Urine -Derived Bacteriophages as Biocontrol Agents: In Vitro Evaluation of Their Lytic Activity Against Uropathogenic *Escherichia coli* Clinical Isolates and Implication for Phage Therapy in UTIs.

Shaymaa M. Hashim[1], Assist. Prof. Dr. Abdulrahman M. Geeran Al Fahdaw[2], Prof. Dr. Waleed Nassar Jaffal[3]

[1,2] Department of Microbiology, College of Medicine, University of Anbar, Anbar, Iraq.

[3] College of Medicine, University of Anbar, Anbar, Iraq.

[1]sha21m0014@uoanbar.edu.iq, [2]abdulrahmanmohammed34@uoanbar.edu.iq, [3]med.waleed.aljaffal@uoanbar.edu.iq

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Abstract

The rising cases of multidrug-resistant (MDR) infections have necessitated the World Health Organization (WHO) to reiterate the need to find alternative antimicrobial measures. Bacteriophage therapy is one of such options that has gained popularity. This study evaluates the therapeutic capabilities of bacteriophages that have been directly obtained from the urine of patients with the clinical diagnosis of urinary tract infections (UTIs). Two out of five positive cultures of urine samples that produced positive cultures of the bacteria were lysogenized to obtain lytic phages. The temperate bacteriophages were found to have a strong lytic capability against 13 out of 15 MDR *Escherichia coli* clinical isolates. The presence of resistance in two isolates highlights the interplay of forces that are complex to determine phage-bacterial interactions that may be mediated by genetic processes (CRISPR-Cas systems or mutations in receptors). Notably, the lytic spectrum was expanded by the use of phage cocktails. Transmission electron microscopy (TEM) analysis has shown that the phages that have been isolated are of *Myoviridae* and *Podoviridae* families. While purified phages exhibited specificity for *Escherichia coli*, raw urine samples contained phages capable of lysing other uropathogens, such a*s Klebsiella pneumoniae, Enterobacter spp., and Serratia spp.* This indicates that the reservoir of human urine can be varied and individualized bacteriophages in extreme infection, hence highlighting the prospect of urine-derived phage to develop patient-specific targeted therapies, of special importance in areas where resistance to antibiotics is a major health concern on the part of the population.

1. Introduction

Urinary tract infections (UTIs) are a common set of bacterial diseases that impact individuals at both national and international levels and create an enormous cost to health care systems. UTI occurs commonly in the clinical setting in Iraq, particularly in female patients, and it leads to increased morbidity and rising costs of treatment [1, 2]. Among the various etiologic agents of bacteria, *Escherichia coli*, especially the uropathogenic strains (UPEC), is recognized as the most common causative pathogen in all forms of community-acquired and hospital-acquired UTIs and causes about 90 percent of reported cases [3].

Despite empirical antibiotic treatment remaining a pillar of clinical practice, with the advent and spread of multidrug-resistant strains of Escherichia coli, the effectiveness of first-line antimicrobial agents, such as

ampicillin, trimethoprim, sulfamethoxazole, as well as fluoroquinolones, has been significantly undermined [4], [5]. This alarming trend of the increasing rate of antimicrobial resistance is further highlighted by recent annotations by healthcare facilities in Iraq and that the situation has resulted in higher treatment failure and recurrence of infection [6].

However, the situation has changed with the growing menace of antimicrobial resistance, in which the investigation of novel sustainable alternatives to traditional antibiotics has been encouraged by the World Health Organization (WHO). Among those alternatives, there is Bacteriophage therapy, which uses viruses that infect and lyse bacterial cells selectively. The accuracy with which phages can attack drug-resistant strains makes them an even more viable and appealing alternative in tackling multidrug-resistant strains [6].

In most instances, bacteriophage isolation has relied on high density microbial environments of sewage and wastewater. Other clinical samples, such as urine and other body fluids in infected patients, however, have not been studied to a relative extent, which is amenable, even though they may contain phages that have been naturally selected to survive in uropathogenic bacteria. The exploration of these poorly used sources can produce therapeutic phages with better applicability in urinary tract infections [7], [8].

In light of these considerations, the current study seeks to separate and characterize bacteriophages directly from the urine of patients diagnosed with UPEC-related UTIs. By targeting a clinically relevant but rarely explored sample type, this research aims to assess the potential of urine-derived phages as targeted therapeutic agents and to contribute to the development of phage-based strategies tailored to UTI management.

