

ORIGINAL RESEARCH ARTICLE

A safety-prioritized pipeline for therapeutic phage discovery: Isolation of a novel phage NLE251 from soil using *Escherichia coli* DH5α as a host

Xiting Lin^{1,2}, Noura M. Bin Yahia^{1,2}, Bingxian Li^{1,2}, Yiming Chen^{1,2}, Jing Ma^{1,2}, Shengjie Wu^{1,2}, Sihao Zhao^{1,2}, Xiaofan He^{1,2}, Ying Xu^{1,2}, Huihui Mu^{1,2}, and Yanhui Yang^{1,2*}

¹Ningxia Key Laboratory of Infection and Immunity, School of Basic Medical Sciences, Ningxia Medical University, Yinchuan, Ningxia, China

²Ningxia Key Laboratory of Clinical and Pathogenic Microbiology, General Hospital of Ningxia Medical University, Yinchuan, Ningxia, China

Abstract

The relentless spread of antimicrobial-resistant *Escherichia coli* underscores the critical need for alternative therapeutics, such as bacteriophages. A major translational hurdle is the efficient and safe initial isolation of lytic phages with defined therapeutic potentials. This study established and validated a safety-conscious pipeline using the non-pathogenic laboratory strain *E. coli* DH5α (BSL-1) for environmental phage screening, a strategy designed for accessibility and to circumvent early biosafety concerns. After continuous screening, we isolated a novel lytic phage, vB_EcoS_NLE251, from the soil. Comprehensive phenotyping revealed robust stability (tolerant up to 60 °C) and efficient replication, with an optimal multiplicity of infection (MOI) of 100, yielding a high titer of 2.1×10^{10} PFU/mL. Crucially, host-range determination using a panel of 35 clinical *E. coli* isolates showed that NLE251 only exhibited lytic activity in spot assays against a single biliary tract infection-derived strain (54959124), though subsequent quantitative infection efficiency data indicate extremely low productive infection against this target strain. Genomic analysis (49,336 bp dsDNA, belonging to the class Caudoviricetes) further confirmed the absence of virulence or antibiotic resistance genes, fulfilling a key safety prerequisite for therapeutic use. Although NLE251 is a narrow-spectrum agent, this study provides a validated methodological framework that prioritizes safety and specificity from the initial isolation step, offering a safety-prioritized, standardized pipeline for accelerating phage discovery against multidrug-resistant bacterial infections.

*Corresponding author:

Yanhui Yang
(yangyanhui@nxmu.edu.cn)

Citation: Lin X, Bin Yahia NM, Li B, et al. A safety-prioritized pipeline for therapeutic phage discovery: Isolation of a novel phage NLE251 from soil using *Escherichia coli* DH5α as a host. *Microbes & Immunity*. 2026;3(4):025010144. doi: 10.36922/MI025010144

Received: December 31, 2025

Revised: May 4, 2026

Accepted: May 26, 2026

Published online: June 19, 2026

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Keywords: *Escherichia coli* DH5α; *Escherichia* phage NLE251; Antimicrobial resistance; Phage screening

1. Introduction

The global rise in antimicrobial resistance (AMR) represents one of the most pressing public health crises.^{1,2} *Escherichia coli*, a ubiquitous Gram-negative bacterium, is a paradigm of this challenge.³ While commensal strains are found in the gut, pathogenic

strains are the leading cause of both intestinal and extraintestinal infections, ranging from urinary tract infections to life-threatening sepsis.^{4–9} The therapeutic arsenal against these infections is rapidly eroding. According to World Health Organization reports, the clinical resistance rates of *E. coli* to cornerstone antibiotics, such as third-generation cephalosporins and fluoroquinolones, exceed 50% in many regions, severely limiting treatment options.^{10,11} This alarming scenario has spurred the urgent exploration of alternative antimicrobial strategies, with bacteriophage (phage) therapy re-emerging as a promising complementary or alternative approach.^{12,13}

Phages, which are viruses that specifically infect and lyse bacteria, offer several theoretical advantages over broad-spectrum antibiotics. Their high host specificity allows for the targeted elimination of pathogens while sparing commensal microbiota, potentially reducing collateral damage and dysbiosis.¹⁴ Furthermore, their lytic activity is independent of conventional bacterial resistance mechanisms, making them effective against multidrug-resistant strains.¹⁵ Historical precedents and recent clinical successes in compassionate-use cases have reaffirmed the potential of phage therapy.^{16,17}

However, the translational pathway from environmental isolation to a validated therapeutic candidate remains fraught with methodological and safety issues.¹⁸ A critical, yet often overlooked, initial step is host isolation. Many studies screen for phages using the clinical and pathogenic strains they aim to target. Although direct, this approach necessitates a stringent biosafety level 2 (BSL-2) or higher containment from the outset, limiting accessibility and increasing the complexity and cost of early-stage discovery research in non-specialized laboratories.¹⁹ Moreover, it does not inherently address whether a safe, non-pathogenic model host can serve as a reliable proxy for discovering phages active against related pathogens, a strategy that could streamline and democratize the initial discovery phase.

In addition to isolation, a standardized characterization pipeline is essential. Key phenotypic properties, including burst size, thermal stability, and ion dependence, determine the practicality of a phage in formulation and application.²⁰ Most critically, rigorous host-range profiling against a panel of clinically relevant isolates is necessary to define the spectrum of activity, distinguishing broadly lytic from precisely targeted agents.²¹ Finally, genomic sequencing is a non-negotiable safety checkpoint to confirm the absence of lysogeny determinants, bacterial virulence genes, and antibiotic resistance genes, ensuring the suitability of the candidate for therapeutic development.²²

It is worth noting that the core logic of using non-

pathogenic laboratory strains of *E. coli* as surrogate hosts to isolate phages and screen for those active against pathogenic bacteria was established by Maffei et al.²³ Using the non-pathogenic laboratory strain *E. coli* K-12 as the host, they isolated 68 virulent phages from environmental samples, such as river water, compost, and sewage, and systematically characterized the host ranges of these phages against pathogenic *E. coli* strains, including urinary tract pathogenic *E. coli* (UPEC) and enteropathogenic *E. coli* (EAEC). The core objective of this study is to establish a reference phage library for basic research on phage–host interactions, rather than to develop a standardized workflow for the discovery of therapeutic phages; this distinction holds critical practical significance. Maffei et al.'s workflow did not incorporate the therapeutic-oriented characterization and safety screening steps necessary for identifying viable therapeutic candidate phages. There was a lack of standardized phenotypic assays for key parameters, including optimal multiplicity of infection (MOI), thermal stability, and host density tolerance, and the lysis profile of the isolated phages was not systematically validated using a set of clinically relevant isolates.

To bridge this gap between the feasibility of basic research and the demands of therapeutic applications, we proposed and validated a safety-first, standardized pipeline for the discovery of therapeutic phages. We hypothesized that using a well-characterized, non-pathogenic laboratory strain (*E. coli* DH5 α , BSL-1) for initial environmental screening can effectively yield lytic phages with subsequent activity against specific clinical pathogens. As a proof of concept, we employed this strategy to isolate a novel *E. coli* phage, vB_EcoS_NLE251. We then subjected it to comprehensive phenotypic characterization (determining optimal MOI, stability, and host range) and whole-genome sequencing with bioinformatic safety screening. This study demonstrates that a methodical, accessible, and safety-conscious approach from the initial isolation can efficiently advance environmental phages toward potential therapeutic applications, providing a standardized, safety-first protocol specifically tailored for the initial screening of therapeutic phage candidates in the future.

