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Bacteriophage-Mediated Control of Extensively Drug-Resistant (XDR) Uropathogens

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Abstract

Urinary tract infections (UTIs) are the most common bacterial infections worldwide and are primarily caused by *Escherichia coli* and *Pseudomonas aeruginosa*. In developing countries like Bangladesh, the rapid emergence of antibiotic resistance is largely driven by irrational antibiotic use, over-the-counter availability of antimicrobial drugs, and limited diagnostic facilities. This study aimed to identify uropathogenic bacteria and study the potential of bacteriophages as an alternative antimicrobial strategy. Four bacterial isolates from urine samples of suspected UTI patients were identified as *E. coli* and *P. aeruginosa* using Gram staining, biochemical tests, 16S rRNA gene sequencing, and NCBI BLAST analysis. Antibiotic susceptibility testing against 14 antibiotics revealed extensive multidrug resistance; one *P. aeruginosa* isolate was pandrug-resistant, while the others showed resistance to 7–12 antibiotics. To explore an alternative treatment approach, bacteriophages were isolated from sewage samples using *E. coli* and *P. aeruginosa* as host bacteria. Five lytic bacteriophages (Phage-1 to Phage-5) were successfully isolated and confirmed by spot and double agar layer assays. These phages produced clear lytic zones and distinct plaques, showing effective antibacterial activity. Stability tests showed that the phages remained active between −20 °C and 50 °C and within a pH range of 5–9, although activity decreased under extreme temperature and pH conditions. These results suggest that bacteriophage therapy shows great promise as an alternative or adjunct therapy for UTIs caused by extensively drug-resistant uropathogens. Further studies on phage genomics, standardized production, clinical safety, and regulatory approval are needed before therapeutic application in humans.

Keywords: Multidrug-Resistant, Uropathogens, Bacteriophage Therapy, *E. coli*, *P. aeruginosa*

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1. Introduction

Urinary tract infections are the most treated bacterial infections in humans worldwide, especially in women, the elderly, and immunocompromised individuals. Epidemiological data indicate that UTIs represent 15.5% of hospitalizations and 6.2% of deaths in people >65 years [1]. These infections occur due to uropathogens, the bacteria that can colonize the urinary tract and cause infection. Of these, Uropathogenic *Escherichia coli* (UPEC) dominates as the cause of infection and accounts for 60–80% of cases [2]. *Klebsiella pneumoniae*, *Proteus mirabilis, Pseudomonas aeruginosa,* and *Staphylococcus saprophyticus* are other critical uropathogens [3].

UPEC and other uropathogens have many virulence factors, including adhesins, toxins, iron-acquisition systems, and immune evasion mechanisms, which enable them to survive in the urinary tract [3]. Some infections are resolved spontaneously, but many require antimicrobial treatment, particularly once infection has progressed to pyelonephritis or bacteremia. In addition, uropathogens are clinically significant as urinary infections due to E. coli contribute to a large fraction of E. coli-associated sepsis cases in humans [4],[5].

Infections solely due to uropathogens are increasingly more difficult with the emergence of extensively drug-resistant (XDR) bacterial strains, which limit available treatment options [6]. This problem is exacerbated in Bangladesh as well as in other countries of South Asia by unregulated usage of antibiotics, scarcity of clinical microbiology facilities, and easy access to obtain antibiotics without prescription [7]. Consequently, resistance to commonly available antibiotics is emerging in uropathogens. Antimicrobial resistance is known to spread quickly via selective pressure exerted by the antibiotic and horizontal gene transfer mediated by mobile genetic elements such as plasmids, transposons, or other small mobile genetic elements [8]. Due to the fact that resistance is emerging more rapidly than new antibiotics are being discovered, there is a critical and urgent need for alternative antimicrobial strategies. One approach that shows potential is using phage therapy as bacteriophages infect and kill specific bacterial cells. Phages adhere to the bacterial host, deliver genetic material via injection into cells, replicate intracellularly, and ultimately lyse the bacterium [9]. Phage therapy is widely accepted to be preferable to common antibiotics on multiple fronts. Phages are extremely specific to their bacterial hosts; this means they target pathogenic bacteria but cause less disruption to the normal beneficial microorganisms [10],[11]. They can be isolated from environmental sources (e.g., sewage, wastewater) and selected against multidrug-resistant clinical isolates as well. Lytic phages are particularly useful for therapeutic applications as they kill the bacterial cells [12].

