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# Antibacterial Activity of River Water Bacteriophage against Multidrug-resistant Gram-negative Bacteria, An *In vitro* Study

**Mohammed R. Mohaisen[1,2]*, Shehab A. Lafi[3] and Sawasan Q.T. Al-Quhli[3]**

[1]Molecular Microbiology, College of Dentistry, University of Anbar, Ramadi, Iraq.
[2]Institute of Infection, Veterinary, and Ecological Sciences, University of Liverpool, Liverpool, United Kingdom.
[3]Medical Microbiology, Microbiology Department, College of Medicine, University of Anbar, Ramadi, Iraq.

## Abstract

**Microbes show a high antimicrobial resistance due to a high rate of mutations predisposed by many factors, especially the abuse of antibiotics. Therefore, there is a great need for an alternative therapeutic agent for infectious diseases caused by microbes resistant to antibiotics. Bacteriophages are viruses parasitizing microbes, that got a big scientist's attention due to their ability as an alternative therapy for severe bacterial infections. This study is devoted to identifying bacteriophage from river water on tested pathogenic isolates isolated from clinical cases of UTI *in vitro* and finding out the effect of phage on these bacterial isolates as an initial step of further *in vivo* phage therapeutic study on the same tested isolates. The results showed a significant bactericidal effect of the isolated bacteriophages against the pathogenic bacterial isolates.**

**Keywords:** Phage, Bacteriophages, Phage Therapy and Resistant Bacteria

***Correspondence:** den.m.radhi@uoanbar.edu.iq

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## INTRODUCTION

Many bacterial species over several years are able to avoid the effect of antimicrobial agents, because of bacterial genomic DNA transfer or spontaneous bacterial genome mutations due to the abuse and overuse of antibiotics. These microbial mechanisms defect the activity of certain antibacterial antibiotics turning them ineffective.[1] Infectious bacterial pathogens that are resistant to antibiotics are categorised as Mutable Drug-Resistant pathogens (MDR) and they become a significant health threat worldwide widely.[2] This turned out to be even more problematic, especially with the slower production of new antibiotic agents over recent years.[3] Therefore, it became urgent to use an alternative agent for treating diseases caused by infectious bacteria to decrease antibiotic resistance emerging and disseminating.[4,5]

Bacteriophages are viruses that infect and replicate inside bacterial cells.[6] They got big scientists' attention due to their ability as an alternative therapy for severe bacterial infections. Bacteriophage or simply phage infection can lead to the production of progeny phage via the lytic replication cycle inside bacterial cells. Lytic replication describes the process by which the phage uses the bacterial host cell as a factory to produce new phage capsids, tails and other structural proteins to form new phage particles within the bacterium.[7] Recent theoretical developments have revealed that bacteriophages could take the role of solving the pathogenic bacterial resistance problem as alternative antibacterial therapy.[8,9] The mechanisms used by phage are promiscuous compared to therapeutic regimes of antibiotics. Phage infection is so specific, each bacterial species has its specific phage that could be infected with, even if those bacteria are Mutable Drug-Resistant pathogens.[10,11] In addition to the highly precise specificity of its bacterial host, the phage is abundant, effective in small doses and they are safe for humans and animals. Furthermore, phage particles decreased in their number with their host declined.[12]

A large number of therapeutic, *in vitro* and *in vivo,* approaches using phage particles have been developed over the last few decades testing different bacterial species and have achieved some very good results.[13] For example, bacteriophage was evaluated for the treatment of infection caused by *Klebsiella pneumoniae* in mice.[14] Moreover, several *in vitro* assays reported the efficiency of host-specific bacteriophage in the control of antibiotic-resistant bacteria for vancomycin-resistant *Enterococcus faecium* in experimental animals*,* as well as the efficiency of bacteriophage species isolated from some river water or swage water in the treatment against some enteric pathogens.[15,16] These are examples of using a host-specific phage as an alternative treatment against Mutable Drug-Resistant bacterial pathogens.

In Iraq, antimicrobial resistance among pathogenic bacteria becomes predominant, and little effort has been forced to control this problem.[17] At the same time, this country possesses very good water recourses with two big rivers; therefore, looking for lytic phage in river water or sewage water could be an alternative solution for antimicrobial resistance.

In this study, we examined the effect of phage from Euphrates River water, near Ramadi city, Iraq, against multidrug-resistant bacteria isolated from patients with urinary tract infections (UTI).