2. Materials and Methods

This study was carried out from May to August 2024, in the microbiology departments of Al-Ramadi Teaching Hospital and Al-Mahmudiya General Hospital.

2.1 Isolation of bacteria and preservation

The midstream urine samples were taken from patients with clinically suspected infection of the urinary tract. Only specimens that showed growth of the E. coli were taken to undergo further analysis. The isolates of the bacteria were identified by using the traditional phenotypic methods and these methods included the colony morphology on a MacConkey agar, lactose fermentation and Gram staining of the bacterial isolate.

To confirm the identifications and test the antimicrobial sensitivity of the isolates, each of the isolates was evaluated using the VITEK -2 Compact system (bioMérieux, France). The operations were done following the guidelines of the manufacturer, and the results were interpreted according to the standards of the Clinical and Laboratory Standards Institute (CLSI 2024) [9,10]. The isolates were stored at -20 °C in brain heart infusion (BHI) broth containing 15% glycerol to maintain their long-term stability and availability in future bacteriophage assays.

2.2 Isolation of Bacteriophages:

Isolation of bacteriophages was done using urine samples of five patients who were clinically diagnosed with urinary tract infections. The sample included a group of people of different ages: a 6-month-old boy, a 13-year-old boy, a 25-year-old woman, a 35-year-old woman, and a 40-year-old woman. Each subject would provide 5 ml of urine in aliquots, which were done separately. Centrifugation of the urine samples was done using 5000 rpm centrifugation for 7 minutes to get the cellular debris out. A 1:10 (chloroform: supernatant) chloroform mixture was then added to the cleared supernatant and transferred. This was mixed in a shaking incubator (8 min) to facilitate homogenization and to remove any remaining cells of bacteria [11].

Following incubation, the treated mixture was centrifuged again at room temperature at 5,000 rpm for 7 minutes. This modified protocol was based on standard phage enrichment procedures. The resulting supernatants were screened for the presence of bacteriophages by using the spot test method. Drops of each treated lysate (10µL) were spotted onto previously prepared (0.05 McFarland of *E. coli)* lawns by separating 1 ml of *E. coli* derived from UTI patient isolates after drying for 10-20 min at room temperature, then incubated overnight. The appearance of a clear lytic zone indicated the presence of lytic bacteriophages. The remaining lysate was saved in a refrigerator temp for many days [12], [13].

2.3 Plaque Assay and Phage Characterization:

To evaluate the key characteristics of the isolated lytic bacteriophages, a standard double-layer agar (DLA) plaque assay was employed. Isolated bacteriophages from the urine samples were saved in prepared SM-buffered saline (SM buffer contains:200 mM NaCl2,10 mM MgSO4, 50 mM Tris-HCl, pH 7.5). A 100 µL aliquot was mixed with 100 µL of an overnight E. coli culture (approximately 10^8 CFU/mL) and incubated at 37°C for 10 minutes to allow phage adsorption. After incubation, each mixture was combined with 3 mL of molten soft

agar (0.4% agar of nutrient medium) maintained at 45°C, gently vortexed, and poured over pre-warmed nutrient agar plates. Cultures were incubated at 37 °C after the solidification of the top layer took place, 18-24h. Obvious lysis spots (plaques) were then noted, and their sizes, periphery, and transparency of each phage isolate were measured [12].

To further clarify the characteristics of isolated phages, the host range was determined using the spot assays on a panel of *E.coli* isolates obtained from different patients with urinary tract infections. The least dose of lysate that worked (MED) was also determined by serial dilution of the lysate used on the test strains compared to the host strain. These methodology procedures are consistent with protocols reported before by investigations conducted in the area of Iraq, and also emphasized the importance of complete phage characterization in the therapeutic use [12-15].

2.4 Transmission Electron Microscopy (TEM) Analysis

Morphological examination of the isolated phages was performed using transmission electron microscopy (TEM). For transmission electron microscopy (TEM), negative staining was performed using Uranyl acetate stain. A droplet (~10 µL) of the purified bacteriophage suspension was placed on a piece of parafilm. Then, a formvar-coated copper grid was carefully placed on the phage droplet using fine-tipped forceps and allowed to adsorb the sample for approximately 1 minute. The grid was then gently blotted using filter paper to remove excess liquid. Subsequently, the grid was transferred onto the UranyLess droplet (~10 µL) for 1 minute to allow staining, followed by a second blotting step to remove excess stain. The stained grids were left to air-dry and imaged at 80 kV under the transmission electron microscope [16]. Phages were classified morphologically according to ICTV guidelines.

