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Evaluating Indigenous Lytic Bacteriophages for Activity Against Multi-Resistant *Enterobacteriaceae*

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Abstract

The widespread and indiscriminate use of antibiotics is increasing drug resistance. The study isolated, characterized, and assessed the bioactivity of bacteriophages against some multi-resistant *Enterobacteriaceae*. Using standard protocols, eight wastewater samples were obtained and used to isolate and characterize potential host enteric bacteria and bacteriophages. The wound-healing effect of the phage cocktail (PC) was evaluated by treating coinfected incision 4-cm wounds in animal groups. One hundred bacterial isolates were obtained. Six: U1, U2, S1, S2, P1, and P2, resulted in polyvalent bacteriophages. The cocktailing increased the host range to seventeen. Morphologically, the six bacterial colony sizes ranged from 1 to 6 mm and exhibited different responses to biochemical tests. The PC characterization indicated the presence of constituent phages of the host bacteria. The observed plaque morphology included small, medium, and large-sized plaques, as well as diffuse and clear plaques. The PC concentrations ranged from 3.6×10^3 to 5×10^5 PFU/ml. The adsorption times ranged from 9 to >10 min, with different burst sizes and latent periods. The bacterial isolates showed high resistance, with high multi-antibiotic resistance indices. The host bacteria were identified as *Klebsiella aerogenes* (U1), *Citrobacter freundii* (U2), *Salmonella enterica* (S1 and S2), and *Escherichia coli* (P1 and P2). Phage therapy with the PC showed significant differences in wound size ($p = 0.000$), with the PC groups outperforming the negative control groups. The study provides promising evidence for the potential use of phages as alternatives to antibiotics, though further research and clinical trials are necessary to establish their efficacy and safety.

Keywords: Lytic polyvalent bacteriophages, PC, Multi-resistant Enterobacteriaceae, Antibiotic resistance, Wound-healing effect

Purpose, Rationale, and Limitations

The purpose of this work was to isolate, characterize, and evaluate locally sourced lytic bacteriophages active against six important drug-resistant bacterial species. With antimicrobial resistance continuing to undermine the effectiveness of conventional antibiotics, there is a growing need for alternative biological agents that can target and reduce the burden of multidrug-resistant pathogens. Our goal was therefore to assemble a panel of indigenous phages with potential therapeutic and environmental applications, while also establishing baseline biological features that could support future cocktail formulation or *in vivo* studies.

The rationale for focusing on locally isolated phages is that phages are highly specific to the

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bacterial strains present in a given ecological niche. Using phages collected from the same environments where resistant bacteria thrive increases the likelihood of obtaining robust and highly lytic candidates capable of targeting regionally prevalent strains. At a time when many infections no longer respond reliably to standard antibiotics, phages offer several attractive properties, including self-amplification at the site of infection, minimal disturbance to the normal microbiota, and the ability to evolve alongside their hosts. Identifying phages that naturally interact with multidrug-resistant (MDR) bacteria found in wastewater, clinical sources, and community environments provides a relevant starting point for exploring how these agents might support infection control, wound management, or decontamination efforts.

Despite these strengths, the study does have limitations. Our isolation approach, which relied on enrichment and single-host screening, may have favoured fast-replicating or dominant phage types while missing lower-abundance or strain-restricted variants. The absence of whole-genome sequencing means that more profound insights into phage taxonomy, virulence genes, or lysogenic potential could not be fully resolved at this stage. Additionally, while we identified promising lytic activity in vitro, further work, including genomic analysis, in vivo testing, and assessment of stability, immunogenicity, and formulation requirements, will be needed before any of these phages can be considered for therapeutic use. These limitations do not diminish the value of the initial discovery but highlight the steps necessary to translate local phage candidates into practical tools for addressing antimicrobial-resistant infections.

Introduction

With the increasing menace of multi-resistant pathogenic bacteria, which is of public health concern and arising from the mismanagement of synthetic antibiotics, including the overuse and administration, self-care/medication, and improper and random prescription of antibiotics (1–4); significant research effort in pharmaceutical science is geared towards alternative therapies, especially from natural products, including the exploitation and optimisation of bacteriophages using differing techniques (5).

Bacteriophages (phages) are viruses that infect and replicate within bacteria and archaea (6). They can nearly always be found where living bacteria exist, as they are the most prevalent living organisms and play an essential role in controlling bacterial populations and affecting all ecosystems. Phages have been used for many years as a natural antibiotic substitute, especially in Eastern Europe and the former Soviet Union, to treat bacterial illnesses. Bacteriophages have been utilised as effective agents for regulating the composition of prokaryotic communities, particularly pathogenic ones, due to their host-specific infection characteristics (7). All forms of phages can be isolated from the environment for therapeutic use, including soil, sewage, and animal secretions (8–10). They fall into the virulent or temperate categories (11). Virulent phages reproduce through the lytic cycle, in which phage infection triggers DNA synthesis and structural protein expression, capsid and ultrastructure assembly, up-regulation of the lytic enzyme, bacterial host death, and (ultimately) the release of progeny phage, while temperate phages entering a host to multiply can start either a lytic or lysogenic lifestyle (11). While transcriptional repressors prevent most phage genes from being expressed and the production of new infectious particles, the lysogenic cycle enables the replication of the phage genome alongside that of the host bacterium (12). The phage genome is packaged into a protein shell, cut off from the bacterial genome, and the mature phages lyse the cells. However, under specific physiological conditions and environmental circumstances, the life cycle of these phages may transition to the lytic cycle (13), as seen in the bacteriophage λ RexA and RexB functions reported by Thomason *et al*. (14).

Bacteriophages have been widely used in therapeutic applications to prevent and treat bacterial infections (15,16). The use of lytic bacteriophages, the natural enemies of pathogenic bacteria, to selectively kill pathogenic bacterial cells is known as phage therapy (17). The primary benefits of phage therapy include their capacity to multiply at the site of application/infection, low production costs, host specificity, lack of adverse effects, and low allergenicity (18). They are also safe for normal microflora. There are also diverse delivery and administration routes,

including oral, subcutaneous, intranasal, and intravenous (19–21). They can also be paired with various therapies and medications, such as other phages, cocktails, or antibiotics (22). Developing new alternatives against resistant bacteria is also faster and cheaper than developing new antibiotics.

The Centers for Disease Control and Prevention (CDC) has warned about the emergence of multidrug-resistant organisms (MDROs) and the potential for infections resistant to available treatment options (23). Antimicrobial resistance is an ongoing public health challenge that threatens the efficacy of antibiotics and hinders the treatment of bacterial infections, and is contributed to by the poor and indiscriminate use of antibiotics, leading to the bacterial agents developing mechanisms to escape antibiotic agents' actions, which leads to treatment failures and has enormous impacts, including economic ones (24). Antibiotics are also often allergenic and have multiple side effects. Although many enteric bacteria play functional roles in the gut, some become pathogenic when infection conditions are established, especially in immunocompromised individuals (25). Research has shown patterns of resistance to conventional antibiotics by these enteric bacteria, some of which include the Campylobacter (*C. jejuni* and *C. coli*), Shigella (*S. flexneri, S. sonnei, S. boydii,* and *S. dysenteriae*), *Salmonella enterica* (typhoidal and non-typhoidal), *Vibrio cholerae*, *Clostridioides difficile*, *Bacteroides fragilis* group, *Escherichia coli*, Enterococci (*E. faecium* and *E. faecalis*), and *Klebsiella* (*K. aerogens, K. quasipneumoniae* and *K. pneumoniae*) species and are often found in abundant amount in reservoirs such as food, water, animals, and humans (26,27,25). The need for alternative therapies to antibiotics to control these bacteria is imminent, utilising fewer toxic substances as seen in natural products, including bacteriophages. The use of bacteriophages and their associated products, including endolysins, as an alternative or complementary treatment for bacterial infections is a promising area of research that requires further exploration (28,29). Bacteriophage research lags in Africa, including Nigeria. While there is a need to close the gap, it is good to start by isolating and profiling indigenous bacteriophage strains directly associated with indigenous enteric pathogens that cause various diseases.

Enterobacteriaceae, used interchangeably with enteric bacteria in this report, are a family of Gram-negative bacteria belonging to Enterobacterales and are facultative anaerobes commonly found in the gastrointestinal tract of animals and humans and can also be found in soil, water, and plants (30). While many species are harmless and form part of the normal gut flora, some can cause diseases, such as urinary tract infections, pneumonia, sepsis, and gastrointestinal infections in humans, including those in the *E. coli*, *Salmonella*, and *Klebsiella* genera (31). Some members of this family can also be used in industrial applications, such as in the production of antibiotics or other chemicals. The therapeutic management of these pathogens involves antibiotics, and the selection of an appropriate antibiotic depends on the specific organism, the severity of the infection, and the patient's characteristics, such as age and underlying medical conditions (32,33). The commonly used antibiotics for treating *Enterobacteriaceae* infections include cephalosporins (e.g., ceftriaxone, cefotaxime, and cefepime), which inhibit the cell wall synthesis, leading to bacterial cell death, fluoroquinolones (e.g., ciprofloxacin and levofloxacin), which inhibit the bacterial DNA synthesis, aminoglycosides (e.g., gentamicin and tobramycin), often used in combination with other antibiotics to treat applicable infections and binds to the bacterial ribosome, leading to the inhibition of protein synthesis and the subsequent bacterial cell death, and carbapenems (e.g., imipenem and meropenem) which inhibit the cell wall synthesis and have broad-spectrum effects against many pathogens, including those resistant to other antibiotics (34–36). Aside from using infection-control therapeutic agents, it is essential to practice appropriate infection-control measures, such as hand hygiene, to prevent the spread of infectious agents.

The emergence of antibiotic-resistant Enterobacteriaceae strains is a growing concern in healthcare settings worldwide. The development of antibiotic resistance occurs when bacteria undergo genetic changes that enable them to survive exposure to antibiotics, and can happen through different mechanisms, including mutation and the acquisition of resistance genes from

other bacteria (37). Treatment options for infections caused by these resistant strains have become limited, increasing morbidity, mortality, and healthcare costs, emphasising the need for safer and more therapeutic options. Thus, the study aimed to isolate, characterize, and assess the wound-healing activity of an indigenous lytic, polyvalent bacteriophage (phage) cocktail (PC) isolated from wastewater samples against some multi-resistant *Enterobacteriaceae*.

Materials and methods

Equipment

The equipment used in the study include; incubator (FANEM), microcentrifuge, tubes, centrifuge (model Optima TLX micro-ultracentrifuge), TLA-55 Fixed-Angle centrifuge Rotor (Indianapolis, IN, USA), membrane filters filter (0.45 and 0.22 µm), swab sticks, petri dishes, micropipettes and tips, cotton wool, test tubes, spread rods, Eppendorf tubes (1.5 mL), laminar flow cabinet (type B2, class II, SP-LABOR, Presidente Prudente, SP, Brazil), Master System All MS2000 (Gehaka, São Paulo, SP, Brazil), Ultra-low temperature freezer (MDF-U56VC, Panasonic, USA), Quant-IT Picogreen dsDNA assay kit (Life Tehcnologies, USA), 2100 Bioanalyzer (Agilent, Palo Alto CA).