Therefore, the present study aims to isolate and characterize bacteriophages from environmental sources and determine their efficacy against XDR uropathogens isolated from patients in Rajshahi, Bangladesh. This research, integrating classical microbiological techniques and morphological characterization, may deliver helpful insights for the creation of phage-based alternatives against resistant uropathogens. In the context of increasing antimicrobial resistance, phage therapy has emerged as a relevant and innovative treatment for infections caused by MDR, XDR, and PDR uropathogens.

2. Materials and Methods

2.1. Sample collection

Urine samples were collected from urinary tract infection-suspected patients at Rajshahi Medical College and Kushtia Medical College. The genital area was scrubbed with antiseptic solution to minimize external contamination before sample collection. Sterile leak-proof containers were then used to collect midstream urine. Samples were transported to the laboratory and processed no later than 2 h from sampling. Contaminated samples that could not be processed immediately were kept at 4 °C to ensure bacteria were still alive and did not outgrow any contaminating organisms.

2.2. Isolation and identification of bacteria

Detection of pathogens was done by standard bacteriological procedures [13]. To start, each of the samples was serially diluted from 10^{-2} to 10^{-8} in autoclaved distilled water for analysis, with the application of a calibrated loop (100 μL) to spread onto appropriate nutrient culture media. Plates were then incubated aerobically at 37 °C for 18–24 h. Following incubation, growth of bacteria was examined based on colony count, morphology, and characteristics. Biochemical tests, 16S rRNA gene sequencing, and phylogenetic analysis based on the NCBI database also confirmed the substantial growth of bacteria.

2.2.1. Biochemical tests of isolated bacteria

Following primary isolation, single, well-isolated bacterial colonies were selected and subcultured onto fresh culture media to obtain pure isolates for subsequent identification and characterization. The isolates that were purified were identified further using routine bacteriological methods by means of standard biochemical tests, which included the oxidase test, catalase test, coagulase test, and Triple Sugar Iron (TSI) Test [13],[14]. Isolated bacteria were characterized by their morphology and Gram stain, as a standard microbiological protocol [15],[16]. To maintain these isolates for preservation, pure stocks of bacterial cultures were stored in 50% (v/v) glycerol at −80 °C.

2.2.2. Molecular characterization of bacteria

DNA extraction from bacterial isolates was performed by the boiling method [17]. One colony from an overnight LB agar culture was resuspended in 30 μL distilled water and heated at 100 °C for 10 min. The suspension was promptly moved to ice and permitted to chill for 5 min, then centrifuged at 13,000 × g for 10 min. The supernatant containing genomic DNA was used as the PCR template. Two universal bacterial primers, F2 (AGAGTTTGATCCTGGCTCAG) and R1391

(GACGGGCGGTGTGTRCA), were used to amplify 16S rDNA [18]. The PCR product was sequenced at a sequencing facility using an Illumina DNA Prep kit (Illumina, San Diego, CA, USA) to obtain high-quality sequences for taxonomic profiling and microbial diversity analysis based on sequence information available in the NCBI database.

2.2. Antibiotic susceptibility profiling

Antibiotic profiling of isolated bacteria was done according to Clinical and Laboratory Standards Institute (CLSI) guidelines [19]. Multiple classes of antibiotics (n=14) were tested through the disc diffusion method (**Table 4**). Isolated bacterial cultures (100µL) were evenly swabbed on LB agar plates, and antibiotic discs were placed at equal distances from one another. The plates were left to incubate overnight at 37 °C, and then zones of inhibition of bacterial susceptibility and resistance patterns were measured.

2.3. Isolation, purification, and efficacy testing of bacteriophages

Bacteriophages targeting multidrug-resistant bacteria were isolated from sewage samples collected from distinct geographical areas (**Figure 1**) using a modified enrichment and isolation method [20]. A mixture of 4.5 mL sewage water, 0.5 mL 10× LB broth, and 1 mL of mid-log phase bacterial host was incubated at 37 °C and 180 rpm for 24 h. Centrifugation of the culture was done at 6,000×g for 10 min, and the supernatant was filtered through a 0.22 µm syringe filter. The crude phage lysate was kept at 4 °C, and lytic phages were detected on LB agar plates using spot plating assays (spreading 100 µL of bacterial culture and spotting 5–10 µL of phage lysate). Double-layer agar was used to determine phage titration and plaque morphology. The plates were incubated overnight at 37 °C, and clear zones of lysis indicated phage activity. Phage lysates (10^{10}–10^{12} PFU/mL) were purified by a modified ammonium acetate method [21],[22],[23].

Figure 1. Geographical Location of Collecting Sewage Water Sample.

