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ORIGINAL ARTICLE

Lytic Bacteriophage as an Alternative to Antibiotics for Controlling Multidrug-Resistant Bacteria Isolated from Human, Animal, and Environmental Samples in Tanzania

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ABSTRACT

Background: The rise of multidrug-resistant bacteria poses a significant global health threat, outpacing the development of new antibiotics and potentially reverting medical practices to a pre-antibiotic era. Despite advancements in antibiotic therapies for various diseases, rising antibiotic resistance rates have shifted focus towards alternative treatments, including the use of bacteriophages, capable of targeting antibiotic-resistant bacteria. Therefore, this study aimed to investigate the use of lytic bacteriophages as an alternative to antibiotics in controlling bacteria isolated from clinical and environmental samples in Moshi, Kilimanjaro, Tanzania.

Methodology: A cross-sectional study was conducted from May to July 2023, involving clinical and environmental components. Clinical bacterial isolates were obtained from Kilimanjaro Christian Medical Centre (KCMC), while environmental bacteria were collected from soil, animal waste, and wastewater samples. Standard microbiological methods were used for bacterial isolation, identification, and antimicrobial susceptibility testing. Bacteriophages were isolated from wastewater samples using enrichment techniques, and their lytic activity was evaluated against bacterial isolates by spot assays. Data were analysed using descriptive statistics to summarise the distribution of bacterial isolates and bacteriophage activity across different sample types.

Results: A total of 7, 20, 16, and 26 bacterial isolates were obtained from clinical samples, soil, animal waste, and wastewater, respectively. The predominant bacterial species included ***Escherichia coli***, ***Pseudomonas aeruginosa***, ***Klebsiella pneumoniae***, and ***Staphylococcus aureus***. Bacteriophages isolated from wastewater showed lytic activity against bacteria from soil, animal waste, and clinical samples, with activity mainly observed at lower dilutions (10^{0}–10^{-2}). A decline in bacteriophage activity was observed with increasing dilution, with most isolates showing resistance at higher dilutions ($\geq 10^{-3}$). Environmental isolates were generally more susceptible to bacteriophage activity compared to clinical isolates.

Conclusion: Bacteriophages isolated in this study exhibited lytic activity against multidrug-resistant bacteria from environmental and clinical sources, particularly at lower dilutions. These findings suggest their potential as alternative or adjunct antibacterial agents within a One Health framework. However, further molecular characterisation and in vivo studies are required before therapeutic application.

BACKGROUND

Emergence of multidrug resistance (MDR) is an ever-increasing public health threat worldwide today, as drug resistance accumulates and accelerates at an alarming pace.[1] The global antimicrobial resistance (AMR) is expected to increase significantly by the year 2050, whereby antibiotic-resistant infections are envisaged to kill approximately 10 million people annually 2, and most of the resistant bacteria affect developing countries, accounting for 4.73 to 4.15 million deaths in Asia and Africa, respectively.[2]

In many countries, antibiotics are given without professional oversight and are inappropriately used in both humans and animals.[3] For instance, consumption of antibiotics by people with viral infections or common colds[4] and use of antibiotics for disease prevention and growth promotion in animals.[3] The use of antimicrobial agents plays a critical role in reducing the morbidity and mortality due to communicable diseases[2]; however, the emergence and spread of MDR bacteria are negating their effectiveness.

Over the past decades, there have been several reports on the growing rates of MDR in specific bacterial strains in different geographic locations.[5–9] The increased drug resistance to the most potent

antibiotics used in the treatment of infections makes control of pathogens very difficult and costly.[10] Such bacteria include methicillin-resistant *Staphylococcus aureus* (MRSA),[5,11] Vancomycin-Resistant *Enterococcus* (VRE).[12,13] Extended spectrum β- Lactamase (ESBL),[5] multidrug-resistant *Tuberculosis* (MDR-TB),[8] extensively drug-resistant *Mycobacterium Tuberculosis* (XDR-TB) and totally drug-resistant *Mycobacterium tuberculosis* (TDR-TB)[14,15] both strains are difficult to control and pose significant threat to medical practices.

The fight against MDR bacteria have been shifted towards alternative therapies in human as well as bio-control in animal.[16] Several approaches has resulted in promising results in fighting against drug resistant bacteria such as antibodies, lysins, probiotics, antimicrobial peptides and bacteriophage therapy.[16–19] Therefore, there is an urgent need to develop a promising alternative treatment modalities for use in the prevention or therapy of both humans infections[10,20]. Bacteriophage therapy came back into the scientists' visions because of the increasing AMR in the most parts of the world, which not only strengthened the use of bacteriophage therapy for the clinical purpose, but also accelerated its application to control pathogenic bacteria in bio-technology.[21]

Bacteriophages are naturally occurring antimicrobial agents that can infect and kill bacteria including antibiotic-resistant bacteria. Bacteriophages were discovered by a French Canadian microbiologist called Felix d'Herelle, even before the discovery of penicillin in 1928[22]. Bacteriophages are remarkably diverse, numerous and widespread in nature. The emerging AMR crisis has caused a resurgence of interest in bacteriophage therapy. Bacteriophages can kill their host bacteria regardless of whether or not the host is resistant to multiple antimicrobial compounds.[23] Bacteriophages can be isolated from clinical and environmental samples; and these bacteriophages have a perfect action against MDR bacteria causing various infections such as septic wounds.[24,25] Therefore, this study aimed to investigate the use of lytic bacteriophages as alternative to antibiotics in controlling bacteria isolated from clinical and environmental samples in Moshi, Kilimanjaro, Tanzania.