Reagents and chemicals

Gram staining and biochemical characterization reagents, polyethylene glycol (PEG) 600, phenol, uranyl acetate, isoamyl alcohol, isopropanol, sodium acetate, 70% ethanol, distilled water, media Muller-Hinton broth/agar, antibiotic discs (tetracycline (30 µg), meropenem (10 µg), sulphamethoxazole/trimethoprim (25 µg), azithromycin (15 µg), amoxicillin (15 µg), ofloxacin (5 µg), erythromycin (15 µg), aztreonam (30 µg), chloramphenicol (30 µg), and gentamicin (10 µg), calcium chloride ($CaCl_2$), cetrimide agar (CA), salmonella-shigella agar (SSA), eosin methylene blue agar (EMB), salt-magnesium (SM) buffer, phosphate-buffered saline, tryptic soy agar (TSA) and -broth (TSB), ethylenediaminetetraacetic acid (EDTA), phenol, absolute ethanol, sodium acetate (NaAc), chloroform (Sigma-Aldrich, MO, USA), general purpose agar (Gibco Diagnostics), ultra-purified tap water (final resistivity = 18.18 MΩ × cm, conductivity of 0.05 µS/cm), RNAse (10 mg/mL), DNAse (20 mg/mL), and Proteinase K (20 mg/mL) (Transgen Biotech, China).

Sample collection and processing

Twenty (20) milliliter volume of different environmental wastewater samples (in triplicate), including residential, fishery, abattoir, farmland, hospital, piggery, chicken rearing, and central sewage of the University of Nigeria, Nsukka, were collected in and around Nsukka, Town, Enugu State, Nigeria using sterile amber colored bottles. The samples were taken to the laboratory immediately. They were first filtered through coarse filter paper to remove debris, after which 10 mL of each filtrate was collected and centrifuged at 6500 rpm for 15 min. The resulting supernatants were filtered through a membrane filter (size: 0.45 µm) into a sterile bottle. The samples were stored at 4 ºC pending further use. All the sample processing and manipulation of bacteria and bacteriophages were done in the laminar flow cabinet. All experiments involving water, including environmental sample processing and the preparation of media and buffers, were performed using ultra-purified water.

Isolation, morphological, and biochemical characterization of bacterial isolates

The pulled environmental sample was appropriately homogenized and serially diluted up to a dilution factor of 10^{-5} using SM buffer. Following dilution, a drop of the 10^{-5} dilution was spotted on prepared sterile SSA, EMB, and CA plates, after which a sterile glass rod was used to spread the drop evenly across the agar lawns. The plates were incubated for 24 hours at 37 ºC, after which characteristic colonies were sub-cultured for proper isolation. The bacterial isolates were stored at 4°C pending characterization and used as bacterial hooks (38) for bacteriophage screening. Successful bacteriophages that host bacterial strains were distinctively coded.

The isolates' morphological characterization was performed by observing their characteristic appearances on the respective selective media (SSA, EMB, and CA), noting attributes such as size, shape, elevation, surface appearance, transparency, and color. The preliminary confirmation of the bacterial isolates was performed using biochemical tests, including citrate, hydrogen sulfide (H_2S), motility, indole, triple sugar Iron

(TSI), and oxidase, according to standard protocols.

Antibiotic profiling of bacterial isolates and multiple antibiotic resistance (MAR) index

Using sensitivity discs (Oxoid), the antibiotic profile of the isolates to relevant conventional antibiotics, including tetracycline (30 µg), meropenem (10 µg), sulphamethoxazole/trimethoprim (25 µg), azithromycin (15 µg), amoxicillin (15 µg), ofloxacin (5 µg), erythromycin (15 µg), aztreonam (30 µg), chloramphenicol (30 µg), and gentamicin (10 µg) were done by placing the discs on the bacterial lawn (OD_{600} = 0.5, equivalent to 1.5×10^8 CFU/mL) of the isolates. Inhibition zone diameters (IZD) in millimetres were recorded, and the results were used in the classification of the isolates as 'sensitive,' 'intermediate,' or 'resistant,' as recommended by the most recent Clinical and Laboratory Standards Institute (CLSI) Classification (39). The multiple antibiotic resistance index (MAR index) was determined using the procedure described by Arbab *et al*. (40) using the formula below:

MAR index = number of antibiotics to which the isolate is resistant (a) / Total number of antibiotics against which the isolate was tested (b)

Molecular characterization of host bacteria

DNA extraction, electrophoresis, and polymerase chain reaction

Two (2) milliliters of bacterial cell broth in a ZR BashingBeadTM Lysis Tube received 750 µL of lysis solution. It was processed at maximum speed for 5 min and held in a bead with a 2 mL tube holder assembly. The ZR BashingBeadTM Lysis Tubes were centrifuged at > 10,000 × g for one minute in a microcentrifuge. After being transferred to a Zymo-Spin IV Spin Filter in a collecting tube, 400 µL of supernatant was centrifuged at 7,000 × g for one minute. The resulting filtrate was mixed with 1,200 µL of bacterial DNA binding buffer before 800 ul of the mixture was transferred to the Zymo-SpinTM IIC column in a collection tube. This process was repeated after discarding the flow through the collection tube. The Zymo-Spin TM IIC column in the new collection tube was filled with DNA pre-wash buffer, then centrifuged at 10,000 × g for 1 minute. The Zymo-SpinTM IIC column was washed with 500 µL of bacterial DNA wash buffer and then centrifuged at 10,000 × g for 1 minute. A clean 1.5 ml microcentrifuge tube was transferred to the Zymo-SpinTM IIC column, and a 100 µL DNA Elution Buffer was added directly to the column matrix. The column was centrifuged at 10,000 × g for 30 sec to elute the DNA.

Standard procedures were used to prepare and analyze DNA by agarose gel electrophoresis and to perform polymerase chain reaction (PCR) analysis.

Sequencing

Using an Applied Biosystems Genetic Analyser 3130xl sequencer and the BigDye Terminator v3.1 cycle sequencing kit, the amplified fragments were sequenced according to the manufacturer's instructions. All genomic analyses were performed using MEGA 11 and BioEdit.

Evolutionary relationships of taxa

The Neighbour-Joining technique inferred the evolutionary history (41). The ideal tree is displayed, with a branch length sum of 1.08784864. With branch lengths in the same units as the evolutionary distances used to estimate the phylogenetic tree, the tree is rendered to scale. The evolutionary distances, measured as the number of base substitutions per site, were calculated using the Maximum Composite Likelihood method (42). There were six nucleotide sequences in this investigation. Included were the first, second, third, and non-coding codon locations. For each sequence pair, all ambiguous regions were removed (pairwise deletion option). The final dataset contained 1224 locations altogether. In MEGA X, evolutionary analyses were performed (43).

Sample enrichment, isolation, and production of bacteriophages

The pulled environmental sample also served as the source of bacteriophages (44). Protocols, as described by Pereira *et al*. (45), with tailored modifications, were adopted for the isolation of bacteriophages. The sample was employed in an enrichment system of 50 mL samples, 50 µL of

an overnight broth culture of the potential bacterial host, 0.05 g/L $CaCl_2$, and 50 mL of double-strength (2×) TSB. The mixture was incubated at 37 ºC while agitating at 120 – 180 rpm for 48 h. Following incubation, the enriched sample was placed in a 50 mL tube and centrifuged at 9,000 × g for 10 min, after which the supernatant was passed through a 0.22 µm filter and the filtrate collected in another sterile 50 mL tube.

Ten (10) µL of both the filtrate and the potential host bacteria overnight broth culture was added into a soft TSA (50%), overlaid on hard lower TSA plates, and allowed to gel, after which the plate was incubated at 37 ºC for 18 – 24 h, after which the presence of plaques indicative of the presence of phages were observed.

Distinct plaques were picked using a sterile toothpick and transferred into SM buffer, then, using a sterile toothpick, onto double-overlay TSA agar plates, as explained above, for observation of purer plaques after incubation. These were repeated until pure phage isolates were obtained.

Bacteriophage propagation was performed by dipping a pick into SM buffer and inserting it uniformly, horizontally, and at several locations into 20 host bacterial double-layer agar bed plates (4 mL soft-top TSA (50%) with bacteria/20 m; hard lower TSA (100%). A sterile paper strip was also horizontally passed through the phage inserts across the entire bacterial bed. The plates were incubated at 37 ºC for 18 - 24 h, after which 5 ml of SM buffer was introduced into the plates, sealed with parafilm tape, and incubated at 4 ºC for 24 h, after which the liquid in the media was recovered and passed through a 0.22 µm filter, collected in sterile bottles and stored at 4 ºC pending further use.

This phage-hunting process was repeated for all 100 bacterial isolates, after which successful susceptible bacterial hosts (U1, U2, S1, S2, P1, and P2) were characterized and used for further studies.

The six (6) phage suspensions against the host bacteria were combined to form a PC, and the PC was used to characterize the phages against the susceptible bacterial isolates (U1, U2, S1, S2, P1, and P2). The characterizations on PC include spot assay and phage morphology, phage titration, host specificity/host range, one-step growth and adsorption curves, and *an in vivo wound*-healing assay**.**

Spot assay and phage morphology

Five (5) µL of the phage suspensions were spotted on the double-layer agar impregnated with the bacteria ($OD_{600\ nm}$ = 0.5) and incubated at 37 ºC for 18 – 24 h, after which spots/plaques/lytic zones were noted and observed for morphological appearances, including size and shape.

Phage titration

The PC was serially diluted up to the tenth (10^{-10}) dilution using SM buffer, after which five (5) µL of all the dilutions (10^{-1} – 10^{-10}) were spotted on the bacterial bed (using broth optical density at adsorption of 600 nm ($OD_{600\ nm}$ = 0.5) of the host bacterial isolates (U1, U2, S1, S2, P1, and P2), incubated at 37 ºC for 18 – 24 h, after which, the number of plaque of the lowest concentration with positive lytic zone to determine the plaque forming unit per ml (PFU/mL), using the equation below.

PFU/ml = (Plaque number * Reciprocal of the dilution factor)/volume of phage dilution spotted

Phage specificity/host range

The PC was spotted against all 100 bacteria initially isolated from the environmental samples using the double overlay TSA bacterial bed (made using overnight broth culture adjusted to an optical density at adsorption of 600 nm (OD_{50}) of 0.5), incubated at 37 ºC for 18 – 24 h, after which the positive presence of plaques or lytic regions was observed.