2. Materials and methods

2.1. Bacterial strain and culture conditions

The non-pathogenic *E. coli* strain DH5 α was selected as the host for the phage isolation and propagation. This strain was cultivated in lysogeny broth (LB) medium (Yinchuan Runyan Biotechnology Co., Ltd., China) at 37 °C with shaking at 250 rpm until the optical density at 600 nm (OD₆₀₀) reached 0.5 (mid-log phase). For long-term storage, bacterial cultures were mixed with 40% (v/v) glycerol and preserved at –80 °C.

2.2. Phage isolation

2.2.1. Sample collection and processing

Soil samples were collected from the shore of Yanhu Lake at Ningxia Medical University (GPS coordinates: 38.4134 °N, 106.2789 °E). The samples were transported in sterile plastic bags and processed within 24 h.

2.2.2. Phage extraction and enrichment

Phages were extracted from the soil using a modified protocol based on those of Nikkhahi *et al.*²⁴ and Lee *et al.*²⁵ Briefly, 5 g of soil was mixed with 15 mL of LB broth and agitated at 180 rpm for 2 h at room temperature. The mixture was centrifuged at 8,000× g for 10 min, and the supernatant was filtered through a 0.22 µm pore-size membrane filter (Millipore, United States [US]).

For enrichment, 1 mL of mid-log phase *E. coli* DH5α culture (OD₆₀₀ = 0.5) was added to 50 mL of filtered supernatant and incubated at 37 °C with shaking at 160 rpm for 16 h. The enriched lysate was centrifuged and filtered as above to remove bacterial debris.

2.2.3. Plaque assay and phage detection

The presence of lytic phages was confirmed using a double-layer agar plaque assay.²⁶ Briefly, 100 µL of the enriched lysate was mixed with 500 µL of host bacteria and incubated for 10 min at 37 °C. This mixture was combined with 5 mL of soft LB agar (0.7% agar) and overlaid on LB agar plates. After solidification, plates were incubated at 37 °C for 12–24 h and examined for plaque formation. The number of plaques from initial screening was recorded, and single plaques with distinct morphological characteristics were selected for subsequent purification.

2.3. Phage purification and amplification

Single, well-isolated plaques were picked and subjected to three rounds of plaque purification using the serial dilution method.²⁷ Phage stocks were amplified by inoculating purified plaques into liquid cultures of *E. coli* DH5α at an MOI of 0.1 and incubating until complete lysis was observed. Lysates were treated with chloroform (1% v/v), centrifuged, filtered, and stored in saline magnesium (SM) buffer (50 mM Tris-HCl, 100 mM NaCl, 8 mM MgSO₄, pH 7.5) at 4 °C.

2.4. Determination of phage titer

Phage titers were determined using the double-layered agar method. Serial dilutions of the phage lysate (10⁻¹ to 10⁻¹⁰) were prepared in SM buffer, and 100 µL of each dilution was mixed with the host bacteria before plating. Plaques were counted after overnight incubation, and the

titer units were expressed as plaque-forming units per milliliter (PFU/mL).

2.5. Transmission electron microscopy

High-titer phage lysates (≥10¹⁰ PFU/mL) were negatively stained with 2% (w/v) uranyl acetate and imaged using a Hitachi HT-7800 transmission electron microscope (Japan) at 100 kV. Morphometric analysis was performed using the ImageJ software (National Institutes of Health, US). The candidate bacteriophage strains were initially classified by transmission electron microscopy (TEM). If morphological parameters such as head diameter and tail length of the two phages showed no significant differences, they were regarded as repeated isolates of the same phage and eliminated.

2.6. Biological characterization

2.6.1. Host-range determination

The lytic spectrum of phage NLE251 was assessed against 35 clinical *E. coli* isolates using a spot assay.²⁶ Clinical bacterial isolates and associated patient information (diagnoses and antibiotic susceptibility profiles) were obtained from the routine diagnostic laboratory at the First Affiliated Hospital of Ningxia Medical University and fully anonymized before analysis. This study was approved by the Ningxia Medical University Ethics Committee (2025-Z031). Briefly, 5 µL of phage lysate (10⁸ PFU/mL) was spotted onto the lawns of the test strains. Sensitivity was determined based on the formation of clear zones after incubation.

2.6.2. Host density

We blended logarithmic phase cultures of *E. coli* DH5α with LB broth. Incubated on a shaking incubator at 37 °C and 250 rpm for varying durations to obtain bacterial suspensions with OD₆₀₀ values of 0.1, 0.5, 1, and 1.5. Then, we prepared double-layer plates with bacterial cultures at different OD₆₀₀ values and determined phage PFU using the spot titer method.

2.6.3. Optimal multiplicity of infection

The optimal MOI was determined by infecting mid-log phase *E. coli* DH5α at MOIs ranging from 0.001 to 100. After 6 h of incubation, the lysates were titrated, and the MOI yielding the highest phage titer was identified.

2.6.4. Thermal stability

Phage stability was evaluated by incubating aliquots at temperatures ranging from 4 to 65 °C (4, 25, 37, 45, 60, and 65 °C) for 1 h. Residual infectivity was assessed using a plaque assay.

2.6.5. Calcium dependence

To assess the calcium ion dependence of phage NLE251, we employed the method outlined by Satish and Desouza²⁸ with minor modifications. LB agar plates containing varying concentrations of CaCl_2 (0, 5, 10, 15, and 20 mM) were prepared. Similarly, the SM buffer used for phage dilution was supplemented with the corresponding concentrations of CaCl_2 . A control plate without CaCl_2 was also included. Each plate was swabbed with a host bacterial suspension ($\text{OD}_{600} = 0.5$), followed by spot inoculation with 5 μL of serially diluted phage suspensions (10^{-1} to 10^{-10}). After incubation at 37 °C for 6–12 h, the plates were examined for plaque formation.

2.6.6. Bacterial lysis curves

To evaluate the bactericidal activity of phage NLE251 against the laboratory host *E. coli* DH5 α and the target clinical *E. coli* strain, we performed in vitro bacterial lysis curve assays following the protocol described by Chen *et al.*²⁹ with minor modifications. Phage and host preparation were conducted as follows: both host strains were cultivated to mid-log phase ($\text{OD}_{600} = 0.5$), and their concentrations were measured prior to the assay, with *E. coli* DH5 α at 4×10^8 CFU/mL and purified NLE251 at 4×10^9 PFU/mL. As the phage titer was determined using DH5 α as the host, MOIs for both the DH5 α group and the clinical strain group were calculated based on the NLE251 titer obtained from DH5 α , with gradient MOIs set at 10, 10^2 , 10^3 , 10^4 , and 10^5 . For 96-well plate preparation, each subgroup (including the negative control [NC] group and each MOI group for both hosts) was set up with three technical replicates: the NC group received 270 μL of host bacterial suspension and 30 μL of LB broth, while MOI groups received 270 μL of host suspension at the corresponding concentration and 30 μL of NLE251 suspension at 4×10^9 PFU/mL. The plate was then loaded into a microplate reader (Synergy 2, BioTek, United States) pre-adjusted to 37 °C, with moderate shaking applied at 1-s intervals, and OD_{600} was measured every 30 min for a total of 8 h to monitor bacterial growth dynamics.

