

EFFICACY OF BACTERIOPHAGE AGAINST *PROTEUS MIRABILIS* IN URINARY TRACT INFECTIONSLaiba Javed^{*1}, Kainat Abbas², Zahra Batool³, Shaista Hamid⁴, Hafiz Nida Ullah⁵^{*1}University of Sialkot²University of Central Punjab, Lahore³Riphah International University⁴Jinnah University for Woman⁵Department of Microbiology, University of Veterinary & Animal Sciences, Swat^{*1}laibajaved325@gmail.comDOI: <https://doi.org/10.5281/zenodo.17119040>**Keywords**

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Abstract

Background: The rising prevalence of multi-drug resistant (MDR) pathogens in urinary tract infections (UTIs) poses a significant public health challenge, highlighting an urgent need for alternative therapeutic strategies. Phage therapy, which uses lytic bacteriophages to specifically target and eliminate bacteria, represents a promising alternative to conventional antibiotics.

Objectives: This study aimed to isolate and evaluate the lytic activity of bacteriophages against clinical isolates of multi-drug resistant *Proteus mirabilis* obtained from patients with urinary tract infections.

Methods: We collected 16 wastewater samples from various locations in Kohat to screen for bacteriophages. Ten clinical samples of MDR *P. mirabilis* were isolated from 400 urine specimens cultured from UTI patients at District hospital Kohat. Standard microbiology techniques, including the Kirby-Bauer disk diffusion method, were used for antibiotic susceptibility testing. Phage lytic activity was initially screened using a spot assay, followed by a double-layer agar (DLA) assay to confirm activity and quantify the phage populations.

Results: Four of the sixteen wastewater samples (Samples No. 4, 9, 7, and 11) demonstrated clear zones of inhibition, confirming the presence of bacteriophages with lytic activity against *P. mirabilis*. The plaque assays revealed varied plaque sizes and morphologies, suggesting the presence of diverse phage populations.

Conclusion: The successful isolation of lytic bacteriophages against clinical MDR *P. mirabilis* isolates indicates that these phages have strong potential as a therapeutic agent. Phage therapy could serve as a viable alternative or an effective adjunct to conventional antibiotics for treating UTIs caused by multidrug-resistant organisms.

INTRODUCTION

Urinary tract infections (UTIs) represent one of the most common bacterial conditions globally, with an estimated prevalence of over 150 million cases per year. These infections pose a significant public health and economic burden, accounting for millions of

clinical visits and hospitalizations annually. While *Escherichia coli* is the most frequent causative pathogen of uncomplicated UTIs, other microorganisms, particularly *Proteus mirabilis*, are

major contributors to complicated and catheter-associated infections (Foxman, 2002).

Proteus mirabilis is a facultative anaerobic, Gram-negative bacterium characterized by several key virulence factors that facilitate its role in complicated UTIs. Its most notable virulence factor is the enzyme urease, which hydrolyzes urea to ammonia, raising the urine pH and promoting the formation of struvite (magnesium ammonium phosphate) stones. These crystals not only complicate treatment but also provide a surface for the bacterium to form a tenacious crystalline biofilm (HCAI Technology Innovation Programme, 2008). Biofilms serve as a protective extracellular matrix, shielding the bacteria from both host immune responses and antibiotic penetration, thereby contributing significantly to persistent and recurrent infections (Foxman, 2002).

The widespread and often inappropriate use of antibiotics has fueled a global crisis of antimicrobial resistance (AMR), which further complicates the management of UTIs. *P. mirabilis* has demonstrated a remarkable ability to acquire and spread resistance genes through both vertical evolution and horizontal gene transfer (HGT), a process primarily mediated by mobile genetic elements like plasmids and transposons (Foxman, 2002). This genetic flexibility has led to the emergence of multidrug-resistant (MDR) strains that are often resistant to multiple classes of antibiotics, including cephalosporins and fluoroquinolones, leaving clinicians with limited therapeutic options.

Given the escalating threat of AMR and the unique challenges posed by biofilm-associated infections, alternative therapeutic strategies are urgently needed. Bacteriophages, or phages, are viruses that specifically target and kill bacteria, offering a promising alternative or adjunct to conventional antibiotics. Phages are the most abundant biological entities on Earth, found wherever their bacterial hosts exist. They are highly specific, targeting only their host bacteria without harming human cells or beneficial commensal flora. Lytic phages, which replicate within and then burst their host cell, are particularly effective as bactericidal agents. This mechanism of action, combined with their ability to overcome bacterial resistance and biofilm barriers, makes them

a compelling candidate for treating infections caused by MDR bacteria like *P. mirabilis*.

