranging from 6 to 10 (Figure 2C). However, at pH 5, the phage titers were significantly reduced compared to those at pH 7. The phage was nearly completely inactivated at pH 4. Thermal stability tests revealed that phage N22 is sensitive to elevated temperatures (Figure 2D). No significant difference in phage titer was observed between incubation temperatures of 20 °C and 37 °C for a duration of 1 h. However, a marked reduction in titer was noted at 65 °C when compared to 20 °C, and complete phage inactivation occurred at 80 °C within 10 min. Despite this, phage N22 exhibited commendable temperature and pH tolerance (Figure 2C and D), making it a promising candidate for future phage therapy applications.



**Figure 1** Visual representations of plaques and transmission electron microscopy of phage N22. (A) Plaque formation by phage N22. (B) Transmission electron micrograph of phage N22, with a scale bar indicating 100 nm.



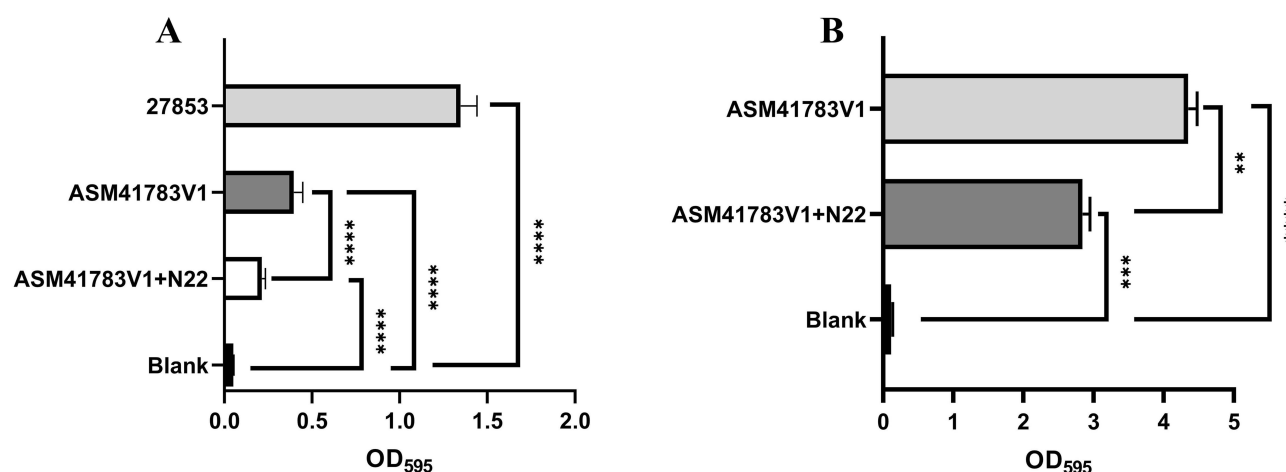


**Figure 2** Biological characteristics of phage N22. **(A)** Optimal multiplicity of infection (MOI) of KP-ASM with phage N22. **(B)** One-step growth curve of phage N22 on KP-ASM. **(C)** pH sensitivity: Phage titer of N22 following incubation at various pH values at 37 °C for 1 hour. **(D)** Thermal stability: Phage titer of N22 measured after 1-hour incubation under different temperature conditions. Data represent the mean of three independent experiments.

## Phage N22 Inhibits KP-ASM Biofilm Formation

To evaluate the efficacy of the phage cocktail in eradicating biofilms under static conditions, 48-h biofilms formed by KP-ASM were cultivated in 96-well polystyrene microtiter plates. These established KP-ASM biofilms were subsequently treated with phage cocktail N22. The biomass density was assessed by crystal violet staining and OD<sub>600</sub> measurements at 48 h (Figure 3A). Treatment with KP-ASM + phage N22 resulted in a 46% reduction in biofilm biomass compared with that in the untreated KP-ASM group. The experimental results indicated that phages were significantly more effective in rapidly reducing biofilm biomass.