OSGC

The OSGC of Phage suspensions against the corresponding bacterial hosts (U1, U2, S1, S2, P1, and P2) was performed using standard procedures (46, 47, 45). The overnight broth cultures of the respective host bacterial isolates were standardized ($OD_{600\ nm}$ = 0.5) and serially diluted twice, after which 50 µL of the second bacterial dilution (10^{-2}) was introduced into a sterile 10 ml TSB broth (in a test tube) termed

'Treatment' and was incubated for 10 min. Following incubation, the phage suspensions were diluted two times, after which 40 µl of the dilutions (10^{-2}) were introduced into the treatment to infect and obtain the optimal multiplication of infection (MOI). The therapy was incubated; 50 µL of the sample was withdrawn at time 0 and serially diluted to the fifth (10^{-5}) dilution. The corresponding sampling and dilutions were repeated after 5, 10, 15, 20, 25, 30, 40, 50, 60, 70, 80, 90, 100, 110, 120, 130, 140, 150, 160, and 170 phage-bacterial infection times (in minutes). Following the end of sampling, different five (5) µl of the samples and dilutions were spotted in triplicates on the respective bacterial lawn prepared by the addition of 100 µl of the bacterial suspensions ($OD_{600\ nm}$ = 0.5) in 4 mL soft top (50%) TSA, which were overlaid on the stiff lower (100%) TSA in Petri plates incubated at 37 ºC overnight. The average number of plaques per dilution was recorded and used to plot the one-step growth curves (OSGC), essentially PFU/mL versus infection time. All the experiments were conducted on three independent days. A direct plot of the average PFU/mL versus time, including 0, 15, 20, 30, 40, 50, 60, and 70 min, was used to determine the phage growth parameters, including burst sizes and latent periods.

Adsorption curves

The adsorption curves (AC) of the constituent phage particles against the corresponding bacterial hosts (U1, U2, S1, S2, P1, and P2) were generated using standard procedures (45–47)**.** Overnight broth cultures of the respective host bacterial isolates were standardized ($OD_{600\ nm}$ = 0.5) and centrifuged at 7000 g for 5 min; the supernatant was discarded, and the pellets were resuspended in 2 mL TSB in Falcon tubes labeled 'Treatment.' The phage suspensions were diluted to the eleventh dilution (10-11). Afterward, 100 µL of the last dilution was added to the resuspended host bacteria in the treatment tube to obtain an MOI of 0.0001, and the mixture was gently homogenized with a micropipette. At time 0, 50 µL of the treatment sample was withdrawn and placed in a sterile Eppendorf tube containing 400 µL of SM buffer. 50 µL of chloroform was added, yielding the first dilution, which was subsequently diluted to the fifth (10^{-5}) dilution. No chloroform was added to the Eppendorf tubes for the 10^-2 to $10^{\wedge-5}$ dilutions. The remaining 50 µL aliquot from the first (10^{-1}) dilution Eppendorf tube was immediately retrieved after mixing with 400 µL of buffer and 50 µL of chloroform to kill bacterial cells in the 10^{-1} dilution. This procedure was repeated every 30 seconds and lasted for 10 minutes.

Following the end of sampling, different five (5) µL of the samples and dilutions were spotted in triplicates on the respective bacterial lawn prepared by the addition of 100 µL of the bacterial suspensions ($OD_{600\ nm}$ = 0.5) in 4 mL soft top (50 %) TSA, which were overlaid on the hard lower (100 %) TSA in Petri plates incubated at 37 ºC overnight. The average number of plaques per dilution was recorded and used to plot the AC, essentially a plot of PFU/mL versus infection time; however, phage adsorption was expressed as the decrease in phage titer in the supernatant relative to time 0. All the experiments were conducted on three independent days. A direct plot of average PFU/mL versus time, including 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, and 10 min, was used to determine the adsorption time.

In vivo wound-healing assay

A total of thirty (30) laboratory albino rats weighing 100 to 150 g were procured from the animal house, Department of Veterinary Parasitology and Entomology, Faculty of Veterinary Medicine, University of Nigeria, Nsukka. They were divided into six (6) groups of five (5) rats each and acclimatised for two (2) weeks with adequate water and air. Wounds measuring 4 cm each were aseptically created on the backs of the rats on Day 0, after which the wounds were inoculated with a mixture of six bacterial isolates (U1, U2, S1, S2, P1, and P2) and allowed to establish infection for 1 day. The contaminated wounds (CWs) of the animals in Groups two to four were treated from Day 1 to Day 10 using 25, 50, 75, and 100 % PC of the six (6) host bacterial isolates, respectively. Animals in Group 5 were not treated and served as the negative control, while those in Group 6 were treated with a standard drug (ciprofloxacin [25 %]). Wound sizes were measured every 2 days starting on Day 2 and recorded in centimetres. The grouping and treatment of the animals are illustrated on the next page.

Group 1: CW + 25% PC
Group 2: CW + 50% PC
Group 3: CW + 75% PC
Group 4: CW + 100% PC
Group 5: CW + No treatment.
Group 6: CW + Ciprofloxacin (25%)

At the end of the study, the animals were euthanized according to the recommended procedures (48).

Statistical analysis

Different statistical tools in Microsoft Excel (Microsoft 365) and the Statistical Package for the Social Sciences (SPSS) (Version 26) were used to evaluate the data. Values were presented as the mean ± standard error of the mean. Formulas in Microsoft Excel were used to aggregate and plot graphs for in vitro bacteriophage characterization. Analysis of variance (ANOVA) was used to analyze the level of significance ($p < 0.05$) between and among the *in vivo* study treatment groups.

Results

Isolation, morphological, and biochemical characterization of the host bacteria

The isolation yielded a total of one hundred (100) distinct bacterial isolates that were obtained and served as potential hosts for sourcing bacteriophages, with only six (6) leading to positive results, which were designated as U1, U2, S1, S2, P1, and P2. U1, U2, S1, and S2 were isolated and morphologically (Table 1) characterized using SSA, while P1 and P2 were characterized using CA and EMB. While all six (6) isolates were convex in elevation and opaque, U1 was ≈ 2–3 mm in size, rod-shaped, mucoid, and pink-colored. U2 is ≈ 2 - 6 mm, straight rod-shaped, smooth, and light brown/grey; S1 and S2 were both ≈ 2 – 3 mm, circular, smooth, and transparent with black centres (on EMB). P1 and P2 were ≈ 1 – 2 mm, rod-shaped, smooth, and yellow to amber (on CA) and sheer metallic green (on EMB) colored.

The biochemical tests (Table 2) showed that all six bacterial isolates were motile and negative for oxidase. U1, U2, S1, and S2 were positive and negative for citrate and indole, respectively, and interchanged for P1 and P2. While U1, P1, and P2 showed adverse reactions to H_2S in TS1 agar, the results were reversed for U2, S1, and S2, respectively.

Both morphological and biochemical profiles suggest that the isolates belong to the genera *Klebsiella*, *Citrobacter*, *Salmonella*, and *Escherichia*.

Table 1: Morphological characterization

Code	Size (mm)	Shape	Elevation	Surface	Opacity	Color
U1	2–3	Rod	Convex	Mucoid	Opaque	Pink (on SSA)
U2	2-6	Straight rod		Smooth		Light brown/grey (on SSA)
S1	2-3	Circular				Transparent with a black (on SSA)
S2						
P1	1 - 2	Rod			Extremely opaque	Yellow to amber (on CA), Sheer metallic green (on EMB)
P2						

Antibiotic profiling and MAR index

Ranging inhibition zone diameters (IZDs, Supporting information, Table S1) were recorded across the ten (10) antibiotics, except tetracycline, sulphamethoxazole/trimethoprim, and amoxicillin, which recorded 0.0 ± 0.0 mm. Ofloxacin recorded the best IZD, inhibiting all but P1 and P2, with values ranging from 11.83 ± 0.17 to 23.70 ± 0.30 mm, followed by chloramphenicol (10.00 ± 0.00 to 17.00 ± 0.58 mm). Gentamicin

had the broadest spectrum activity, inhibiting all six (6) isolates with values ranging from 2.00 ± 0.17 mm. U2 and U1 were the most sensitive, recording 25.0000 ± 0.1732 mm and 23.70 ± 0.33 mm against ofloxacin, respectively. P1 and P2 recorded no IZDs against the antibiotics, except against aztreonam (1.00 ± 0.12 against P1) and gentamicin (2.00 ± 0.12 and 2.00 ± 0.17 against P1 and P2, respectively).

The CLSI classification of the isolates' resistance profiles (CLSI, 2023) (Supporting information, Table S2) showed that all isolates were resistant to most antibiotics, with breakpoints below the required margin for classification as intermediate or sensitive.

Table 2: Biochemical tests

Isolates	Citrate	H_2S	Motility	Indole	TSI	Oxidase
U1	+	-	+	-	A/A	-
U2	+	+	+	-	K/A	-
S1	+	+	+	-	K/A	-
S2	+	+	+	-	K/A	-
P1	-	-	+	+	A/A	-
P2	-	-	+	+	A/A	-

NB: '+' = Positive; '-'= Negative, 'K/A' = Alkaline/Acid, 'A/A' = Acid/Base

While U1 was sensitive to ofloxacin, intermediate to chloramphenicol and gentamicin, presenting a MAR index of 0.7, U2 was sensitive/susceptible to both ofloxacin and chloramphenicol, showing a MAR index of 0.8. The other isolates (S1, S2, P1, and P2) showed MAR index values of 1.

Molecular characterization of host bacteria

DNA extraction, electrophoresis, and PCR

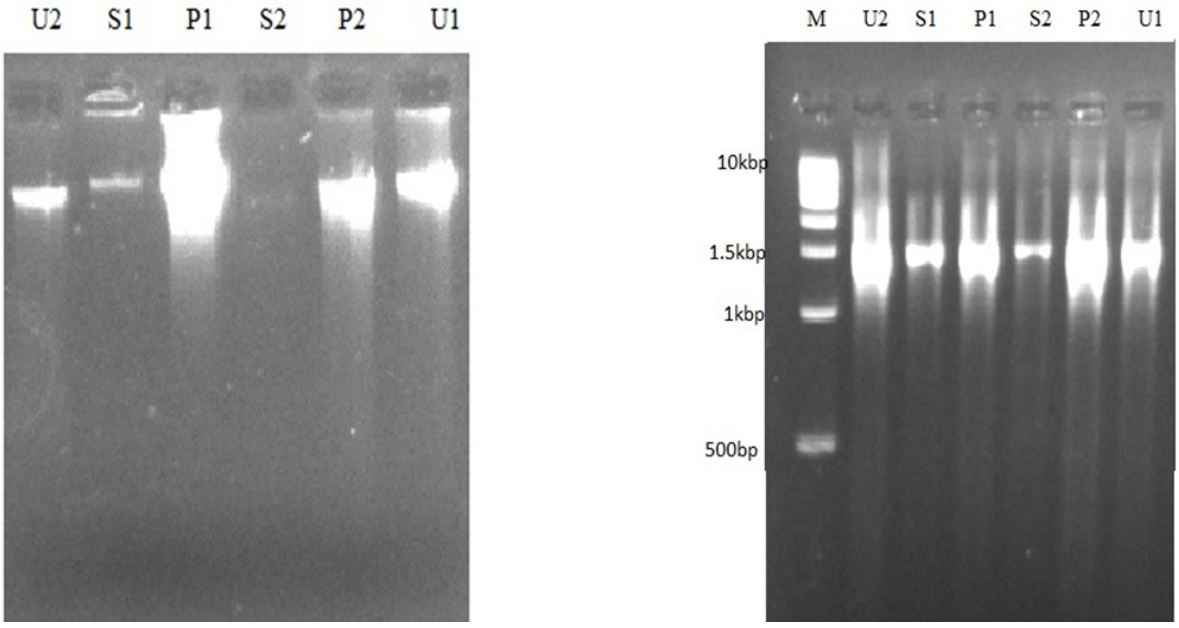

Figure 1: Left shows the gel electrophoresis of the DNA and the PCR amplification of the 16S rRNA gene for the host bacterial isolates U1, U2, S1, S2, P1, and P2, respectively, following DNA isolation. A 1 kb DNA ladder was used to estimate the size of the amplified 16S rRNA gene sequences, which were approximately 1500 bp.

