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Isolation and identification of *Klebsiella pneumoniae* phage ΦK2046: optimizing its antibacterial potential in combination with chlorhexidine

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Abstract

Background Hospital-acquired infections (HAIs) significantly increase morbidity and mortality worldwide, with *Klebsiella pneumoniae* (*K. pneumoniae*) being a leading HAI pathogen requiring targeted eradication in healthcare settings. The growing bacterial tolerance to chemical disinfectants, like chlorhexidine, highlights an urgent need for novel disinfection strategies. Bacteriophages, which employ unique mechanisms to lyse bacteria, offer a potential solution. Combining phages with disinfectants could reduce the use of chemical agents and delay the development of bacterial resistance. However, the use of phages for contamination control in clinical environments remains underexplored.

Methods ΦK2046 was isolated from hospital wastewater and characterized by transmission electron microscopy, one-step growth curve, optimal multiplicity of infection, and stability analysis. Whole-genome sequencing was performed to identify the genomic characteristics of ΦK2046. The antibacterial and antibiofilm effects of ΦK2046 combined with chlorhexidine were assessed through growth curves, time-kill assays, crystal violet staining, and scanning electron microscopy. A contaminated medical device model was established to assess the ΦK2046-chlorhexidine combination's biofilm reduction efficacy, and different dosing sequences and timing intervals were evaluated for their impact on biofilms formed on urinary catheters.

Results ΦK2046, characterized by a short latency period, strong environmental stability, safety, and tolerance to chlorhexidine, significantly enhanced the antibacterial and antibiofilm effects of chlorhexidine against FK2046, and reduce the emergence of resistant strains. In contaminated medical device models, the combination of ΦK2046 and chlorhexidine diminished bacterial load and biofilm formation on surfaces. A "phage-first" dosing sequence, particularly with a 90-min interval before chlorhexidine treatment, showed superior efficacy in biofilm reduction.

Conclusions This study, using ΦK2046 as an example, demonstrates the potential of phages to enhance the antibacterial and antibiofilm effects of chlorhexidine and their feasibility in medical device disinfection. This innovative approach not only improves chlorhexidine's disinfecting power but also effectively tackles the issue of reduced susceptibility of *K. pneumoniae* to chlorhexidine. The research advances the development and application of phage-based disinfectants and lays a foundation for establishing a phage library with adjuvant properties for disinfectants.

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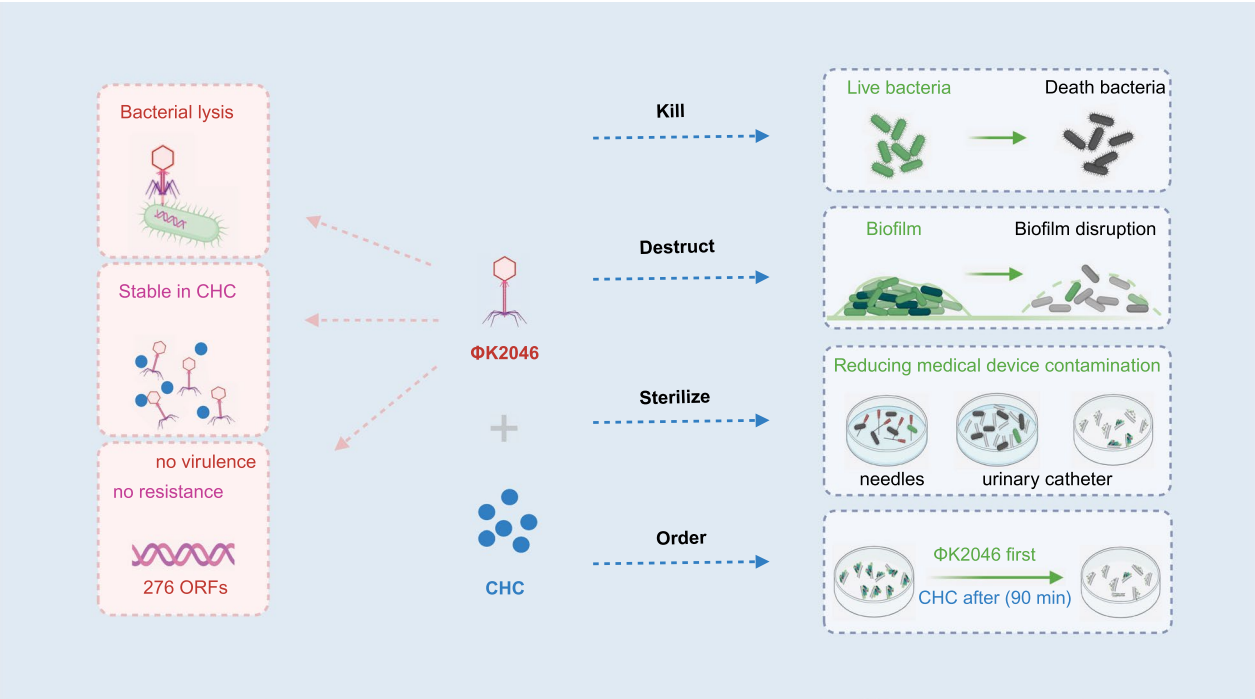
Keywords Bacteriophages, Chlorhexidine, Composite disinfectant, *Klebsiella pneumoniae*, Low-susceptibility

Graphical abstract



Introduction

Klebsiella pneumoniae (*K. pneumoniae*), a major healthcare-associated pathogen, causes increasing morbidity and mortality worldwide [1]. Once nosocomial infection occurs, *K. pneumoniae* invades the host and causes ventilator-associated pneumonia, catheter-associated urinary tract infection, and even bloodstream infection, which increases the length of hospital stay, healthcare costs, and in-hospital mortality, especially in intensive care unit (ICU) patients and immunocompromised individuals, such as cancer patients [2]. *K. pneumoniae* can form biofilm to enhance its protection barrier and cause persistent environmental contamination in hospital settings [3]. Therefore, controlling *K. pneumoniae* and its biofilm in the hospital environment is crucial.

Chlorhexidine is one of the most common biguanide disinfectants with a broad-spectrum bactericidal effect. It is widely used in the care of critically ill patients in hospital intensive care units, for antiseptic bathing, and for the disinfection of medical devices such as catheters [4–6]. However, bacteria have a strong ability to respond to the stress of external chemical disinfectants. The overuse and misuse of chemical disinfectants in hospitals and public environments have led to bacteria becoming increasingly resistant to these agents, including chlorhexidine [7]. At the same time, the"chlorine residual effect"caused by the usage of high concentrations of chlorhexidine will not only induce strain tolerance to chlorhexidine but also promote the spread of resistance genes. Furthermore, it may pollute the aquatic environment and produce toxic effects on aquatic organisms [8]. In addition, a close correlation has been found between the development of chlorhexidine resistance and the development of bacterial resistance to clinically used antimicrobial drugs. For example, studies have found that continuous exposure to chlorhexidine can induce cross-resistance to colistin in *K. pneumoniae*, making it more difficult to treat [9]. Therefore, it is urgent to develop new disinfectant formulations that are safe, low-toxic, and effective as alternatives to traditional disinfectants to reduce the use of chlorhexidine.

Bacteriophages are highly specific viruses with potent lytic capabilities that effectively infect and kill bacteria, while being non-toxic to human cell. They have demonstrated efficacy in removing bacterial biofilms as well [10, 11]. Disinfectant products based on bacteriophages have garnered increasing attention from researchers, being utilized in virus-oriented integrated pest management systems [12] and as disinfectants to reduce contamination of food contact surfaces or poultry carcasses under industrial conditions [13]. Furthermore, an FDA-approved disinfectant product containing bacteriophages active against *Escherichia coli* O157:H7 and *Salmonella* has been developed [14]. Chlorhexidine exerts its bactericidal effect by releasing positively charged "CH" components at physiological pH, which bind to the negatively charged phospholipids on bacterial cell membranes. This interaction disrupts the normal osmotic balance, leading to leakage of low-molecular-weight substances such as potassium and phosphorus, protein coagulation, and ultimately cell death [15]. In contrast, bacteriophages attach to specific receptors on the bacterial surface—such as membrane proteins and polysaccharides—inject their genomes into the host cell for replication and proliferation, and ultimately cause bacterial lysis, releasing progeny phages. These agents employ distinct mechanisms to combat bacteria. Applying multiple and different pressures to bacteria is more effective than using a single pressure and helps inhibit the development of bacterial resistance. For instance, a study illustrated that combining a *Pseudomonas aeruginosa* bacteriophage with chlorhexidine enhanced the killing efficacy of *P. aeruginosa* [16]. In the food industry, a combination of Listeria-specific bacteriophages and disinfectants such as sodium hypochlorite, hydrogen peroxide, and lactic acid can be employed as an innovative antibacterial and antibiofilm disinfection strategy [17]. The synergistic mechanism between phages and disinfectants is not yet well defined. It may be related to the various enzymes released by bacteriophages, which degrade the bacterial biofilm matrix and facilitate the deeper penetration of both bacteriophages and disinfectants into the biofilm to kill bacteria [17], the evolutionary trade-off caused by bacterial resistance to disinfectants may increase their sensitivity to bacteriophages [18], and the selective pressure exerted by bacteriophages accelerates bacterial resistance mutations, but the associated fitness cost leads to reduced bacterial virulence and increased sensitivity to drugs [19].

In summary, this study aims to isolate and identify a *K. pneumoniae* phage, ΦK2046, that can enhance the antibacterial and anti-biofilm of chlorhexidine, delay the development of bacterial resistance to chemical disinfectants, and demonstrate the feasibility and potential of phage combined with chemical disinfectants for medical device disinfection in hospitals. We believe that this idea and evaluation in line with the disinfectant formulation will help to improve the disinfection effect of chlorhexidine and solve the problem of reduced susceptibility of *K. pneumoniae* to chlorhexidine, as well as to promote the establishment of phage libraries with the role of disinfectant supplements and the rapid development and application of compound disinfectant formulations.

Material and methods

Bacterial strains

K. pneumoniae strains used in this study was isolated from the First Affiliated Hospital of Wenzhou Medical University, China. The strains were identified using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF/MS; BioMérieux, Lyon, France) and kept at −80 °C. Before each experiment, the strain was revived and streaked onto Columbia Blood Agar plates to obtain single colonies, which were then inoculated into Luria–Bertani broth and cultured with shaking to obtain logarithmic phase bacteria.