2.7. Genome sequencing and bioinformatics analysis

Phage genomic DNA was extracted using the phenol-chloroform method and sequenced on an Illumina NovaSeq platform (Chengdu Phagetimes Biotech Co., Ltd., China). De novo assembly was performed using SPAdes, and open reading frames (ORFs) were predicted using Prokka. Functional annotation was performed using BLASTp against the National Center for Biotechnology Information (NCBI) non-redundant (NR) database, the Prokaryotic Virus Remote Homologous Groups (PHROG)

database, and the Virulence Factors Database (VFDB). Genes were identified using metaSPAdes from SPAdes (v3.10, St. Petersburg State University, Russia). Phylogenetic analysis was performed based on the major capsid protein sequences. The sequencing results were uploaded to the China National GeneBank Database (CNCBdb). The data that support the findings of this study have been deposited in the China National GeneBank Sequence Archive (CNSA) under accession number N_AAJSBX01000000.³⁰ Phage taxonomy was assigned following the 2025 ratified International Committee on Taxonomy of Viruses (ICTV) taxonomy standards for bacterial viruses³¹, based on whole-genome Virus Classification and Tree Building Online Resource (VICTOR) classification across D0 (strictest species demarcation), D4, and D6 similarity thresholds, combined with major capsid protein phylogeny.^{32–34} After BLASTn alignment with the NCBI database, strains with extremely high genomic similarity were identified as repeated isolations of known phages and eliminated.

2.8. Statistical analysis

All experiments were performed in triplicate. Data are presented as the mean $\pm$ standard deviation (SD). Statistical significance was determined using one-way ANOVA with Tukey's post hoc test ($p < 0.05$).

3. Results and discussion

3.1. Isolation and purification of phage NLE251 and morphological observation under an electron microscope

Screening of the initial soil samples yielded dozens of morphologically similar but differently sized phage plaques (Figure 1a). Three independent plaques were randomly selected for plaque purification, resulting in three candidate phage strains. TEM revealed that two of these strains showed no significant differences in morphological parameters, including average head diameter and tail length. They were therefore determined to be duplicate isolates of the same phage, and one strain was excluded. The remaining two phages (Figure 1b and d), which exhibited significant morphological differences under TEM, underwent whole-genome sequencing. BLASTn alignment results showed that one of them (Figure 1b) had 100% genomic similarity to the previously reported *E. coli* phage 899; it was determined to be a duplicate isolation of a known phage and was therefore excluded. Ultimately, only one phage strain was retained and named vB_EcoS_NLE251 for subsequent phenotypic and genomic characterization studies. Phage NLE251's lytic plaques exhibited well-defined, regular edges with a diameter of 0.9 cm, accompanied by a halo (Figure 1a).

The TEM images revealed that phage NLE251 possessed a regular icosahedral head, with an average diameter of 46.8 ± 3.8 nm, and a long tail, with a diameter of 102.8 ± 2.1 nm, matching the morphological features of siphovirus-like phages. The former order Caudovirales and family Siphoviridae based on morphological classification were abolished in the 2021 ICTV update³⁵, and NLE251 is currently classified within the class Caudoviricetes under the 2025 ratified ICTV bacterial virus taxonomy³¹ (Figure 1b). Through electron microscopy, detailed morphological characteristics of bacteriophages can be observed, such as the size and shape of the head, as well as the length and structure of the tail. This information is crucial for the classification, identification, and understanding of the biological properties of bacteriophages.

3.2. Biological characteristics of phage NLE251

3.2.1. Analysis of the host range

We conducted host-range studies to evaluate selection criteria for strains, including clinical isolates, antibiotic-resistant strains, and model strains.

As shown in Table 1, phage NLE251 has a narrow lytic spectrum. Among 35 tested clinical bacterial strains isolated from patients at the First Affiliated Hospital of Ningxia Medical University, NLE251 exclusively lysed the *E. coli* strain 54959124.

Clinical antimicrobial susceptibility testing revealed that *E. coli* strain 54959124 remained susceptible to antibiotics prone to resistance development, such as ampicillin and cephalosporins (Table 2). Most clinically

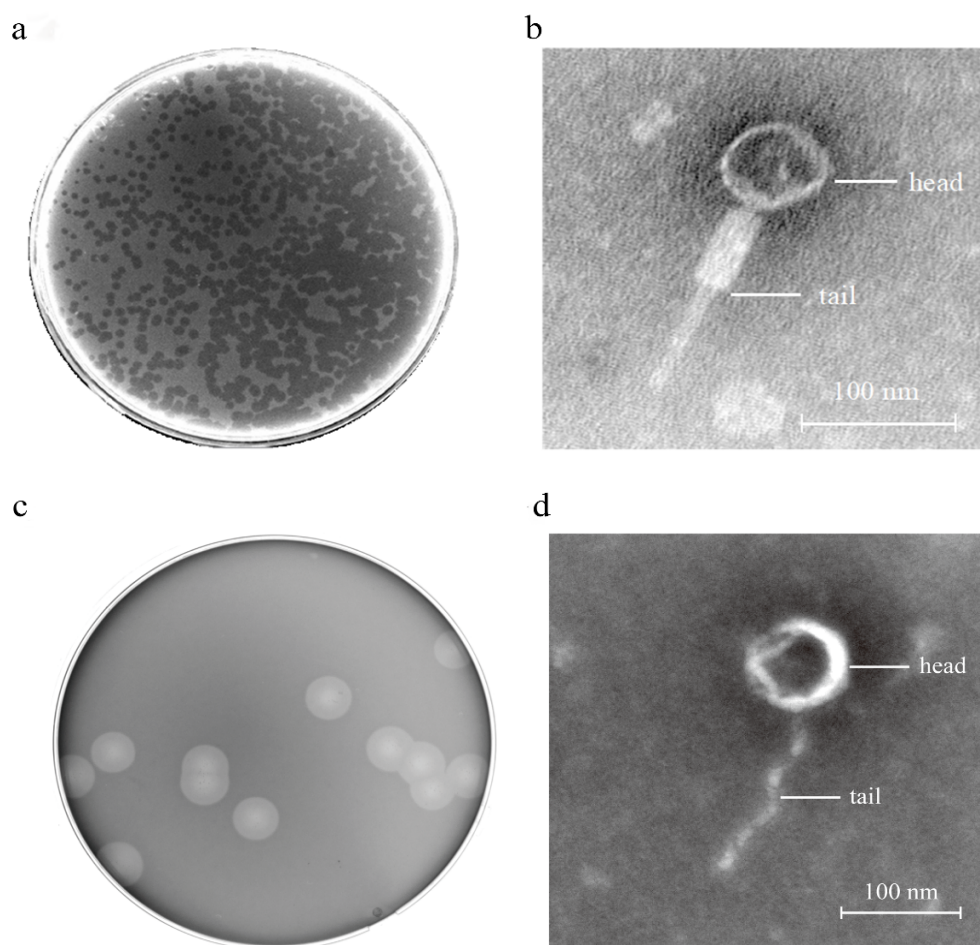


Figure 1. Isolation, identification, and screening of the novel lytic phage NLE251 from soil samples. (a) Initial phage plaque screening from soil samples. (b) Electron micrograph of another phage strain identified in the initial screening. (c) Plaque characteristics of phage NLE251; (d) Electron microscopy features of phage NLE251. Scale bars: 100 nm; magnifications: 120,000 $\times$.

isolated *E. coli* strains often exhibit resistance to multiple antibiotics, particularly to third-generation cephalosporins and fluoroquinolones. In contrast, *E. coli* strain 54959124 is susceptible to these commonly used antibiotics according to our results, indicating its lower antibiotic resistance. Other clinical strains may originate from various infection types, such as urinary tract infections, acute appendicitis, peritonitis, and skin infections. Meanwhile, *E. coli* strain 54959124 is associated with biliary tract infections (common bile duct stones complicated by acute cholecystitis), demonstrating its pathogenicity within the biliary system.

Previous studies have reported that sensitive strains isolated from patients with common bile duct stones are prone to forming biofilms and increase the risk of chronic infection.^{36,37} Obstruction and cholestasis caused by common bile duct stones provide bacteria with stable attachment surfaces (e.g., stones, damaged bile duct mucosa). This environment is highly conducive to bacteria secreting extracellular polysaccharides, proteins, and other matrix components to form biofilms, thereby evading host immunity and antibiotic killing. Moreover, antimicrobial therapy alone had limitations. In this case of common bile duct stones, the patient received potent antibiotics (cefotiam) but still required endoscopic retrograde cholangiopancreatography to relieve the obstruction and drain bile. This demonstrates that, for infections involving biofilms, endoscopic stone removal and biliary drainage must be combined to achieve complete clearance of the lesion.