To replicate the efficacy of cocktail phages in eradicating biofilms within pipeline systems, a 48-h biofilm was cultivated using KP-ASM in a PVC infusion tube. *Staphylococcus aureus* 27853 was excluded from the analysis because of its high biofilm formation. The established KP-ASM biofilms were subsequently treated with phage cocktail N22. After a 24-h treatment period, biofilm biomass density was assessed using crystal violet staining and OD<sub>600</sub> assays (Figure 3B). The biofilm biomass demonstrated a 35% reduction following treatment with the combination of KP-ASM and phage N22. The results demonstrated that the bacteriophages effectively reduced biofilm accumulation within the pipelines. However, although phages significantly mitigate biofilm formation, they do not entirely eradicate biofilms or eliminate pathogenic bacteria.



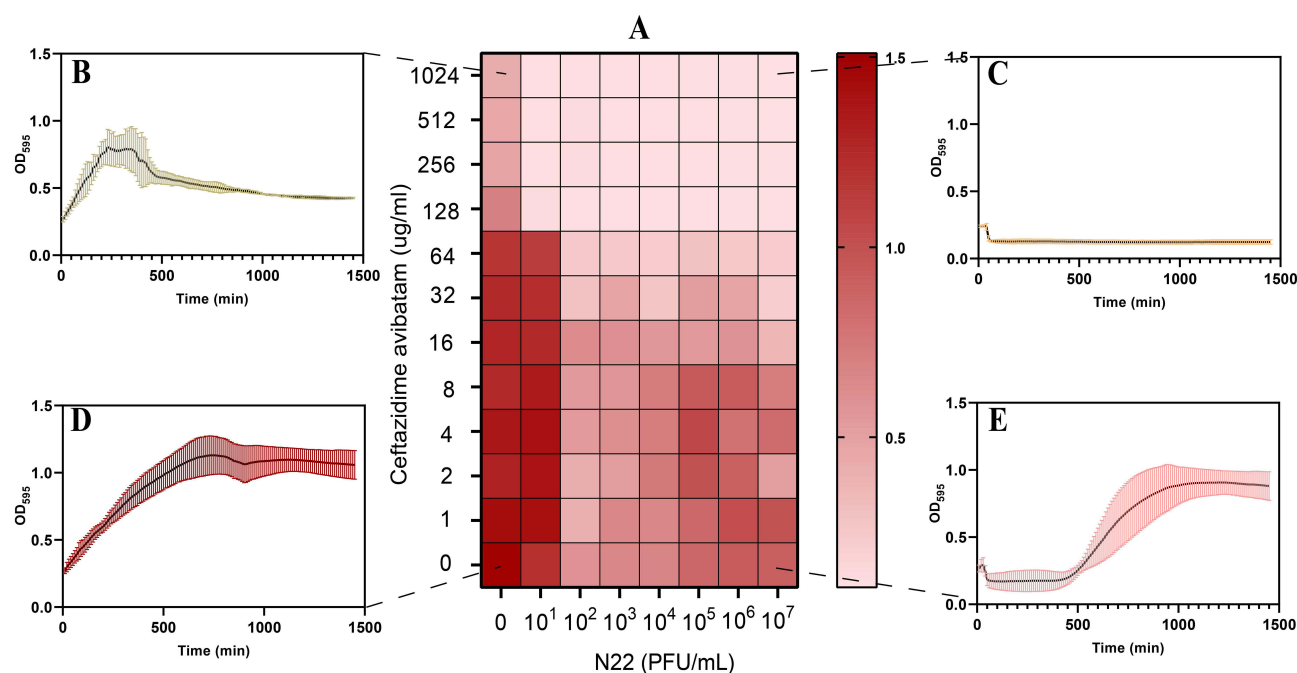
**Figure 3** Impact of Phage Treatment on Mature Biofilms. **(A)** The optical density (OD) of the biofilm was measured after incubating well plates for 24 hours with the *Staphylococcus aureus* model strain ATCC 27853, KP-ASM, KP-ASM combined with phage N22, and LB as a blank control. This experiment was conducted in triplicate. **(B)** The experiment was also performed using a catheter culture biofilm containing KP-ASM, KP-ASM combined with phage N22, and LB as a control. The biofilm system was connected to a flow rate pump operating at 60 mL/h, and this experiment was similarly conducted in triplicate (\*\*\*;  $p < 0.0001$ , \*\*;  $p = 0.0003$  and \*;  $p = 0.0017$ ).