METHODOLOGY

Study Design, Period and Area

A cross-sectional study design was employed. The study was conducted between May and July 2023 in Moshi, Kilimanjaro Region, Tanzania. Moshi is a semi-urban area with both clinical and diverse environmental settings. The study incorporated both clinical and non-clinical components. Clinical isolates were obtained from KCMC laboratory, while environmental samples (soil, animal waste, and wastewater) were collected from selected sites within the Moshi area. This approach allowed assessment of bacterial diversity and antimicrobial resistance patterns across interconnected human, animal, and environmental sources within a One Health framework during the study period.

Study Population and Eligibility Criteria

The study population included bacterial isolates obtained from clinical and environmental sources within Moshi, Kilimanjaro Region, Tanzania. Clinical bacterial isolates were obtained from routine diagnostic cultures at KCMC, while environmental isolates were derived from soil, animal waste, and wastewater samples collected from selected sites within the study area.

For clinical samples, only bacterial isolates that were confirmed through standard microbiological identification procedures were included in the study. Environmental samples were eligible if they yielded cultivable bacteria using standard culture techniques. All bacterial isolates that were successfully identified and showed viable growth on culture media were included for antimicrobial susceptibility testing and bacteriophage assays. Non-viable cultures or contaminated samples were excluded from the analysis to ensure data reliability.

Sample Collection

Soil samples were collected aseptically from agricultural fields and pasture lands within a 1 to 3 km radius of Moshi. Surface debris was first removed, and approximately 20–50 g of soil was collected from a depth of 5 to 15 cm using sterile stainless-steel spatulas into sterile sampling containers. Animal waste samples were collected directly from freshly voided cattle and goat feces using sterile spatulas and transferred into sterile screw-capped containers. Wastewater samples (250–500 mL) were collected aseptically from drainage channels and public wastewater outlets using sterile sampling bottles. Human clinical bacterial isolates were obtained from routine diagnostic specimens processed at Kilimanjaro Christian Medical Centre (KCMC), Moshi, Tanzania. All samples were transported to the laboratory under cold-chain conditions (4–8°C), processed within 6 h of collection, and subjected to standard microbiological identification procedures.[26]

Bacterial Isolation from Clinical Samples

Upon arrival at the microbiology laboratory, each clinical specimen was first examined for its appearance and recorded on a laboratory data sheet. The specimens were then inoculated onto nutrient agar (NA) and MacConkey agar (MCA), both obtained from Becton Dickinson and Company (Cockeysville, MD, USA). After incubation, colony morphology was observed, and isolates were subjected to Gram staining, biochemical tests, and antimicrobial susceptibility testing. All procedures followed standard protocols, ensuring accurate identification and characterisation of the bacterial isolates.

Bacteria Isolation and Identification of Bacterial Species

Waste water samples were serially diluted from 10^1 to 10^8 in Phosphate Buffered Saline (PBS). For soil and animal dung samples, 100 grams of the sample was mixed with 50 mL of PBS, and allowed to settle at room temperature for 30 minutes. Then the resulting solution was serially diluted from 10^1 to 10^8 in PBS. The diluted samples (wastewater, soil and animal waste) were separately mixed with sterile, molten Luria Bertani (LB) agar medium which was then allowed to solidify and incubated for 18 to 24 h at 37 °C. Then number of colonies was counted in each plate and recorded as Colony Forming Unit per millilitre (CFU/mL). The viable titer was determined by counting colonies (CFU's) and multiplying by the dilution factor:

$$\text{CFU/mL} = \frac{\text{Number of colonies}}{\text{Dilution factor}}$$

To obtain pure culture and study morphological characteristics of bacteria colonies, specimens were sub-cultured on NA, MCA and incubated at 37 °C aerobically for 24 hours. Preliminary identification of bacteria was based on colony morphology, changes in the physical appearance of the differential media, enzyme activities of the organisms and Gram staining. Biochemical tests were performed on pure-culture colonies from the plates using the catalase test, oxidase test, and sulphur-indole-motility (SIM) test.