Through the efficiency of plating (EOP) analysis, phage NLE251 exhibited significantly lower infection efficiency on the clinical strain than on the control host DH5 α . The experiment yielded an EOP value of 5×10^{-7} , indicating that NLE251's productive infection efficiency on this clinical strain was approximately 2 million-fold lower than on the control host. This value is far below the thresholds for therapeutic utility established in prior studies: Mirzaei and Nilsson³⁸ defined $\text{EOP} \leq 0.001$ as indicative of inefficient infection unsuitable for therapeutic use, while Gibson *et al.*³⁹ classified only phages with $\text{EOP} > 0.1$ (strong killers) or $0.001 < \text{EOP} < 0.1$ (moderate killers) as candidates with therapeutic potential. The extremely low EOP value suggests the positive lysis signal observed in the initial spot assay may reflect lysis from without (high-concentration phage-mediated cell wall disruption without productive intracellular replication) rather than genuine productive infection, consistent with the finding of Mirzaei and Nilsson³⁸ that spot assays alone overestimate host range and cannot be used as the sole criterion for therapeutic phage selection. This study further confirmed that

although phages may exhibit lytic activity in spot assays, their replication efficiency was significantly constrained in quantitative liquid infections, suggesting potential limitations for therapeutic applications.

To confirm the lytic activity of phage NLE251 and the closely related phage phiD2-2 against clinical strains, we performed spot tests. As shown in Figure 2, no phage spots appeared in the NC. Figure 2a–c shows the lysis of DH5 α by phage NLE251 solutions at concentrations of 10^{-1} – 10^{-2} , 10^{-4} – 10^{-6} , and 10^{-7} – 10^{-9} , respectively (each dilution was repeated three times; a single row in the plate represents one concentration). It can be observed that phage plaques formed at concentrations ranging from 10^{-1} to 10^{-7} , while at 10^{-8} , phage plaques appeared in only one dilution. As shown in Figure 2d, when undiluted phage NLE251 was spotted onto a plate uniformly inoculated with clinical strain 54959124, distinct lysis spots were observed; when a 10-fold dilution of phage NLE251 was applied as a droplet onto the plate, the lysis spots were less distinct, and no lysis spots were detected in the 100-fold dilution. In contrast, regardless of dilution, no lysis spots were detected when phage NLE251 was spotted onto plates inoculated with the non-susceptible strain 54985313 (Figure 2e) or when phage phiD2-2 was spotted onto plates inoculated with the clinical strain 54959124 (Figure 2f).

3.2.2. Host density

Measuring phage titer at varying host bacterial densities clarifies the effect of host bacterial concentration on phage proliferation while assessing the phage's tolerance range for host bacterial density. As shown in Figure 3a, the phage titer exhibited a slight increase when the OD₆₀₀ value was equal to 1.5, but the overall trend indicated that host density had no significant effect on its activity. One-way ANOVA analysis confirmed that host density had no statistically significant effect on NLE251 titer ($F_{(3,8)} = 1.24$, $p = 0.36$).

3.2.3. Optimal multiplicity of infection

The core objective of MOI is to establish the optimal infection ratio between phage and host bacteria. One-way ANOVA analysis showed that MOI had a highly significant effect on NLE251 titer ($F_{(6,14)} = 127.83$, $p < 0.001$). Tukey's post hoc test results showed that the titer of the MOI = 100 group was significantly higher than that of all other MOI groups ($p < 0.001$), and the titer of the MOI = 10 group was significantly higher than that of all groups with MOI ≤ 1 ($p < 0.01$); there were no significant differences in titer between groups with MOI ≤ 1 ($p > 0.05$ for all pairwise comparisons). At an MOI of 100, phage NLE251 exhibited the highest titer, reaching approximately 2.13×10^{10} PFU/mL (Figure 3b). Therefore, the optimal MOI for this phage

Table 1. Host range of phage NLE251

Strains	Strains ID	Year of isolation	Source of isolation	Plaque formation
Clinical <i>Escherichia coli</i> type 1	54989908	2025	Patients with urinary tract infections and type 2 diabetes	–
Clinical <i>E. coli</i> type 2	54994808	2025	Patients with acute appendicitis and acute peritonitis	–
Clinical <i>E. coli</i> type 3	54959124	2025	Patients with common bile duct stones	+
Clinical <i>E. coli</i> type 4	54985313	2025	Patients with incomplete intestinal obstruction and abdominal pain	–
Clinical <i>E. coli</i> type 5	54959141	2025	Patients with sequelae of cerebral hemorrhage	–
Clinical <i>E. coli</i> type 6	54975295	2025	Patients with skin infections	–
Clinical <i>E. coli</i> type 7	55530132	2025	Patients with respiratory tract infections	–
Clinical <i>E. coli</i> type 8	55513625	2025	Patients with acute diffuse peritonitis	–
Clinical <i>E. coli</i> type 9	55516715	2025	Patients with L3-4 lumbar tuberculosis	–
Clinical <i>E. coli</i> type 10	55496903	2025	Patients with malignant tumors of the cervix	–
Clinical <i>E. coli</i> type 11	55501560	2025	Patients with gallbladder stones and acute cholecystitis	–
Clinical <i>E. coli</i> type 12	55539372	2025	patient with acute gangrenous appendicitis accompanied by perforation	–
Clinical <i>E. coli</i> type 13	55538864	2025	Patients with respiratory failure	–
Clinical <i>E. coli</i> type 14	55532026	2025	Patients with acute myeloid leukemia	–
Clinical <i>E. coli</i> type 15	55520041	2025	Patients with coronary atherosclerotic heart disease	–
Clinical <i>E. coli</i> type 16	55497312	2025	Patients with acute suppurative appendicitis	–
Clinical <i>E. coli</i> type 17	55529708	2025	Patients with diabetic ketoacidosis, sepsis, and acute renal failure	–
Clinical <i>E. coli</i> type 18	55526477	2025	Patients with septic shock and sepsis	–
Clinical <i>E. coli</i> type 19	55538712	2025	Patients with common bile duct stones	–
Clinical <i>E. coli</i> type 20	55526387	2025	Patients with acute hemorrhage of the right middle cerebral artery	–
Clinical <i>E. coli</i> type 21	55509824	2025	Patients with malignant pancreatic tumors and abdominal infections	–
Clinical <i>E. coli</i> type 22	55517078	2025	Patients with adrenal tumors	–
Clinical <i>E. coli</i> type 23	55492050	2025	Patients with the extremely rapid progression type of liver cancer in the right lobe of the liver	–
Clinical <i>E. coli</i> type 24	55528055	2025	Patients with abdominal pain and type 2 diabetes	–
Clinical <i>E. coli</i> type 25	55526478	2025	Patients with septic shock and sepsis	–
Clinical <i>E. coli</i> type 26	55535784	2025	Patients with acute peritonitis	–
Clinical <i>E. coli</i> type 27	55490868	2025	Patients with hemiplegia	–
Clinical <i>E. coli</i> type 28	55508787	2025	Patients with malignant tumors of the cervix	–
Clinical <i>E. coli</i> type 29	55525434	2025	Patients with coronary atherosclerosis	–
Clinical <i>E. coli</i> type 30	55491737	2025	Patients with malignant tumors of the cervix	–
Clinical <i>E. coli</i> type 31	55480909	2025	Patients with rheumatoid arthritis	–
Clinical <i>E. coli</i> type 32	55526654	2025	Patients with abdominal pain and acute cholecystitis	–
Clinical <i>E. coli</i> type 33	55521011	2025	Patients with acute myeloid leukemia	–
Clinical <i>E. coli</i> type 34	55517696	2025	Patients with hydronephrosis, ureteral dilation, and urinary tract infection	–

(Cont'd...)