Despite the increasing evidence supporting the potential of phage therapy, there remains a critical need for localized research to identify and characterize phages effective against regional clinical isolates. This study aims to address this gap by isolating and evaluating the lytic activity of bacteriophages against clinical MDR *P. mirabilis* strains from local wastewater sources. Our findings will provide a scientific basis for the potential use of phage therapy as an effective tool in combating the rising problem of antibiotic resistance in UTIs.

Methodology:

Materials and Methods

Study Design, Setting, and Ethical Approval

This study employed a cross-sectional and experimental design to evaluate the in vitro lytic efficacy of bacteriophages against clinical isolates of *P. mirabilis*. The research was conducted at the University of Kohat, Research Laboratory. Clinical urine samples were collected from patients visiting the District General Hospital, Kohat. The study was conducted in accordance with the ethical standards.

Sample Collection

A total of 502 mid-stream urine samples were collected from patients with clinical signs and symptoms of a urinary tract infection (UTI) over a one-month period. Samples (4–5 mL) were collected in sterile containers using the clean-catch mid-stream technique. Each sample was labeled with patient demographics, and all patients provided informed consent. In parallel, 12 wastewater samples (approximately 500 mL each) were collected from various sites, including hospitals, wastewater treatment plants, and residential sewage system of Kohat, Pakistan. All samples were transported to the laboratory on ice and processed within 24 hours of collection.

Bacterial Isolation, Identification, and Maintenance

Urine samples were cultured on selective and differential media, including MacConkey agar and blood agar, for the isolation of uropathogenic bacteria. After a 24-hour incubation at 37°C, colonies with characteristic morphology were sub-cultured and purified. Presumptive *P. mirabilis*

isolates were identified based on standard microbiological techniques, including Gram staining and colony morphology. Final confirmation of the isolates was performed using the API 20S biochemical kit (BioMérieux, France) according to the manufacturer's instructions. Confirmed isolates were stored in a glycerol stock at -80°C for long-term preservation.

Antibiotic Susceptibility Testing (AST)

The antibiotic susceptibility profile of all confirmed *P. mirabilis* isolates was determined using the Kirby-Bauer disk diffusion method on Mueller-Hinton agar. The inoculum turbidity was adjusted to a 0.5 McFarland standard. A panel of clinically relevant antibiotic disks was tested, including fluoroquinolones (levofloxacin), macrolides (azithromycin), beta-lactams (amoxicillin-clavulanic acid, cefuroxime, ceftazidime), polymyxins (polymyxin B), and aminoglycosides (amikacin). The plates were incubated at 37°C for 18–24 hours. Inhibition zone diameters were measured and interpreted as susceptible, intermediate, or resistant according to the Clinical and Laboratory Standards Institute (CLSI) and European Committee on Antimicrobial Susceptibility Testing (EUCAST) 2021 guidelines.

Bacteriophage Isolation and Purification

Bacteriophages were isolated from the collected wastewater samples using a phage enrichment protocol. Each wastewater sample was filtered through a $0.22\mu\text{m}$ membrane to remove bacterial cells. The filtrate was then mixed with a log-phase bacterial culture of a multidrug-resistant (MDR) *P. mirabilis* isolate and incubated at 37°C with shaking for 24 hours to promote phage replication.

Initial screening for lytic activity was performed using a spot assay. A bacterial lawn of a target *P. mirabilis* strain was prepared on a nutrient agar plate. Small drops of the enriched phage lysate were spotted onto the lawn, and the plate was incubated overnight. The presence of clear zones of lysis indicated potential phage activity.

Positive lysates were then subjected to a double-layer agar plaque assay to quantify and isolate individual phages. Serial dilutions of the lysate were mixed with soft agar containing the host bacterium and poured over a solidified nutrient agar base. After incubation, single, isolated plaques were picked with a sterile

pipette tip, re-suspended in a phage buffer, and re-assayed. This plaque purification cycle was repeated three consecutive times to ensure a monoclonal phage population. Purified phages were then characterized and stored at 4°C .

Data Analysis

All data were recorded and analyzed using Microsoft Excel. Results from the bacterial isolation, identification, and antibiotic susceptibility testing were presented as frequencies and percentages. The plaque assay results were quantified as plaque-forming units per milliliter (PFU/mL). Descriptive statistics were used to summarize the findings.

Results:

A total of 502 UTI-positive samples were included in this study. The age of the patients ranged from 1 to 80 years, with a mean age of 35 ± 15 years. Of these positive cases, 68.8% ($n = 345$) were female, and 31.2% ($n = 157$) were male.