Isolation, propagation, and titer determination of ΦK2046

ΦK2046 was isolated and purified from a wastewater sample using *K. pneumoniae* FK2046 as the host bacterium. Briefly, raw wastewater samples collected from a hospital were filtered through a 0.22 μm polyethersulfone filter (Millipore Stericup-GP) to remove bacteria, supplemented with LB medium, and cultured overnight with a log-phase bacterial suspension. After centrifuging the mixture at 5000 rpm for 15 min, the supernatant was separated and subsequently passed through a 0.22 μm filter. The filtrate was placed on a double-layer agar plate with the host strain and incubated at 37 °C for 8–10 h. Single phage plaques formed on the top agar layer were picked, eluted in SM buffer (50 mM Tris–HCl, pH 7.5, 100 mM NaCl, and 8 mM $MgSO_4$) overnight, and serially diluted. 100 μL of the phage dilutions were mixed with 100 μL of a log-phase bacterial suspension for 10 min, and double-layer agar was prepared and incubated at 37 °C for 8–10 h. Single plaques were picked and propagated as described above until a single phage was obtained [20].

To propagate the bacteriophage, a mixture of phage lysate (200 μL), 1 mL of log-phase FK2046 suspension, and LB (19 mL) was prepared and incubated at 37 °C and 180 rpm for 24 h. The culture was then centrifuged at 5000 rpm for 15 min, and the supernatant was collected and filter-sterilized to obtain the phage suspension [20].

The phage titer was determined using the double-layer agar method [21]. Briefly, a mixture of log-phase bacterial suspension (100 μL), serially diluted phage solution (100 μL) and 0.4% molten agar (8 mL) was poured onto

solidified 1.5% agar plates. Phage titer (PFU/mL) = the number of plaques × 10 × the reciprocal of the dilution.

Transmission electron microscopy observation of ΦK2046

Bacteriophages are enriched, deposited onto a copper mesh, and subjected to a 1-min precipitation. They are then stained with 2% phosphotungstic acid for 15 s, followed by another 1-min precipitation, and air-dried. Phage morphology is examined following negative staining, utilizing a Hitachi HT7800 electron microscope (Hitachi, Japan) [22].

Phage genome sequencing and annotation

The genomic DNA of ΦK2046 was extracted utilizing the Bio-Spin Bacterial Genomic DNA Extraction Kit (BioFlux, Tokyo, Japan) and sequenced on the Illumina Hiseq 2500 platform (~ 1 Gbp/sample). Subsequently, the data underwent quality control employing fastp (https://github.com/OpenGene/fastp), sequence assembly employing A5-MiSeq and SPAdes (http://spades.bioinf.spbau.ru) prior to genome annotation. ORFs were predicted, and potential virulence genes and antibiotic resistance genes were excluded using Pharokka [23]. To analyze the similarity and family relationships among bacteriophage genomes, a viral proteomic tree was constructed using the online tool viptree (http://www.genome.jp//viptree).

Analysis of bacteriophage biological characteristics

The optimal multiple infection (MOI) of bacteriophages ΦK2046 was determined using the method described previously [24]. A mixture of bacteriophage titers to host bacterial counts (1×10^8 CFU/mL) ranging from 0.0001 to 10 was prepared, and incubated at 37 °C with agitation at 180 rpm for 5 h. Afterward, the supernatant of each ratio was collected by centrifugation at 10000 rpm for 6 min and sterilized using a 0.22 μm filter membrane filtration. The titers of progeny bacteriophages in the obtained supernatant were determined using a double-layer agar plate method.

The procedure for the phage adsorption kinetics experiment referred to previously published studies [25, 26]. Mix 1 mL of logarithmic phase host bacterial suspension with phage suspension at the optimal MOI, and incubate in a 37 °C incubator for the following time points: 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25, and 30 min. After incubation, quickly centrifuge the samples at 12000 rpm, carefully remove the supernatant, and filter it through a 0.22 μm filter membrane to collect all unadsorbed free phages. The phage titer in the collected samples is then determined using the double-layer agar plate method.

The growth pattern of ΦK2046 is characterized by a one-step growth curve, as previously described [27]. This involves mixing the FK2046 bacterial suspension in the log phase with bacteriophages at the optimal MOI concentration and incubating them at 37 °C for 30 min. Following this, centrifugation is performed at 10000 rpm for 6 min The bacteria are then resuspended in 2 mL of LB, with the washing process being repeated twice, to remove the supernatant containing free bacteriophages. Finally, the bacteria are resuspended in 10 mL of LB culture medium and incubated at 37 °C with agitation at 180 rpm. Samples are collected at different time points, filtered, and the phage titers are measured. Phage burst size: Average phage number during the plateau phase/Average phage number during the latent period (PFU/mL) in a single phage life cycle [28].

Phage stability analysis

The stability of bacteriophage ΦK2046, reflecting its survival ability, as outlined in a previous study [24]. For the temperature stability experiment, 1 mL of phage lysate with the same initial titer was immersed in water baths set to temperatures ranging from 4 to 90 °C for 1 h. Subsequently, the phage lysate was cooled to room temperature, followed by the evaluation of the phage titer using the double-layer plate method. In the pH stability experiment, the pH of 1 mL of phage lysate with the same initial titer was adjusted to the range of 2–12. The lysate was then incubated in a water bath at 37 °C for 1 h before assessing the phage titer using the double-layer plate method. In the chlorhexidine acetate (CHC) stability experiment, bacteriophages with the same initial titer were mixed with equal volumes of CHC solutions at different concentrations, resulting in final CHC concentrations of 0, 4, 8, 16, and 32 μg/mL. The lysate was then incubated in a 37℃ water bath for 1 h, and the phage titer was evaluated using the double agar layer method [29]. This section used the same initial phage titre (8×10^7—1×10^8 PFU/mL).

Growth curve test

This experiment referred to previous reports [30] and adjusted the overnight cultured bacteria to a concentration of 1.5×10^6 CFU/mL. Each group was then treated with an equal amount of the corresponding concentration of CHC or ΦK2046. The groups were categorized as follows:

1) Fixed CHC concentration, including control, 1/2 MIC CHC, 10^5, 10^6, 10^7, 10^8 PFU/mL ΦK2046, 1/2 MIC CHC + 10^5 PFU/mL ΦK2046, 1/2 MIC CHC + 10^6 PFU/mL ΦK2046, 1/2 MIC CHC + 10^7 PFU/mL ΦK2046, 1/2 MIC CHC + 10^8 PFU/mL ΦK2046;

2) Fixed phage concentration, including Control, 10^8 PFU/mL ΦK2046,1/8, 1/4, 1/2, MIC CHC, 10^8 PFU/mL ΦK2046 + 1/8 MIC CHC, 10^8 PFU/mL ΦK2046 + 1/4

MIC CHC, 10^8 PFU/mL ΦK2046 + 1/2 MIC CHC, 10^8 PFU/mL ΦK2046 + MIC CHC.

The samples were cultivated in a 37 °C incubator, and the absorbance values of each sample were measured at OD_{600} at time points 0, 2, 4, 6, 12, and 24 h to generate the growth curve.

Time-kill assay

This experiment was conducted following previous reports [31]. Overnight cultured bacteria were inoculated into clean centrifuge tubes containing LB (final concentration = 1.5 × 10^6 CFU/mL). Each group was treated with an equal amount of the corresponding concentration of CHC and/or ΦK2046 bacteriophage, with grouping conditions identical to those used in the growth curve experiment. The test tubes were then incubated at 37 °C and 180 rpm. Samples were collected at designated time points, and live bacterial counts were performed. Synergistic activity: after 24 h, the colony count of the combined group was reduced by ≥ 2 Log_{10} CFU/mL compared to the most effective single-agent group; Bactericidal activity: after 24 h, the colony count decreased by ≥ 3 Log_{10} CFU/mL compared to the initial count.

Biofilm formation inhibition test and formed biofilm removal test

The biofilm formation inhibition test was conducted as described, with slight modifications [32]. Briefly, 100 μL of corresponding concentrations of PBS, CHC, bacteriophages, or CHC combined with bacteriophages were added to a 96-well plate. Fresh bacterial solutions (1.5 × 10^6 CFU/mL) were prepared and incubated at 37 °C for 24 h. The liquid was then discarded, and the wells were washed with PBS (Beijing Solarbio Science & Technology Co.,Ltd., China) to remove planktonic bacteria before air-drying at room temperature. Next, 1% crystal violet (CV) solution (Beijing Solarbio Science & Technology Co.,Ltd., China) was added to stain the biofilms for 10 min. After discarding the liquid and washing with PBS, the wells were dried. An ethanol acetone solution (95:5 vol/vol) was added and incubated for 10 min. The absorbance values of the different groups were measured at OD_{595} nm using a microplate reader (Thermo Scientific ™ Multiskan FC).

Formed biofilm removal test was conducted as described previously, with slight modifications [32]. In summary, fresh bacterial solution (200 μL, 1.5 × 10^6 CFU/mL) was added to each well of a 96-well plate and incubated at 37 °C for 48 h to allow for the formation of mature biofilms. After discarding the bacterial solution, the wells were washed with PBS and air-dried at room temperature. Subsequently, 200 μL of corresponding concentrations of PBS/CHC/ΦK2046, or CHC combined with ΦK2046 were added, and then incubated at 37 °C for 24 h. The subsequent washing and staining procedures were performed following the protocol outlined in the biofilm formation inhibition test.

Scanning electron microscope (SEM)

The experimental groups included: blank control (PBS), 1/2 MIC CHC group, phage group (1 × 10^7 PFU/mL, 1× 10^8 PFU/mL), and combination group (1 × 10^7 PFU/mL phage + 1/2 MIC CHC, 1× 10^8 PFU/mL phage + 1/2 MIC CHC).

Sterile square silicon wafers (5 × 5 mm) were placed in 1 mL of freshly cultured bacterial solution containing 1.5 × 10^6 CFU/mL and a mixture of 1 mL of corresponding drugs. The samples were then incubated at a constant temperature of 37 °C for 24 h. Following incubation, the silicon wafers were carefully removed with tweezers, gently rinsed to remove floating bacteria from the surface, and air-dried. The wafers were then immersed in 2.5% (v/v) glutaraldehyde and fixed in the dark for 4 h. Subsequently, the wafers were dehydrated using a series of ethanol solutions (30%, 50%, 70%, 90%, and 100%) for 10 min each. After air-drying naturally, the wafers were coated with gold and observed using a SEM (Hitachi Reguius8100, Japan).