Table 1. (Continued)

Strains	Strains ID	Year of isolation	Source of isolation	Plaque formation
Clinical <i>E. coli</i> type 35	55500245	2025	Patients with malignant tumors or ulcers of the stomach	–
<i>E. coli</i> DH5α	-	2025	-	+

Note: “+” indicates that the phage could lyse bacteria, “–” indicates no lysis.

Table 2. Antibiotic susceptibility test results for clinical *Escherichia coli* strain 54959124

Drugs	KB (mm)	MIC (μg/mL)	Result
Ampicillin	-	≤2.0	Sensitivity
Piperacillin	-	≤4.0	Sensitivity
Ampicillin/Sulbactam	-	≤2.0	Sensitivity
Piperacillin/Tazobactam	-	≤4.0	Sensitivity
Ticarcillin/Bacitracin	-	≤8.0	Sensitivity
Cefoperazone/Sulbactam	-	≤8.0	Sensitivity
Cefazolin (urine)	23	-	Sensitivity
Cefazolin (other)	23	-	Sensitivity
Cefuroxime	-	4.0	Sensitivity
Cefuroxime axetil	-	4.0	Sensitivity
Ceftriaxone	-	≤1.0	Sensitivity
Ceftazidime	-	≤0.12	Sensitivity
Cefepime	-	≤0.12	Sensitivity
Cefotetan	-	≤4.0	Sensitivity
Aztreonam	-	≤1.0	Sensitivity
Imipenem	-	≤0.25	Sensitivity
Meropenem	-	≤0.25	Sensitivity
Amikacin	-	≤2.0	Sensitivity
Gentamicin	-	≤1.0	Sensitivity
Tobramycin	-	≤1.0	Sensitivity
Minocycline	-	≤1.0	Sensitivity
Doxycycline	-	1.0	Sensitivity
Levofloxacin	-	1.0	Intermediary
Ciprofloxacin	-	≤0.25	Sensitivity
Trimesulf	-	≤20.0	Sensitivity
Macrodantin	-	≤16.0	Sensitivity
Tigecycline	-	≤0.5	Sensitivity
Colistin	-	≤0.5	Intermediary

Abbreviations: KB: Kirby–Bauer disk diffusion method; MIC: Minimum inhibitory concentration.

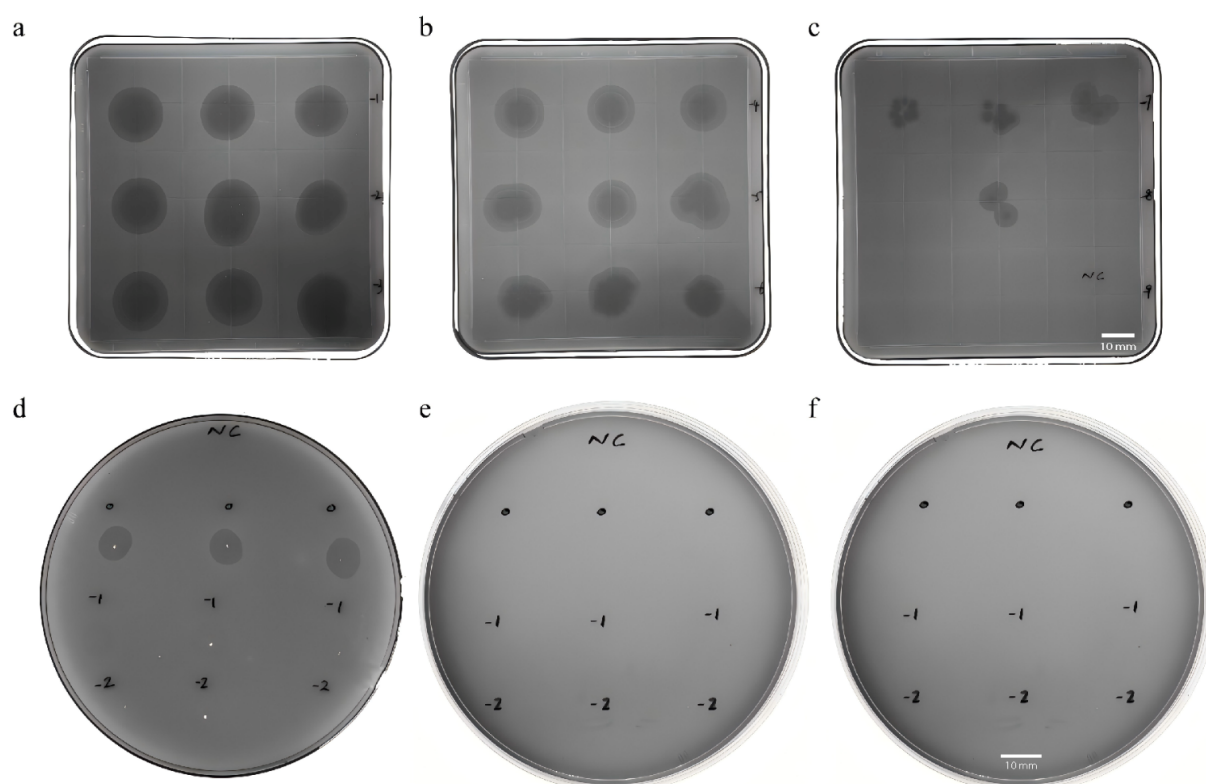


Figure 2. Comparison of phage plaque formation results. (a–c) The spot assay image was obtained using the drop-on-plate method to visualize the lysis of DH5α by phage NLE251 at dilutions from (a) 10^{-1} to 10^{-3} , (b) 10^{-4} to 10^{-6} , and (c) 10^{-7} to 10^{-9} . (d–e) Spot assay image was obtained using the drop-on-plate method to visualize the lysis of (d) clinical strain 54959124, (e) non-susceptible strain 54985313, and (f) clinical strain 54959124 by phage phiD2-2 with the dilution from 0 to 10^{-2} . Scale bars: 10 mm; magnifications: 0.4×.

was 100, as this ratio maximized phage infection efficiency and yielded the highest progeny production.

3.2.4. Thermal stability

Thermal stability studies aimed to understand and predict the activity of NLE251 during storage and operation, providing a basis for evaluating its therapeutic potential and revealing its structural resilience. One-way ANOVA analysis showed that incubation temperature had a highly significant effect on NLE251 infectivity ($F_{(5, 12)} = 89.67$, $p < 0.001$). Tukey's post hoc test showed no significant differences in titer between the 4 °C control group and groups incubated at 25, 37, 45, or 60 °C ($p > 0.05$ for all pairwise comparisons), indicating that NLE251 is highly stable at temperatures up to 60 °C, with optimal stability at 37 °C (physiological temperature). After 1 h of incubation at 65 °C, the phage titer decreased significantly ($p < 0.001$; Figure 3c). The results indicate that NLE251 is highly stable

at 37 °C (body temperature) and tolerates temperatures up to 60 °C, but its titer decreases significantly at 65 °C.