Antibiotics Susceptibility Patterns

Antimicrobial susceptibility pattern of isolated bacterial species was performed by the Kirby-Bauer disc diffusion method according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI).[27] Bacteria isolated were tested against meropenem (10 µg), cefotaxime (30 µg), amoxyllin/clavulanicacid (30 µg), ampicillin (10 µg), azithromycin (15 µg), vancomycin (30 µg), gentamycin (30 µg), ciprofloxacin (5 µg), amikacin (30 µg), and imipenem (10 µg). all the antibiotic discs were from oxoid (Thermo Fisher Scientific, USA). Antibiotic sensitivity was reported as resistance, intermediate, and sensitive according to the CLSI.[27] For clinical samples, the choice of the antibiotic agent was varied depending on the range of antibiotics available at the laboratory. Bacteria were classified as MDR if a particular strain is resistant to at least one agent in three classes of first-line antimicrobial agents.[28] Bacteria were stored at -20 °C until further use. The isolated bacteria were used for analysis of the effectiveness of isolated bacteriophages.

Bacteriophage Isolation and Purification

Bacteriophages were isolated from all samples except clinical samples. Protocol for bacteriophage isolation was adopted,[29] with few modifications.[30] About 200-400 g of soil/animal waste in conical flask was taken, 400 mL of bacteriophage buffer was added, mixed and, allow to settle for 30 minutes at the room temperature. Then, the mixture was filtered by pouring into the new flask to remove the debris. Then to all filtrates (samples) from wastewater, soil and animal waste, LB powder (2gm in 100 mL) was added to make 2%. All samples were filtered through 0.22 µm Syringe filters. Then, in a new sterile tube the following was added: 10 mLof the filtered sample, 10 mL of LB liquid media, 40 mL of the 1M of calcium chloride and 100 mL of overnight culture of bacteria (known bacteria). The mixture was incubated for 24 h at 37 °C. After between 24 and 48 h, 5 mLof sample was filtered and label as stock and stored for further use.

Spotting and Bacteriophage Effectiveness

Briefly, isolated bacteriophages were tested against each bacterium. Ten-fold serial dilutions in eight 1.5 mL Eppendorf tubes were prepared and marked as 10^{-1} to 10^{-8}. About 100 µL of the overnight bacterial culture was mixed with 0.7% LB medium. Then the mixtures were poured onto one of the marked LB agar plates and allowed to solidify. About 10 µL of each diluted bacteriophage lysate was added to the marked sections (10^{-1} to 10^{-8}) on the duplicate agar plates. Plates were incubated overnight at 37 °C. Plaques from different bacterial hosts with different sizes and morphologies were picked from the overlays and placed individually in bacteriophage buffer and placed at 4 °C overnight. Plaques were counted from all positive agar plate and recorded as a plaque forming unit (PFU/mL after 24 hrs.

$$\text{PFU/mL} = \frac{\text{number of plaques} * \text{dilution factor}}{\text{Volume plated (mL)}}$$

Quality Control

The T4 bacteriophage was used as the positive control for the bacteriophage isolation. The T4 was kindly donated by Dr Shawna McCallan from Bulgiest University Hospital, University of Zürich, and Zürich, Switzerland. American Type Culture Collection (ATCC) standard reference strains *Escherichia coli* ATCC-25922, *Staphylococcus aureus* ATCC 25923 and *Pseudomonas aeruginosa* ATCC 25853 was used for quality control.

Data Analysis

Data were entered into the computer, cleaned, and checked for completeness before analysis. Data nalysis was performed using IBM SPSS Statistics for Windows version 25 (IBM Corp, Armonk, NY, USA). Descriptive statistics were used to summarize the data, and results were presented as frequencies and percentages for bacterial distribution, antimicrobial resistance patterns, and bacteriophage lytic activity across different sample types (clinical and environmental). Findings were displayed using tables and figures for clear interpretation.

Ethical Considerations

The study was approved by the KCMC University ethical committee with certificate number UG 04/2021. Permission to conduct the study was granted by the Kilimanjaro Christian Medical Centre administration. Confidentiality of participants' information was strictly maintained throughout the study. Data were anonymized by assigning unique identification codes instead of personal identifiers, and all collected information was accessible only to the research team. The data were securely stored and used solely for research purposes.

RESULTS

Distribution of Bacterial Isolates from Soil, Animal Waste, and Wastewater Samples

A total of 62 bacterial isolates were recovered from soil, animal waste, and wastewater samples, with variations in both frequency and species composition across the different environmental sources. In soil samples (n=20), the most frequently isolated organisms were *Escherichia coli* and *Salmonella* spp., each accounting for 4 (20%) of isolates. *Campylobacter* spp. was the least frequently isolated organism. In animal waste samples (n=16), *Staphylococcus aureus* was the predominant isolate, representing 7 (43.7%) of all bacteria recovered. This was followed by coagulase-negative staphylococci (CNS) at 5 (31.2%). Wastewater samples (n=26) were mainly dominated by *Escherichia coli* (34.6%), followed by *Pseudomonas aeruginosa* 7 (26.9%) and *Staphylococcus*

aureus 6 (23.1%). *Klebsiella pneumoniae* was the least frequently isolated organism from wastewater samples, (Table 1).