2.4. Thermal and pH stability

Phage stability was evaluated by exposing suspensions to various temperatures (–20 °C, 4 °C, 25 °C, 37 °C, 50 °C, 70 °C, 90 °C) and different pH levels (5, 7, 10, 12) using sterile 1 N HCl or 1 N NaOH for 1 h. Samples were cooled or neutralized to pH 7 before titration. Residual infectivity was measured by the double-layer agar method, with titers expressed as PFU/mL. Experiments were done in triplicate, and mean values were reported [24].

3. Results

3.1. Isolation of bacterial strains

Four bacterial strains were isolated from urinary tract-infected patients (**Table 1**).

3.2. Biochemical assay analysis

In this study, several biochemical tests were performed (**Table 2**) to identify the isolated bacteria from urine samples. Strain-1, Strain-2, and Strain-4 were oxidase negative, Catalase positive, and coagulase negative. Strain-3 was Oxidase positive and showed similar results in the rest of the tests. All isolated samples were Gram-negative. Strain-1, Strain-2, and Strain-4 showed A/A (Acid slant/acid butt) with gas and no H_2S, indicating sugar fermentation with gas production but no hydrogen sulfide. Strain-3 showed K/NC (Alkaline slant/no change butt) with no gas and no H_2S, indicating non-fermentation and no hydrogen sulfide production.

Table 1. List of isolated bacteria and their respective sources.

Isolates	Patient Name	Age (Years)	Sex	Specimen
Strain-1	Monira	35	Female	Urine
Strain-2	Liton	38	Male	Urine
Strain-3	Anisur Rahman	41	Male	Urine
Strain-4	Afia	09	Female	Urine

Table 2. Presumptive identification of bacterial isolates based on biochemical properties.

	Test Name					
Isolates	**Oxidase**	**Catalase**	**Coagulase**	**Gram-Staining**	**TSI**	**Identified Bacteria**
Strain-1	–	+	–	–	A/A, gas produce, no H_2S	*E. coli*
Strain-2	–	+	–	–	A/A, gas produce, no H_2S	*E. coli*
Strain-3	+	+	–	–	K/NC, no gas, no H_2S	*P. aeruginosa*
Strain-4	–	+	–	–	A/A, gas produce, no H_2S	*E. coli*

3.3. Characterization of isolated bacteria

Four isolated bacteria were identified through 16S rDNA sequencing, NCBI BLASTing, and phylogenetic analysis. The results are shown in **Table 3** and **Figure 2**(**A, B, C, D**).

Figure 2. Phylogenetic analysis of bacterial isolates. (A) Strain-1 showed similarity with *Escherichia coli* strains. (B) Strain-2 showed similarity with *Escherichia* sp. strains. (C) Strain-3 showed similarity with *Pseudomonas* sp. strains. (D) Strain-4 showed similarity with *Escherichia coli* strains.

3.4. Antibiotic susceptibility profiling

For antibiotic profiling, 14 antibiotics from different classes were tested against the bacterial isolates (**Table 4** and **Figure 3**). Strain-3 showed the highest level of resistance, being resistant to all 14 antibiotics tested. Strain-4 was resistant to 12 antibiotics, while Strain-1 showed resistance to 9 antibiotics and intermediate sensitivity to 2 antibiotics. Strain-2 showed resistance to 7 antibiotics and intermediate sensitivity to 2 antibiotics. Overall, Strain-3 exhibited the strongest pan-drug resistance pattern among the tested isolates (**Figure 4**).

3.5. Bacteriophage isolation and confirmation of their lytic activity

Five bacteriophages designated as Phage-1, Phage-2, Phage-3, Phage-4, and Phage-5 were isolated from wastewater samples, where *E. coli* and *P. aeruginosa* were used as hosts. Phage presence was first screened using the direct spot assay and was later verified through the double-layer agar assay (**Figure 5**). The isolated lytic phage formed clear, well-defined

plaques on the lawn of host bacteria, which confirms its ability to lyse the host cells. The plaques displayed diversity in size, shape, and clarity, highlighting variations in the lytic potential of the isolated pages. Some isolates generated large, sharply defined plaques, indicative of strong lytic activity [25],[26].

Table 3. Similarity of the isolated bacterial strain after NCBI BLAST.

Isolates	Sources	Length (bp)	% of Identity	Accession no.	Organisms
Strain-1	Urine	1409	100.00	OK271886.1	*Escherichia* sp.
Strain-2	Urine	1120	98.25	KJ803892.1	*Escherichia coli*
Strain-3	Urine	1373	100.00	MK367615.1	*Pseudomonas aeruginosa*
Strain-4	Urine	1420	97.68	OK271886.1	*Escherichia* sp.

