# Isolation of various local Bacteriophages via Simple Methods and their Effects against Multidrug Resistance Bacterial Isolates

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**Abstract:**

Recently, dissemination of antibiotic resistant bacteria considered as a major public health concern. Therefore, bacteriophages gained great attention as a promising alternative therapy for resistant infectious diseases.

The current study aimed to isolate bacteriophages by simple methods and determining their efficiency against bacteria isolated from clinical samples.

Bacteria were first isolated from clinical specimens and identified using standard bacteriological and biochemical procedures. antibiogram of the isolated bacteria were determined using different antibiotic discs and the results were interpreted according to the clinical laboratory standards institute (CLSI) guidelines. Then sewage samples were processed using two protocols to isolate specific bacteriophages. Finally, antibacterial effect of bacteriophages was determined using Double Layer Agar(DLA) method.

The highest lytic activity of the isolated bacteriophages was seen using the first protocol. However, both methods showed antibacterial effect.

In conclusion, bacteriophages could be isolated using very simple methods.

**Keyword**: Bacteriophage, DLA, Multidrug resistant bacteria

## عزل عاثيات محلية مختلفة بواسطة طرق بسيطة وتأثيرها على العزلات البكتيرية المقاومة لمضادات حيوية متعددة

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**الخلاصة**

ان انتشار البكتيريا المقاومة للمضادات الحيوية في الآونة الأخيرة ، يعتبر أحد الاهتمامات الرئيسية للصحة العامة . لذلك ، اكتسبت العاثيات اهتماما كبيرا كعلاج بديل واعد للأمراض المعدية المقاومة للمضادات.

هدف الدراسة الحالية إلى عزل العاثيات البكتيرية بواسطة طرق بسيطة وتحديد فعاليتها ضد البكتيريا المعزولة من العينات السريرية .

بداية ,تم عزل البكتيريا من العينات السريرية وتم تشخيصها باستخدام الطرق البكتريولوجية والبيوكيميائية النموذجية . كما تم تحديد مقاومة البكتيريا المعزولة للمضادات الحيوية باستخدام أقراص مضادات حيوية مختلفة وتم تفسير النتائج وفقًا لـ (CLSI) وقد تم أخذ عينات من مياه الصرف الصحي لغرض عزل العاثيات باستخدام طرق مختلفة . وأخيرا ،تم دراسة التأثير المضاد للعاثيات المعزولة باستخدام طريقة الـ (DLA) .

تم عزل عاثيات مختلفة لها تاثير مضاد للبكتريا المقاومة للمضادات الحيوية كما لوحظ أعلى تاثير للعاثيات المعزولة باستخدام الطريقة الأولى. نستنتج انه يمكن عزل عاثيات بكتيرية من مياه الصرف الصحي بواسطة طرق بسيطة .

**كلمات مفتاحية:** العاثيات البكتيرية, DLA, البكترية المقاومة لمضادات متعددة.

## Introduction:

Antibiotics overuse has led to the emergence and dissemination of antibiotic resistance bacteria worldwide which represent a major public health concern.Unfortunately, the rate of new drug development is not rapid enough despite the great efforts to replace less effective drugs[1]. Subsequently, higher morbidity and mortality rates has been documented [2]. Therefore, this necessitates the development ofnew promising alternative antimicrobial drugs for treating infectious diseases and reduce the dissemination of antibiotic resistant strains [3, 4].

Bacteriophages (or phages; viruses that infect bacteria) are the most diverse and numerous microorganism on earth because it is thought to be found in the environment as ten times more than their bacterial host cells[5]. Additionally, phages are thought to be economical, self-replicating, safe and effective bactericidal agents[6].

like all viruses, Phages consist of nucleic acid (DNA or RNA) and capsid (protein coat). But, are not enveloped (unlike some plant and animal viruses). Some phages have elaborate structures for attaching to the bacterial surface and injecting nucleic acid into the cytoplasm[7].

In recent years, bacteriophages gained special attention as an alternative therapeutic regimen as they impose antibacterial effect and self-replicate during infection[8, 9]. Hence, there is new start in the use of bacteriophages to get rid from resistant pathogens[10]. Because of widely distribution in the environment, bacteriophage can be isolated from different sources like fresh water, soil and sewage ecosystems. [11] The high prevalence of bacterial pathogens found in sewage water makes it an important reservoir for isolation of various phages. Although there were different previous studies that aimed to isolate phages from sewage using different methods[12, 13], isolation of phages from sewage water in Kerbala province, Iraq had been not curried out yet. Hence, the current study aimed to isolate phages from sewage water using simple protocols and to explore the effectiveness of these phages by using Double Layer Agar technique (DLA).