Evaluation of medical device disinfection

The grouping of all experiments in this section is as follows: blank control (PBS), 1/2 MIC CHC group, phage group (1 × 10^7 PFU/mL, 1× 10^8 PFU/mL), and combination group (1 × 10^7 PFU/mL phage + 1/2 MIC CHC, 1× 10^8 PFU/mL phage + 1/2 MIC CHC). The operational method for the contaminated needle disinfection model was adopted from published literature [16]. In summary, a sterile needle was immersed in a 1 × 10^6 CFU/mL FK2046 bacterial suspension and contaminated for 1 h. Subsequently, the needle was dried and divided into six groups. The contaminated needles were then disinfected for 2 h. After disinfection, the needles were removed and immersed in equal amounts of PBS. They were sonicated for 5 min to completely remove the bacteria from the needle surface, and the colonies were counted on LB agar plates.

The operational method for the urinary catheter disinfection model was adapted from published literature [33] with slight modifications. For the 2-h short-term sterilization test of the contaminated urinary catheter, the procedure is identical to the disinfection model of the contaminated needle mentioned earlier. For the biofilm sterilization test in the urinary catheter, the procedure is

as follows: A uniformly prepared and sterile 1 cm urinary catheter is fully immersed in fresh FK2046 bacterial solution and exposed to 37 °C for 48 h to allow biofilm formation. The urinary catheter is then removed, the surface rinsed with PBS, and dried. The catheters are disinfected for either 2 or 24 h. After disinfection, the urinary catheters are removed and immersed in equal amounts of PBS. They are then sonicated for 10 min to completely remove the biofilm bacteria from the surface. The solution is gradient diluted, and colony counting is performed to assess bacterial viability.

Effect of different phage and disinfectant addition sequences on the reduction of formed biofilms

The experimental protocol was developed based on the procedures outlined in the referenced article [34, 35]. Fresh FK2046 (0.5 McFarland Turbidity) was spread on plates and incubated at 37 °C for 48 h to obtain mature biofilms. The planktonic bacteria were washed away, and 1×10^8 PFU/mL ΦK2046 or 1/2 MIC CHC was added in a simultaneous or sequential order, with an interval of 10 min or 90 min before and after the addition of 1×10^8 PFU/mL ΦK2046 or 1/2 MIC CHC. After the completion of the addition of 1×10^8 PFU/mL ΦK2046 or 1/2 MIC CHC, the plates were incubated at 37 °C for 24 h, and stained with crystal violet. Biofilm reduction rate = (control group absorbance—experimental group absorbance)/control group absorbance × 100%

Similarly, 1 mL of fresh FK2046 was incubated in the 24-well plate, incubated at 37℃ for 48 h to obtain mature biofilm, and the same sequence of dosing was carried out, incubated at 37℃ for 24 h, discarded the liquid, cleaned the planktonic bacteria, added 1 mL of PBS, sonicated for 15 min to make the bacteria in the biofilm dispersed, and mixed the liquid sufficiently to carry out the colony counting.

According to the catheter disinfection modeling method described herein, the catheter contaminated with FK2046 biofilm was obtained, and 1×10^8 PFU/mL ΦK2046 or 1/2 MIC CHC was added at the same time or in sequential order, with an interval of 90 min before and after, and incubated at 37 °C for 24 h. The liquid was discarded, the planktonic bacteria were cleaned, and 1 mL of PBS was added, and the bacteria in the biofilm were dispersed and mixed thoroughly by ultrasonication for 10 min, and then counted. The bacteria in the biofilm were dispersed by sonication for 10 min, and the liquid was mixed well for colony counting.

Statistical analysis

Statistical analysis was performed utilizing GraphPad Prism version 9.0 software. The findings are depicted as means ± standard deviations (SD) from a minimum of three independent experimental trials. Statistical significance was assessed using Student's t-test and one-way analysis of variance (ANOVA). $P< 0.05$ were considered statistically significant, denoted as follows: *, $P< 0.05$; **, $P< 0.01$; ***, $P< 0.001$; ****, $P< 0.0001$."ns"indicates no significance ($P> 0.05$) for all analyses.

Results

Isolation and biological characterization of *K. pneumoniae* phage ΦK2046

FK2046, a clinical isolate derived from sputum samples of patients at the First Affiliated Hospital of Wenzhou Medical University, carries the efflux pump gene *cepA* [36], associated with chlorhexidine (CHX) tolerance. The epidemiological cutoff value for CHX resistance, as reported in the literature, is MIC ≥ 64 μg/mL [37], with strains exhibiting MIC ≥ 16 μg/mL considered to have reduced susceptibility to CHX. Based on its MIC results for CHC, FK2046 qualifies as a strain with reduced susceptibility to CHC (MIC = 16 μg/mL) (Table S1).

Subsequently, using FK2046 as the host strain, specific bacteriophage ΦK2046 was isolated and purified from hospital wastewater. The plaque morphology of the bacteriophage is illustrated in Fig. 1A, characterized by small plaque diameters predominantly comprising a central clear plaque (diameter 0.5–1 mm) with no discernible zones of lysing enzymes. Furthermore, transmission electron microscopy images (Fig. 1B) reveal that ΦK2046 exhibits distinct head–tail structures, with a head diameter ranging from 110 to 120 nm, displaying symmetrical icosahedral geometry. The MOI, defined as the ratio of bacteriophage to bacteria yielding the maximum progeny, is depicted in Fig. 1C, with ΦK2046 demonstrating an optimal MOI of 0.001 (Fig. 1C).

The phage adsorption test revealed that ΦK2046 requires a relatively long adsorption time, with an adsorption rate exceeding 70% after 30 min (Fig. 1D). Based on this, a one-step growth curve experiment was established. A one-step growth curve can assess the latent period and burst size of the bacteriophage. As shown in Fig. 1E, at the optimal MOI for ΦK2046, the latent period is 8 min, with titers reaching stability after 90 min. Additionally, the burst size of the bacteriophage is determined to be 5623 plaque-forming units (PFU) per cell.

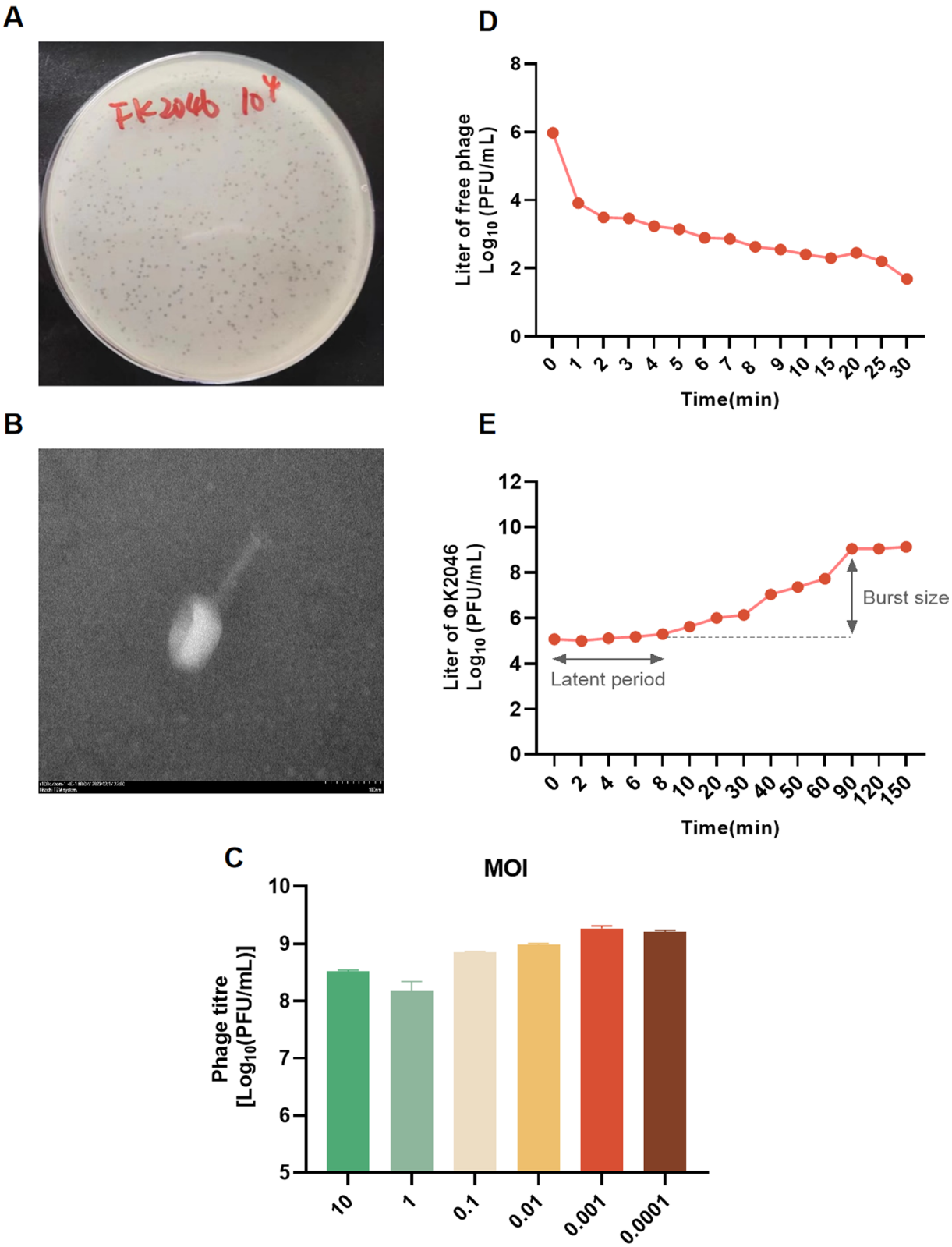


Fig. 1 Isolation, purification and biological characterization of *Klebsiella pneumoniae* phage ΦK2046. **A** Observation of phage plaque morphology by double-layer agar method; **B** Transmission electron microscopy of phage morphology; **C** Optimal multiplicity of infection (OMOI); **D** Phage adsorption kinetics experiment; **E** One-step growth curve. Abbreviations: MOI, multiplicity of infection; PFU, plaque forming unit