3.2.5. CaCl_2 stability

To investigate the efficacy of CaCl_2 in adsorbing NLE251 and its correlation with host physiology, and to elucidate the infection mechanism of NLE251, CaCl_2 stability was tested. As shown in Figure 3d, at 0 mM CaCl_2 , the phage titer was at a high level, while it decreased slightly at 5 mM CaCl_2 . As the CaCl_2 concentration increased further, the phage titer rose marginally. The results of the one-way ANOVA showed that CaCl_2 concentration had no significant effect on the residual titer of NLE251 ($F_{(4, 12)} = 0.78$, $p = 0.56$). These results indicate that NLE251 remains stable within a CaCl_2 concentration range of 0–20 mmol/L, and Ca^{2+} has no significant effect on its infectivity. Overall, CaCl_2 had little effect on phage titer, indicating that phage adsorption proteins do not depend on CaCl_2 .

3.2.6. Bacterial lysis curves

Comparison of bacterial lysis curves (Figure 3e and f) indicated that DH5 α was more susceptible to phage NLE251, as complete inhibition was achieved with a minimal MOI of 10^2 . The clinical strain showed higher resistance, requiring an MOI of 10^5 for complete inhibition. At lower MOIs (10 and 10^2 for the clinical strain), bacterial recovery was observed, suggesting the possible development of resistance or the presence of phage-resistant subpopulations. Phage NLE251 exhibited potent bactericidal activity against both DH5 α and the clinical strain 54959124, with the latter demonstrating higher resistance. One-way ANOVA analysis confirmed that the MOI required for complete sterilization of the clinical strain was significantly higher than that for DH5 α ($F_{(1,8)} = 214.56$, $p < 0.001$), consistent with the higher resistance of the clinical strain to NLE251. The results highlight the importance of selecting an appropriate MOI based on the bacterial strain to achieve effective phage therapy while minimizing the risk of resistance development.

3.3. Phage sequence

Genome sequencing confirmed the absence of virulence or antibiotic resistance genes as a baseline safety measure for future applications, and comparative genomics were conducted to determine evolutionary relationships with known phages. Despite high similarity, we highlighted unique features at specific gene loci (e.g., lytic modules) and discussed their significance.

The NLE251 genome (Figure 4) is a double-stranded circular DNA molecule, 49,336 bp in length, with a guanine–cytosine (GC) content of 50.39% and contains 79 valid ORFs. The ORFs of NLE251 were annotated using Prokka and further analyzed by Blastp against the NR database. The functional annotations revealed the presence of key genes involved in phage morphogenesis, DNA replication, and host lysis. Genes encoding structural proteins, such as the major head protein (ORF79), minor capsid protein (ORF75), portal protein (ORF74), and tail proteins (ORFs 2–14), were identified. These proteins are essential for the assembly of the phage particle. Genes involved in DNA replication and repair, including DNA helicase (ORF37), DNA primase (ORF21), exonuclease VIII (ORF17), and DNA repair exonuclease (ORF30), were detected. These proteins facilitate the replication and maintenance of the phage genome. Genes responsible for host cell lysis, such as endolysin (ORF33), holin (ORF32), and Rz-like spanin (ORF34), were also found. These proteins work together to disrupt the host cell membrane and release the phage progeny. Other genes with diverse functions were also annotated, including RNA-binding protein (ORF61),

polynucleotide kinase (ORF63), and terminase subunits (ORFs 72–73), playing roles in RNA metabolism, DNA modification, and phage genome packaging, respectively.

The genome of NLE251 was organized in a modular fashion, with genes involved in similar functions clustered together. The structural protein genes are located toward the end of the genome, the DNA replication and repair genes are positioned in the middle, and the host lysis genes are found near the end of the genome, ensuring efficient release of phage progeny upon host cell lysis.

Using DeePhage, the lifestyle of NLE251 was predicted to be virulent, with a lifestyle score of 0.62, indicating that NLE251 is a lytic phage that actively lyses its host cells to release progeny, rather than integrating into the host genome as a temperate phage. Analysis using VFDB and ResFinder revealed that NLE251 did not carry any known virulence factors or antibiotic resistance genes, making it a promising candidate for phage therapy and unlikely to contribute to the spread of virulence or resistance traits in bacterial populations.

3.4. Genome linear comparison

Through gene alignment, the phylogenetic relationship between NLE251 and other known phages can be determined, enabling its accurate classification and identification. Furthermore, it helps predict the functional genes of this bacteriophage. For phages with potential application value, gene alignment helps identify the unique functions and characteristics, thereby providing a foundation for developing new phage applications. As shown in Figure 5a, transcription direction is indicated by arrows in the coding DNA sequence. The BLASTn results showed that phage NLE251 exhibited 99.7% homology with *E. coli* phage phiD2-2 (taxonomy for taxid 3110555) and 99.5% homology with *E. coli* phage FCWL1 (taxonomy for taxid 3136680), indicating that the core genes of phage NLE251 exhibit high similarity to those of phage phiD2-2 and phage FCWL1. Whole-genome taxonomic classification using VICTOR across D0, D4, and D6^{32–34} (Figure 5b–d) similarity thresholds further confirmed that NLE251 belongs to the class Caudoviricetes (realm Duplodnaviria), family Demerecviridae, subfamily Markadamsvirinae, genus *Tequintavirus*, according to the ICTV 2025 taxonomy.³¹ The former order Caudovirales and family Siphoviridae, based on morphological classification, were formally abolished in the 2021 ICTV update.³⁵ This classification is consistent with that of its closest relatives, *E. coli* phage phiD2-2 and *E. coli* phage FCWL1 (Figure 5). The GC content (50.39%) and genome length (49,336 bp) of NLE251 fall within the typical range of Caudoviricetes members clustering with these two