Antibiotic Resistance Patterns of Bacterial Isolates

i) Soil-Derived Bacterial Isolates

Soil-derived bacterial isolates exhibited high levels of resistance to commonly used antibiotics (Table 1). All bacterial species exhibited multidrug resistance; however, the extent of resistance varied among the isolates. Campylobacter spp. demonstrated the broadest resistance profile, including resistance to cefotaxime, amoxicillin-clavulanic acid, ampicillin, vancomycin, and amikacin. In contrast, the remaining bacterial species exhibited resistance to fewer antimicrobial agents, with amoxicillin-clavulanic acid, ampicillin, and vancomycin being the most commonly observed resistance pattern. (Table 2).

ii) Animal Waste Isolates

Bacterial isolates from animal waste also demonstrated multidrug resistance patterns. *Proteus vulgaris* was resistant to amoxicillin-clavulanic acid, ampicillin, and azithromycin, and showed intermediate susceptibility to ciprofloxacin. *Serratia* spp. exhibited resistance to cefotaxime, amoxicillin-clavulanic acid, ampicillin, and vancomycin, with intermediate susceptibility to azithromycin. (Table 2).

iii) Wastewater Isolates

Wastewater-derived isolates showed extensive resistance to multiple antibiotic classes. *Escherichia coli* was resistant to cefotaxime, amoxicillin-clavulanic acid, ampicillin, and azithromycin, with intermediate susceptibility to amikacin. *Klebsiella* spp. were resistant to cefotaxime, amoxicillin-clavulanic acid, ampicillin, vancomycin, gentamicin, and imipenem. *Pseudomonas aeruginosa* showed resistance to meropenem, amoxicillin-clavulanic acid, ampicillin, and vancomycin, with intermediate susceptibility to azithromycin and ciprofloxacin. *Staphylococcus aureus* was resistant to vancomycin and showed intermediate susceptibility to amoxicillin-clavulanic acid. (Table 2).

iv) Clinical Isolates

Clinical isolates also demonstrated high levels of antimicrobial resistance. *Citrobacter* spp. were resistant to cefotaxime, amoxicillin-clavulanic acid, ampicillin, and ciprofloxacin. Coagulase-negative staphylococci (CNS) were resistant to cefotaxime, amoxicillin-clavulanic acid, and ampicillin. *Staphylococcus aureus* showed resistance to amoxicillin-clavulanic acid, ampicillin, and ciprofloxacin, with intermediate susceptibility to cefotaxime. *Klebsiella pneumoniae* was resistant to amoxicillin-clavulanic acid and ampicillin, with intermediate susceptibility to cefotaxime. *Escherichia coli* was resistant to amoxicillin-clavulanic acid and ampicillin, with intermediate susceptibility to azithromycin and cefotaxime. *Pseudomonas aeruginosa* exhibited resistance to amoxicillin-clavulanic acid and ampicillin, with intermediate susceptibility to cefotaxime and vancomycin. *Proteus mirabilis* was resistant to amoxicillin-clavulanic acid, ampicillin, and ciprofloxacin, with intermediate susceptibility to amikacin. (Table 2).

Bacteriophage Activity Against Soil-Derived Bacterial Isolates

Bacteriophages isolated from wastewater demonstrated varying lytic activity against bacteria obtained from soil samples. High bacteriophage activity was observed at lower dilutions (10^0–10^{-1}), where multiple bacterial species, including *Campylobacter* spp., *Serratia* spp., *Salmonella* spp., *Escherichia coli*, and *Pseudomonas aeruginosa*, showed susceptibility as indicated by plaque formation (Figure 1A). As dilution increased, a gradual decline in bacteriophage activity was observed. At higher dilutions ($\geq 10^{-4}$), most bacterial isolates exhibited resistance, with no plaque formation detected. Notably, *Klebsiella pneumoniae* and *Proteus mirabilis* showed consistent resistance across most dilutions. (Table 3).

Bacteriophage Activity Against Animal Waste Bacterial Isolates

Bacteriophage activity against bacterial isolates obtained from animal waste samples followed a similar trend. Strong lytic activity was observed at lower dilutions (10^0–10^{-1}), particularly against *Serratia* spp., *Staphylococcus aureus*, and Coagulase-negative Staphylococci (CNS). (Figure 1B).

However, *Proteus vulgaris* demonstrated early resistance, with no plaque formation observed beyond the lowest dilution (10^0). As dilution increased ($\geq 10^{-4}$), all tested bacterial isolates exhibited resistance, indicating reduced bacteriophage effectiveness at lower concentrations. (Table 4).