Table-4. Antibiotic resistance profile of four (4) isolated bacteria according to CLSI standard.

Antibiotic Disk (µg)	Bacterial strain with sensitive (S), resistance (R), and intermediate (I) zone of inhibition (ZI) in mm							
	Strain-1	ZI	Strain-2	ZI	Strain-3	ZI	Strain-4	ZI
Penicillin (P10)	R	0	R	0	R	0	R	0
Gentamicin (CN10)	R	0	S	20	R	0	R	0
Azithromycin (AZM10)	S	14	R	0	R	0	R	0
Meropenem (MEM10)	S	25	S	26	R	0	S	15
Co-Trimoxazole (COT25)	R	0	S	30	R	0	S	30
Ceftriaxone(CRO30)	I	15	I	20	R	0	R	0
Cefixime (CFM5)	I	10	I	15	R	0	R	0
Amikacin (AK30)	S	20	S	20	R	0	R	0
Nitrofurantoin (F300)	R	0	R	0	R	0	R	0
Erythromycin (E15)	R	0	R	0	R	0	R	0
Ciprofloxacin (CIP5)	R	0	R	0	R	0	R	0
Imipenem (IMP10)	R	0	R	0	R	0	R	0
Ampicilin (AMP10)	R	0	R	0	R	0	R	0
Tetracycline (TE30)	R	0	S	15	R	0	R	0

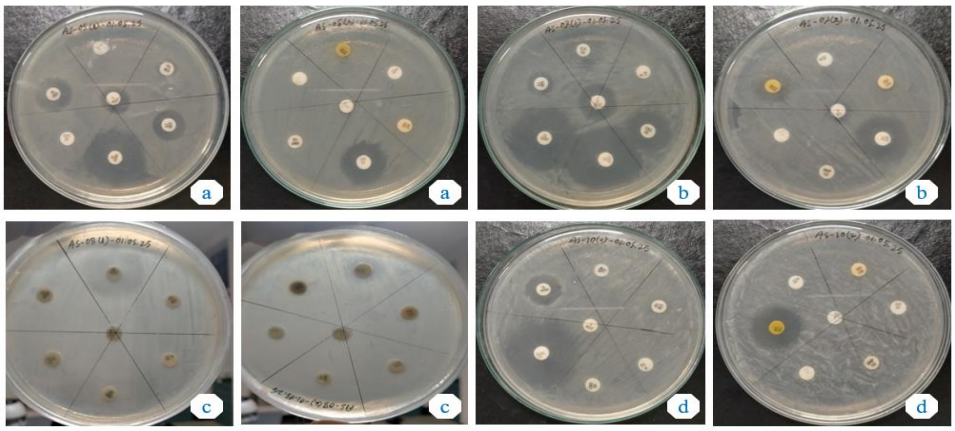

Figure 3. Antibiotic resistance profile of four isolated bacteria. Where **a** = strain-1**, b** = Strain-2**, c** = Strain-3**, d** = Strain-4.

3.6. Thermal and pH stability:

Based on the lysis capacity and host specificity, two phages (Phage-1 and Phage-5) were selected for further characterization. In case of the thermal stability test, both phages showed normal growth after 1 h incubation at -20 °C, 4 °C, 25 °C, 37 °C, and 50 °C but failed to grow at 70 °C and 90 °C (**Figure 6**). In the pH stability test, Phage-1 and Phage-5 showed no growth at pH 1 and pH 3 in LB liquid media. But both phages showed normal growth at pH 5, pH 7, and pH 9, and the growth decreased at pH 12 (**Figure 7**).