Sequencing

The sequence results (Supporting information, Table S3) enabled the matching and identification of the host bacterial isolates U1, U2, S1, S2, P1, and P2, with pairwise similarities to *K. aerogenes*, *C. freundii, S. enterica, S. enterica, E. coli,* and *E. coli,* respectively.

Table 5: Sequence results

Code	Pairwise Similarity	NBCI Accession Number	Isolate
U1	89.17% *K. aerogenes* strain GB25	MT664185	*K. aerogenes*
U2	89.75% *C. freundii* strain R2A5	MT103108	*C. freundii*
S1	74.91% *S. enterica* strain ZD2	ON661792	*S. enterica*
S2	78.93% *S. enterica* subsp. enterica serovar Brandenburg strain FSIS1607501	CP082464	*S. enterica*
P1	95.82% *E. coli* strain Rizhao_490_1	MN314233	*E. coli*
P2	90.27% *E. coli strain* AF1	ON653022	*E. coli*

U1 has 89.17% pairwise similarity to *K. aerogenes* strain GB25 (NCBI accession MT664185). Details of the isolated sequences are shown in the supporting information.

Evolutionary relationships of taxa

The phylogenetic tree showing the relatedness among the host bacterial isolates U1, U2, S1, S2, P1, and P2 is shown in Figure 2.

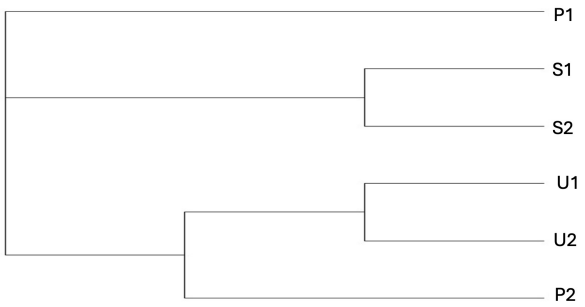

Figure 2: The phylogenetic tree of the host bacteria

Bacteriophage isolation, spot assay, and morphology

The characterization of the phage isolates cocktail showed that the plaques are morphologically small in size on the bacterial lawn (OD_{600nm} = 0.5) relative to the volume spotted (Figure 3) and confirmed the successful isolation of bacteriophage(s) and the presence of at least one strain of bacteriophage with lytic action against the six bacterial isolates.

Of the one hundred (100) initial bacterial isolates subjected to phage hunting, only six (6) coded U1, U2, S1, S2, P1, and P2 showed a positive response, indicated by the presence of plaques, as indicated in Figure 4, from which the different bacteriophages were isolated, purified, propagated, and constituted into a cocktail (PC).

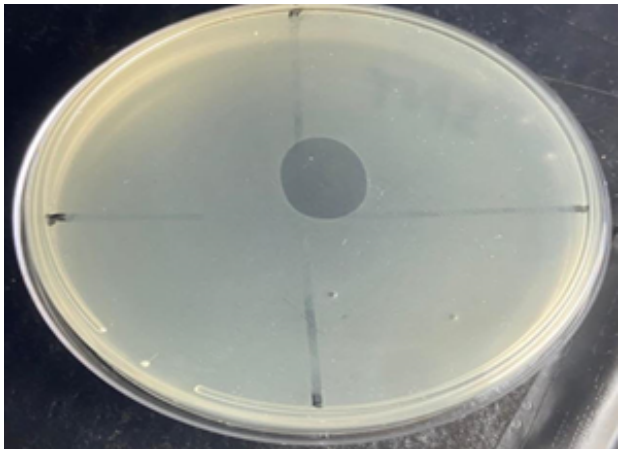

Figure 3: Spot morphology of the PC against the host bacteria.

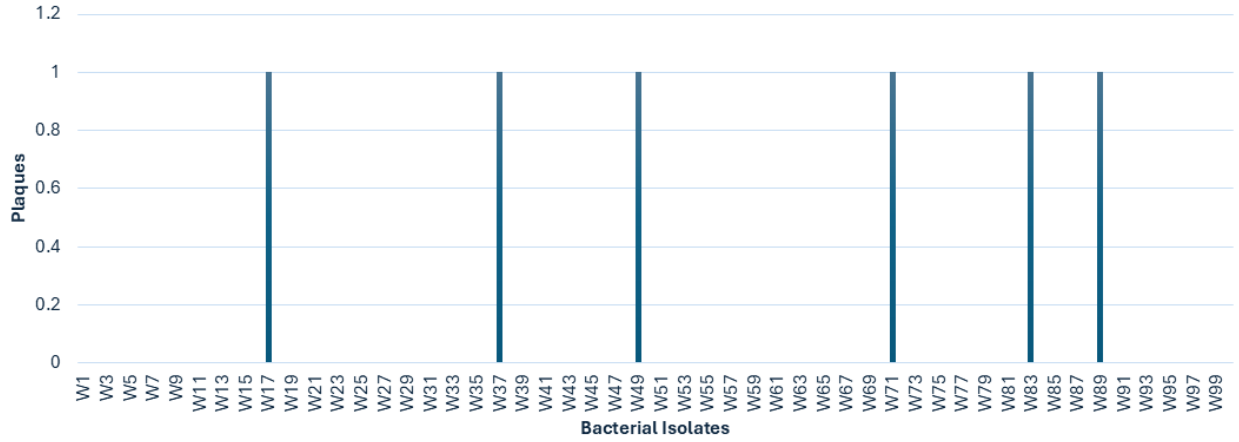

Figure 4: Positive host bacterial isolates yielding bacteriophages. NB: '1' – Presence of plaques; '0' = Absence of plaque; Initial coding of the bacterial samples was 'W and the serial number', thus, for U1, U2, S1, S2, and P1, P2, represents W17, W37, W49, W71, W83, and W89, respectively.

Phage titration

Phage titration results are presented in Tables 6 and 7. For PC, the least dilutions (Table 6) with the presence of plaques were 10^{-6}, 10^{-4}, 10^{-3}, 10^{-4}, 10^{-2}, and $10^{-3,}$ corresponding to U1, U2, S1, S2, P1, and P2, respectively, of which the plaques were counted (shown in Table 7) and used in the mathematical determination of the constituent phage suspension titers (PFU/ml) (Table 7) which corresponded to 4 x 10^7, 4.6 × 10^5, 5.6 × 10^4, 5 × 10^5, 3.6 × 10^3, and 3 × 10^4, respectively. Thus, the results showed that in the PC, the constituent phage concentration of U1 was the highest, followed by S2, U2, S1, P2, and P1.

Table 6: Results of spot test of PC titration dilutions

Isolates	Dilutions									
	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}	10^{-10}
U1	+	+	+	+	+	+	-	-	-	-
U2	+	+	+	+	-	-	-	-	-	-
S1	+	+	+	-	-	-	-	-	-	-
S2	+	+	+	+	-	-	-	-	-	-
P1	+	+	-	-	-	-	-	-	-	-
P2	+	+	+	-	-	-	-	-	-	-

Table 7: Phage titers of the constituent phages

S/N	Isolate	Dilution	No. of Plaques	PFU/mL
1	U1	10^{-6}	20	4×10^7
2	U2	10^{-4}	23	4.6×10^5
3	S1	10^{-3}	28	5.6×10^4
4	S2	10^{-4}	25	5×10^5
5	P1	10^{-2}	18	3.6×10^3
6	P2	10^{-3}	15	3×10^4

Phage specificity/host range

The spot of the PC on all the bacterial lawns of all the 100 bacteria initially isolated from the environmental samples showed an increased host specificity, with an additional five (5) bacterial isolates susceptible to the PC, as represented by the spikes in Figure 5.

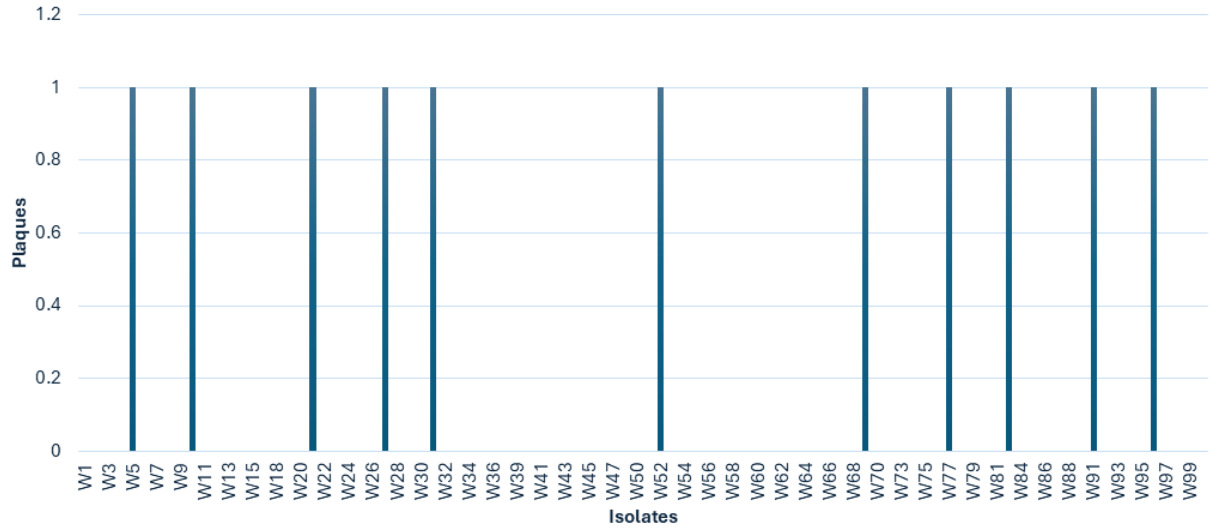

Figure 5: Host range of PC against the 100 environmental bacterial isolates. NB: '1' – Presence of plaques; '0' = Absence of plaque

One Step Growth Curves (OSGC)

The OSGC results are presented in Figure 6. The respective curves of PC against the host bacterial isolates (U1, U2, SI, S2, P2, and P2) used in the determination of the growth parameters and shows for the PC, the burst sizes for the isolates (*K. aerogenes*, *C. freundii*, and two (2) different strains each of *S. enterica*, and *E. coli*) to range from 31 – 62 and the latent periods to range from 15 – 20 min. In details, the burst sizes and latent periods against U1, U2, S1, S2, P1, and P2 were 62 and 15 min, 56 and 15 min, 44 and 20 min, 40 and 20 min, 41 and 20 min, and 39 and 20 min, shown in Figures 6A, B, C, D, E, and F, respectively. With the results, the largest burst size was observed against U1, followed by U2, then S1, S2, P1, and P2, while the latent periods were the same for U1, U2, and S1, S2, P1, and P2

Adsorption Curves

The adsorption curves (Figure 7) were applicable against U1, U2, S1, S2, P1, and P2. The phage cocktail showed a high adsorption rate, with adsorption times ranging from ≈ 9.5 to > 10, with the shortest times recorded for *C. freundii*, followed by *K. aerogenes*, the two *E. coli isolates,* and the two *S. enterica* isolates. Specifically, the adsorption times obtained against U1, U2, S1, S2, P1, and P2 were in the order: ≈ 10, > 10, ≈ 9.5, ≈ 9.5, ≈ 9.75, and ≈ 9.5 min, and shown in Figures 7A, B, C, D, E, and F, respectively.