Bacteriophage Activity Against Wastewater-Derived Bacterial Isolates

Bacteriophages showed broader lytic activity against bacterial isolates derived from wastewater samples. At lower dilutions (10^0 to 10^{-2}), most Gram-negative bacteria, including *P. vulgaris*, *K. pneumoniae*, *P. mirabilis*, and *Pseudomonas aeruginosa*, were susceptible to bacteriophage activity. (Figure 1C and 1D). In contrast, *Staphylococcus aureus* remained resistant across all tested dilutions. A decline in bacteriophage activity was observed with increasing dilution, with most isolates becoming resistant at dilutions$\geq 10^{-4}$. Interestingly, variability in susceptibility was noted between *Pseudomonas aeruginosa* isolates. (Table 5).

Bacteriophage Activity Against Clinical Bacterial Isolates

Bacteriophage activity against clinical isolates was comparatively limited. Lytic activity was observed only against *Klebsiella pneumoniae* and *Escherichia coli* at lower dilutions (10^0–10^{-3}), as evidenced by plaque formation. (Figure 1E and 1F). All other clinical isolates, including *Staphylococcus aureus*, *Citrobacter* spp., Coagulase-negative Staphylococci (CNS), *Klebsiella oxytoca*, and *Proteus mirabilis*, showed resistance across all dilutions tested. At higher dilutions ($\geq 10^{-4}$), no bacteriophage activity was detected against any clinical isolate. (Table 6).

FIGURE 1A: Bacteriophage Clearance and Individual plague Against *Pseudomonas Aeruginosa*

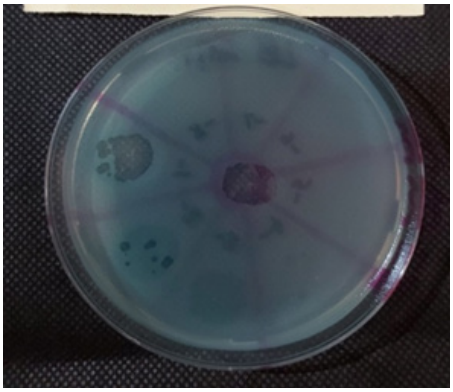

FIGURE 1B: Bacteriophage Lysis Against *Salmonella spp*. from Soil Sample at Dilution 10^0,10^{-1}, and 10^{-2}

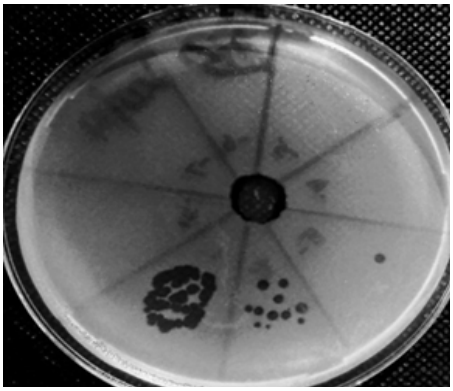

FIGURE 1C: Bacteriophage Lysis Against *Serratia spp*. from Animal Waste at Dilution 10^0,10^{-1},10^{-2},10^{-3} & 10^{-4}

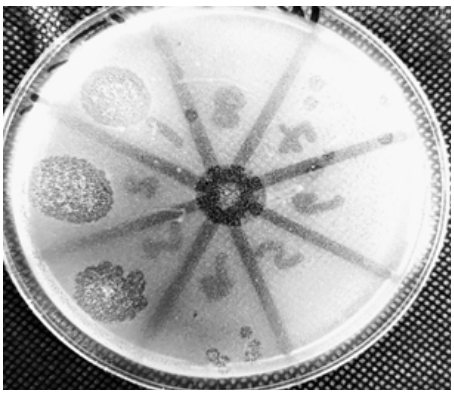

FIGURE 1D: Bacteriophage Lysis Against *Pseudomona aeruginosa* from Waste Water Sample at Dilution 10^0,10^{-1} & 10^{-2}

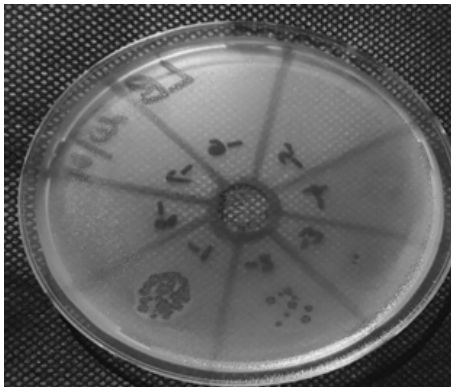

FIGURE 1E: Bacteriophage Lysis against *E. coli* from Clinical Sample at Dilution 10^0,10^{-1} and 10^{-2}

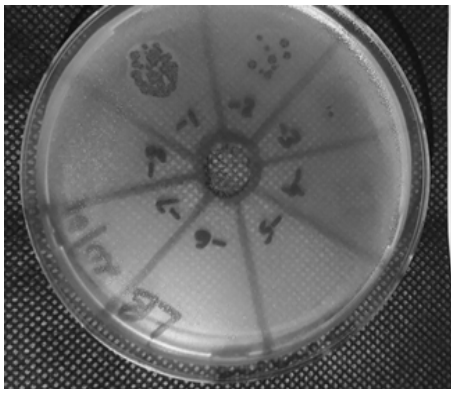

FIGURE 1F: Figure1F: Bacteriophage Lysis Against *K. pneumonia* from Clinical Sample at Dilution 10^0,10^{-1} & 10^{-2}