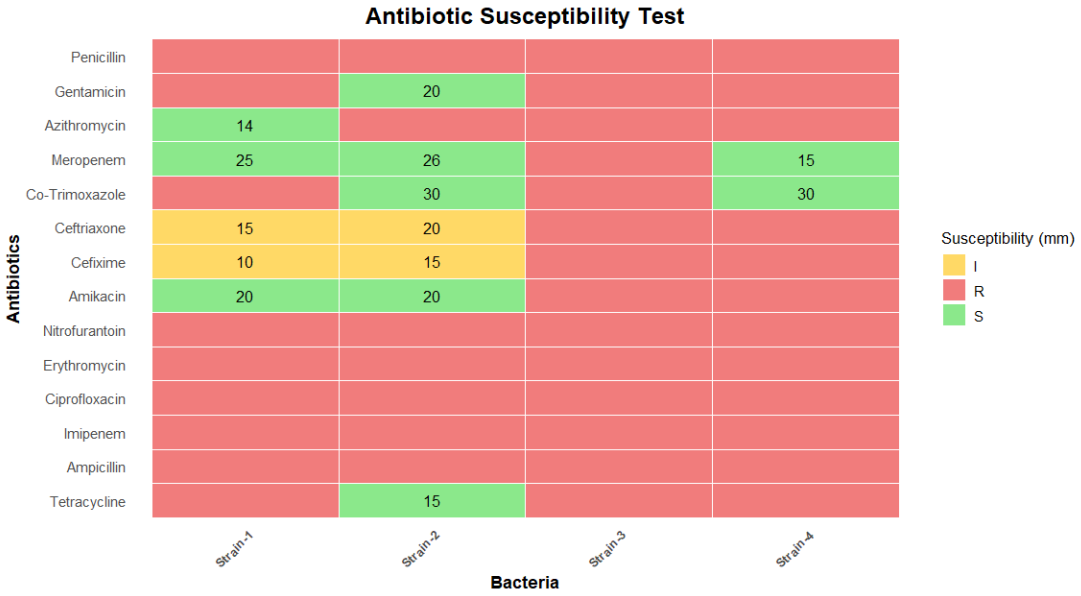

Figure 4. Heatmap showing zone of inhibition (mm) for four bacterial strains. Yellow indicates I = Intermediate, red indicates R = Resistant, and green indicates S = Sensitive.

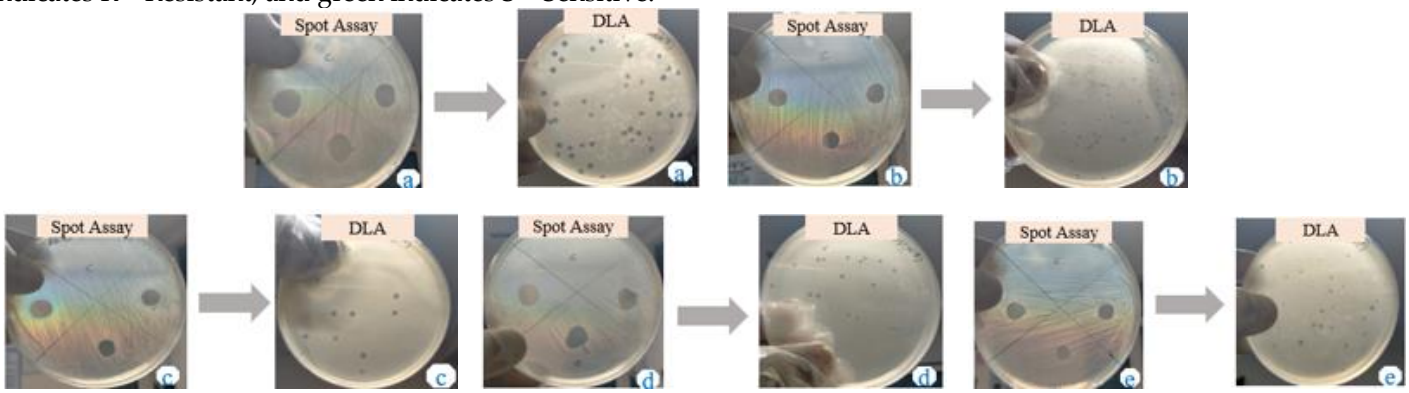

Figure 5. Lytic activity of isolated bacteriophages was demonstrated by spot assay and double-layer agar (DLA) assay. The spot assay plates showed clear zones of bacterial cell lysis, while the DLA assay showed distinct plaque formation, confirming successful bacteriophage infection and lysis of susceptible host bacteria. The tested phages were designated as: (a) Phage-1, (b) Phage-2, (c) Phage-3, (d) Phage-4, and (e) Phage-5. Among them, Phage-5 was tested against *Pseudomonas aeruginosa*, whereas the remaining phages were tested against *Escherichia coli*.