3. Results

3.1 Antimicrobial Resistance Profiles of *E. coli* Isolates

The antimicrobial susceptibility patterns of the 15 urinary *E. coli* isolates are summarized in Table 1. Resistance reached 100% for trimethoprim–sulfamethoxazole. High levels of resistance were also observed for ciprofloxacin (86.7%), ceftazidime (80.0%), and gentamicin (66.7%). Carbapenems remained the most effective drugs, with imipenem and meropenem showing susceptibility rates of 93.3% and 92.9%, respectively. Nitrofurantoin showed moderate effectiveness (80.0%). All isolates (100%) fulfilled the definition of multidrug resistance (resistance to ≥3 drug classes).

3.2 Isolation of Bacteriophages from Urine Samples

Five urine specimens were processed for phage isolation as shown in Table 2. Two samples (40%), both culture-positive for E. coli, yielded phages. Samples without detectable E. coli showed no phage recovery

Table 1: Antibiotic susceptibility profiles of 15 *E. coli* isolates tested using the VITEK 2 system.

Antibiotic	Class	Tested (n)	Susceptible n (%)	Intermediate n (%)	Resistant n (%)
AK	Aminoglycoside	15	9 (60.0)	4 (26.7)	2 (13.3)
CN	Aminoglycoside	15	5 (33.3)	0 (0.0)	10 (66.7)
CIP	Fluoroquinolone	15	2 (13.3)	0 (0.0)	13 (86.7)
IMP	Carbapenem	15	14 (93.3)	1 (6.7)	0 (0.0)
MEM	Carbapenem	14	13 (92.9)	0 (0.0)	1 (7.1)
STX	Folate inhibitor	15	0 (0.0)	0 (0.0)	15 (100.0)
CFZ	Cephalosporin	13	5 (38.5)	0 (0.0)	8 (61.5)
CAZ	Cephalosporin	15	3 (20.0)	0 (0.0)	12 (80.0)
F	Nitrofuran	15	12 (80.0)	0 (0.0)	3 (20.0)

Abbreviations: AK = Amikacin, CN = Gentamicin, CIP = Ciprofloxacin, IMP = Imipenem, MEM = Meropenem, STX = Trimethoprim–sulfamethoxazole, CFZ = Cefazolin, CAZ = Ceftazidime, F = Nitrofurantoin. All isolates (100%) met the MDR definition (resistance in ≥3 drug classes).

Table 2: Summary of 5 urine samples used for bacteriophage isolation, including demographic data, turbidity, phage detection, and *E. coli* culture results.

No.of sample	Age group	Sex	Turbidity	Phage Detection	Bacterial culture result for (*E. coli)*
1	6m	male	moderate	negative	negative
2	13y	male	moderate	negative	negative
3	25 y	female	high	positive	positive
4	35 y	female	High	negative	negative
5	40 y	female	High	Positive	Positive

Abbreviations: m: month, y: year

3.3 Phenotypic Characterization of Isolated Phages

Nine phages were recovered from the two positive urine samples. They displayed diverse plaque morphologies, ranging in size from 1 to 3 mm, with variable shapes, margins, clarity and Quantitative spot assays determined minimum effective doses (10^{-3}–10^{-8}), as shown in Table 3, and Figs. 1, 2).

3.4 Host Range and Lytic Activity

The nine phages and their cocktails were tested against all 15 E. coli isolates, as shown in Table 4. Sensitivity varied across isolates. Some isolates (e.g., Pt. 3, Pt. 5, Pt. 10) were highly susceptible, whereas Pt. 11 and Pt. 15 were completely resistant. Phage cocktails (Sh.cok, Sa.cok) demonstrated broader host ranges compared to individual phages, as displayed in Fig. 3.

Table 3: Morphological and functional characterization of bacteriophage isolates.