## Materials and Methods:

In this study, Sewage water samples was collected and processed in the Department of Clinical laboratories, College of Applied Medical Science, University of Kerbala, during the period from October 2017 to April 2018.

### Isolation of bacteria from clinical samples:

Different clinical samples (including CSF, Swabs) taken from patients attending Al-Kafeel hospital in Kerbala governorate were analyzed aseptically. After initial culturing in the hospital, Bacterial cultures were transferred to the microbiology laboratory. Sub-culturing and Gram staining were performed to ensure purity of the isolates. Other biochemical test including Oxidase, Catalase, Coagulase tests were performed. Antibiogram for the identified isolates was done. Media that used include: Mannitol salt agar, Eosin Methylen Blue (EMB), peptone water, Simmone Citrate agar, MR-VP media, Muller Hinton Agar (Hi Media Laborataries / India), MacConkey agar, Nutrient agar, Nutrient broth, Brain Heart infusion broth, DNase agar (Lab M Limited Topley House/ United Kingdom). For Antibiotics susceptibility testing (AST), The Kirby Bauer disk diffusion method were applied according to the

CLSI guidelines[14] using the specific antibiotics for each isolated bacteria as in table (1).

**Isolation of bacteriophage**

Sewage water sample was collected using screw-capped bottles from ecosystem in Kerbala. Sample processing and isolation of bacteriophage were done using two methods.First strategy was applied as described by O'Flaherty*et al.* with few modifications[15]. Briefly, the sample was separated to 4 parts and each part mixed with overnight bacterial culture and Brain heart infusion Media. The Mixture was incubated overnight at 37º c. In the next day, 1% v/v Chloroform was added to the mixture for 15 min at room temperature. Then, Mixture was centrifuged at 6,000 rpm for 15 minutes, and a supernatant was filtered using a 0.20 µm syringe filter (chm SHIFT filtration by CHMLAB group/ USA). The final filtrate was examinedfor lytic activity by means of double layer agar method (DLA).

The second method was applied as described previouslyby Bhetwal*et al.*, with few modifications[16]. Briefly, sewage samples were centrifuged at 6000 rpm for 20 minutes, the supernatant was slowly filtered through a syringe filter with a pore size of 0.20 $\mu$m. Then the phage filtrate examined for the presence of phages by the DLA method. Serial dilution has been made for the phage filterate.

**Double Layer Agar Technique (DLA)**

DLA technique were done as described by Sambrook and Russell[17]. Briefly, 100 µl of phagefilterate was added to 100µl of a bacterial suspension grown overnight at 37°C. This solution was added to 3-5 ml of the top agar (Nutrient broth with 0.7% Agar- Agar base), mixed gently, and poured into a nutrient agar petri dish which were previously prepared. The plates were gently swirled, dried for 10 min at room temperature, and then incubated at 37°C overnight.

**Results**

**Isolation of Bacteria**

Four bacterial isolates were identified, *S. aureus, Klebsiella peumoniae, E. coli, Pseudomonas spp*. Antibiotic Susceptibility testing were performed for these isolates using specific types of antibiotics recommended by CLSI. After interpretation of the results, three bacterial isolates were found to be Multidrug resistant bacteria and the forth one was non, as shown in table (2).

Table 1. Antibiotics used for AST for four bacteria

| Antibiotics | *K. peumoniae* | *E. coli* | *S. aureus* | *Pseudomonas spp.* |
|---|---|---|---|---|
| Amikacin (AK) | S | S | S | S |
| Trimethoprim (TR) | S | R | R | N |
| Nalidixic Acid (NA) | S | R | N | N |
| Amoxicillin (AMC) | R | R | N | N |
| Gentamicin (GM) | S | R | R | S |
| Tygicycline (TGC) | S | S | N | S |

| | | | | |
|---|---|---|---|---|
| Aztreonam (AZI) | R | S | N | S |
| Ciprofloxacin (CIP) | S | R | R | I |
| Levofloxacin (LEV) | S | R | R | S |
| Tobramycin (TOB) | R | R | R | R |
| Netilmicin (NET) | N | N | S | S |
| Imipenem(PM) | N | N | N | S |
| Meropenem(MEM) | N | N | N | S |
| Norfloxacin(NOR) | N | N | N | S |
| Clindamycin (CD) | N | N | R | N |
| Erythromycin (E) | N | N | R | N |
| Norfloxacin(NOR) | N | N | R | N |

S: sensitive, R: resistant, I: intermediate, N: not performed

Table 2. Multidrug Resistant Bacteria (MDR)

| Drug resistance | Bacteria | Classes of antibiotic resistant by bacteria |
|---|---|---|
| MDR | *Klebsiella* | Pencillins<br>Monobactams<br>Aminoglycosides |
| | *Staphylococcus aureus* | Sulphonamides<br>Aminoglycosides<br>quilnolones<br>Macrolides<br>Lincosamides |
| | *E. coli* | Pencillins<br>Aminoglycosides<br>quinolones<br>Sulphonamides |
| Non-MDR | *Pseudomonas* | aminoglycosides |