## MATERIALS AND METHODS

### Water samples

Samples of river water were taken from virial places of the Euphrates River near Ramadi city, Iraq. The static surface of the side water was used to collect samples in 100 ml sterile containers. Samples were centrifuged to remove big particulates from the water for 30 minutes at 3000 rpm. The supernatant was poured into a sterile wight tube after it was filtered using a 0.22µm syringe filter.

### Bacterial Isolates

Eighty-six Bacterial isolates were isolated from 123 patients suffering from urinary tract infections at the Ramadi Teaching Hospital for Maternity and Children. Bacterial isolates were then investigated for their bacterial species and antibiotic sensitivity using VITEK 2 system (bioMeriex). Clinical bacterial isolates were the only strains used in this study as the availability

of commercial strains could not be achieved. MacFarland standards of turbidity on 0.5 O.D. for each isolate were obtained. This was by inoculating two or three bacterial colonies in 20 ml of Nutrient broth after 4 hours of incubation at 37°C.

**Detection of bacteriophage effect against host bacteria**

The host range of possible water bacteriophage was identified with 86 bacterial isolates using the spot test. Bacterial lawn culture was made using a sterile cotton swab, after being moistened with bacterial broth culture with 0.5 O.D. on tryptic soy agar plates from each bacterial isolate. A drop of five microliters of each filtered water sample was spotted on the agar plate. After they were allowed to dry, the plates were incubated at 37°C for 24 hours. The plates were then examined for the effectiveness zone on the spotted places.[16]

**Plaque assay for bacteriophage isolation**

After it was filtrated, one ml of phage fluid was added to a sterile, 100 ml conical flask already containing 50 ml of a bacterial suspension of the respective host. The mixture was mixed very well before being left for 10 minutes at an appropriate temperature. The mixture was then mixed well with 3 ml of 07% molten agar at 50°C, before it was poured into the top of a nutrient agar plate. After incubation at 37°C, the plates were absorbed for plaque formation over the surface of the top agar for each plate.[18]

## RESULTS

To find out whether the study hypothesis is verified or not, eighty-six (86) clinical bacterial isolates were isolated from 123 patients suffering from urinary tract infections. These clinical isolates are *Escherichia coli*(60 isolates), *Enterobacter cloacae* (23 isolates), *Klebsiella pneumoniae* (24 isolates), *Pseudomonas aeruginosa* (12 isolates), *and Proteus mirabilis* (27 isolates). On another hand, eight samples of river water were taken from the static surface of the riverside to find out whether these water samples contained phage species or not.

The antibiotic sensitivity of bacterial isolates was tested using VITEK 2 system (BioMeriex), this enabled the detection of the bacterial phenomenon of multidrug resistance (Figure 1).

The results revealed 88.33 % of *Escherichia coli* isolates were resistant to a wide range of antibiotics. Similarly, a high range of resistance was found when we tested *Pseudomonas aeruginosa* against used antibiotics. The antibiotic-sensitivity test of *Enterobacter cloacae and Proteus mirabilis* isolates showed medium resistance, with 34.68

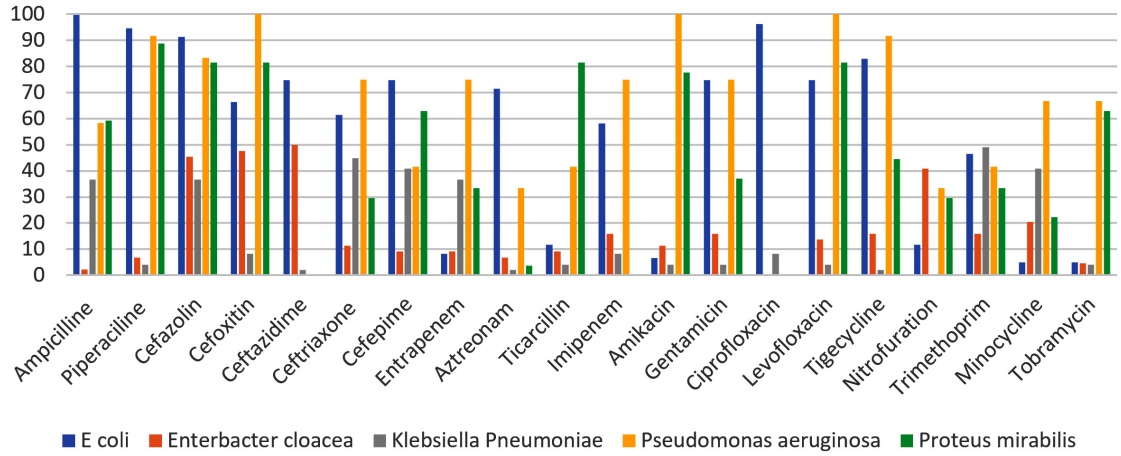


**Figure 1.** The sensitivity pattern of bacterial isolate used in this study.*Escherichia coli* and *Pseudomonas aeruginosa* showed high resistance to the used antibiotics, while medium antibiotics resistance was reported with *Enterobacter cloacae and Proteus mirabilis*. And *Klebsiella pneumoniae* isolates were the most sensitive isolates to antibiotics