Among the isolated pathogens, *E. coli* was the most prevalent, accounting for 67.0% ($n = 336$) of the cases. *Klebsiella* and *S. aureus* were the next most common, each representing 8.0% ($n = 40$) of the positive samples. *P. mirabilis* was identified in 2.0% ($n = 10$) of the samples, with a gender distribution of 87% ($n = 9$) female and 13% ($n = 1$) male. The mean age for patients with a UTI-positive diagnosis was 34.6 years, with the majority of cases occurring in middle-aged and older individuals. The demographic information, age, and gender distribution for the study population are summarized in Table 5.1.

From the 14 wastewater samples collected from the twin cities, four confirmed positive for bacteriophage activity via spot plaque assay. Notably, no positive results were obtained from samples collected in Rawalpindi. The double-layer agar overlay method for plaque purification yielded a final phage titer of 10.0×10^3 PFU/mL.

Growth and Identification of Microorganisms

The bacterial isolates were grown on various culture media to facilitate identification and characterization. On MacConkey agar, *P. mirabilis* formed characteristic pale, colorless colonies due to its inability to ferment lactose (Figure 4.6.2.1). In contrast, on Blood agar, the isolates produced large, distinct swarming colonies across the surface of the

medium, with some strains displaying a hemolytic pattern (Figure 4.6.2.2).

Microscopic examination using Gram staining confirmed the isolates were Gram-negative, rod-shaped bacteria, which appeared pink or red under the microscope (Figure 4.6.5.1). The final biochemical confirmation of *P. mirabilis* was performed using the API 20S kit.

Antibiotic Susceptibility and Phage Characterization
Antibiotic susceptibility testing (AST) was performed using the Kirby-Bauer disc diffusion method on Mueller-Hinton agar. The results showed that the *P.*

mirabilis isolates exhibited resistance to multiple antibiotics, including cefotaxime (CTX), ceftriaxone (CRO), azithromycin (AZM), piperacillin (PI), amikacin (Ak), and levofloxacin (LEV). However, the isolates demonstrated susceptibility to polymyxin B (PB) and meropenem (MEM).

From the wastewater samples, bacteriophage activity was initially screened using the spot assay, which produced clear zones of lysis, confirming the presence of active phages. A subsequent double-layer plaque assay was performed to purify and quantify the phages, revealing distinct plaques.