## Synergistic Effect of CAZ/AVI and Phage N22 Against KP-ASM

Our findings indicated that the combination of CAZ/AVI and phage N22 produces an additive antimicrobial effect against KP-ASM. In this study, we employed an in vitro model utilizing the gold standard time-kill curve and a traditional checkerboard assay, both of which have been validated for evaluating phage-antibiotic combinations. Using the synogram, we examined the antimicrobial efficacy of CAZ/AVI at concentrations ranging from 0 to 1024  $\mu\text{g/mL}$  and phage N22 at concentrations from  $10^1$  to  $10^7$  PFU/mL. Various combinations of these agents were tested as non-treatment controls. The measured outcomes included bacterial population size at 24 h (Figure 4A) and the area under the bacterial growth curve (Figures 4B–4E). Analysis of the individual wells in the synogram revealed that all phage concentrations led to the emergence of resistance. Importantly, even sub-inhibitory doses of the phages and CAZ/AVI, when used in combination, resulted in sustained bacterial suppression (Figure 4A). In contrast, the use of antibiotics alone failed to inhibit bacterial growth, even when the CAZ/AVI concentration was increased to 1024  $\mu\text{g/mL}$ . Furthermore, increasing the concentration of phages above 100 PFU/mL did not yield improved outcomes. The data indicated that a phage concentration of 100 PFU/mL was adequate to produce enhanced antibacterial effects when used in conjunction with 64  $\mu\text{g/mL}$  of CAZ/AVI, confirming the presence of a synergistic interaction between CAZ/AVI and N22 against KP-ASM, at least in vitro.

## Efficacy of Phage N22 and CAZ/AVI Combination Therapy in *G. mellonella*

The in vivo efficacy of KP-ASM phage N22 was evaluated using *G. mellonella* as a model organism (Figure 5). PBS injection (50  $\mu\text{L}$ ) resulted in 100% survivability, confirming that the injection procedure itself did not cause any harm. The experimental groups included KP-ASM (Control), KP-ASM + N22 (N22), KP-ASM + N22 + CAZ/AVI at 128  $\mu\text{g/mL}$  (Combination 128  $\mu\text{g/mL}$ ), and KP-ASM + N22 + CAZ/AVI at 64  $\mu\text{g/mL}$  (Combination 64  $\mu\text{g/mL}$ ). Following KP-ASM injection, all larvae succumbed within 24 h. In contrast, N22 administration resulted in a 50% survival rate of the larvae at 48 h post-injection. Upon administration of a single dose of phages, specifically at concentrations of 128  $\mu\text{g/mL}$  and 64  $\mu\text{g/mL}$ , a 100% survival rate was observed up to 24 h. However, at the 48-h mark, there was a notable increase in melanization, a reduction in movement, and a diminished capacity for cocoon formation. In bacterial infections, statistically significant outcomes were achieved with PBS treatment alone, as evidenced by 100% larval survival for up to 24 h compared with the infected group. This study, utilizing the *G. mellonella* infection model, demonstrated promising results with phage therapy, particularly when combined with CAZ/AVI treatment.