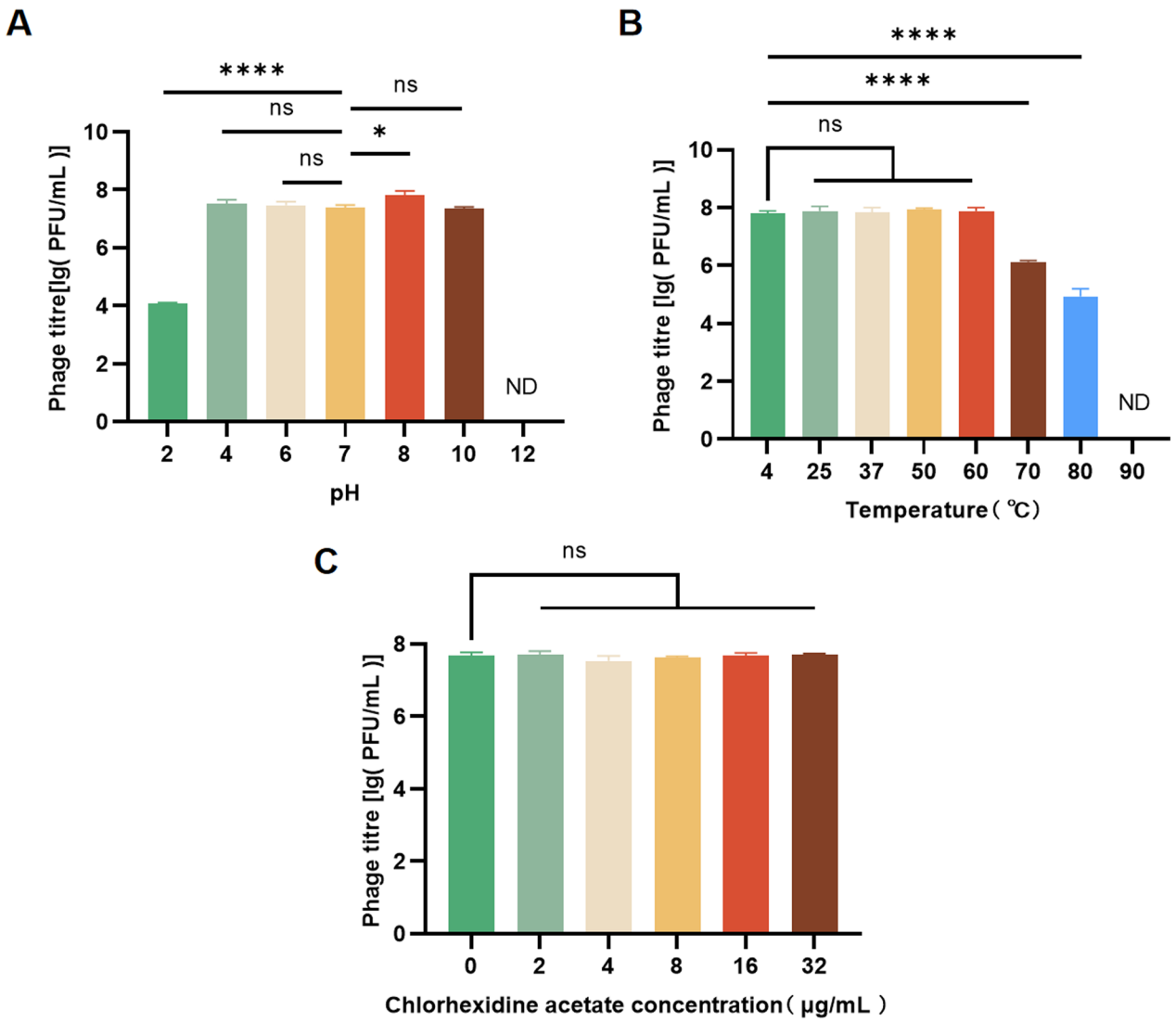


Fig. 2 Stability of *Klebsiella pneumoniae* phage ΦK2046. **A** pH stability (compared to the pH = 7 group); **B** Temperature stability (compared to the 4℃ group); **C** Stability at different concentrations of chlorhexidine acetate (compared to the group without chlorhexidine acetate). Abbreviations: PFU, plaque forming unit; ND, not detected; $P > 0.05$ (ns), $P < 0.05$ (*), $P < 0.0001$ (****)

Stability of bacteriophage ΦK2046

The practical applicability of ΦK2046 was evaluated by assessing its stability under different pH conditions and temperature. The bacteriophage titer did not show a significant decrease within the pH range of 4.0–10.0 (with pH 7.0 as the control), and increased at pH 8.0, suggesting that pH 8.0 might be more suitable for bacteriophage survival (Fig. 2A). The bacteriophage titer did not show a significant decrease within the temperature range of 4–60℃ (with 37℃ as the control) (Fig. 2B). The titer of bacteriophage ΦK2046 did not rapidly decrease in the presence of CHC at different concentrations ranging from 2 to 32 μg/mL (Fig. 2C).

Whole genome characterization of ΦK2046

Whole genome sequencing results showed that phage ΦK2046 possessed a relatively large dsDNA genome with 41.92% G+C content and did not carry virulence genes or drug resistance genes, suggesting that it is safe for application. Genomic annotation showed that ΦK2046 has 276 open reading frames (ORFs), mainly including structural proteins, replication and recombination proteins, auxiliary metabolic genes, and phage endolysin proteins used for lysis, including genes mediating lysis inhibition (Fig. 3A), which is very similar to the classification of the phage defined as a "giant phages" [38]. In addition, phylogenetic tree analysis showed that ΦK2046

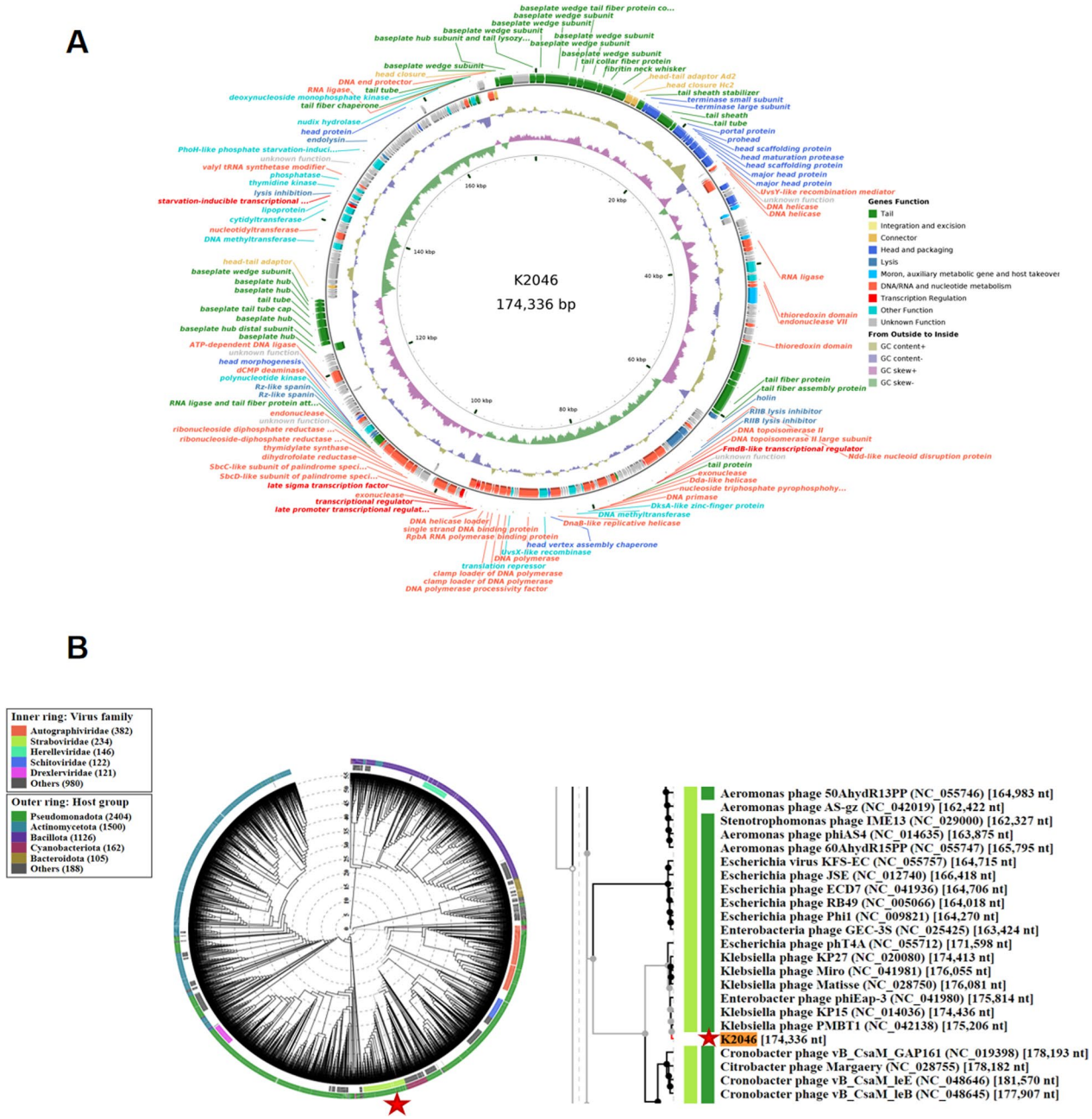


Fig. 3 Genetic characterization of phage ΦK2046. **A** Genome annotation of bacteriophage; **B** Evolutionary developmental tree of the proteome, the red star indicates the position where phage ΦK2046 is located. Abbreviations: bp, base pairs; K2046, *Klebsiella pneumoniae* phage ΦK2046

belongs to *Caudovirales*, *Straboviridae*, and *Slopekvirus*, and is closely related to T4-like phages (Fig. 3B). The sequence number of ΦK2046 has been uploaded to the NCBI database as PP736830.