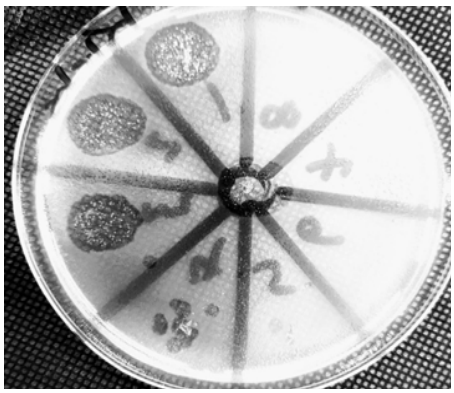

TABLE 1: Distribution of Bacterial Isolates from Soil, Animal Waste, and Wastewater Samples

Sample source	Type of Bacteria Isolated	Frequency (n)	Percentage (%)
Soil (n=20)	*Campylobacter spp*	1	5.0
	P.mirablis	3	15.0
	Salmonella spp	4	20.0
	Serratia spp	2	10.0
	K.pneumoniae	3	15.0
	E.coli	4	20.0
	P.aureginosa	3	15.0
Animal waste (n=16)	*P.vulgaris*	1	6.2
	S.aureus	7	43.7
	Serratia spp	3	18.7
	CNS	5	31.2
Waste water (n=26)	*E.coli*	9	34.6
	K.pneumoniae	4	15.4
	P.aureginosa	7	26.9
	S.aureus	6	23.1

Abbreviation key: CNS, coagulase-negative ***staphylococci***; spp, species.

TABLE 4: Bacteriophage Spotting Assay Results of Bacteriophages Isolated from Wastewater Against Animal Waste Bacterial Isolates

Bacteriophage source	Dilution	*P.vulgaris*	*S.aureus*	*Serratia spp*	*CNS*
Waste water	10^{0}	S	S	S	S
	10^{-1}	R	S	S	S
	10^{-2}	R	R	S	S
	10^{-3}	R	R	S	S
	10^{-4}	R	R	R	R
	10^{-5}	R	R	R	R
	10^{-6}	R	R	R	R
	10^{-7}	R	R	R	R
	10^{-8}	R	R	R	R

Abbreviation key: S, presence of plaques (indicating bacterial susceptibility to bacteriophage activity); R, no plaque formation (indicating resistance). CNS, coagulase-negative ***staphylococci***. Dilutions represent ten-fold serial dilutions of bacteriophage lysate (10^{0} to 10^{-8}).

TABLE 2: Susceptibility Pattern of Multidrug-Resistant (MDR) Bacteria Isolated from Soil, Animal Excreta and Wastewater Samples

Sample source	Bacteria isolated	MEM	CTX	AMC	AMP	AZM	VA	CN	CIP	AK	IPM	MDR
Soil	*Camplylobacter spp*	S	R	R	R	S	R	S	S	R	S	Yes
	Serratia spp	S	S	R	R	R	R	S	S	S	S	Yes
	K.pneumoniae	S	I	R	R	R	R	S	S	S	S	Yes
	Salmonella spp	S	S	R	R	S	R	S	S	S	S	Yes
	P.mirabils	S	S	R	R	S	S	S	S	S	S	No
	E.coli	*S*	R	I	R	I	R	S	S	S	S	Yes
	P.aureginosa	S	S	R	R	S	R	S	S	S	S	Yes
Animal	*P. vulgaris*	S	S	R	R	R	R	S	I	S	S	Yes
	S. aureus	S	S	S	S	S	S	S	S	S	S	No
	Serratias	S	R	R	S	I	R	S	S	S	S	No
	CNS	S	S	S	S	S	S	S	S	S	S	No
Waste water	*E.coli*	S	R	R	R	R	R	S	S	I	S	Yes
	Klebsiella spp	S	R	R	R	S	R	S	R	S	R	Yes
	S.aureus	S	S	I	S	S	R	S	S	S	S	No
	P.aureginosa	S	R	R	R	S	R	S	S	S	S	Yes
Clinical	*Citrobacter*	S	R	R	R	S	I	S	R	S	S	Yes
	CNS	S	R	R	R	S	S	S	S	S	S	Yes
	S.aureus	S	I	R	R	S	S	S	R	S	S	Yes
	K.pneumoniae	S	I	R	R	S	S	R	S	S	S	Yes
	E.coli	S	I	R	R	I	S	S	S	S	S	No
	P.aureginosa	S	I	R	R	S	I	S	S	S	S	No
	P.mirabilis	S	S	R	R	S	S	S	R	I	S	Yes

Abbreviation key: R, resistant; S, susceptible; I, intermediate; MDR, multidrug-resistant (defined as resistance to at least one agent in three or more antimicrobial classes). VA, vancomycin; CIP, ciprofloxacin; AK, c; AZM, azithromycin; CN, gentamycin; AMC, amoxicillin; IPM, imipenem; AMP, ampicillin; MEM, meropenem; CTX, ceftriaxone/cefotaxime; CNS, Coagulase Negative *Staphylococcus*; MDR, Multi drug resistance.