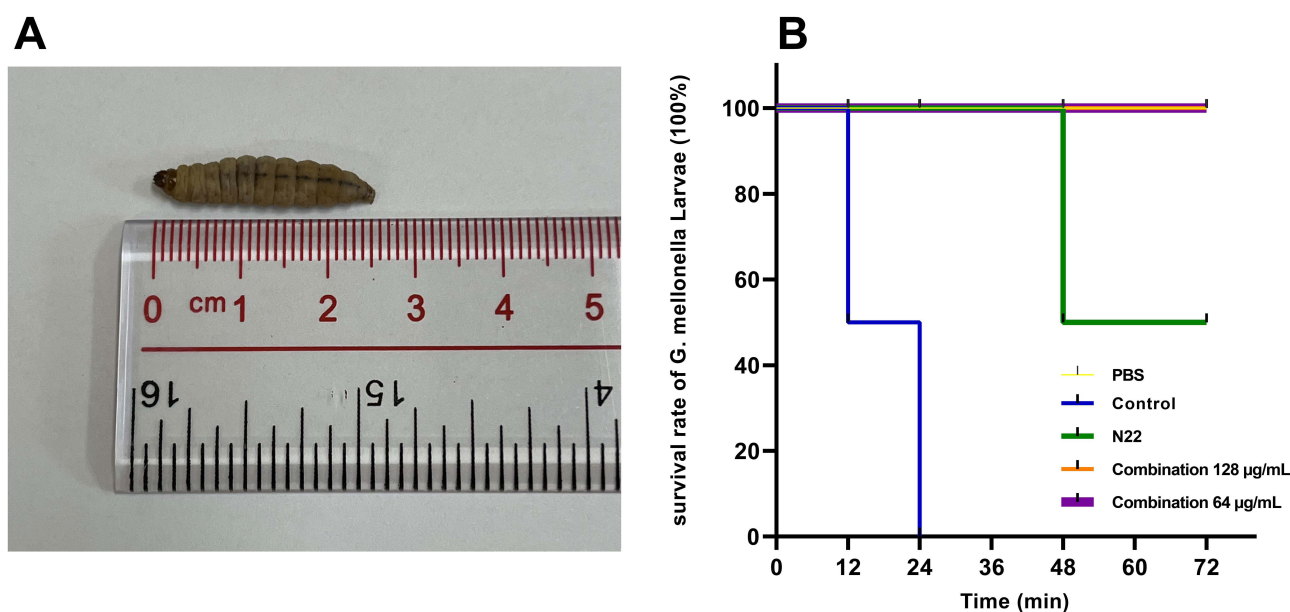
**Figure 4** Growth characteristics and interaction plots illustrating phage-antibiotic synergy and the growth curve of KP-ASM under various conditions: **(A)** depicts a heat map representing the combined effect of phage N22 and CAZ/AVI on KP-ASM, the scale corresponds to optical density measurements (OD<sub>595</sub>) at a wavelength of  $\lambda = 595$  nm, where the depth of the colour represents an increase in the number of bacteria. **(B)** illustrates growth curves with CAZ/AVI alone (1024 µg/mL), **(C)** demonstrates growth curves with the combined addition of CAZ/AVI (1024 µg/mL) and phage N22 (10<sup>7</sup> PFU/mL), **(D)** displays growth curves in the absence of phage N22 and CAZ/AVI, and **(E)** shows growth curves with the addition of phage N22 alone (10<sup>7</sup> PFU/mL).

## Discussion

Although necessary, the clinical transformation of new therapeutic strategies for KP can be challenging. Here, we advanced the transformation of a phage-antibiotic combination strategy, taking advantage of phage knowledge and the emergence of phage resistance in KP. CZA preserves the efficacy of ceftazidime by inhibiting  $\beta$ -lactamase activity, whereas bacteriophages can directly lyse the bacterial cell wall, potentially enhancing the bactericidal effect of ceftazidime by different mechanisms.<sup>28</sup> In strains with resistance due to alterations in membrane porins or the presence of metalloproteinases, bacteriophages can circumvent the resistance mechanisms associated with conventional antibiotics and provide additional killing pathways.<sup>29</sup> There are many factors that affect or limit the potential application value of bacteriophage, including the presence of lysogenicity, virulence, antimicrobial resistance or integrase-related genes in the genome.<sup>22,30</sup> Our analysis did not detect the presence of these genes in phage N22, indicating that bacteriophage N22 has potential application value. In this study, we successfully demonstrated that the combination of CAZ/AVI with phage N22 was more effective than each agent individually in a *G. mellonella* model (Figure 5). The superiority of this approach can be elucidated by examining the interactions among phages, their bacterial and insect hosts, and their antibiotics. Additionally, the intraperitoneal administration route, which may not perfectly correlate with the intravenous route employed in humans, could affect phage bioavailability and efficacy at specific anatomical sites.

In this study, we provided insights that significantly contribute to the advancement of combination-based therapeutic strategies employing phages against the high-priority pathogen KP. In previous studies targeting mouse models of multidrug-resistant *Klebsiella pneumoniae* (ST258-type), the use of phage P1, P2, or combinations thereof alone significantly reduced bacterial loads in blood and tissues and improved survival after infection, nevertheless, it was observed that certain bacterial strains were able to recover and develop resistance to the phages following treatment, indicating the limitations associated with monotherapy.<sup>31</sup> Initially, we confirmed that the combination of CAZ/AVI and phage N22 exhibited synergistic rather than additive effects in vitro. Furthermore, we demonstrated superior therapeutic outcomes of phage-antibiotic synergistic therapy targeting KP pathogens, with particular emphasis on carbapenem-resistant *K. pneumoniae* (CRKP). The results of the optimization experiments revealed that the number of phage