Synergistic bactericidal effect of bacteriophage ΦK2046 in combination with CHC

To investigate whether there exists a synergistic bactericidal effect between bacteriophage ΦK2046 and CHC, the growth inhibition of FK2046 was assessed upon exposure to different concentrations of ΦK2046 and CHC. In the CHC-only treatment groups, the optical density at 600 nm (OD_{600}) of bacterial growth

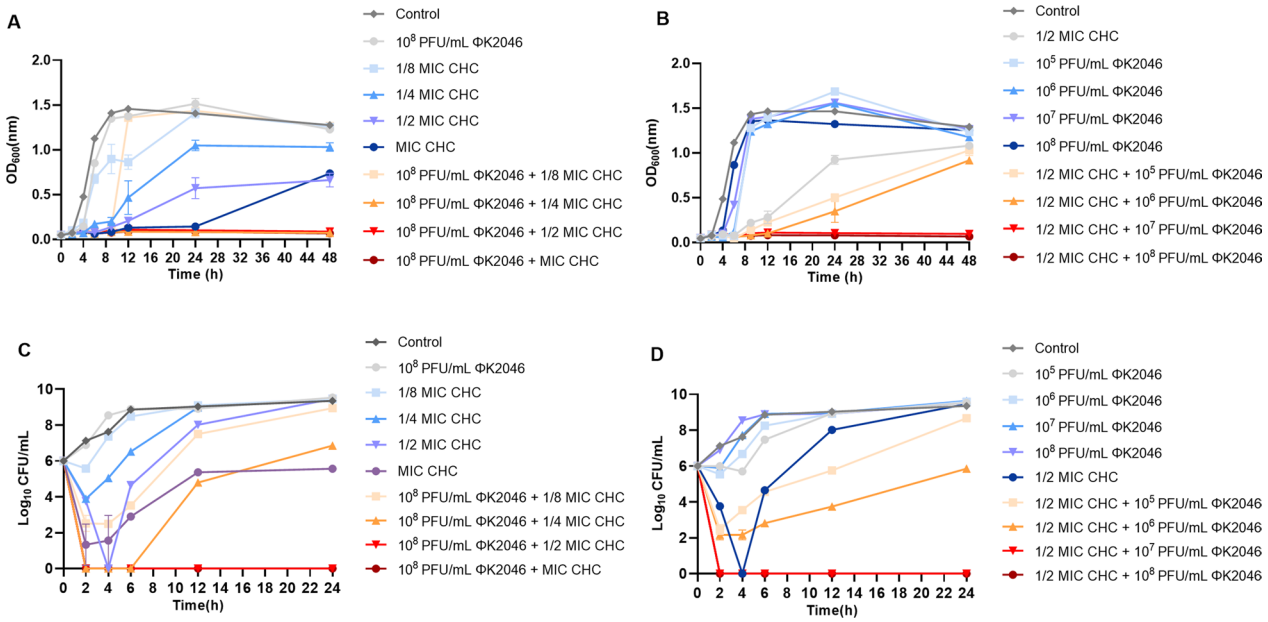


Fig. 4 Growth curve and time-kill tests with different treatment groups. **A** Growth curves of FK2046 after treatment with fixed phage concentration of 10^8 PFU/mL and different CHC concentrations alone and in combination; **B** Growth curves of FK2046 after treatment with fixed CHC concentration of 1/2 MIC and different phage concentrations individually and in combination; **C** Dynamic killing of FK2046 after treatment with fixed phage concentration of 10^8 PFU/mL, individually and in combination with different CHC concentrations; **D** Dynamic killing of FK2046 after treatment with fixed CHC concentration of 1/2 MIC, individually and in combination with different phage concentrations. OD_{600}, optical density at 600 nm; CFU, colony-forming unit; h, hour; CHC, Chlorhexidine acetate; MIC, Minimum inhibitory concentration

decreased in a concentration-dependent manner with increasing CHC concentrations. After 24 h treatment with MIC CHC, although the OD_{600} value of bacterial growth were suppressed to 0.143, by 48 h, the OD_{600} value had increased to above 0.5, indicating that CHC alone could not completely eradicate the bacteria (Fig. 4A). When different concentrations of ΦK2046 or 1/2 MIC CHC were used alone, the bacterial OD_{600} values reached over 1 after 48 h. However, when ΦK2046 was added to the 1/2 MIC CHC treatment, the results showed a synergistic effect. As the concentration of ΦK2046 increased, the OD_{600} values of bacterial growth decreased further. Combinations of 10^7 PFU/mL and 10^8 PFU/mL of ΦK2046 with 1/2 MIC of CHC exhibited potent synergistic bactericidal effects against FK2046. After 48 h, the bacterial optical density remained below 0.1, indicating suppressed bacterial growth (Figs. 4B).

To further examine the bactericidal kinetics of the combination treatment, a time-kill assay was conducted to enumerate changes in bacterial counts within 24 h. As shown in Figs. 4C–D, combinations of 10^7 PFU/mL and 10^8 PFU/mL of ΦK2046 with 1/2 MIC CHC also exhibited a synergistic antibacterial effect under dynamic culture conditions, resulting in the reduction of over 3 Log_{10} CFU/mL bacteria within 2 h (achieving an bactericidal activity) [39], and maintaining bacterial growth inhibition for up to 24 h. In the CHC-only treatment groups, a decrease in bacterial numbers was observed during the first 2–4 h, especially in the 1/2 MIC and MIC CHC groups, where bacterial counts were reduced by more than 3 Log_{10} CFU/mL within the first 4 h. However, this effect was not sustained beyond 6 h, and by 24 h post-treatment, bacterial counts had rebounded to 5.5–9.4 Log_{10} CFU/mL (Figs. 4C). In the groups treated with different concentrations of ΦK2046, the bacterial count at 24 h was similar to the control group, with a difference of less than 0.5 orders (Figs. 4D).

The results of bacterial resistance mutation counts indicated that the combination of ΦK2046 and CHC significantly reduced phage-resistant bacteria (Figure S1) and CHC-resistant mutants (Figure S2) ($P < 0.05$). Additionally, the frequency of resistant mutants was dependent on the initial bacterial concentration. When the initial bacterial concentration was 10^5 or 10^6 CFU/mL, no resistant bacteria were detected after combined treatment. However, when the initial bacterial concentration was 10^8 CFU/mL, a certain number of resistant bacteria remained, suggesting that an increase in the concentration of the combined treatment or an increase in treatment frequency may be necessary. Overall, the combination of ΦK2046 and CHC effectively kills bacteria

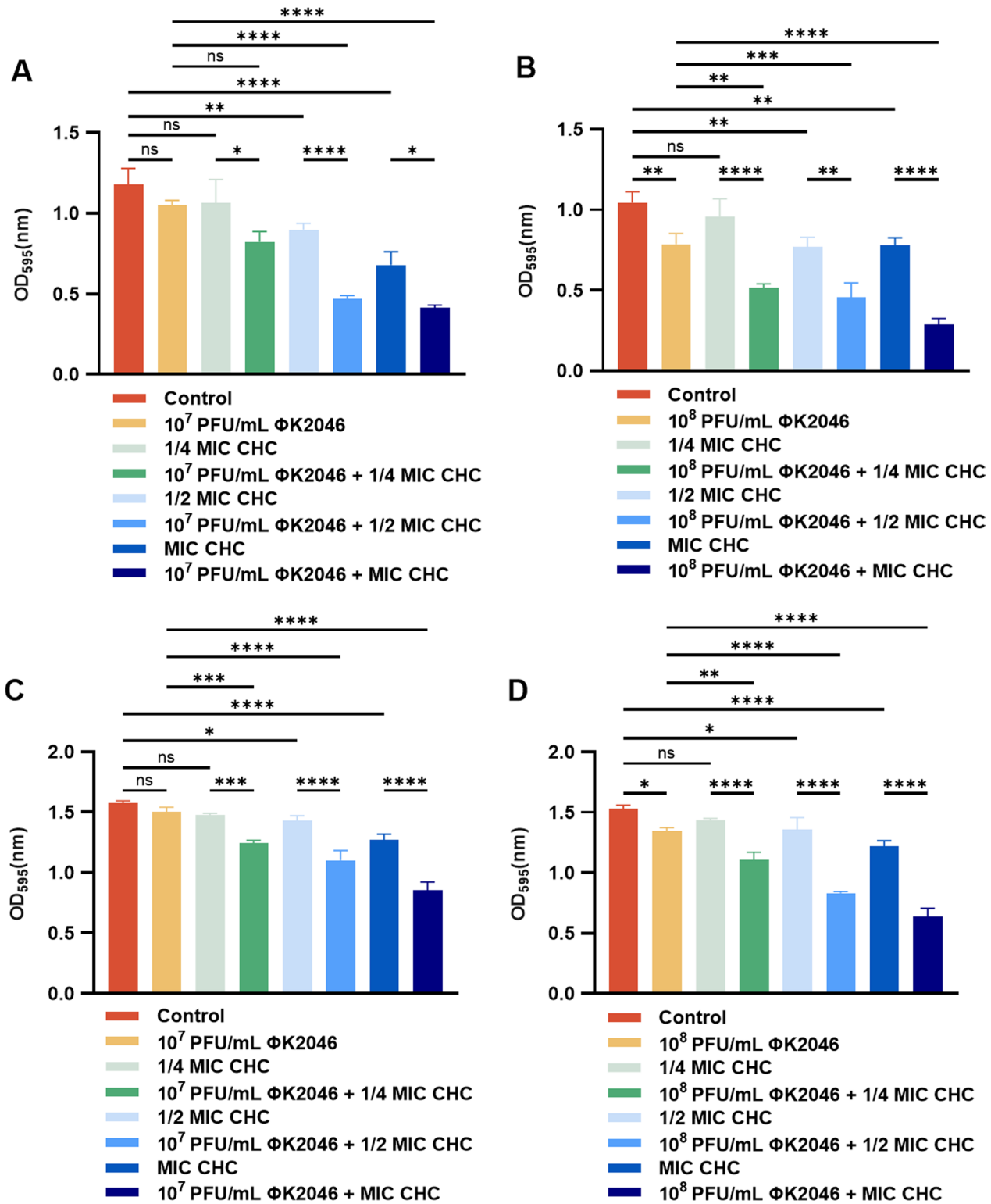


Fig. 5 CHC alone or in combination with ΦK2046 Antibiofilm effect. **A** Inhibition effect of adding 10^7 PFU/mL of ΦK2046 to different concentrations of CHC on the biofilm formation of FK2046; **B** Inhibition effect of adding 10^8 PFU/mL of ΦK2046 to different concentrations of CHC on the biofilm formation of FK2046; **C** The effect of adding 10^7 PFU/mL of ΦK2046 to different concentrations of CHC on the removal of FK2046 biofilm formed; **D** The effect of adding 10^8 PFU/mL of ΦK2046 to different concentrations of CHC on the removal of FK2046 biofilm formed. OD_{595}, optical density at 595 nm; PFU, plaque forming unit; CHC, Chlorhexidine acetate; MIC, Minimum inhibitory concentration; $P > 0.05$ (ns), $P < 0.05$ (*), $P < 0.01$ (**), $P < 0.001$ (***), $P < 0.0001$ (****)

while inhibiting the emergence of bacterial resistance mutants.