TABLE 3: Bacteriophage Spotting Assay Results of Bacteriophages Isolated From Wastewater Against Soil-Derived Bacterial Isolates

Bacteriophage source	Dilution	Campylobacter spp	Serratia spp	K.pneumoniae	Salmonella spp	P.mirabilis	E.coli	P.aureginosa
Waste water	10^{0}	S	S	R	S	R	S	S
	10^{-1}	S	S	R	S	R	S	S
	10^{-2}	R	S	R	S	R	R	S
	10^{-3}	R	S	R	R	R	R	S
	10^{-4}	R	R	R	R	R	R	S
	10^{-5}	R	R	R	R	R	R	R
	10^{-6}	R	R	R	R	R	R	R
	10^{-7}	R	R	R	R	R	R	R
	10^{-8}	R	R	R	R	R	R	R

Abbreviation key: S, presence of plaques (indicating susceptibility of bacteria to bacteriophage activity); R, no plaque formation (indicating resistance). Dilutions are expressed as ten-fold serial dilutions of bacteriophage lysate (100 to 10^{-8}); *C. spp.* = *Campylobacter spp.; S. spp.* = *Serratia spp.; K. pneumoniae* = Klebsiella *pneumoniae; Salmonella spp.* = *Salmonella* species; *P. mirabilis* = *Proteus mirabilis; E. coli* = *Escherichia coli; P. aeruginosa* = *Pseudomonas aeruginosa*.

TABLE 5: Bacteriophage Spotting Assay Results of Bacteriophages Isolated From Wastewater Against Wastewater-Derived Bacterial Isolates

Bacteriophage source	Dilution	*P.vulgaris*	*K.pneumoniae*	*P.mirabilis*	*P.aureginosa*	*S.aureus*
Waste water	10^{0}	S	S	S	S	R
	10^{-1}	S	S	S	S	R
	10^{-2}	S	S	S	S	R
	10^{-3}	S	R	S	R	R
	10^{-4}	R	R	R	R	R
	10^{-5}	R	R	R	R	R
	10^{-6}	R	R	R	R	R
	10^{-7}	R	R	R	R	R
	10^{-82}	R	R	R	S	R

Abbreviation key: S, presence of plaques (indicating bacterial susceptibility to bacteriophage activity); R, no plaque formation (indicating resistance). Dilutions represent ten-fold serial dilutions of bacteriophage lysate (10^{0} to10^{-8})

TABLE 6: Bacteriophage Spotting Assay Results of Bacteriophages Isolated from Wastewater Against Clinical Bacterial Isolates

Bacteriophage source	Dilution	*Citrobacter*	*CNS*	*S.aureus*	*K.pneumonia*	*E.coli*	*K.oxytoca*	*P.mirablis*
Waste water	10^{0}	R	R	R	S	S	R	R
	10^{-1}	R	R	R	S	S	R	R
	10^{-2}	R	R	R	S	S	R	R
	10^{-3}	R	R	R	S	S	R	R
	10^{-4}	R	R	R	R	R	R	R
	10^{-5}	R	R	R	R	R	R	R
	10^{-6}	R	R	R	R	R	R	R
	10^{-7}	R	R	R	R	R	R	R
	10^{-8}	R	R	R	R	R	R	R

S = Presence of plaques (indicating bacterial susceptibility to bacteriophage activity); R = No plaque formation (indicating resistance). CNS = Coagulase-negative Staphylococci. Dilutions represent ten-fold serial dilutions of bacteriophage lysate (10^{0}–10^{-8}).

DISCUSSION

This study demonstrated a high prevalence of multidrug-resistant (MDR) bacteria across clinical, animal, and environmental sources, with dominant species including *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus*. These findings are consistent with global reports identifying these organisms among the most important MDR pathogens, particularly within the ESKAPE group.[31,32] Previous studies have also shown that these bacteria are widely distributed in both clinical and environmental settings due to their ability to acquire resistance genes through horizontal gene transfer.[33,34] This widespread distribution is further facilitated by selective pressure from extensive antibiotic use in human, animal, and environmental sectors, which accelerates the emergence and dissemination of resistant strains.

The high level of resistance observed in this study, particularly to commonly used antibiotics such as ampicillin and amoxicillin-clavulanic acid, is consistent with findings from Sub-Saharan Africa and other low- and middle-income countries, where antibiotic misuse in humans and livestock contributes significantly to resistance development.[35,36] Compared to earlier studies, which reported moderate resistance levels, the current findings suggest a broader and more established resistance pattern across multiple ecological niches, reinforcing the role of environmental reservoirs in AMR dissemination.