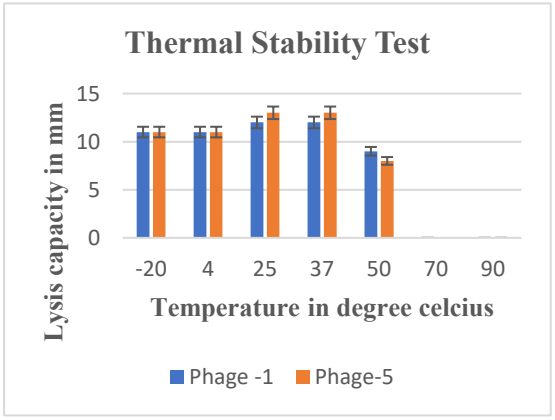

Figure 6. Thermal stability at different temperatures.

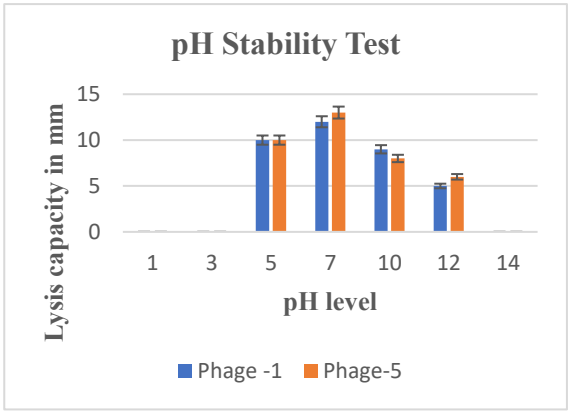

Figure 7. pH stability at different pH levels.

4. Discussion

The current study indicates that multidrug-resistant bacterial isolates from patients suspected of urinary tract infection are common and illustrates the potential use of bacteriophages as alternative antibacterial agents. Four bacterial strains were isolated from urine samples and characterized chiefly as *Escherichia coli* and *Pseudomonas aeruginosa* through biochemical tests, Gram staining, 16S rRNA gene sequencing, and phylogenetic analysis using NCBI. The phenotypic methods utilized in this study, together with molecular confirmation, have increased the reliability of species identification. This is particularly important in clinical microbiology where the accurate detection of urinary pathogens is crucial for informing appropriate treatment decisions [27],[28].

Antibiotic susceptibility testing showed that all four isolates had resistance to multiple antibiotics. Only Strain-3, named *Pseudomonas aeruginosa*, turned out to be the most resistant, as it exhibited pan-drug resistance (PDR). This result is alarming since *P. aeruginosa* has an elegant strategy of developing resistance, betraying its intrinsic mechanisms, biofilms, efflux pumps, and acquisition of genes responsible for antibiotic resistance [29],[30]. Strain-4 was also the most resistant, with 12 antibiotics resisted, while Strain-1 and Strain-2 were able to resist 9 and 7 antibiotics, respectively. These findings suggest that multidrug resistance is a serious issue among the uropathogens assessed.

The high resistance of these isolates to common antibiotics indicates that treatment options may be limited for infections caused by these strains. The above trend is consistent with earlier reports of rising antimicrobial resistance among uropathogens, mainly attributed to inappropriate use of antibiotics, over-the-counter availability, and poor clinical diagnostic practice in low- and middle-income countries like Bangladesh [6],[7],[31]. This study has also identified the degree of multidrug-resistant isolates, which clearly indicates that alternative antibacterial strategies are essential.

Five phages isolated from wastewater exhibited strong lytic activity against *E. coli* and *P. aeruginosa*, forming clear zones of lysis on bacterial lawns. The capacity of the phage to lyse susceptible bacterial cells, as reflected by variations in plaque size and clarity, suggested that these phages may have therapeutic or biocontrol potential [20],[25],[26]. Phage-1 and Phage-5 were selected due to their strong lytic activity with specificity, stabilized through the normal temperature (−20 °C to 50 °C) and pH range (5–10) yet lost activity under extremely high temperatures as well as highly acidic or alkaline conditions. Such robustness and stability, as suggested by previous studies of stability profiles in other phages can enhance the practical use of phage in varying environmental conditions [24]. This is consequential for further therapeutic targeting against uropathogens.

In general, this study provides a firm basis for the potential use of bacteriophages as alternative treatment systems against XDR uropathogens. However, additional studies are required, especially clear regulatory guidelines and clinical safety assessment before validating their therapeutic application.

5. Conclusion

The isolation of XDR *E. coli* and *P.aeruginosa* from the urine samples validates the increasing problem of antibiotic resistance among uropathogens. The isolation of lytic bacteriophages from wastewater and their persistence at different temperatures and pH also shows that these phages have potential for the development of phage-based control strategies. Consequently, bacteriophage therapy may be a potential option for the treatment of multidrug-resistant uropathogens in Bangladesh and similar settings with limited resources.

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