Enhanced anti-biofilm effect of ΦK2046 in combination with CHC

Based on previous growth curves as well as time-kill experiments, combinations of 10^7 PFU/mL and 10^8 PFU/mL of ΦK2046 with varying concentrations of CHC were tested for their ability to inhibit biofilm formation and eradicate mature biofilms (Fig. 5). In the monotherapy groups, treatment with 10^7 PFU/mL ΦK2046 or 1/4 MIC CHC did not significantly affect biofilm formation or the removal of pre-formed biofilm compared to the control group ($P > 0.5$). However, treatment with 10^8 PFU/mL ΦK2046 or 1/2 MIC/MIC CHC significantly reduced biofilm formation and effectively cleared pre-formed biofilms ($P < 0.05$). When combined, the ability to inhibit FK2046 biofilm formation was significantly enhanced ($P < 0.05$) (Fig. 5A–B). Notably, the combination of 10^8 PFU/mL phage and MIC CHC reduced biofilm formation by 72.5% compared to the control group. Similarly, after phage addition, CHC at different concentrations exhibited a more pronounced biofilm eradication effect on pre-formed FK2046 biofilm ($P < 0.05$) (Fig. 5C–D), with the combination of 10^8 PFU/mL phage and MIC CHC reducing pre-formed biofilm by 58.2%.

SEM was employed to visually assess the effect of the combination of ΦK2046 and CHC on FK2046 biofilm. The results showed that 10^7 PFU/mL or 10^8 PFU/mL of ΦK2046 and CHC alone was not effective in reducing the biofilm. The biofilm appeared similar to that of the control group (Fig. 6A–B), showing a intact biofilm morphology, tight fitting and overlapping between the bacteria as well as the presence of nanotube-like structure formation [40] (Fig. 6C–H). In contrast, after the combined treatment, there was basically no biofilm formation, few and lysed bacteria, and lack of intact bacterial morphology (Fig. 6I–M). The results demonstrated that the combination of phage and CHC was more effective in reducing biofilm formation compared to monotherapy.

Simulation disinfection of medical devices

Short-term disinfection (2 h) and biofilm eradication abilities of 10^7 and 10^8 PFU/mL of ΦK2046 in combination with 1/2 MIC CHC were tested using needles and urinary catheters contaminated with FK2046 bacterial suspension (Figure S3). The combined of phage and CHC significantly reduced the bacterial load on the surfaces of needles and urinary catheters compared to individual

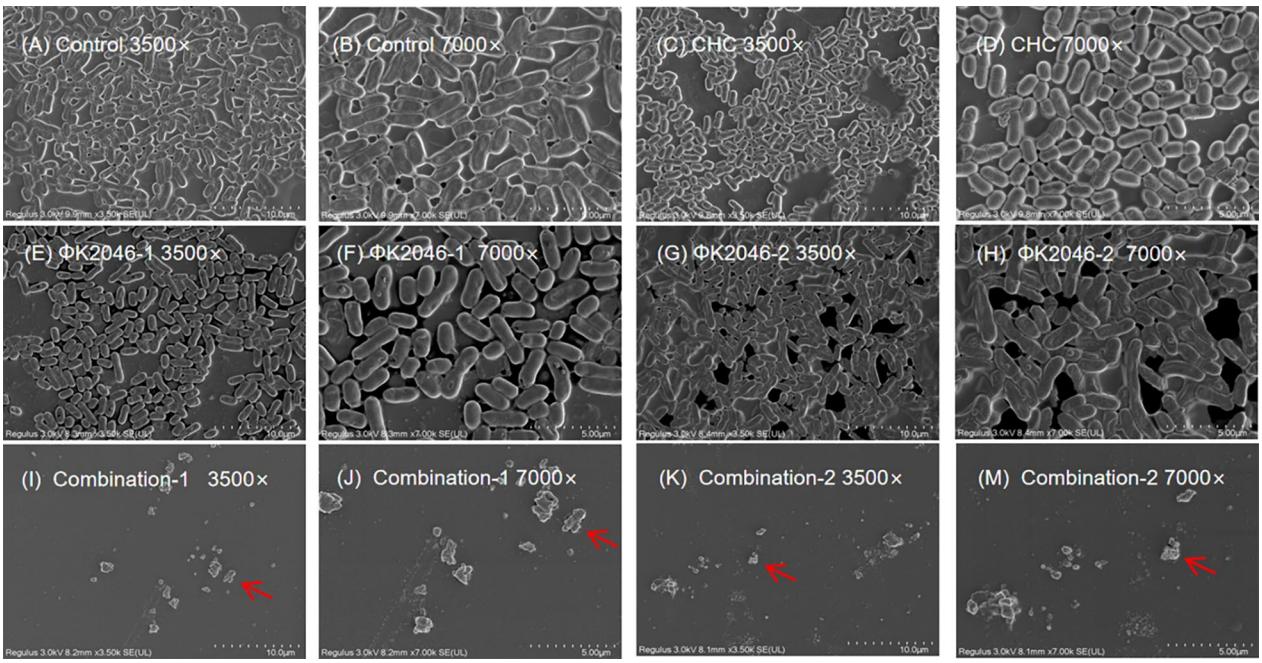


Fig. 6 Scanning electron microscopic observation of the enhanced anti-biofilm effect of the combination of ΦK2046 and CHC. (**A**–**B**) PBS-treated images of FK2046 biofilms magnified 3500 × or 7000 × as controls; (**C**–**D**) 1/2 MIC of CHC-treated FK2046 biofilm magnified 3500 × or 7000 ×; (**E**–**F**) 10^7 PFU/mL of ΦK2046 treated FK2046 biofilm magnified 3500 × or 7000 ×; (**G**–**H**) 10^8 PFU/mL of ΦK2046 treated FK2046 biofilm magnified 3500 × or 7000 ×; (**I**–**J**) 10^7 PFU/mL of ΦK2046 co-treated with 1/2 MIC of CHC in FK2046 biofilm magnified 3500 × or 7000 ×; (**K**–**M**) 10^8 PFU/mL of ΦK2046 co-treated with 1/2 MIC of CHC in FK2046 biofilm magnified 3500 × or 7000 ×. Biofilm images formed at 24 h for all groups

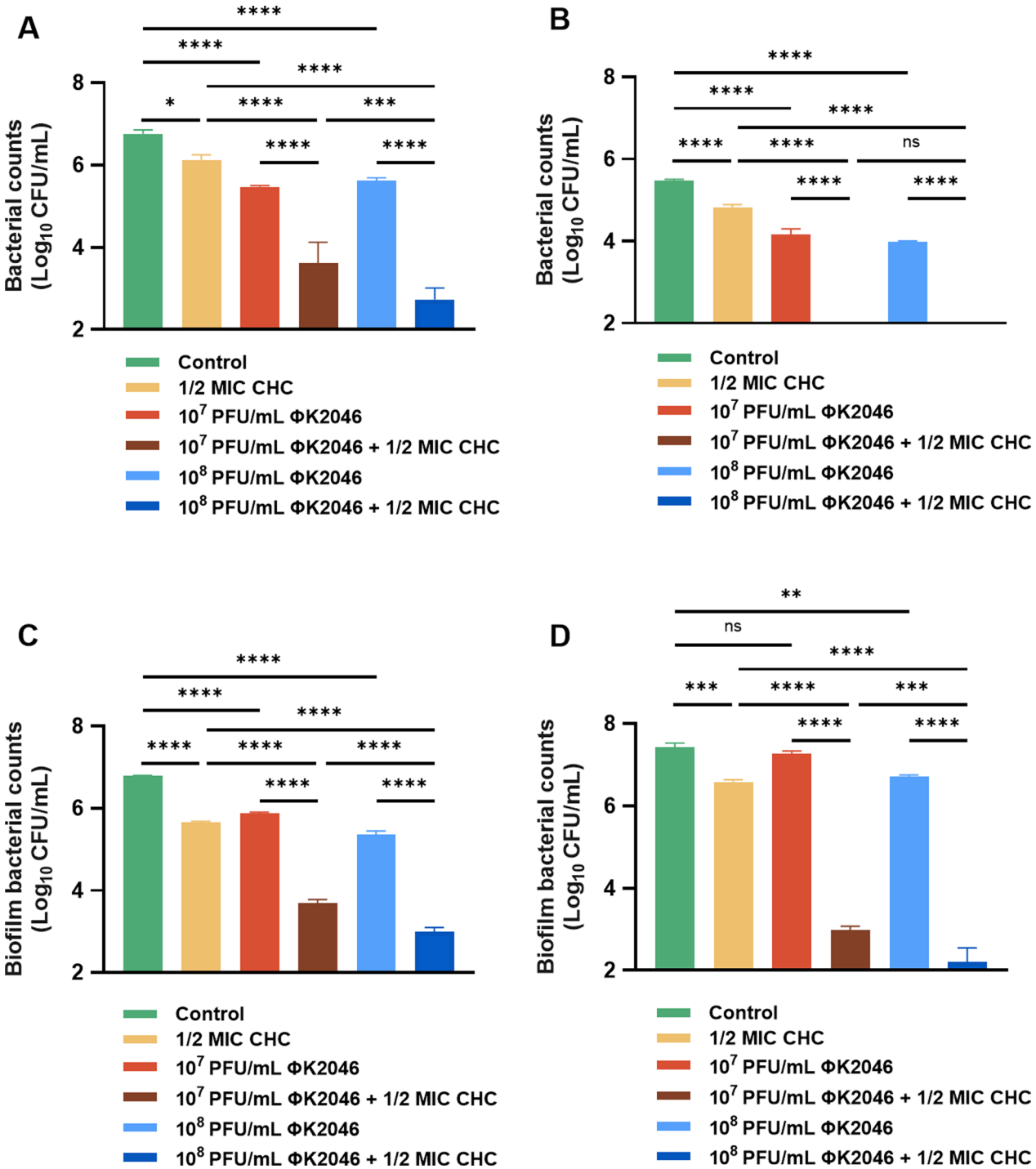


Fig. 7 Effects of ΦK2046 and CHC combination on the removal of bacteria attached to the surface of medical devices. **A** Disinfect the needle contaminated with FK2046 (drugs treatment for 2 h); **B** Disinfect the Urinary catheter contaminated with FK2046 (drugs treatment for 2 h); **C** Disinfect the urinary catheter contaminated with biofilm attached by FK2046 (drugs treatment for 2 h); **D** Disinfect the urinary catheter contaminated with biofilm attached by FK2046 (drugs treatment for 24 h). PFU, plaque forming unit; CFU, colony-forming unit; CHC, Chlorhexidine acetate; MIC, Minimum inhibitory concentration; $P > 0.05$ (ns), $P < 0.05$ (*), $P < 0.01$ (**), $P < 0.001$ (***), $P < 0.0001$ (****)

treatment by 1.9 and 2.9 orders of magnitude, respectively (Fig. 7A–B). Regarding the eradication of biofilm-contaminated urinary catheters, the number of biofilm bacteria after short-term (2 h) combined treatment was reduced by 2.2–2.4 orders of magnitude compared to single treatment, and by 4.3–4.5 orders of magnitude after long-term (24 h) treatment (Fig. 7C–D). In the combined treatment group, a phage concentration of 10^8 PFU/mL exhibited a more significant bacterial killing effect than 10^7 PFU/mL, both against planktonic and biofilm bacteria (Fig. 7A,C,D).