Bacteriophage assays in this study showed variable lytic activity, with greater effectiveness observed at lower dilutions and higher susceptibility among Gram-negative bacteria compared to Gram-positive bacteria. This observation agrees with previous studies demonstrating that bacteriophages are often more effective against Gram-negative bacteria due to easier access to outer membrane receptors.[37,38] In contrast, reduced susceptibility among Gram-positive bacteria such as *Staphylococcus aureus* has been widely reported and is attributed to structural differences in the bacterial cell wall and more complex bacteriophage-host interactions.[39,40] This difference may be explained by the thick peptidoglycan layer in Gram-positive bacteria, which can act as a physical barrier limiting bacteriophage adsorption and penetration, thereby reducing lytic efficiency.

Interestingly, this study found that environmental isolates were more susceptible to bacteriophages than clinical isolates. This contrasts with several studies that have reported successful bacteriophage activity against MDR clinical isolates, including *Klebsiella pneumoniae* and *Pseudomonas aeruginosa*.[41,42] The reduced susceptibility observed among clinical isolates in this study may be explained by prolonged antibiotic exposure, which promotes adaptive resistance mechanisms such as biofilm formation and receptor modification, thereby limiting bacteriophage effectiveness.[43,44] The reduced susceptibility among clinical isolates in the present study may be explained by prolonged antibiotic exposure, which can enhance bacterial defense mechanisms, biofilm formation, and potential bacteriophage resistance. This highlights a critical difference between environmental and clinical bacterial populations and suggests that bacteriophage therapy effectiveness may depend on bacterial origin and prior selective pressure.

The decline in bacteriophage activity with increasing dilution observed in this study is consistent with fundamental bacteriophage biology, where reduced bacteriophage concentration lowers the probability of host infection and plaque formation.[21,45] Similar findings have been reported in experimental studies emphasizing the importance of optimal bacteriophage dosing for effective bacterial control.[46] Additionally, recent studies suggest that bacteriophage cocktails and bacteriophage-antibiotic combinations can enhance antibacterial activity and overcome limitations associated with single-bacteriophage therapy.[39,40] This supports the concept that increasing bacteriophage diversity or combining therapeutic modalities may improve bacterial targeting efficiency and reduce the risk of resistance development.

Despite the promising findings, the results of this study align with growing evidence that, while bacteriophages are effective antibacterial agents, several challenges remain. These include narrow host range, potential development of bacteriophage resistance, variability in pharmacokinetics, and lack of standardized clinical protocols.[40,47] Recent reviews further emphasize the need for molecular characterization, personalized bacteriophage selection, and regulatory frameworks to enable clinical translation.[48] Furthermore, Advanced approaches, including engineered bacteriophages and bacteriophage-antibiotic synergy, are currently being explored to address these limitations and improve therapeutic outcomes.[39,43] Generally, the findings of this study support the growing body of evidence indicating that bacteriophages are promising alternative or adjunct antimicrobial agents, particularly against Gram-negative MDR pathogens. However, in contrast to some studies reporting high clinical success rates, the variability in bacteriophage effectiveness observed here underscores the need for further research, including molecular characterization, host range determination, and in vivo validation, before widespread clinical application.

Strengths and Limitations of the Study

This study applied a One Health approach by investigating bacterial isolates from clinical, animal, and environmental sources, providing a comprehensive comparison of multidrug-resistant (MDR) bacteria across interconnected settings. The inclusion of multiple sample types (soil, animal waste, wastewater, and clinical specimens) strengthened the study by allowing comparative analysis of bacterial distribution and resistance patterns. In addition, the isolation and functional assessment of bacteriophages provided preliminary evidence of their lytic activity against MDR bacteria, and the use of standard microbiological and antimicrobial susceptibility testing methods enhanced the reliability of the findings. However, the study also had several limitations, including the absence of molecular characterization of bacteriophages, which limited insights into their genetic diversity and novelty. The study was further restricted to in vitro assays without in vivo validation of efficacy or safety, and bacteriophage host range was not comprehensively evaluated across a wider panel of bacterial strains. Moreover, the study was conducted within a limited geographical area with a relatively small

sample size, which may affect generalizability, and only descriptive statistical analysis was performed, limiting deeper inferential interpretation of the data.

CONCLUSION

This study demonstrates a high prevalence of multidrug-resistant bacteria across clinical, animal, and environmental sources, highlighting the interconnected nature of antimicrobial resistance within a One Health framework. Bacteriophages isolated from environmental samples exhibited variable lytic activity against these bacteria, with greater effectiveness observed against Gram-negative organisms and reduced activity against clinical isolates. These findings suggest that bacteriophages hold promise as potential alternative or complementary antibacterial agents; however, further studies involving molecular characterization, broader host range analysis, and in vivo validation are necessary before clinical application.

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