In vivo wound-healing assay of PC

Phage therapy with the PC showed a significant difference in wound size ($p = 0.0001$). The best activity was, however, observed with the 75% PC, which had a wound diameter of 0.12 ± 0.04 cm on the tenth day, followed by 100, 50, and 25% PC with wound diameters of 0.33 ± 0.03 cm, 0.45 ± 0.04 cm, and 0.70 ± 0.03 cm, respectively. The PCs, except for 25 %, outperformed the negative control (untreated group) by 0.62 ± 0.04 cm on day 10 but fell short of the positive control (ciprofloxacin [25 %]). The recorded diameters (cm) of healing wounds are shown graphically in Figure 8.

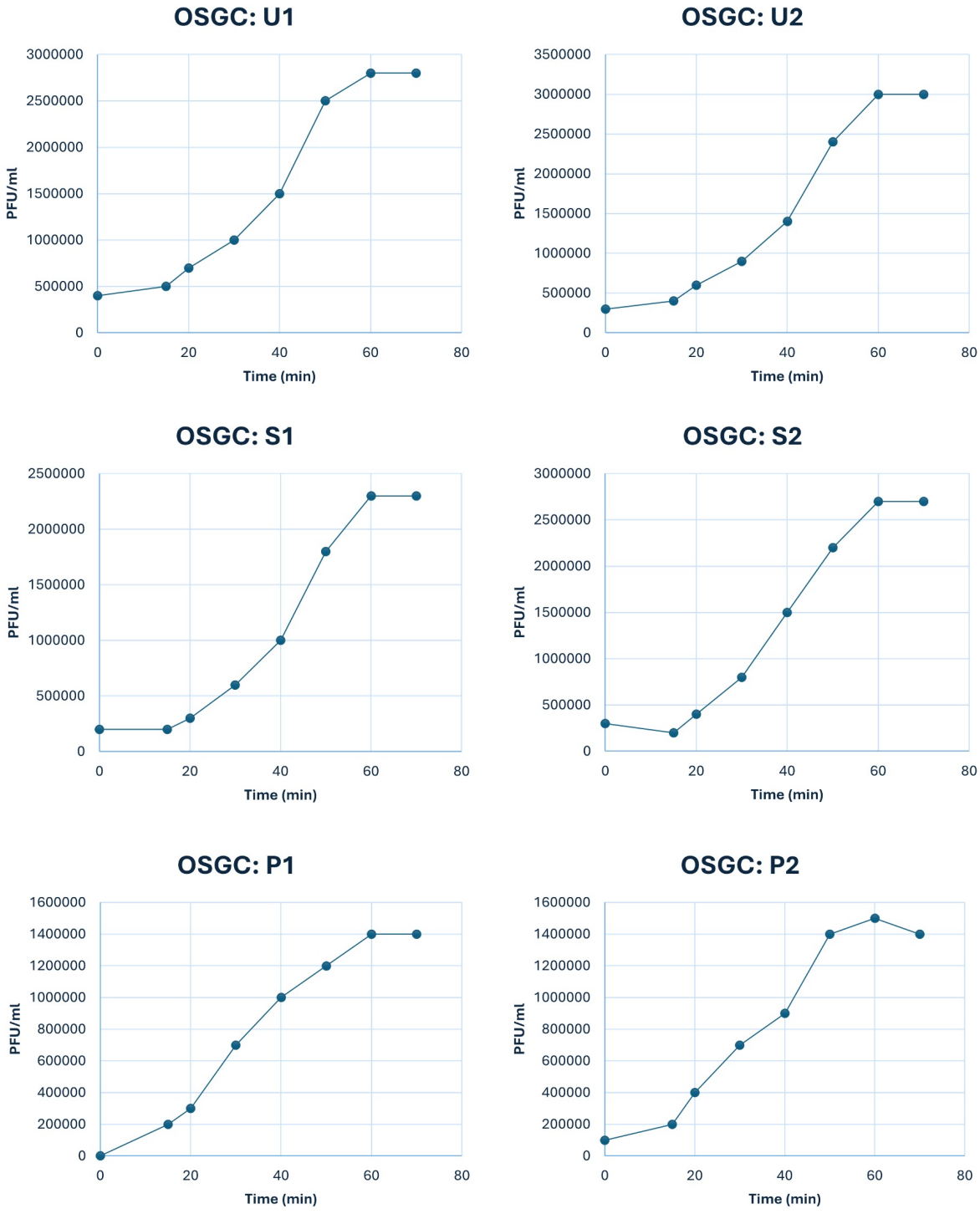

Figure 6: A: One-step growth curves of PC against U1; B: One-step growth curves of PC against U2; C: One-step growth curves of PC against S1; D: One-step growth curves of phage coctail against S2; E: One-step growth curves of PC against P1; F: One-step growth curves of PC against P2

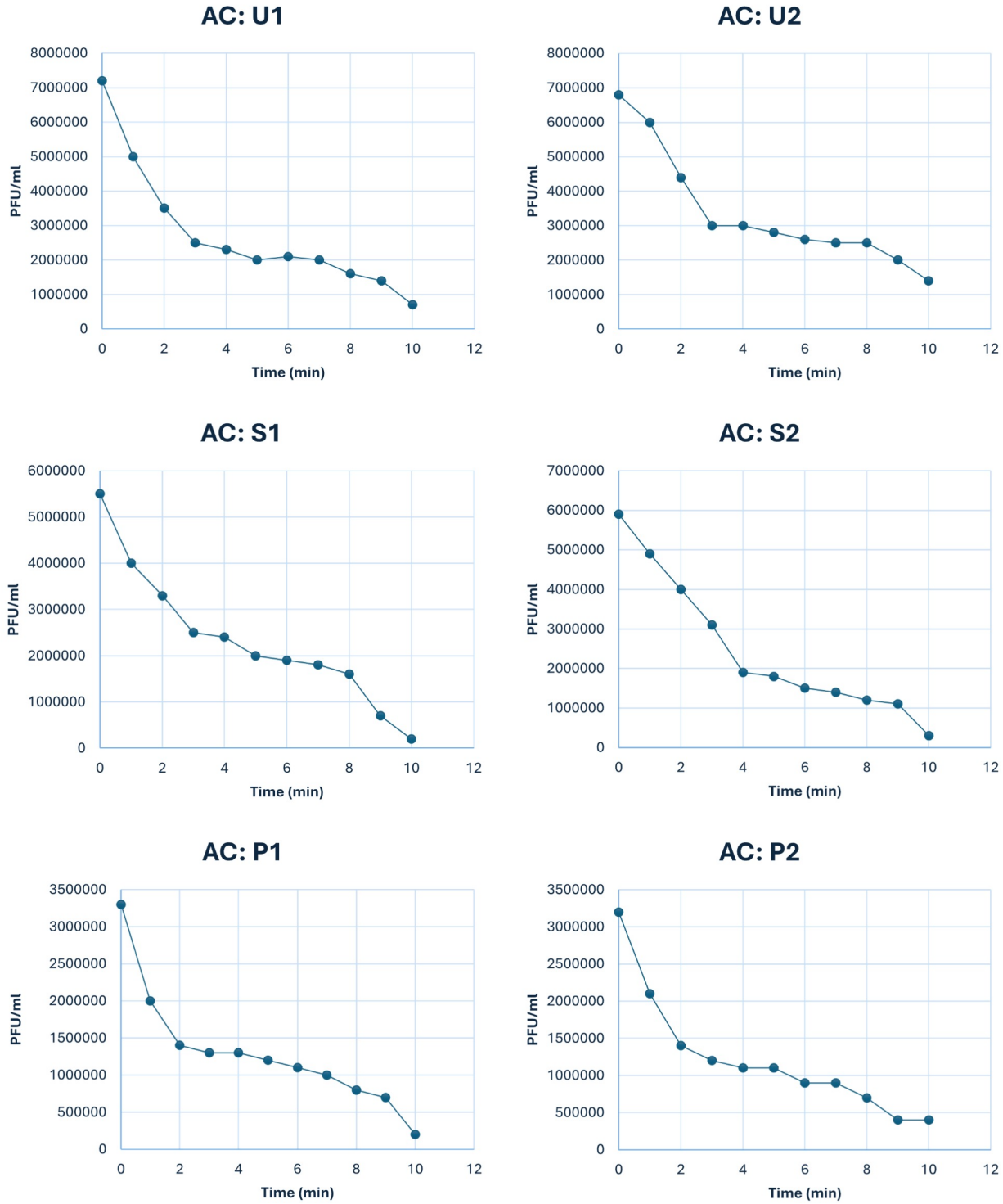

Figure 7: A: Adsorption curve of PC against U1; B: Adsorption curve of PC against U2; C: Adsorption curve of PC against S1; D: Adsorption curve of PC against S2; E: Adsorption curve of PC against P1; F:Adsorption curve of PC against P2.