**Figure 5** Survival plot of *G.mellonella* subjected to various phage-antibiotic combinations. The experimental groups include the PBS control group, the KP-ASM group, the N22 group, the Combination 128 µg/mL group, and the Combination 64 µg/mL group. (A) details the methodology for measuring the length of *G.mellonella* using laboratory animal model. (B) illustrates the survival rates of *G.mellonella* following injection with the specified substances. The Orange line denotes the control group administered with PBS, while the blue line signifies the group treated with KP-ASM. The brown line indicates the group injected with N22, the cyan line represents the group receiving Combination 128 µg/mL, and the purple line corresponds to the group treated with Combination 64 µg/mL. The experiment was conducted in triplicate, and the resulting data were analyzed utilizing GraphPad Prism version 8.0.

cocktails and dosage of antibiotics significantly influenced the antibacterial activity and resistance development (Figures 4 and 5). Previous in vitro studies have demonstrated the efficacy of *Klebsiella*-specific phages<sup>31–35</sup> or phage-antibiotic combinations<sup>28</sup> against a single KP strain. Further investigation in this area is anticipated to reveal novel uses for phage-based combination treatments rather than promoting phages as an alternative to antibiotics.<sup>11</sup> For instance, in the analyzed clinical cases involving the application of phage therapy against *Acinetobacter baumannii*, patients were administered both phages and antibiotics concurrently, albeit for a brief duration.<sup>36–38</sup> In at least one of these cases, therapeutic synergy may have played a role in positive patient outcomes.<sup>11,39</sup> It is plausible to consider that the broader clinical adoption of phage therapy could be facilitated using phage-antibiotic combinations.

Current approaches frequently employ multi-phage cocktails<sup>40</sup> or pre-adapted phages<sup>41</sup> to address resistance issues. Previous studies<sup>42</sup> demonstrated that λ phages, evolved through serial passage over 28 d, exhibited enhanced bacterial suppression, and delayed the emergence of resistance. Another approach involves the use of cocktails composed of multiple phages to expand the antimicrobial activity spectrum and mitigate phage resistance development. Notably, phage-resistant bacteria frequently experience a fitness burden that can lead to re-sensitization to antibiotics.<sup>43,44</sup> Consequently, phage-antibiotic combinations may enhance bacterial growth suppression while minimizing the risk of resistance emergence.

In conclusion, to optimize the efficacy of phage therapy against KP strains, we propose two strategies: 1) selection of broad-host-range bacteriophages targeting diverse KP strains, considering the significant implications of phage host range on interactions and broad-spectrum protection against pathogenic bacteria, and 2) optimization of phage formulations based on their lytic capacity and host-range specificity, to maximize efficacy against diverse bacterial targets.

In summary, the primary limitation of these findings is confined to the antibiotic CAZ/AVI and phage N22, in addition to the small number of animal and biochemical tests that were carried out to confirm that phages effectively prevent biofilm formation. The underlying mechanisms and rationale for the observed inhibitory effect of the phage-antibiotic combination on biofilms remain unclear. Future research should aim to elucidate the specific mechanisms by which phage-antibiotic combinations impede biofilm formation and investigate whether phages, when encapsulated, can be gradually released in a manner analogous to antibiotics to enhance therapeutic outcomes. This study provides a preliminary investigation into the

effects of combining CAZ/AVI with phage therapy, highlighting its potential significance in developing novel therapeutic strategies for bacterial infections resistant to CAZ/AVI. The findings underscore the importance of advancing highly effective phage agents as alternative treatment modalities for patients with infections refractory to conventional antibiotics, a pressing concern in the global battle against bacterial drug resistance.

## Conclusion

This study provides a preliminary investigation into the effects of combining CAZ/AVI with phage therapy, highlighting its potential significance in developing novel therapeutic strategies for bacterial infections resistant to CAZ/AVI. The findings underscore the importance of advancing highly effective phage agents as alternative treatment modalities for patients with infections refractory to conventional antibiotics.

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## Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

## Disclosure

No potential conflict of interest to be declared by the author(s).

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