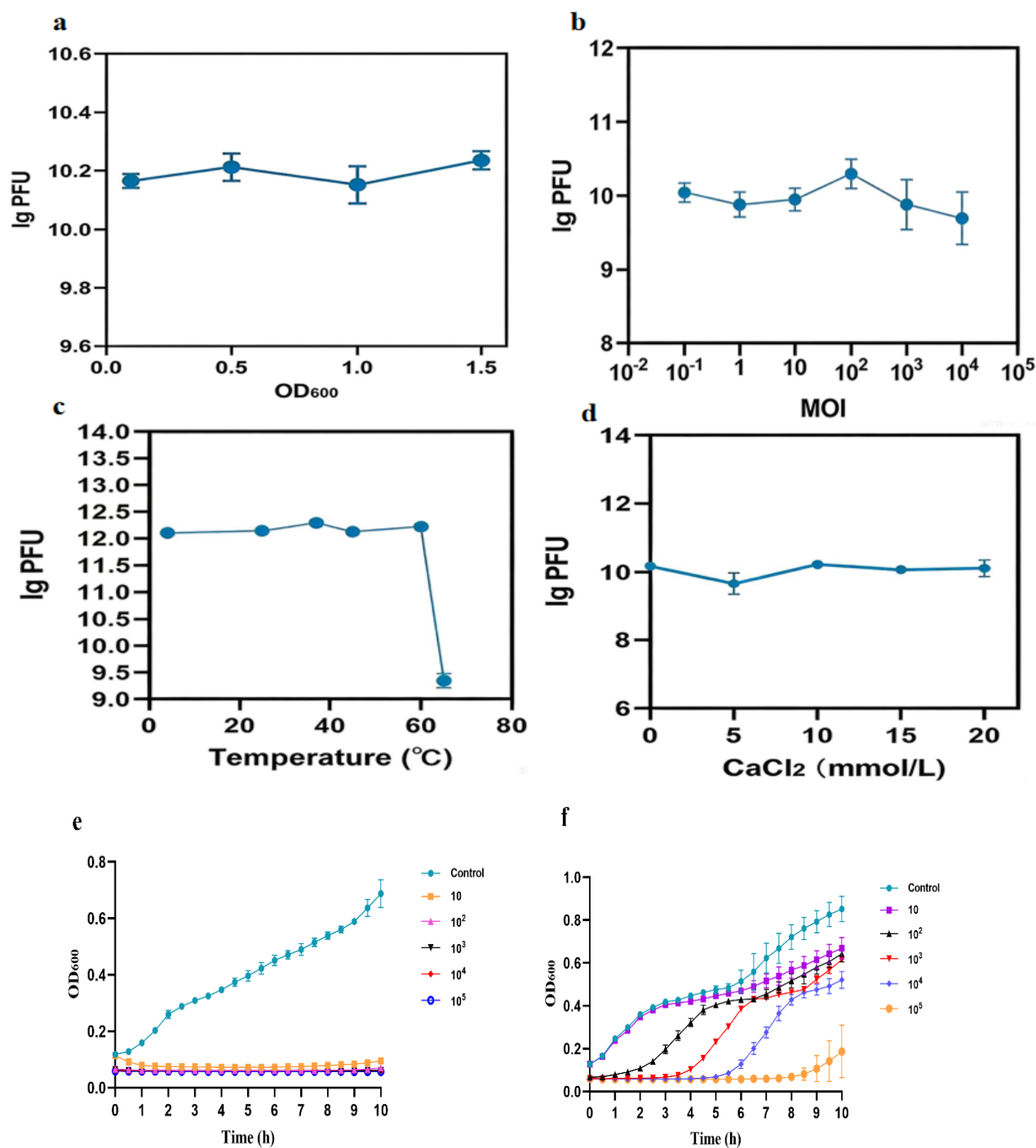


Figure 3. Biological characteristics of phage NLE251. (a) Effects of different host densities on phage NLE251. (b) Determination of optimal multiplicity of infection (MOI) of phage NLE251. (c) Thermal stability of phage NLE251. (d) Effects of different concentrations of CaCl₂ on phage NLE251. (e–f) Comparison of bacterial lysis curves for (e) *Escherichia coli* DH5a and (f) a clinical *E. coli* isolate strain 54959124 infected by phage NLE251 at gradient MOIs.

Abbreviation: OD: Optical density.

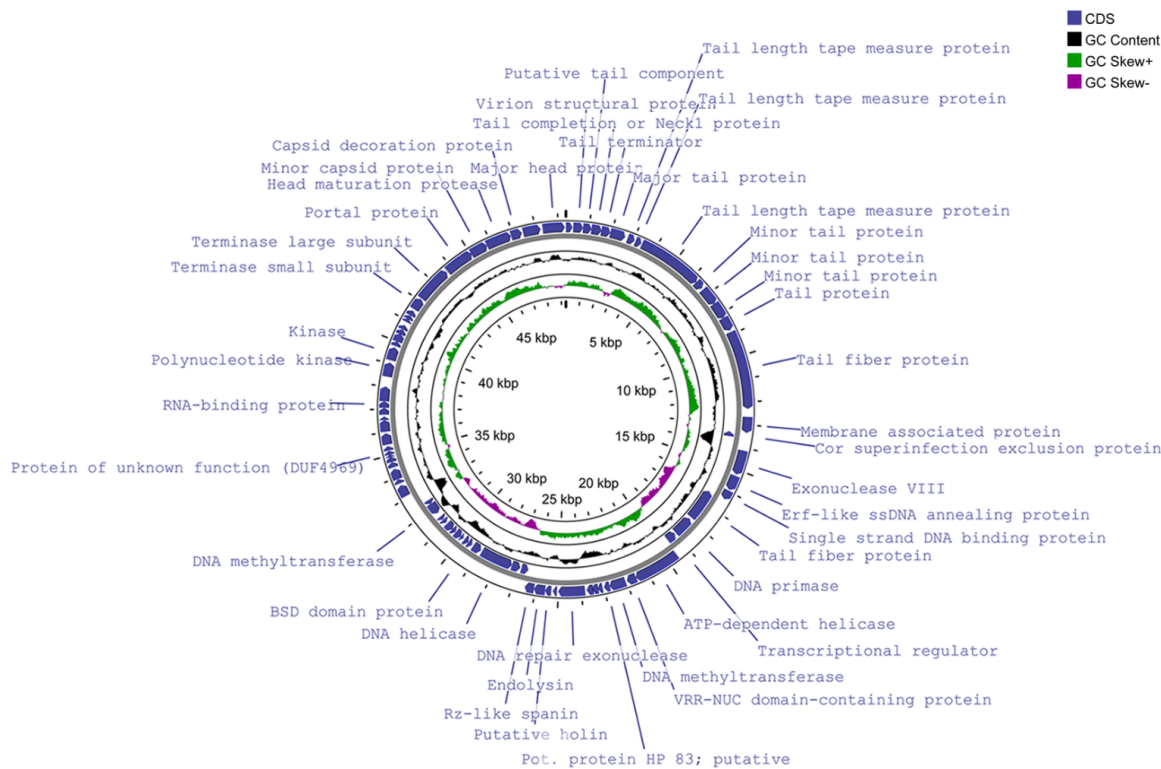


Figure 4. Whole-genome map of phage NLE251
Abbreviations: CDS: Coding DNA sequence; GC: Guanine–cytosine; tRNA: Transfer RNA.

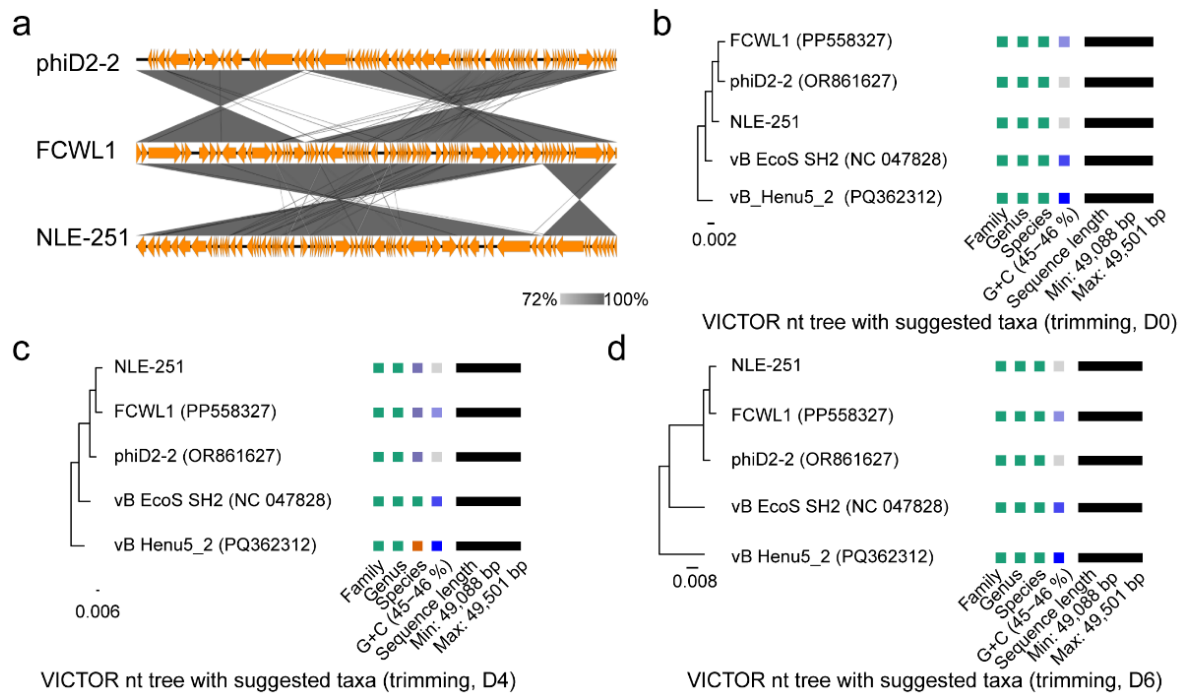


Figure 5. Genomic comparison and taxonomic classification of phage NLE251. (a) Gene alignment among phages NLE251, phiD2-2, and FCWL1. (b–d) Whole-genome phylogenetic tree generated by Virus Classification and Tree Building Online Resource (VICTOR) at the (b) D0 (species-level), (c) D4 (genus-level), and (d) D6 (family-level) similarity thresholds.

phages (GC 45–46%, genome length 49,088–49,501 bp), supporting this provisional classification.