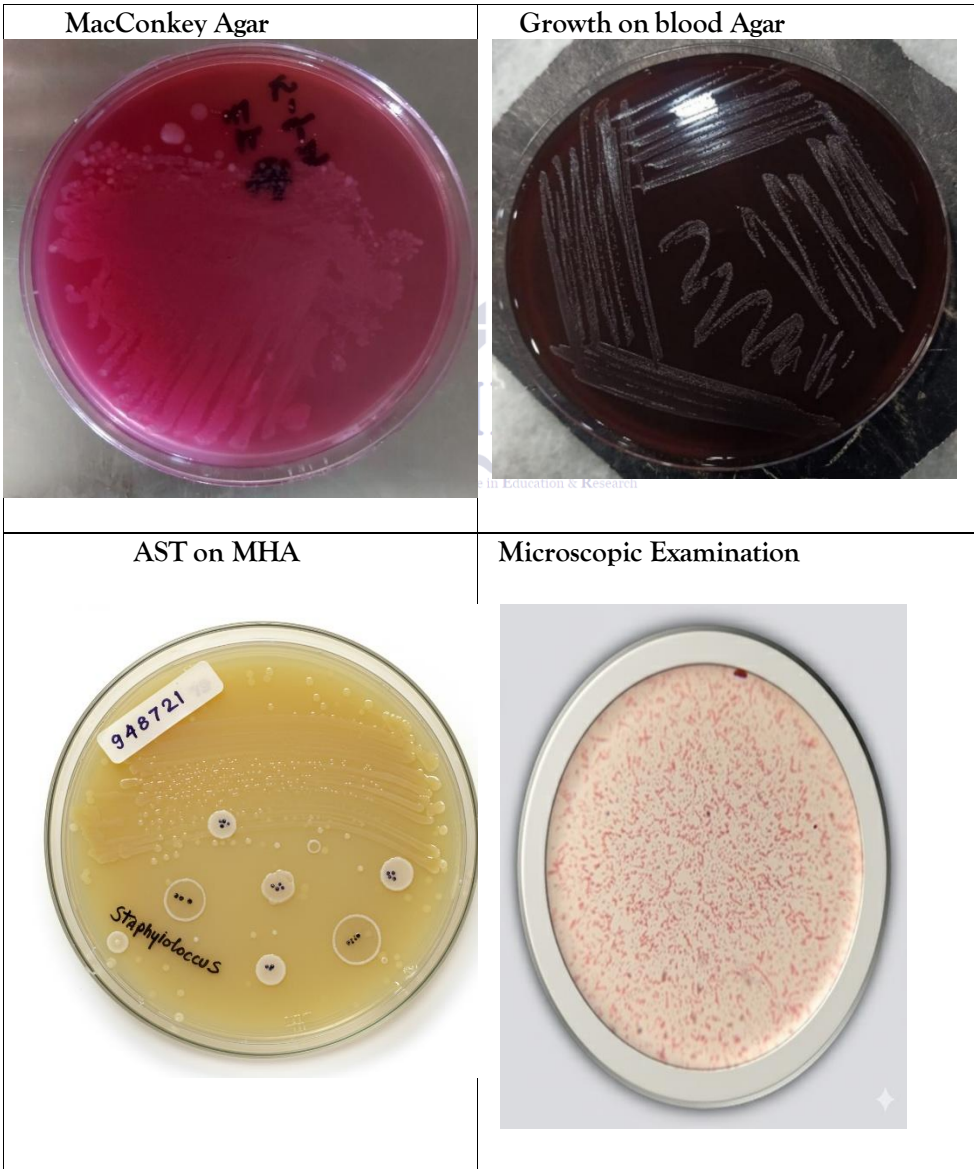


Figure Biochemical Confirmation of *P.mirabilis* through API 20s kit.



Figure Phage Confirmation of *P.mirabilis* through Spot assay

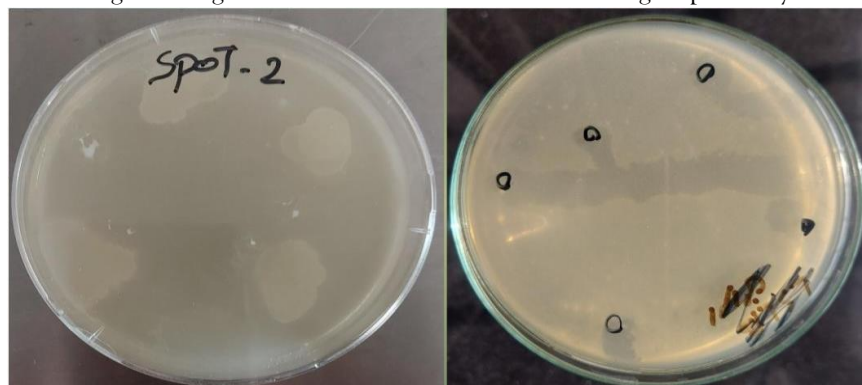


Table: Collected Bacterial Isolates of the current study

Microbe	Positive (%)	Male	Female
E. coli	335 (67.0%)	70	269
Klebsiella	40 (8.0%)	17	23
S. aureus+	40 (8.0%)	17	23
Enterococci-	11 (2.2%)	0	11
Pseudomonas	28 (5.6%)	11	17
Coliform	17 (3.4%)	11	6
Enterobacter	28 (5.6%)	0	28
P.mirabilis	12 (4.0%)	3	9



Figure plaques Appearance in Plaques assay.

Discussion

This study evaluated bacteriophage therapy as a potential alternative treatment for urinary tract infections (UTIs) caused by *P. mirabilis*. Although *P. mirabilis* was a relatively rare isolate in our study (2% of cases), it is a clinically significant pathogen due to its inherent virulence factors, including swarming motility, urease production, and biofilm formation, which make it difficult to treat with conventional antibiotics. Our findings of multidrug resistance in the isolates underscore the critical need for alternative therapeutic strategies.

Bacteriophages, which specifically target and lyse bacteria, offer a promising solution. Unlike broad-spectrum antibiotics, phages can target *P. mirabilis* without disrupting the beneficial native microbiome. Furthermore, their ability to evolve alongside bacteria and to penetrate biofilms provides a significant advantage in treating persistent and chronic infections. The promising results from our in vitro experiments lay the groundwork for a more targeted and effective approach to managing these challenging infections.

Conclusion

This study provides compelling in vitro evidence that bacteriophage therapy is a viable and effective strategy for treating UTIs caused by *P. mirabilis*. The findings demonstrate that phages can effectively lyse strains that are multidrug-resistant and that their high specificity and capacity to target biofilm-forming bacteria are significant advantages over traditional antibiotics. While this research is a crucial first step, the findings must be validated through further studies.

Recommendations and Limitations

This study was limited to in vitro experiments. The efficacy and stability of phages in the complex in vivo environment of the urinary tract, where they may be affected by the host immune system, remains to be determined. Therefore, we recommend that future research focus on animal models and clinical trials to confirm these findings. Additionally, the potential for phage resistance, although less likely than antibiotic resistance, should be managed through the development of phage cocktails or combination

therapies with conventional antibiotics to ensure sustained treatment success.

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