Phage code	Source	Size of plaque	Shape	Margin	Clarity	MED
Sh1	Rasha	3 mm	circular	Irregular	clear	10^{-3}
Sh2	Rasha	2.5 mm	circular	regular	clear	10^{-8}
Sh3	Rasha	2 mm	circular	regular	clear	10^{-4}
Sh4	Rasha	1.5 mm	oval	regular	clear	10^{-5}
Sh5	Rasha	1mm	circular	Irregular	clear	10^{-6}
Sh6	Rasha	2 mm	oval	Irregular	clear	10^{-5}
Sh7	Rasha	1 mm	circular	regular	Semi -clear	10^{-6}
Sa1	Amina	3mm	Circular	irregular	clear	10^{-4}
Sa 2	Amina	2mm	circular	irregular	clear	10^{-5}

Abbreviations: MED = minimum effective dose.

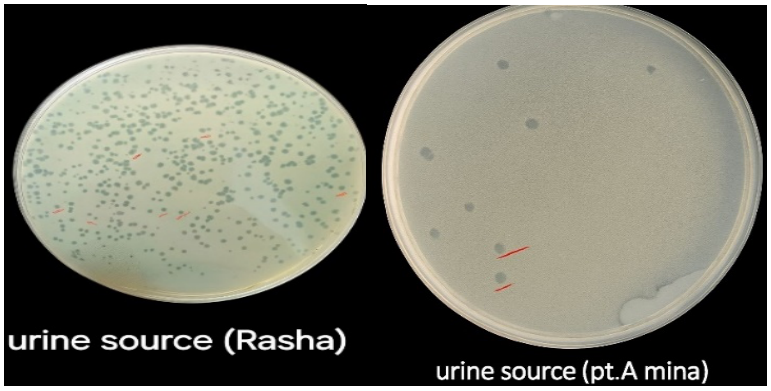

Fig. 1 The double-layer agar (DLA) technique, the first phage-positive sample (from patient Rasha) showed a high abundance of bacteriophages, from which seven distinct phage types were isolated based on plaque morphology. The second phage-positive sample (from Amina) yielded two different phage types.

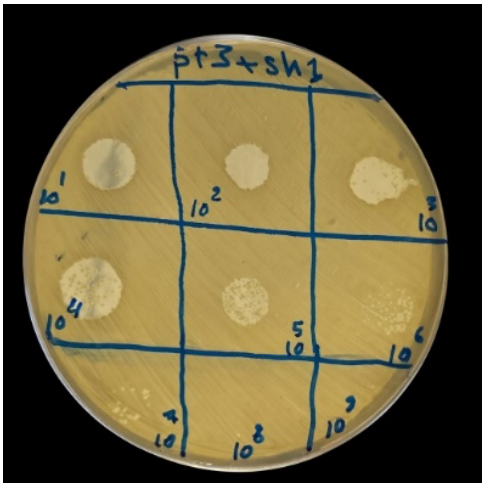

Fig. 2 Minimum effective dose determination by spot assay.

Table 4: Lytic activity of individual phages and cocktails against 15 *E. coli* isolates

Abbreviations: Sh1–Sh7 = Phages isolated from patient Rasha; Sh.cok = Cocktail of Rasha phages; Sa1–Sa2 = Phages isolated from patient Amina;

No.of pt.	Sh1	Sh2	Sh3	Sh4	Sh5	Sh6	Sh7	Sh.cok	Sa1	Sa2	Sa.cok
1	S	S	R	R	S	R	R	S	R	R	R
2	S	R	S	S	R	S	R	S	R	S	S
.3	S	S	S	S	S	S	S	S	S	S	S
4	R	R	R	R	S	R	R	S	R	S	S
5	S	S	S	S	S	S	S	S	S	R	S
6	R	S	R	R	S	S	R	S	S	S	S
7	S	R	S	S	R	S	S	S	R	S	S
8	R	R	R	R	R	R	R	S	R	R	R
9	R	S	R	S	S	S	R	R	R	S	S
10	S	S	S	S	S	S	S	S	R	S	S
11	R	R	R	R	R	R	R	R	R	R	R
12	R	R	R	R	R	R	R	R	S	S	S
13	S	R	R	S	S	R	S	S	S	R	S
14	S	S	R	S	S	R	S	S	R	R	R
15	R	R	R	R	R	R	R	R	R	R	R

Sa.cok = Cocktail of Amina phages; S = Sensitive; R = Resistant.