% and 37.03%, respectively. The most antibiotic-sensitive isolates were *Klebsiella pneumoniae* isolates, with 16.6% bacterial resistance (Figure 1).

Regarding the bacterial isolates' sensitivity to the bacteriophage, the host range of possible water bacteriophage was detected using the plate spot method on bacterial lawns. The results showed a wide sensitivity range of most bacterial isolates to water samples phage, which is considered significant as the P value is 0.024 using T-test. This result was so obvious, as there was a clear zoon usually created in the place of the water spot on each bacterial lawn (Figure 2 and 3).

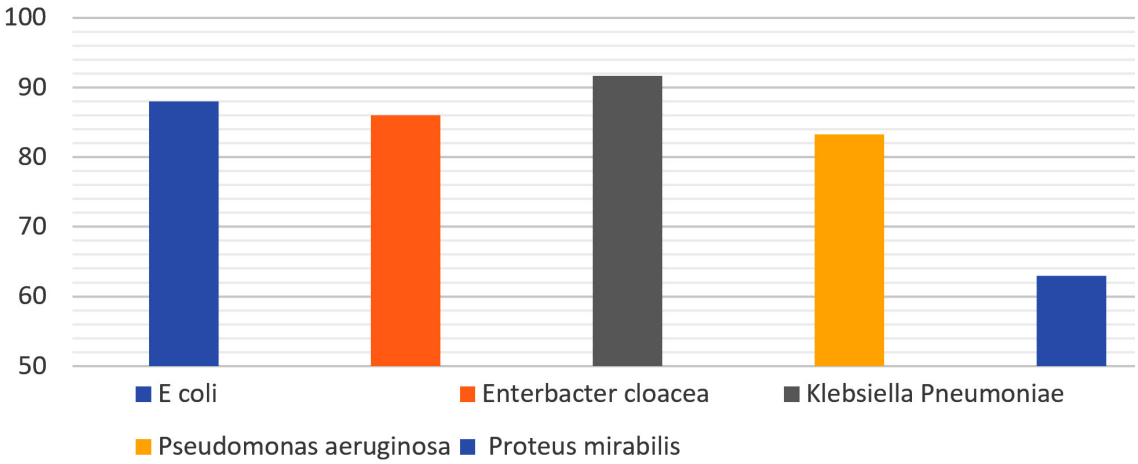


**Figure 2.** The effect of water's phage samples on bacterial isolates used in this study. Bacteria that highly be inhabited by bacteriophage was *Enterobacter cloacae* followed by*E.coli*, while the less be affected bacteria was *Proteus mirabilis*