4. Conclusion

The escalating global crisis of AMR, particularly in *E. coli*, underscores the urgent need for alternative therapeutic strategies.^{40–43} Bacteriophage (phage) therapy, with its potential for targeted bacterial elimination while sparing commensal microbiota, presents a promising solution.^{44,45} However, translating phage therapy from environmental isolation to clinical application is fraught with methodological and safety challenges.^{46,47} This study addresses these challenges by establishing and validating a safety-conscious pipeline for phage discovery using the non-pathogenic laboratory strain *E. coli* DH5 α (BSL-1). Traditional therapeutic phage screening typically uses clinical pathogenic strains as initial hosts. For example, the study by Peng *et al.*⁴⁸ explicitly used the clinically isolated pathogenic strain *P4* (carrying multiple virulence genes) as the initial host for phage isolation. While the study by Abebe *et al.*⁴⁹ directly utilized clinical multidrug-resistant *Klebsiella pneumoniae* isolates resistant to carbapenems, fluoroquinolones, and other antibiotics as screening hosts, further confirming that traditional strategies center on clinical pathogenic bacteria as core targets. This necessitates BSL-2 or higher-level biosafety precautions from the outset. In contrast, our DH5 α -based workflow requires only BSL-1 containment, thereby eliminating the need for high-level biosafety facilities during the initial screening phase.

Regarding the method of using non-pathogenic laboratory *E. coli* as an alternative host for phage isolation, the study by Maffei *et al.*²³ was the first to validate the feasibility of this core approach. Using non-pathogenic *E. coli* K-12 as the host for isolation, they isolated 68 virulent phages from environmental samples and verified their activity against clinically pathogenic *E. coli* strains, including those causing urinary tract infections and intestinal colonization. The core objective was to establish a reference repository for basic research on phage-host interactions, without involving the development of therapy-oriented screening workflows. This study adapted and standardized this approach into an initial screening workflow for therapeutic candidate phages: compared to the work of Maffei *et al.*, this study introduced new core components, including therapeutic phenotype characterization (assessment of optimal MOI, thermal stability, and host density tolerance) and prioritized safety and accessibility from the initial isolation step, circumventing early biosafety concerns and democratizing the discovery process.

As a proof of concept, we used *E. coli* DH5 α as a host and, through screening and elimination, isolated a novel lytic bacteriophage, vB_EcoS_NLE251, from soil. Comprehensive phenotyping revealed that NLE251 possesses robust stability, efficient replication, and a precisely narrow lytic spectrum, targeting only a single biliary tract infection-derived *E. coli* strain (54959124) among 35 clinical isolates tested. Combined with the findings of Mirzaei and Nilsson³⁸, an EOP ≤ 0.001 was clearly defined as a low-efficiency infection unsuitable for therapeutic application. Gibson *et al.*³⁹ categorized only two types of phages as candidate strains with therapeutic potential: those with EOP > 0.1 (highly lytic phages) and those with $0.001 < \text{EOP} < 0.1$ (moderately lytic phages). In the present study, the extremely low infectivity of phage NLE251 against the target clinical strain, with an EOP of 5×10^{-7} , was far below both aforementioned thresholds. Moreover, the discrepancy between positive spot assay results and EOP measurements is highly consistent with the phenomenon that “spot assay overestimates the host range” reported by Mirzaei and Nilsson.³⁸ This indicates that the lytic activity of NLE251 against the target clinical strain is more likely due to direct cell wall destruction induced by high-concentration phages rather than replicative productive infection. Nevertheless, further studies can expand the scale of environmental sample screening to isolate more candidate phage strains. Additionally, engineering strategies, such as directed evolution and tail fiber protein replacement, can be adopted to improve the infectivity of phages against targeted pathogenic strains, thereby verifying the practicality of the established workflow. Genomic analysis confirmed that NLE251 is a class Caudoviricetes under the 2025 ratified ICTV bacterial virus taxonomy³¹, and lacks virulence or antibiotic resistance genes, fulfilling a key safety prerequisite for therapeutic use. Although NLE251 is a narrow-spectrum agent, its isolation and characterization demonstrate the feasibility of our methodological framework for advancing environmental phages toward potential therapeutic applications. Through experimental evolution, phages can co-evolve with bacterial hosts, thereby expanding their host range and effectively targeting multidrug-resistant and extensively drug-resistant clinical isolates.⁵⁰ Meanwhile, yeast-based genome engineering platforms enable straightforward, high-throughput manipulation of phage genomes, allowing simultaneous engineering of multiple sites.⁵¹ By swapping tail fibers or entire tail components, these platforms facilitate the creation of synthetic phages capable of targeting a broad spectrum of bacterial hosts. The application of these technologies holds significant potential for developing precise phage cocktails targeting specific bacteria within mixed microbial populations.

This approach promises to streamline the discovery, manufacturing, and regulatory approval processes for novel phages, offering more effective therapeutic strategies against antibiotic-resistant bacteria.

This study provides a safety-prioritized, standardized pipeline for accelerating phage discovery against multidrug-resistant bacterial infections. By prioritizing safety, specificity, and accessibility from the outset, our pipeline offers a strategic approach to harnessing the potential of bacteriophages in the fight against AMR. Future research can build upon this foundation to develop well-defined phage cocktails with broader host ranges and enhanced therapeutic efficacy, ultimately contributing to the preservation of antibiotic efficacy for future generations. However, it is worth noting that using DH5 α as an alternative host may yield phages biased toward receptors specific to laboratory strains, potentially limiting their activity against clinical isolates. Future studies should consider using multiple alternative hosts or screening directly against clinical strains to mitigate this bias.

Acknowledgments

The authors are grateful to the Ningxia Medical University for the support and coordination, as well as for providing the testing laboratories. The authors would like to express their special thanks to all who participated in data collection and the laboratory activities. We also thank Daniel Cung Tha Thawng for language revision.

Funding

This work was supported by grants from the Research Projects for Social Development of Ningxia Hui Autonomous Region (Grant No. 2026BEG03004), Open Competition Mechanism to Select the Best Candidates for Key Research Projects of Ningxia Medical University (Grant No. XJKF250414) and the Interdisciplinary Research Program of Ningxia Medical University (Grant No. JCXK2025003).

Conflict of interest

The authors declare they have no competing interests.

Author contributions

Conceptualization: Xiting Lin, Noura M. Bin Yahia, Yanhui Yang

Data curation: Bingxian Li, Yiming Chen

Formal analysis: Shengjie Wu, Xiaofan He

Investigation: Jing Ma, Sihao Zhao, Ying Xu, Huihui Mu

Methodology: Xiting Lin, Noura M. Bin Yahia, Yanhui Yang

Writing—original draft: Xiting Lin, Noura M. Bin Yahia

Writing—review & editing: All authors

Ethics approval and consent to participate

This study was approved by the Ningxia Medical University Ethics Committee (2025-Z031). Clinical bacterial isolates and associated patient information (diagnosis and antibiotic susceptibility profiles) were obtained from the routine diagnostic laboratory and fully anonymized before analysis. The requirement for informed consent was waived by the committee because only de-identified data were used.

Consent for publication

Not applicable.

Availability of data

Data used in this work are available from the corresponding author upon reasonable request.

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