Effect of different phage and disinfectant addition sequences on the reduction of formed biofilms

Next, the difference in the order of phage and CHC addition produced different biofilm reduction results, as shown in Fig. 8. The results of crystalline violet staining suggested that compared to the simultaneous addition of phage and CHC, the phage-first group reduced more overall biofilm, whereas there was no significant difference in the CHC-first group ($P > 0.05$); moreover, in the group with phage added first, phage treatment for 10 min (ΦK2046 latency) followed by CHC treatment reduced 46.24% of the formed biofilm (Fig. 8A), while phage treatment for 90 min (ΦK2046 rise phase) followed by CHC treatment reduced 61.59% of the formed biofilm (Fig. 8B), suggesting that phage treatment for 90 min (ΦK2046 rise phase) followed by CHC treatment reduced the overall biofilm ($P > 0.05$). This suggests that treatment with phage for 90 min followed by CHC is a treatment option with stronger ability to reduce formed biofilm.

The results of colony counting in biofilm further suggested that phage-first treatment led to a reduction of bacterial count in biofilm by 0.7 orders of magnitude compared with the simultaneous addition of phage and CHC; moreover, in the phage-first group, pre-treatment with phage for 10 min followed by CHC reduced the viable bacteria in formed biofilm by about 1.5 orders of magnitude compared with that in the blank group (Fig. 8C), while pre-treatment with phage for 90 min followed by CHC treatment reduced the viable bacteria in the formed biofilm by about 2.5 orders of magnitude compared with that of the blank group (Fig. 8D), suggesting that pre-treatment with phage for 90 min before treatment with CHC is also a preferred treatment option to reduce the number of viable bacteria in biofilms.

Next, in a biofilm simulation of biofilm removal from contaminated catheters, the addition of ΦK2046 followed by the addition of CHC (a 92.5% reduction in log-transformed bacterial counts) also produced a better bactericidal effect on bacteria in the biofilm than the simultaneous addition of both (a 66.7% reduction in log-transformed bacterial counts), as shown in Fig. 9. The biofilm and bacteria on the surface of the catheter were observed by scanning electron microscopy, as shown in Figure S4 A–B, the bacteria in the blank control group were stacked, more numerous and with complete morphology. But few bacteria were seen on the surface of the catheter treated with ΦK2046 for 90 min followed by CHC, and the morphology of the bacteria was broken and twisted, which intuitively demonstrated a good ability to reduce the biofilm and to kill the biofilm bacteria (Figure S4 C–D).

Discussion

The importance of hospital-acquired infection prevention and control, mainly caused by Gram-negative bacteria, is increasing. Studies have shown that *K. pneumoniae* can survive for up to 30 months on inanimate surfaces and medical devices (such as bed rails, stethoscopes, ultrasound machines, and catheters), significantly increasing the spread and likelihood of pathogen transmission, evolution, and hospital infection outbreaks [41, 42]. Chlorhexidine is a commonly used disinfectant strategy, but it is now facing challenges: balancing the safety and effectiveness of disinfectant dosages and dealing with the emergence of chlorhexidine-resistant strains as well as cross-resistant strains. In this study, a *K. pneumoniae* bacteriophage ΦK2046 was isolated and identified, capable of lysing the chlorhexidine-resistant *K. pneumoniae* FK-2046 strain, exhibiting good environmental stability, not carrying virulence genes or resistance genes, thus enhancing CHC's antibacterial and anti-biofilm abilities. Moreover, we have demonstrated the feasibility and potential of this combination in medical device disinfection. Additionally, since bacteriophages infect only

(See figure on next page.)

Fig. 8 Differential reduction of formed biofilm by different addition sequences and time intervals of ΦK2046 and CHC. (**A**–**B**) Crystalline violet staining results (the time interval between sequential phage/CHC additions: Figure A of 10 min, Figure B of 90 min); the top measurement is a field photo of the biofilm after staining with crystal violet, and the bottom side is the statistical result of the absorbance value at an OD_{595} nm;(**C**–**D**) Results of viable bacteria counts in biofilms (the time interval between sequential phage/CHC additions: Figure C of 10 min, Figure D of 90 min). PFU, plaque forming unit; CFU, colony-forming unit; CHC, Chlorhexidine acetate; MIC, Minimum inhibitory concentration; "ΦK2046 first", phage ΦK2046 is used first, followed by the disinfectant CHC; "CHC first", CHC is used first, followed by phage ΦK2046, $P > 0.05$ (ns), $P < 0.05$ (*), $P < 0.01$ (**), $P < 0.001$ (***), $P < 0.0001$ (****)

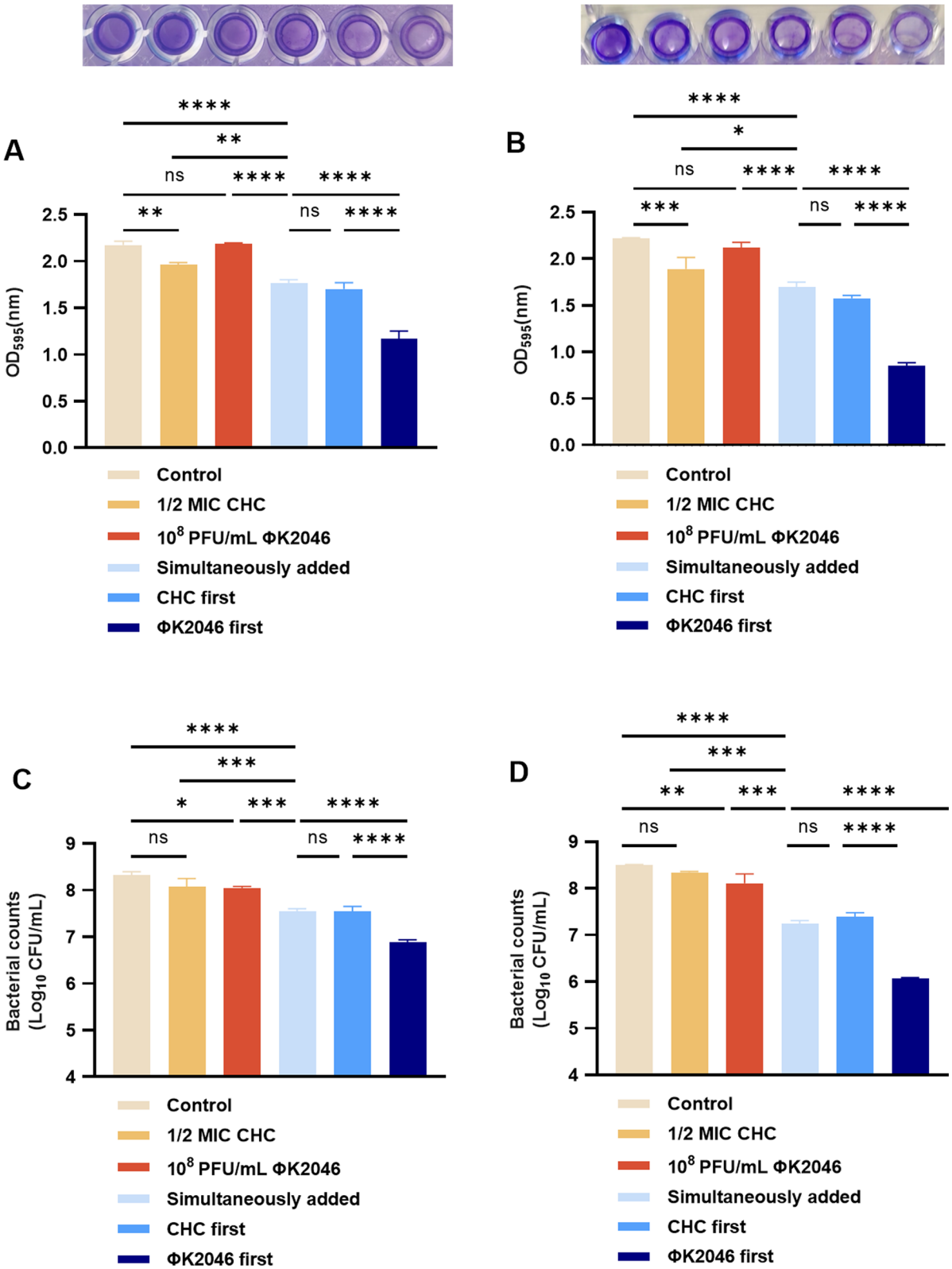


Fig. 8 (See legend on previous page.)