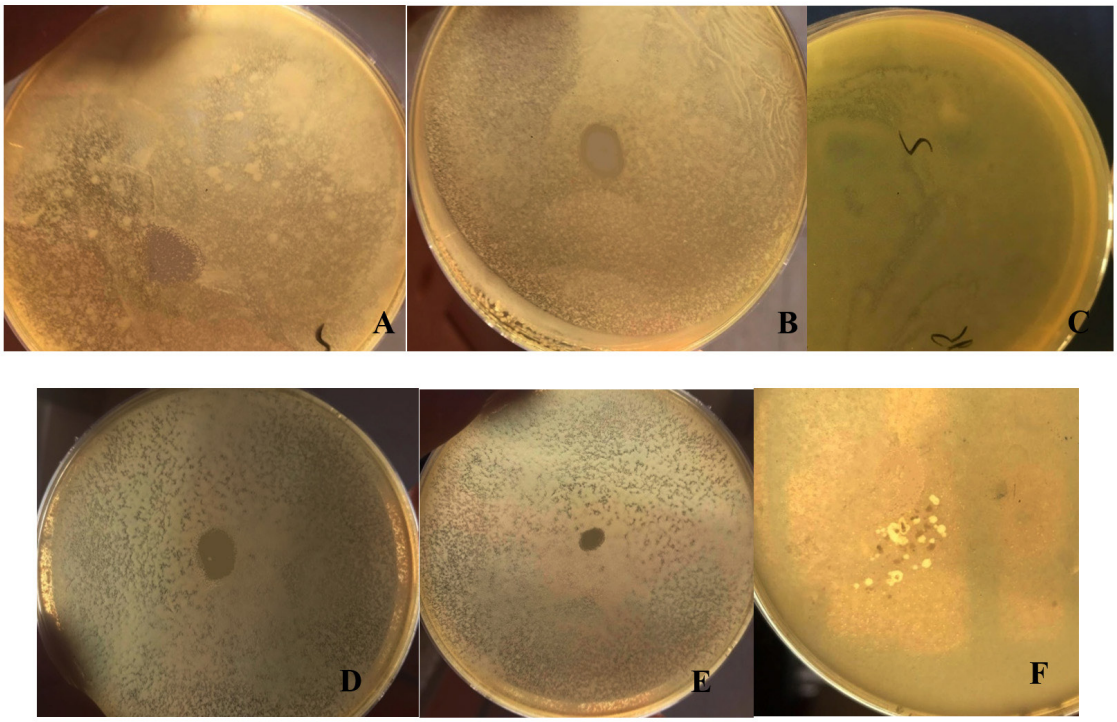


**Figure 3.** The effect of crude water's phage samples on bacterial isolates using the plate spot method on bacterial lawns. (A, B) On *E. coli* lawn plate. (C) On *Pseudomonas aeruginosa* lawn plate. (D) On *Klebsiella pneumoniae* lawn plate. (E) On *Enterobacter cloacae* lawn plate. (F) On *Proteus mirabilis* lawn plate

Although we got a significant detection of water bacteriophage against most bacterial isolates, however, we did not get any positive results of phage plaque assays, this unfortunate result was obtained after ten times repeating plaque assays with several parameter changes throughout about ten months of lab work.

## DISCUSSION

Antibiotics resistant bacteria or what is called multidrug-resistant bacteria (MDR) become a serious problem for the human population throughout the world. Many bacterial pathogens have emerged as new serotypes with high resistance phenomenon to a high range of reliable antibiotics. Moreover, in recent years, the emergence of newly resistant bacterial serotypes was combined with less development of new antibiotics generations, which leads to minimised drug options for some severe bacterial infections.[19]

The use of bacteriophage as an antibacterial infection treatment started even before the discovery of antibiotics. However, the antibiotic emergence in the 1940s especially those with broad-spectrum besides the high bacterial sensitivity to them, at that time, replaced the development of bacteriophage as a therapeutic agent against bacterial infections.[20]

In this study, we examined the effect of suspected phage isolates that might be present in Euphrates River water, near Ramadi city, Iraq, against some Gram-negative bacterial isolates taken from patients with UTI. All bacterial isolates antibiotics sensitivity patrons were tested using VITEK 2 system (bioMeriex). The bacterial isolates' sensitivity to antibiotics was tested against a wide range of antibiotics belonging to groups of Penicillins, several generations of Cephalosporins, Fluoroquinolones, Aminoglycosides, Monobactams, Carbapenems, Folic acid Synthesis Inhibitors and Nitrofurans.

According to the results of this study, most *E. coli* isolates were resistant to antibiotics used. In this study, *E.coli* represents a considerable part of the commensal microbiota of both humans and animals; however, they are also an illness pathogen that could cause serious public health problems.[21] *E. coli* has a variety of antibiotic resistance genes which might be the reason behind their treatment failure in human and animal infections. Significant resistance genes have been detected in *E. coli* genomes recently. Most of these genes were disseminated within the same bacterial generation by horizontal DNA transfer. Furthermore, *E. coli* acts as a resistance genes reservoir for other so related enterobacterial species which can receive these genes and can also act as a donor, so they pass these resistance genes to *E. coli* as well as other enterobacteria.[22] Some of these genes are β-Lactams genes, which are present in numerous numbers in the *E. coli* genome. Many *E. coli* strains have a wide range of extended-spectrum β-lactamases (ESBLs) as well as cephalosporinases genes that could switch off the activity of penicillins, aminopenicillins, and the third and fourth-generation cephalosporins including cefovecin, ceftiofur and cephalosporin cefquinome.[23] Some other *E. coli* strains have the resistance ability to quinolones and fluoroquinolones. The target of these antibiotics is to invalidate the activity of both DNA gyrase, which has two subunits GyrA and GyrB, and topoisomerase, which consists of ParC and ParE subunits, in *E. coli* and other Gram-negative bacteria. Antibiotic resistance to quinolones and fluoroquinolones takes place due to a mutation in the genes for these enzymes in the *E. coli* genome.[24]