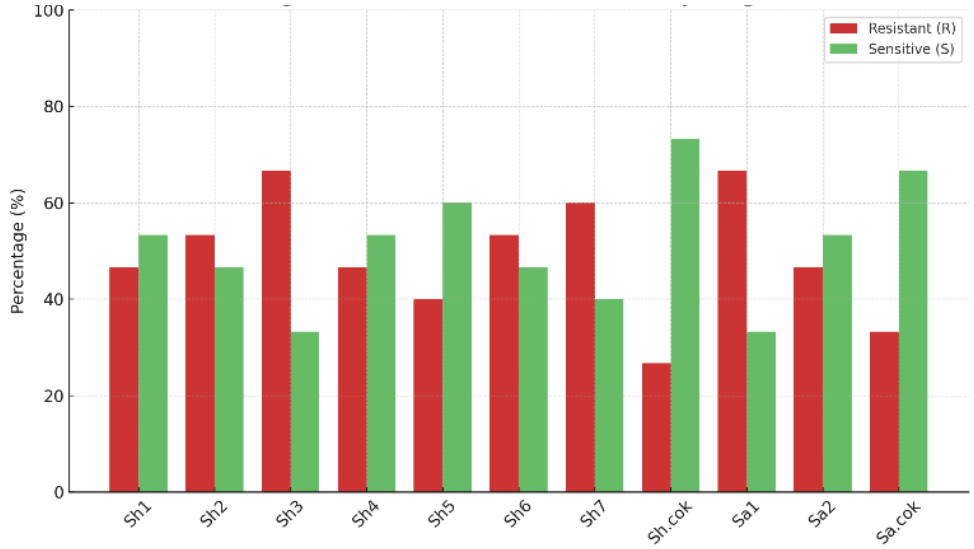

Fig. 3 Percentage distribution of resistant and sensitive responses across tested phages

Notably, the E. coli isolate obtained from patient (Rasha) displayed complete resistance to all evaluated bacteriophages (P1–P7), encompassing both urine- and sewage-derived isolates. No lytic activity or plaque formation was observed on the bacterial lawn. In contrast, the E. coli isolate from patient Amina exhibited clear sensitivity to phages 5B and 6 (originating from sewage) and to phage 7 (isolated from the urine sample of patient Rasha), as evidenced by distinct and well-defined lysis zones on the agar surface as displayed in Fig. 4.

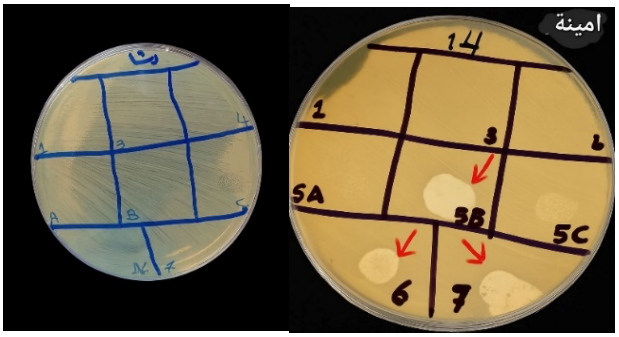

Fig. 4 Phage susceptibility comparison between isolates from patients Rasha and Amina.

3.5 Host Specificity and Unexpected Findings

The nine urine-derived phages showed lytic activity only against *E. coli*. No cross-infectivity was observed with *Klebsiella pneumoniae, Serratia spp., or Enterobacter spp*. However, crude urine samples prior to purification occasionally contained phages infecting other *Enterobacteriaceae*, as displayed in Fig. 5.

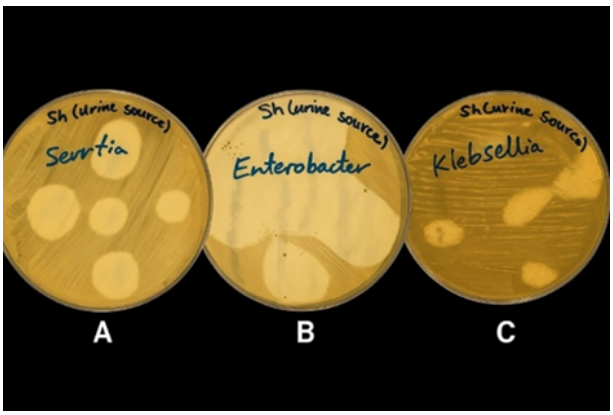

Fig. 5 Phage-induced lysis of *Serratia spp, Enterobacter spp., and Klebsiella pneumoniae*. showing clear spots on bacterial lawns.