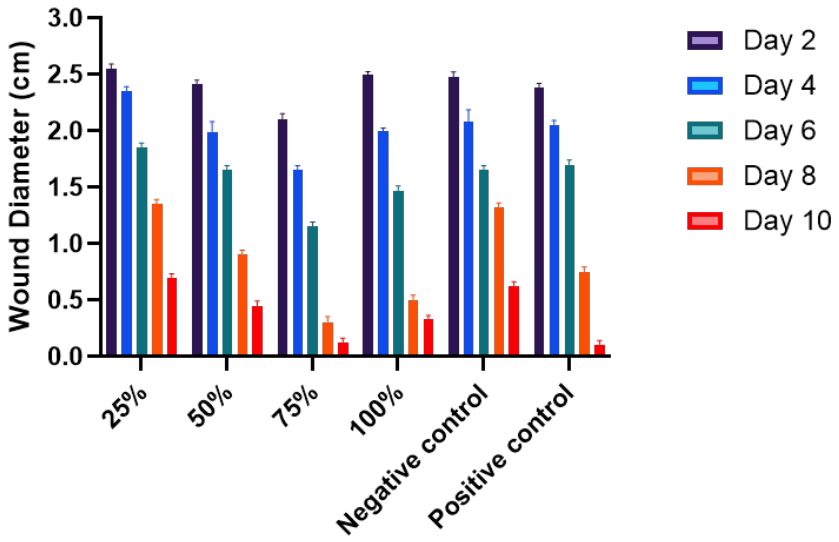

Figure 8: Wound-healing diameters following treatment with the PC. NB: '1' = 25 %, '2' = 50 %, '3' = 75 %, '4' = 100 %, '5' = Negative control, '6' = Positive control.

Discussion

In the era of increasing resistance of microbial pathogens, including enteric bacteria, to conventional antibiotics (25) and associated side effects (49), there is an urgent need for research-driven interventions. Bacteriophages, parasitic viruses of bacteria owing to their desirable features, including specificity and minimal side effects (50), have shown potential for therapeutic use against pathogenic microorganisms, including multi-resistant enteric bacteria (51). The study explored the isolation, identification, and characterization of potent bacteriophages indigenous to and specific for common, prevalent disease-causing enteric pathogenic bacteria. Environmental samples, as recommended in many studies (52), including sewage, swimming pool, abattoir, piggery, fishery, poultry, farmland, and residential wastewater, which are major reservoirs of pathogenic bacteria and bacteriophages, were used to search for bacteriophages targeting *Enterobacteriaceae*. The study's environmental sampling focused on Nsukka, Enugu State, Nigeria, a densely populated community hosting a major public university with over 35,000 students (53). Aside from the university, the community is a major source of many activities that release bacteria into the reservoirs, including farming, domestic waste disposal, and hospital waste disposal. The environmental samples were combined and used for both bacterial and phage isolation to maximise the likelihood of isolating phages from enteric bacteria, given the sensitivity of phage-hunting protocols, which have been shown to harbour enteric pathogen strains (52).

With high MAR indexes, all the isolates have MAR indexes greater than 0.2 (55). The study joins other studies, including those of Kayode *et al*. (56), Beyene *et al*. (57), Shah *et al*. (58), and Duedu *et al*. (59), to expose further the increasing prevalence of resistant enteric bacteria causing common diseases, which invariably increase treatment timelines and cost, morbidity, mortality, pill burden, and side effects and the need for urgent interventions (60,61).

Studies have also identified and profiled bacteriophages against similar isolates prevalent in the gastrointestinal tract across different geographical locations (62). The cocktailing of the study isolated phage particles yielded a PC. Although isolated individually, the PCs increased the lytic spectra, as already mentioned, thus supporting the use of PCs in phage therapy, which would enable the effective management of multi-bacterial-implicated diseases (63,64). The result could be attributed to the synergistic lytic activities of the

constituent phages therein in the PC. The submission is also backed by the reports of Lu *et al*. (65), who suggest that using phages with different infection strategies and host specificities in a cocktail can better suppress the development of phage resistance in a target bacterium.

The bacteriophage titers from the study using the PC against different bacterial isolates showed that the concentration of lytic phage particles in the phage suspension (66) varied across the isolates. However, if the PC contains a single phage strain, it reveals the infection features of the respective bacterial isolate, as shown by the different characterization results. This assertion is scientifically correct, as each bacterium's inherent physical and biological attributes, including cell wall composition, defence mechanisms, and growth cycles, can influence its response to the bacteriophage(s) (67–70).

The one-step growth curve of phages enabled the determination of their growth parameters, including burst sizes, latent periods, intracellular accumulation, and eclipse periods. It provided insights into the phage life cycle, infection dynamics, and phage replication efficiency (71). While the burst size indicates the average number of particles released per successful infection of a bacterial host and the release of daughter progeny cells (72), the latent period is the time required for the release of the daughter cells (72). The study results, compared with some studies, are abundant. Abdelsattar *et al*. (73) reported a burst ofsize as 20 phage progenies and a latent period of 10 min. Royam and Nachimuthu (74) reported two phages (*Citrophage* MRM19 and *Citrophage* MRM57) with burst sizes of 110 ± 20 and 50 ± 5, respectively, and latent periods of 25 ± 3 and 25 ± 2 min, both against Citrobacter sp. Rahimzadeh *et al*. (75), with *E. coli,* showed a burst size of 1,200 with a latent period of 20 min. Alexyuk *et al*. (76) profiled six lytic *E. coli* phages with burst sizes ranging from 117 to 155 and latent periods ranging from 20 to 30 min. Fang & Zong (77) reported a phage, P13, against a carbapenem-resistant K. pneumoniae, with burst sizes and latent periods of 167 and 20 min, respectively. Pertics *et al*. (78) against *K. pneumoniae* 52145, a capsule-reported a defective mutant of a phage with a burst size and latent period of ≈1000 and 10 min, respectively.

The AC enables estimation of phage adsorption times and rates, implying that higher host density can be considered equivalent to a phage with a higher adsorption rate, and vice versa (79). The results of our study are comparable to those of relevant studies, including Kim *et al*. (80), who reported a phage with an adsorption time of 10 min against *Salmonella* sp. Abdelsattar *et al*. (73), with Bacteriophage ZCSE9, presented 20 min against *S. enterica*. Royam and Nachimuthu (74) reported adsorption times of 18 ± 1 and 15 ± 1 min with the two phages (Citrophage MRM19 and Citrophage MRM57) against Citrobacter sp. Alexyuk *et al*. (76), with the six (6) lytic *E. coli* phages, had high adsorption rates, killing 90 % of the bacterial population with adsorption times ranging from 8 to 10 min

In furtherance of the study, the presence of the respective bacterial isolates on CWs, especially Klebsiella spp., and the use of phage therapy in a wound-healing model in experimental animals further support the use of phages in disease management (51). Although limited by the evaluation of potential skin toxicities associated with PC application, the study revealed a relatively dose-dependent healing and closure of the CWs. Also, there was a significant difference ($p < 0.05$) in wound diameter among the animal groups across the treatment period. The superior performance of the 75 % cocktail to the 100 % cocktail likely reflects documented biological and ecological phenomena in phage therapy (81–83). Specifically, high phage concentrations can occasionally lead to lysis-from-without at very high MOI, causing rapid bacterial killing but also transient release of cell debris that may exacerbate local inflammation; density-dependent inhibition, where high phage densities interfere with each other's adsorption efficiency, phage–phage competition or interference, especially in multi-PCs; host immune activation, since high phage loads can increase phagocytic clearance; and reduced diffusion of highly concentrated preparations in wound exudate (81–83). The pharmacodynamics of fluoroquinolones, which have a slower onset but prolonged tissue activity, as well as docu-

mented anti-inflammatory effects during the remodelling phase of wound repair, can explain the 75% PC, which demonstrated superior early wound contraction compared to ciprofloxacin, which eventually produced better final wound closure (84,85). After the initial rapid bacterial reduction by phages, phage titers naturally decline due to immune clearance and reduced host abundance, whereas ciprofloxacin remains active throughout (85,86). The *in vivo* therapeutic application of phages in wound healing has been reported, including Kumari *et al*. (87) who reported a single dose administration of phage Kpn5 (at MOI of 200), mixed with hydrogel, with a significant ($p < 0.0001$) reduction in mortality in *K. pneumoniae* B5055 infected mice burn wound and also outperforming the healing activities of 0.5 % silver nitrate and 1000 mg/L gentamicin. Similar to this study, the use of a PC in the treatment of wounds was reported by Kheljian *et al*. (88) and showed a two phage (PA19 and PB10) constituent cocktail, with respective final concentrations of 4×10^7 and 3×10^7 PFU/ml, and loaded in hydrogel effectively treating a *Pseudomonas aeruginosa* implicated infection and also had better activity in the reduction of the bacterial load than using only the control antibiotics ciprofloxacin (50 µg/mL), however, had best activity in combination with the antibiotic.Reflecting on the study results, it is important to acknowledge both the promise and the limitations of the identified bacteriophages. While the recovery of lytic phages active against six clinically relevant, drug-resistant bacterial species underscores the potential value of local phage discovery, there are clear constraints that shape how these phages may ultimately be used against antimicrobial resistance. Phages naturally possess narrow host ranges, can be rapidly cleared by the immune system, and may allow the emergence of phage-resistant bacterial variants; factors that influence their performance across different infection settings. Similarly, therapeutic applications of these phages can extend to different conditions, including wound infections, urinary tract infections, device-associated infections, and environmental or surface decontamination, depending on host specificity and stability. Also, the methodological limitations of our isolation and screening approach are recognised. Enrichment-based recovery may bias the process toward more aggressive or faster-replicating phages; using single-host strains may underestimate true diversity; and plaque morphology alone cannot substitute for full genomic confirmation.

Conclusion

The study identified and isolated indigenous polyvalent bacteriophages with lytic activity against six proven multi-resistant enteric bacterial isolates, including strains of *K. aerogenes*, *C. freundii*, two *S. enterica*, and two *E. coli.* Characterization of the polyvalent phages revealed the presence of host-specific phages with varying titers, adsorption rates, latent periods, and burst sizes. Phage therapy demonstrated the efficacy of lytic phages against resistant isolates; thus, the study supports the potential use of phages as alternatives to antibiotics, based on the in vivo results. Owing to study limitations, particularly funding, the study lays the foundation for advancing the field. Such advancements can include the thorough *in vivo* and *ex vivo* preclinical and clinical evaluation of phage particles using appropriate models, including determination of safety and toxicological properties, the possible potentiation of therapeutic effects in combination with other therapeutics, including antibiotics and other natural products, and the proper isolation, production, elucidation, and profiling of the expressed phage protein as observed in the study, with the aim of possible patenting, mainstreaming, and commercialization. The study successfully unraveled lytic indigenous bacteriophages sourced from various wastewater samples in Nigeria, with antibacterial activity against common pathogenic Enterobacteriaceae in plants and animals, and provides unique therapeutic options against local multi-resistant bacterial strains. Additionally, the study demonstrates the efficacy of a PC in promoting wound healing in an animal model, supporting the therapeutic application of phages in clinical settings and lending further support for the search for alternative therapies against multi-resistant bacterial pathogens amidst the current wave of antibiotic misuse and abuse and the corresponding drug resistance leading to increasing treatment failures, morbidity, and mortality, thus advocating the use of phage therapy.

Author Contributions: Ebele B. Onuigbo: Conceptualisation, Supervision. Stephen C. Emencheta: Methodology, Data curation, Analysis, Writing-Original draft preparation. Chinonye J. Eneovoa: Data curation, Emmanuel
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Writing- Reviewing and Editing, Supervision.