Regarding the result of antibiotic sensitivity of *Pseudomonas aeruginosa* isolates, a similar result was seen as the result of *E. coli* isolates, as most *P. aeruginosa* isolates have high resistance to antibiotics used in this study. *Pseudomonas aeruginosa* is considered an opportunistic pathogen that is causing a significant health problem, particularly in compromised immunity patients and individuals with cystic fibrosis illness. It was reported that *P. aeruginosa* has a resistance phenomenon to many antibiotics including-lactamase, aminoglycosides, and quinolones.[25,26] These bacteria are remarkably known for their various mechanisms of antibiotic resistance including intrinsic, adaptive, and acquired resistance mechanisms. As intrinsic, the bacteria could make their cells' outer membrane less permeable, they could also drive the antibiotic out of the cell by efflux pump expression, and the creation of enzymes that inactivate antibiotics. As an adaptive ability of resistance, the bacteria

have the ability of biofilm formation particularly in lower respiratory tract infections which limits the access of antibiotics to the bacterial cells. The acquired ability of resistance can be accrued by either mutational change or by antibiotic genes' horizontal transfer.[27,28] Therefore, the use of alternative treatment strategies for the therapy of *P. aeruginosa* infections becomes urgently needed particularly for patients with antibiotic-resistant infections.

The results of this study revealed that both *Enterobacter cloacae and Proteus mirabilis* isolates showed medium antibiotic resistance. *Enterobacter spp.,* are within *Enterobacteriaceae* that resistant to carbapenem antibiotic (CRE) in the United States and this is the reason behind the distribution of carbapenem-resistant infectious diseases.[29] *Enterobacter cloacae* particularly resistant strains area causative agent of a wide range of hospital-acquired infections, including urinary tract infections, pneumonia, and septicaemia.[30,31] It was reported that *Enterobacter cloacae* can acquire antibiotic resistance genes, including wide deferent carbapenems genes.

*Proteus mirabilis* is a member of *Enterobacteriaceae* that is considered as normal bacterial flora of the human genitive tract. However, these bacteria are also opportunistic pathogens that cause serious urinary tract infections. *Proteus mirabilis* has a natural resistance phenomenon to some antibiotics and has low susceptibility to antibiotics like imipenem. *Proteus mirabilis* has the ability to lose porins and reduce the expression of bacterial genes that encode penicillin-binding proteins (PBPs).[32]

*Klebsiella pneumoniae* isolates showed high sensitivity to the most used antibiotics in this study, this is not a bizarre outcome, similar results were obtained by Ameshe *et al*. who stated in their study, in Northwest Ethiopia, in 2022; that *Klebsiella pneumoniae* isolates had highly sensitive patterns to chloramphenicol, gentamicin, ciprofloxacin, cefoxitin, meropenem, ceftazidime, cefotaxime and nitrofurantoin.[33] Nevertheless, many studies reported that multidrug resistance *Klebsiella pneumoniae* become a public health problem in many parts of the world.[34,35]

Pathogenetic bacteria with multi-antibiotic resistance is a significant globule problem that is estimated to be as serious as the reason behind about ten million mortalities annually by 2050.[36] Therefore, new alternative therapeutic approaches are strongly needed. Using bacteriophages therapeutic strategies is one of these approaches. Phage in their nature, like other viruses, are abundant agents that cannot grow or replicate without the use of bacterial cells with all their mechanisms to make new phases and spread after destroying the bacterial cell. Furthermore, phage are innocuous to plants, animals and even humans. For these reasons, they could be used as an antibacterial agent instead of using antibiotics or they could be used in combination with antibiotics to treat bacterial infections in plants, animals and humans.[37]

According to our results using the plate spot method on bacterial lawns, most of the used bacterial isolates showed a high range of sensitivity to water samples' phage. This result confirms many previous studies' outcomes. Similar results were stated by Bhetwal *et al.* as they tested the effect of river-isolated bacteriophage on bacterial isolates that had MDR or ESBL bacteria.[16] Many previous studies confirmed our presented results, as they confirmed the therapeutic effect of bacteriophage on pathogenic bacteria.[38-41]

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## CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

## AUTHORS' CONTRIBUTION

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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None.

## DATA AVAILABILITY

All datasets generated or analysed during this study are included in the manuscript.

## ETHICS STATEMENT

This article does not contain any studies on human participants or animals performed by any of the authors.

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