3.6 Morphological Characterization by TEM:

Transmission electron microscopy revealed structural diversity among isolated phages, including Myoviridae-like and Podoviridae-like morphotypes as displayed in Fig. 6.

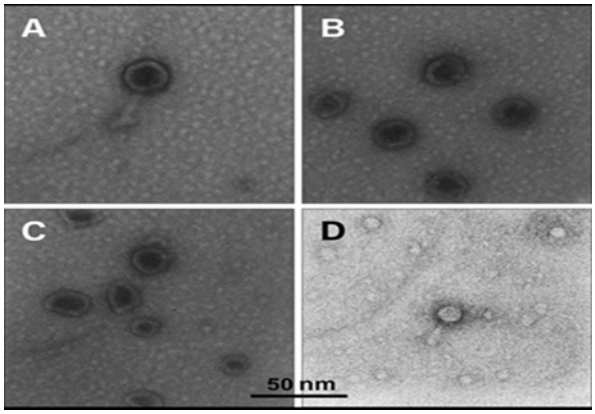

Fig. 6 Transmission Electron Microscopy (TEM) Images of Isolated Bacteriophages (A) *Myoviridae* – large head with contractile tail; (B) *Podoviridae* – short non-contractile tail;(C) *Podoviridae* (tentative) – head only, no visible tail; (D) *Myoviridae* – short tail with icosahedral head. Scale bar = 50 nm.

4. Discussion

The high prevalence of resistance among urinary *E. coli* isolates underscores the growing challenge of MDR pathogens in Iraq. Resistance rates of 100% to trimethoprim–sulfamethoxazole and >80% to fluoroquinolones and cephalosporins are consistent with regional and global studies, highlighting the limited utility of these agents for empirical therapy [17,18]. By contrast, carbapenems retained activity, aligning with international reports that these drugs remain last-resort options, though the threat of carbapenems necessitates strict stewardship [19]. Nitrofurantoin's moderate efficacy (80%) reinforces its value as a urinary-specific antibiotic, as supported by prior studies [20].

The phage isolation was only successful to *E. coli*-positive urine samples, thus, supporting the hypothesis that bacteriophage co-exist with their bacterial hosts in inflamed micro environments [21-23]. This information suggests that urine is not only a diagnostic substance but can be a source of therapeutic phages. The nine isolates exhibited a diverse range of morphologies of the plaque and variable minimum concentrations of the morphology and this is an indication of functional diversity. This type of heterogeneity is a requirement to the development of phage cocktails with a wide host coverage. Host-range studies revealed that single phages often had narrow lytic activities toward individual isolates but phage cocktails have a dramatic lytic breadth. The higher efficacy of combination, rather than that of monophage, is supported by similar results in modern phage-therapy research [24,27].

Full phages resistance in isolates like Pt.11 and Pt.15 can be due to receptor site variants or CRISPR -Cas systems [25], which makes the continuous discovery of new phages with specific receptor targeting expected. The unforeseen seclusion of non- *E. coli* phages in the urine untreated fluid samples implies that urine could possess a larger virome, especially with regards to serious infections. Such observation supports the hypothesis that bacteria phages are usually specific to different *Enterobacteriaceae* in the environment [21]. Morphological heterogeneity, including *Myoviridae*- and *Podoviridae*-like phages, was confirmed using transmission electron microscopy, thus proving the adaptability of the evolution of urine-derived phages. Morphological characterization has remained an important complement to the use of genomic sequencing in taxonomic classification, even though this method now forms the core of the classification process [28].

It is shown here that phages directly recovered from the urine enables for higher patient-specific conformity UPEC strains than sewage-derived sources. Comparable regional background like for example from Iran as similar feasibility although with fewer isolates and lower morphological diversity [29]. Establishing a biobank of urine-derived phages might offer a unique opportunity for targeted therapy against MDR UTI pathogens,

complementing antibiotic stewardship and expanding therapeutic options.

5. Conclusion

Urine from UTI patients serve as one of the quick and effective natural sources for isolation bacteriophages which are lytic to their bacterial hosts. These phages have high lytic efficacy and can provide an alternative approach to treatment of MDR-infected patients. Constructing a heterogeneous phage pool from these clinical sources may provide the basis to produce personalized, phage-therapy in a scope of anti-infective agents which can be used safely and affordably as alternative to conventional antibiotics.

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Conflict of Interest:

The authors declare that there is no conflict of interest regarding the publication of this paper.

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