Ethical approval: Ethical approval (Number: FPSRA/UNN/23/0052) for the use of animal subjects was duly obtained from the Faculty Research Ethics Committee, Faculty of Pharmaceutical Sciences, University of Nigeria, Nsukka. All stated guidelines were followed throughout the study.

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Conflicts of Interest: The authors declare that they have no known competing financial interests or personal relationships to influence the work reported in this paper.

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References

1. Atif M, Asghar S, Mushtaq I, Malik I, Amin A, Babar ZUD, et al. What drives inappropriate use of antibiotics? A mixed methods study from Bahawalpur, Pakistan. Infect Drug Resist. 2019 Mar 26;12:687–99.
2. Bennadi D. Self-medication: A current challenge. J Basic Clin Pharm. 2013 Dec;5(1):19–23.
3. Garofalo L, Di Giuseppe G, Angelillo IF. Self-Medication Practices among Parents in Italy. BioMed Res Int. 2015 Jan 20;2015:e580650.
4. Ocan M, Obuku EA, Bwanga F, Akena D, Richard S, Ogwal-Okeng J, et al. Household antimicrobial self-medication: a systematic review and meta-analysis of the burden, risk factors and outcomes in developing countries. BMC Public Health. 2015 Aug 1;15(1):742.
5. Kiani AK, Anpilogov K, Dhuli K, Paolacci S, Benedetti S, Manara E, et al. Naturally-occurring and cultured bacteriophages in human therapy. Eur Rev Med Pharmacol Sci. 2021 Dec;25(1 Suppl):101–7.
6. Zhan Y, Buchan A, Chen F. Novel N4 Bacteriophages Prevail in the Cold Biosphere. Schaffner DW, editor. Appl Environ Microbiol. 2015 Aug;81(15):5196–202.
7. Rogovski P, Cadamuro RD, Da Silva R, De Souza EB, Bonatto C, Viancelli A, et al. Uses of Bacteriophages as Bacterial Control Tools and Environmental Safety Indicators. Front Microbiol. 2021 Nov 30;12:793135.
8. Artawinata PC, Lorraine S, Waturangi DE. Isolation and characterization of bacteriophages from soil against food spoilage and foodborne pathogenic bacteria. Sci Rep. 2023 June 7;13(1):9282.
9. Ballesté E, Blanch AR, Muniesa M, García-Aljaro C, Rodríguez-Rubio L, Martín-Díaz J, et al. Bacteriophages in sewage: abundance, roles, and applications. FEMS Microbes. 2022 Apr 4;3:xtac009.
10. Shende RK, Hirpurkar SD, Sannat C, Rawat N, Pandey V. Isolation and characterization of bacteriophages with lytic activity against common bacterial pathogens. Vet World. 2017 Aug;10(8):973–8.
11. Gummalla VS, Zhang Y, Liao YT, Wu VCH. The Role of Temperate Phages in Bacterial Pathogenicity. Microorganisms. 2023 Feb 21;11(3):541.

12. Pedersen M, Neergaard JT, Cassias J, Rasmussen KK, Lo Leggio L, Sneppen K, et al. Repression of the lysogenic PR promoter in bacteriophage TP901-1 through binding of a CI-MOR complex to a composite OM-OR operator. Sci Rep. 2020 May 26;10(1):8659.
13. Little JW. Lysogeny, Prophage Induction, and Lysogenic Conversion. In: Phages [Internet]. John Wiley & Sons, Ltd; 2005 [cited 2023 July 1]. p. 37–54. Available from: https://onlinelibrary.wiley.com/doi/abs/10.1128/9781555816506.ch3
14. Thomason LC, Schiltz CJ, Court C, Hosford CJ, Adams MC, Chappie JS, et al. Bacteriophage λ RexA and RexB functions assist the transition from lysogeny to lytic growth. Mol Microbiol. 2021 Oct;116(4):1044–63.
15. Cisek AA, Dąbrowska I, Gregorczyk KP, Wyżewski Z. Phage Therapy in Bacterial Infections Treatment: One Hundred Years After the Discovery of Bacteriophages. Curr Microbiol. 2017 Feb 1;74(2):277–83.
16. Lin DM, Koskella B, Lin HC. Phage therapy: An alternative to antibiotics in the age of multi-drug resistance. World J Gastrointest Pharmacol Ther. 2017 Aug 6;8(3):162–73.
17. Brives C, Pourraz J. Phage therapy as a potential solution in the fight against AMR: obstacles and possible futures. Palgrave Commun. 2020 May 19;6(1):1–11.
18. Samir S. Bacteriophages as Therapeutic Agents: Alternatives to Antibiotics. Recent Pat Biotechnol. 2021;15(1):25–33.
19. Bailey AM, Baum RA, Horn K, Lewis T, Morizio K, Schultz A, et al. Review of Intranasally Administered Medications for Use in the Emergency Department. J Emerg Med. 2017 July;53(1):38–48.
20. Ryan EM, Gorman SP, Donnelly RF, Gilmore BF. Recent advances in bacteriophage therapy: how delivery routes, formulation, concentration and timing influence the success of phage therapy. J Pharm Pharmacol. 2011 Oct;63(10):1253–64.
21. Speck P, Smithyman A. Safety and efficacy of phage therapy via the intravenous route. FEMS Microbiol Lett. 2016 Feb;363(3):fnv242.
22. Łusiak-Szelachowska M, Międzybrodzki R, Drulis-Kawa Z, Cater K, Knežević P, Winogradow C, et al. Bacteriophages and antibiotic interactions in clinical practice: what we have learned so far. J Biomed Sci. 2022 Mar 30;29(1):23.
23. Centers for Disease Control and Prevention (U.S.). Antibiotic resistance threats in the United States, 2019 [Internet]. Centers for Disease Control and Prevention (U.S.); 2019 Nov [cited 2023 July 1]. Available from: https://stacks.cdc.gov/view/cdc/82532
24. Laxminarayan R, Duse A, Wattal C, Zaidi AKM, Wertheim HFL, Sumpradit N, et al. Antibiotic resistance—the need for global solutions. Lancet Infect Dis. 2013 Dec 1;13(12):1057–98.
25. Wallace MJ, Fishbein SRS, Dantas G. Antimicrobial resistance in enteric bacteria: current state and next-generation solutions. Gut Microbes. 2020 Nov 9;12(1):1799654.
26. Karaliute I, Ramonaite R, Bernatoniene J, Petrikaite V, Misiunas A, Denkovskiene E, et al. Reduction of gastrointestinal tract colonization by Klebsiella quasipneumoniae using antimicrobial protein KvarIa. Gut Pathog. 2022 Apr 26;14(1):17.
27. Lindstedt K, Buczek D, Pedersen T, Hjerde E, Raffelsberger N, Suzuki Y, et al. Detection of Klebsiella pneumoniae human gut carriage: a comparison of culture, qPCR, and whole metagenomic sequencing methods. Gut Microbes. 14(1):2118500.
28. Liu H, Hu Z, Li M, Yang Y, Lu S, Rao X. Therapeutic potential of bacteriophage endolysins for infections caused by Gram-positive bacteria. J Biomed Sci. 2023 Apr 26;30(1):29.
29. Özal D, Arndt A, Thomé M. Bacteriophages and related endolysins for reduction of microorganisms in the human body - a systematic review. GMS Hyg Infect Control. 2022;17:Doc01.
30. Xu Y, Liu Y, Liu Y, Pei J, Yao S, Cheng C. Bacteriophage therapy against Enterobacteriaceae. Virol Sin. 2015 Feb 3;30(1):11–8.

31. Guentzel MN. Escherichia, Klebsiella, Enterobacter, Serratia, Citrobacter, and Proteus. In: Baron S, editor. Medical Microbiology [Internet]. 4th edn Galveston (TX): University of Texas Medical Branch at Galveston; 1996 [cited 2023 July 1]. Available from: http://www.ncbi.nlm.nih.gov/books/NBK8035/
32. Bassetti D, Bassetti M, Mantero E. Strategies for antibiotic selection in empirical therapy. Clin Microbiol Infect. 2000 Jan 1;6:98–100.
33. Leekha S, Terrell CL, Edson RS. General Principles of Antimicrobial Therapy. Mayo Clin Proc. 2011 Feb;86(2):156–67.
34. Han R, Teng M, Zhang Y, Zhang T, Wang T, Chen J, et al. Choosing Optimal Antibiotics for the Treatment of Patients Infected With Enterobacteriaceae: A Network Meta-analysis and Cost-Effectiveness Analysis. Front Pharmacol. 2021 June 17;12:656790.
35. Minh NNQ, Toi PV, Qui LM, Tinh LBB, Ngoc NT, Kim LTN, et al. Antibiotic use and prescription and its effects on Enterobacteriaceae in the gut in children with mild respiratory infections in Ho Chi Minh City, Vietnam. A prospective observational outpatient study. PLOS ONE. 2020 Nov 4;15(11):e0241760.
36. Pandey N, Cascella M. Beta-Lactam Antibiotics. In: StatPearls [Internet]. Treasure Island (FL): StatPearls Publishing; 2023 [cited 2023 July 1]. Available from: http://www.ncbi.nlm.nih.gov/books/NBK545311/
37. Reygaert WC. An overview of the antimicrobial resistance mechanisms of bacteria. AIMS Microbiol. 2018 June 26;4(3):482–501.
38. Glonti T, Pirnay J paul. In Vitro Techniques and Measurements of Phage Characteristics That Are Important for Phage Therapy Success. 2022;
39. Clinical & Laboratory Standards Institute [Internet]. [cited 2023 June 25]. M100Ed33 | Performance Standards for Antimicrobial Susceptibility Testing, 33rd Edition. Available from: https://clsi.org/standards/products/microbiology/documents/m100/
40. Arbab S, Ullah H, Wei X, Wang W, Ahmad SU, Zhang J. Drug resistance and susceptibility testing of Gram negative bacterial isolates from healthy cattle with different β – Lactam resistance Phenotypes from Shandong province China. Braz J Biol. 2021 Aug 27;83:e247061.
41. Saitou N, Nei M. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol Biol Evol. 1987 July 1;4(4):406–25.
42. Tamura K, Nei M, Kumar S. Prospects for inferring very large phylogenies by using the neighbor-joining method. Proc Natl Acad Sci U S A. 2004 July 27;101(30):11030–5.
43. Kumar S, Stecher G, Li M, Knyaz C, Tamura K. MEGA X: Molecular Evolutionary Genetics Analysis across Computing Platforms. Mol Biol Evol. 2018 June;35(6):1547–9.
44. Pelzek AJ, Schuch R, Schmitz JE, Fischetti VA. Isolation of Bacteriophages from Environmental Sources, and Creation and Functional Screening of Phage DNA Libraries. Curr Protoc Essent Lab Tech. 2013;7(1):13.3.1-13.3.35.
45. Pereira AO, Barros NMA, Guerrero BR, Emencheta SC, Baldo DÂ, Oliveira Jr. JM, et al. An Edible Biopolymeric Microcapsular Wrapping Integrating Lytic Bacteriophage Particles for Salmonella enterica: Potential for Integration into Poultry Feed. Antibiotics. 2023 June;12(6):988.
46. Balcão VM, Belline BG, Silva EC, Almeida PFFB, Baldo DÂ, Amorim LRP, et al. Isolation and Molecular Characterization of Two Novel Lytic Bacteriophages for the Biocontrol of Escherichia coli in Uterine Infections: In Vitro and Ex Vivo Preliminary Studies in Veterinary Medicine. Pharmaceutics. 2022 Oct 30;14(11):2344.
47. Harada LK, Silva EC, Rossi FP, Cieza B, Oliveira TJ, Pereira C, et al. Characterization and in vitro testing of newly isolated lytic bacteriophages for the biocontrol of Pseudomonas aeruginosa. Future Microbiol. 2022 Jan;17:111–41.
48. National Research Council (US) Committee for the Update of the Guide for theCare and Use of Laboratory Animals. Guide for the Care and Use of Laboratory Animals [Internet]. 8th edn. Washington (DC): National Academies Press (US); 2011 [cited 2023 June 26]. (The National Academies Collection:

Reports funded by National Institutes of Health). Available from: http://www.ncbi.nlm.nih.gov/books/NBK54050/
49. Pancu DF, Scurtu A, Macasoi IG, Marti D, Mioc M, Soica C, et al. Antibiotics: Conventional Therapy and Natural Compounds with Antibacterial Activity—A Pharmaco-Toxicological Screening. Antibiotics. 2021 Apr 7;10(4):401.
50. Koskella B, Meaden S. Understanding Bacteriophage Specificity in Natural Microbial Communities. Viruses. 2013 Mar 11;5(3):806–23.
51. Šivec K, Podgornik A. Determination of bacteriophage growth parameters under cultivating conditions. Appl Microbiol Biotechnol. 2020 Oct 1;104(20):8949–60.
52. Ács N, Gambino M, Brøndsted L. Bacteriophage Enumeration and Detection Methods. Front Microbiol. 2020 Oct 23;11:594868.
53. Nsukka. In: Wikipedia [Internet]. 2023 [cited 2023 July 20]. Available from: https://en.wikipedia.org/w/index.php?title=Nsukka&oldid=1164470260
54. University of Nigeria Nsukka | World University Rankings | THE [Internet]. [cited 2023 July 20]. Available from: https://www.timeshighereducation.com/world-university-rankings/university-nigeria-nsukka
55. Titilawo Y, Sibanda T, Obi L, Okoh A. Multiple antibiotic resistance indexing of Escherichia coli to identify high-risk sources of faecal contamination of water. Environ Sci Pollut Res. 2015 July 1;22(14):10969–80.
56. Kayode A, Okunrounmu P, Olagbende A, Adedokun O, Hassan AW, Atilola G. High prevalence of multiple drug resistant enteric bacteria: Evidence from a teaching hospital in Southwest Nigeria. J Infect Public Health. 2020 Apr 1;13(4):651–6.
57. Beyene AM, Gezachew M, Mengesha D, Yousef A, Gelaw B. Prevalence and drug resistance patterns of Gram-negative enteric bacterial pathogens from diarrheic patients in Ethiopia: A systematic review and meta-analysis. PLOS ONE. 2022 Mar 16;17(3):e0265271.
58. Shah M, Kathiiko C, Wada A, Odoyo E, Bundi M, Miringu G, et al. Prevalence, seasonal variation, and antibiotic resistance pattern of enteric bacterial pathogens among hospitalized diarrheic children in suburban regions of central Kenya. Trop Med Health. 2016 Nov 29;44(1):39.
59. Duedu KO, Offei G, Codjoe FS, Donkor ES. Multidrug Resistant Enteric Bacterial Pathogens in a Psychiatric Hospital in Ghana: Implications for Control of Nosocomial Infections. Int J Microbiol. 2017 Sept 6;2017:e9509087.
60. Infections I of M (US) F on E, Knobler SL, Lemon SM, Najafi M, Burroughs T. Factors Contributing to the Emergence of Resistance. In: The Resistance Phenomenon in Microbes and Infectious Disease Vectors: Implications for Human Health and Strategies for Containment: Workshop Summary [Internet]. National Academies Press (US); 2003 [cited 2023 July 21]. Available from: https://www.ncbi.nlm.nih.gov/books/NBK97126/
61. van Duin D, Paterson D. Multidrug Resistant Bacteria in the Community: Trends and Lessons Learned. Infect Dis Clin North Am. 2016 June;30(2):377–90.
62. Śliwka P, Ochocka M, Skaradzińska A. Applications of bacteriophages against intracellular bacteria. Crit Rev Microbiol. 2022 Mar 4;48(2):222–39.
63. Abedon ST, Danis-Wlodarczyk KM, Wozniak DJ. Phage Cocktail Development for Bacteriophage Therapy: Toward Improving Spectrum of Activity Breadth and Depth. Pharmaceuticals. 2021 Oct 3;14(10):1019.
64. Teklemariam AD, Al Hindi R, Qadri I, Alharbi MG, Hashem AM, Alrefaei AA, et al. Phage cocktails - an emerging approach for the control of bacterial infection with major emphasis on foodborne pathogens. Biotechnol Genet Eng Rev. 2023 Mar 17;1–29.
65. Lu H, Li Z, Elbaz A, Ni SQ. Synergistic action of phages and lytic proteins with antibiotics: a combination strategy to target bacteria and biofilms. BMC Microbiol. 2023 May 23;23(1):149.

66. Anderson B, Rashid MH, Carter C, Pasternack G, Rajanna C, Revazishvili T, et al. Enumeration of bacteriophage particles. Bacteriophage. 2011;1(2):86–93.
67. Daubie V, Chalhoub H, Blasdel B, Dahma H, Merabishvili M, Glonti T, et al. Determination of phage susceptibility as a clinical diagnostic tool: A routine perspective. Front Cell Infect Microbiol. 2022 Sept 21;12:1000721.
68. Ongenae V, Briegel A, Claessen D. Cell wall deficiency as an escape mechanism from phage infection. Open Biol. 2021 Sept;11(9):210199.
69. Ongenae V, Mabrouk AS, Crooijmans M, Rozen D, Briegel A, Claessen D. Reversible bacteriophage resistance by shedding the bacterial cell wall. Open Biol. 2022 June;12(6):210379.
70. Zou X, Xiao X, Mo Z, Ge Y, Jiang X, Huang R, et al. Systematic strategies for developing phage resistant Escherichia coli strains. Nat Commun. 2022 Aug 2;13(1):4491.
71. Kropinski AM. Practical Advice on the One-Step Growth Curve. Methods Mol Biol Clifton NJ. 2018;1681:41–7.
72. Abedon ST, Herschler TD, Stopar D. Bacteriophage Latent-Period Evolution as a Response to Resource Availability. Appl Environ Microbiol. 2001 Sept;67(9):4233–41.
73. Abdelsattar AS, Eita MA, Hammouda ZK, Gouda SM, Hakim TA, Yakoup AY, et al. The Lytic Activity of Bacteriophage ZCSE9 against Salmonella enterica and Its Synergistic Effects with Kanamycin. Viruses. 2023 Apr;15(4):912.
74. Madurantakam Royam M, Nachimuthu R. Isolation, characterization, and efficacy of bacteriophages isolated against Citrobacter spp. an in vivo approach in a zebrafish model (Danio rerio). Res Microbiol. 2020 Dec 1;171(8):341–50.
75. Rahimzadeh G, Resch G, Rezai MS, Nemati Hevelaee E. Characterization and Lytic Activity of Isolated Escherichia coli Bacteriophages against Escherichia coli in vitro. Iran J Med Sci. 2020 July;45(4):298–303.
76. Alexyuk P, Bogoyavlenskiy A, Alexyuk M, Akanova K, Moldakhanov Y, Berezin V. Isolation and Characterization of Lytic Bacteriophages Active against Clinical Strains of E. coli and Development of a Phage Antimicrobial Cocktail. Viruses. 2022 Nov;14(11):2381.
77. Fang Q, Zong Z. Lytic Phages against ST11 K47 Carbapenem-Resistant Klebsiella pneumoniae and the Corresponding Phage Resistance Mechanisms. mSphere. 7(2):e00080-22.
78. Pertics BZ, Kovács T, Schneider G. Characterization of a Lytic Bacteriophage and Demonstration of Its Combined Lytic Effect with a K2 Depolymerase on the Hypervirulent Klebsiella pneumoniae Strain 52145. Microorganisms. 2023 Mar;11(3):669.
79. Shao Y, Wang IN. Bacteriophage Adsorption Rate and Optimal Lysis Time. Genetics. 2008 Sept 1;180(1):471–82.
80. Kim S, Kim SH, Rahman M, Kim J. Characterization of a Salmonella Enteritidis bacteriophage showing broad lytic activity against Gram-negative enteric bacteria. J Microbiol. 2018 Dec 1;56(12):917–25.
81. Abedon ST. Lysis from without. Bacteriophage. 2011 Jan;1(1):46–9.
82. Chan BK, Abedon ST, Loc-Carrillo C. Phage cocktails and the future of phage therapy. Future Microbiol. 2013 June;8(6):769–83.
83. Oechslin F. Resistance Development to Bacteriophages Occurring during Bacteriophage Therapy. Viruses. 2018 July;10(7):351.
84. Chegini Z, Azizi M, Safaiee M, Kalhori F, Zare Shahraki R, Hemmati J, et al. Wound healing properties and antibiofilm activity of hydrogel matrix containing nitric oxide, silver nanoparticles, and ciprofloxacin against Pseudomonas aeruginosa burn wound infection. Sci Rep. 2025 July 21;15(1):26508.
85. Echterhof A, Dharmaraj T, Khosravi A, McBride R, Miesel L, Chia JH, et al. The contribution of neutrophils to bacteriophage clearance and pharmacokinetics in vivo. JCI Insight [Internet]. 2024 Oct 22 [cited 2025 Dec 7];9(20). Available from: https://insight.jci.org/articles/view/181309?utm_source=chatgpt.com

86. Peng H, Rossetto D, Mansy SS, Jordan MC, Roos KP, Chen IA. Treatment of Wound Infections in a Mouse Model Using Zn2+-Releasing Phage Bound to Gold Nanorods. ACS Nano. 2022 Mar 22;16(3):4756–74.
87. Kumari S, Harjai K, Chhibber S. Bacteriophage versus antimicrobial agents for the treatment of murine burn wound infection caused by Klebsiella pneumoniae B5055. J Med Microbiol. 2011;60(2):205–10.
88. Shafigh Kheljan F, Sheikhzadeh Hesari F, Aminifazl MS, Skurnik M, Gholadze S, Zarrini G. Design of Phage-Cocktail–Containing Hydrogel for the Treatment of Pseudomonas aeruginosa–Infected Wounds. Viruses. 2023 Mar;15(3):803.