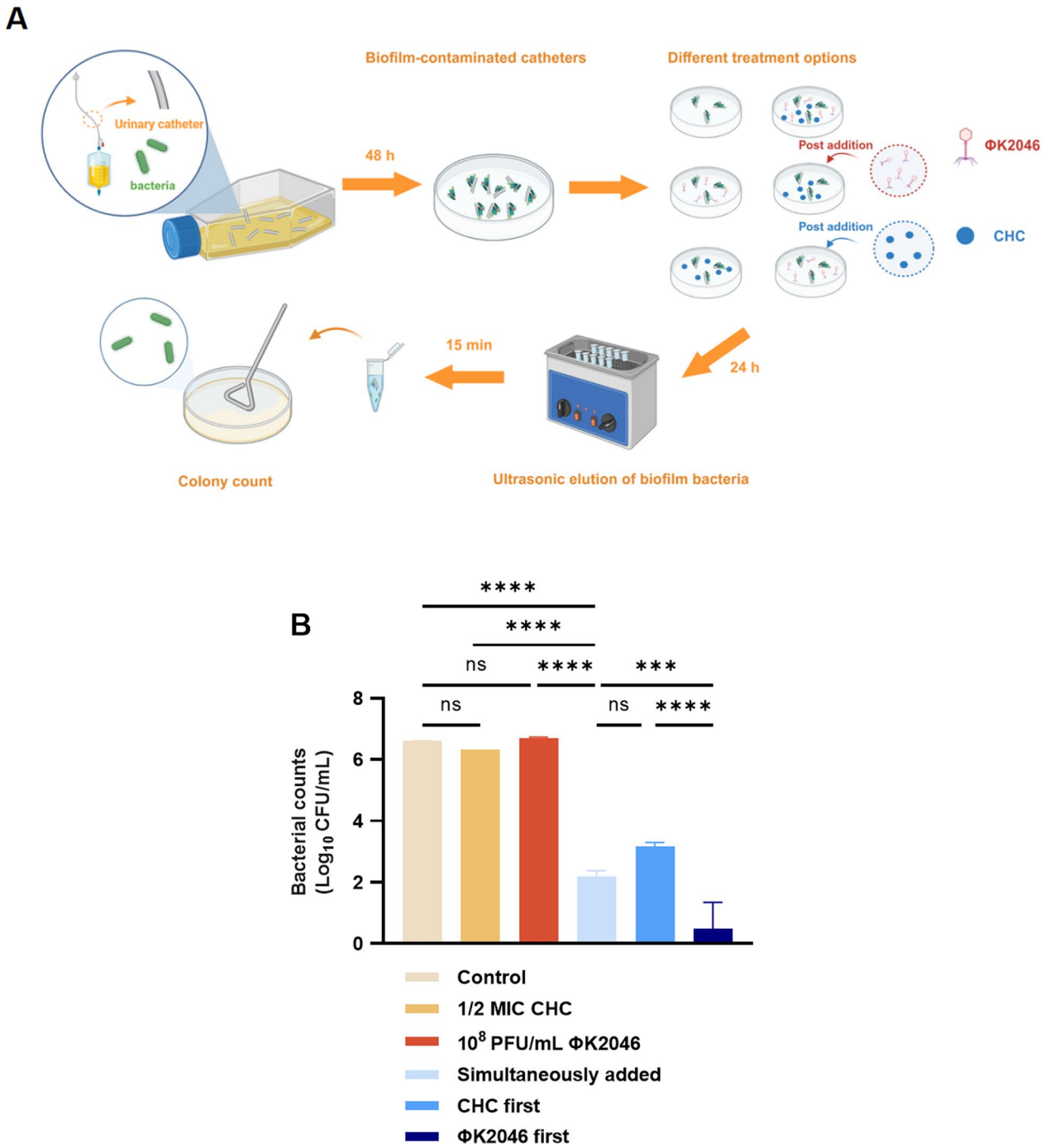


Fig. 9 Effect of different sequences of ΦK2046 and CHC addition on biofilms from contaminated catheters. **A** Schematic diagram of the experimental flow; **B** Killing effect of adding ΦK2046 followed by CHC (90 min interval) on the number of viable bacteria in the biofilm of catheters contaminated with FK2046; $P > 0.05$ (ns), $P < 0.001$ (***), $P < 0.0001$ (****)

bacteria without affecting mammalian cells, the combination formulation can also reduce the concentration of chlorhexidine used, making it safer for human application [43].

The bactericidal effect of bacteriophages is often influenced by disinfectants, with different chemical disinfectants having varying effects on bacteriophage killing (either positive synergy or negative antagonism)

[44, 45]. Through a series of experiments, including growth curve tests and time-kill assays with various concentrations, we determined that the combination of ΦK2046 at 10^7 PFU/mL and 10^8 PFU/mL with 1/2 MIC of CHC exhibited strong synergistic bactericidal effects [29]. Prolonged exposure to CHC often induces the development of resistance in bacterial strains [46]. In this study, after using the combination of ΦK2046 and CHC, their bactericidal actions complemented each other, reducing the emergence of bacteriophage-resistant strains on the one hand and lowering the number of CHC-resistant strains on the other hand, thereby reducing the likelihood of CHC inducing bacterial tolerance, achieving a"two birds with one stone"effect.

In the simulated disinfection tests of medical devices, we selected syringe needles and urinary catheters as the research subjects to evaluate the combined effect of CHC and bacteriophage ΦK2046 on the surface disinfection of medical devices. These items represent different medical device materials, including polypropylene, stainless steel, and polyvinyl chloride, and also encompassing different scenarios of hard surfaces (needles) and soft surfaces (urinary catheter). The results demonstrated that this combination has excellent antibacterial and anti-biofilm effects in both short-term bacterial contamination and the removal of established biofilms, providing a basis for the development of combinations of bacteriophages and traditional disinfectants. This study serves as proof and a valuable addition to existing literature on the effective elimination of bacteria on hard surfaces through the combined use of bacteriophages and chemical disinfectants [29], and the simulation of disinfection using clinically used medical devices may better replicate complex clinical environments.

Due to the low sensitivity of the test strains to CHC, it is difficult for CHC to kill bacteria, let alone bacteria with reduced metabolism in the presence of biofilms in which resistance is greatly enhanced. Therefore, this study also explored the effect of the order of phage and CHC addition on the effectiveness of removing formed biofilms, and found that the addition of phage followed by CHC exhibited a much better ability to reduce the overall level of formed biofilms as well as the inhibition of bacteria in biofilms compared to the addition of both at the same time or the addition of CHC followed by phage. For the time interval between the addition of the two agents, in this study, the end of the eclipse phase and the end of the rise phase were selected based on the results of the one-step growth curve of ΦK2046, both of which showed a better ability to combat the formed biofilm, and 90 min was found to be the better regimen. This could be attributed to the reduction of possible effects of CHC on the process of bacterial lysis by ΦK2046 adsorption, e.g., CHC induces a change in bacterial state and affects phage colonization [47]. One of the major innovations of this paper is in the simulated removal of biofilm from contaminated catheters, where we have similarly demonstrated the feasibility of the clinical application of this disinfection regimen. Compared with the studied sodium hypochlorite-phage hybrid formulations, in which low sodium hypochlorite concentrations (5 or 20 ppm) inactivate phage on contact with ΦJG004 and ΦP1 for 10 min [34], ΦK2046 proposed in this study has a better stability in CHC, and can have a certain reduction of formed biofilm without relying on the need for timely removal of the residual disinfectant after disinfectant treatment before phage can be used in the practical application.

Furthermore, it is worth noting that the bacteriophage ΦK2046 isolated in this study belongs to the *Slopekvirus* virus genus, showing significant similarity to the model bacteriophage T4 and exhibiting typical lytic bacteriophage characteristics with the ability to forcefully dissolve bacteria, hence being referred to as a"T4-like bacteriophage"[48]. Multiple studies have indicated that T4-like bacteriophages have broad host range, strong lytic capabilities, short latent period, lack of virulence genes, making them one of the best candidates for future bacteriophage therapy development [49]. In this study, the T4-like bacteriophage ΦK2046 has also shown promising potential for clinical application in combination with disinfectants, which has not been extensively reported in other studies. However, the time-kill results indicated that ΦK2046 did not exhibit good antibacterial efficacy when used alone, which was closely related to the phage dosage used in this study, at the 4 h of monotherapy, the phage group with a titer closest to the OMOI concentration (10^5 PFU/mL) reduced the bacterial count by 0.3 Log_{10} units. However, after this point, the bacteria in the phage-only treatment group rapidly proliferated, possibly due to the growth of resistant strains. In combination treatments, the bactericidal effect also depended on the differences in phage titer. This phage dose-dependent effect has been observed in phage-antibiotic combination strategies, highlighting the importance of achieving a certain phage concentration. However, excessively high doses of phage exert high survival pressure on pathogens, promoting extreme resistant mutations, which may reduce the effectiveness of the combination treatment. This aspect has not been studied in the present work [19, 50, 51]. Therefore, this study has several limitations: it did not compare the effects of T4-like phages with different characteristics when combined with disinfectants, nor did it investigate whether the synergistic effect is influenced by the type of disinfectant or how a higher phage dose affects antimicrobial efficacy in combination therapies. While phages are easy to proliferate, inexpensive to

produce, and hold great commercialization potential, the challenge of stabilizing their preservation remains unresolved. And the long-term stability of the phage in chlorhexidine remains to be evaluated.

The host range and efficiency of plating test results suggest that the phage is polyvalent. Although it can only lyse *K. pneumoniae*, it exhibits lytic activity against bacterial strains of multiple different sequence types/capsular types (Table S2–S3). The bactericidal action of bacteriophages may be related to the structure of bacteriophage receptors on bacteria, with Gram-negative bacteria bacteriophage receptors being lipopolysaccharides, bacterial outer membrane proteins, capsule polysaccharides, etc. Due to the bacteriophage's inherent targeting specificity towards host bacterial sites, a bacteriophage often exhibits optimal lysing effects on its host bacterium, and because phage have the property of replicating on their own in the host bacterium, their application does not require continuous application of agents, which is beneficial to make up for the depletion of disinfectants, reduce the number of supplemental disinfections, and improve disinfection effects [52]. Therefore, we believe that the combination of chlorhexidine and phage is suitable for site-specific inhibition of the production and colonization of drug-resistant bacteria, especially for sites such as ICUs and operating rooms, as well as for the use of instruments in invasive operations. An interesting avenue for future research would be to delve deeper into exploring the development of a mixed disinfectant containing bacteriophages targeting common clinically relevant K1, K4, K64 capsule types of *K. pneumoniae* associated with hospital-acquired infections and chlorhexidine [53], expanding the variety of bacteriophages that can be added to chlorhexidine, akin to the"cocktail"approach in current bacteriophage therapy [54], thus broadening the clinical applications of composite disinfectants. In addition, the establishment of a phage-disinfectant library of hospital-acquired infection pathogens will allow rapid selection of phage-disinfectant mixtures for patient treatment and environmental disinfection along with routine surveillance of hospital-acquired infections [55].

Conclusion

The results of this study demonstrate that the isolated *K. pneumoniae* bacteriophage ΦK2046 exhibits good environmental stability, does not carry virulence and antibiotic resistance genes, enhances the antibacterial and antibiofilm capabilities of chlorhexidine, and reduces the emergence of mutant strains resistant to chlorhexidine and bacteriophages. These findings suggest that combining specific bacteriophages with chemical disinfectants is an effective and cost-efficient strategy for medical device disinfection in clinical settings.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s13756-025-01548-z.

Supplementary file 1.

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Author contributions

Panjie Hu and Yao Sun were responsible for the conception, experimental design, implementation and manuscript writing, Zeyong Zhong was responsible for the implementation and data analysis, Sichen Liu was responsible for the manuscript writing, Deyi Zhao was responsible for the implementation, Weijun Chen was responsible for the graphical design, Ying Zhang was responsible for the graphical design, Zhexiao Ma was responsible for the manuscript revision, Jianming Cao and Tieli Zhou were responsible for the conception, experimental design, supervision, manuscript revision and financial support. All authors read and approved the final manuscript.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author upon reasonable request.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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