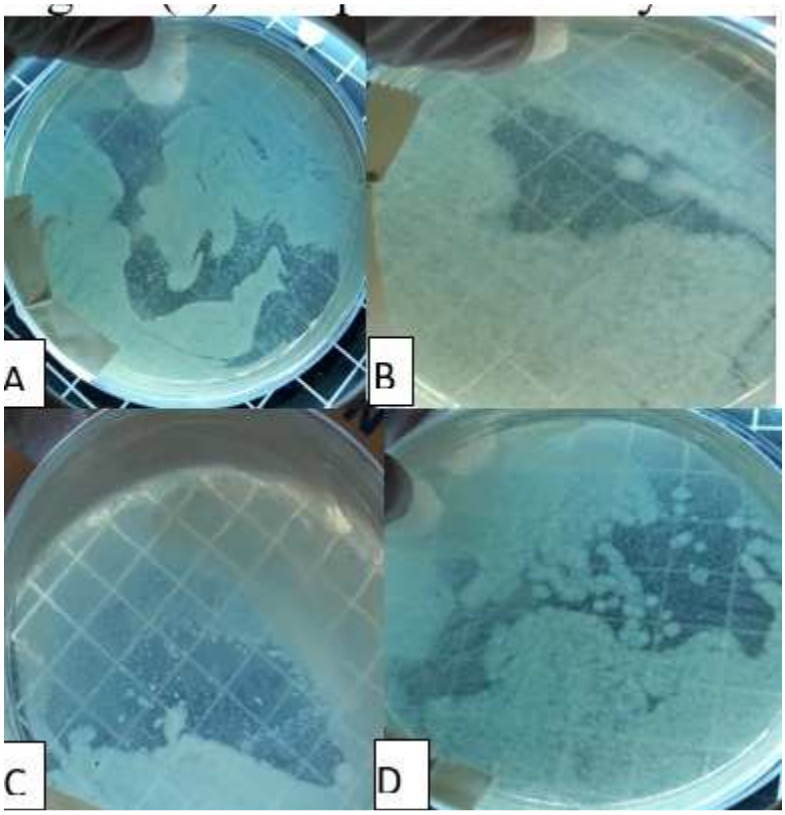
**Bacteriophage Isolation**

Lytic activities were seen in both protocols and for all types of isolated bacteria as shown in Figure (1). However, the plaque size seen were different between protocols. Within the first strategy, the size of plaques was larger than that seen in the second protocol for each isolated bacterium. The diameter of the plaques seen in the first protocols were more than 4 cm whereas the plaques recovered from the second protocols were less than 6 mm in diameter.Concerning the number of plaques, in the first protocol, there were single large plaque and few small plaques, whereas the number of plaques in the second protocols proportional with the dilution of the phage as shown in table (3).

Table 3. Size and number of plaques recovered from 2nd procedure

| Phage filtrate dilution | *E. coli* | *S. aureus* | *Pseudomonas spp.* | *K.pneumoniae* |
|---|---|---|---|---|
| Crud | <65, (1-3mm) | <36 , (1mm) | <80 , (1-4mm) | <50 , (3-6mm) |
| $10^{-1}$ | <52, (1-3mm) | <5 , (3-5mm) | <50 , (1mm) | <40 , (1-3mm) |
| $10^{-2}$ | <46, (1-3mm) | <6 , (3mm) | <4 , (1mm) | Unclear Plaques |
| $10^{-3}$ | <7, (1-4mm) | <3 , (6mm) | Large unclear plaques | <2 , Small unclear plaques |

Figure (1). Plaques formed by DLA covered from first protocol




*A-E. coli, B- K. pneumoniae, C- Pseudomonas spp., D- S. aureus*

Figure 2. Plaques formed by DLA recovered from 2ndprotocol

*A.Klebsiella, B. Pseudomonas, C. S. aureus, D. E coli*

**Discussion**

Nowadays, the misuse of antibiotics resulted in the spreading of antibiotic resistance strains of bacteria. Phages are a potential alternative for antibiotics in the treatment of bacterial infections.The currentstudy aimed to isolate phages from sewage water sources and to assess their effectivenessagainst different types of bacteria.

Four bacterial isolates (*S. aureus, Pseudomonas spp., E. coli, K.pneumonia*) were identified from different clinical samples. Susceptibility testing was performed using Kerby-Bauer testing method and the results were interpreted according to the CLSI. AST revealed that there are three bacterial isolates were resistant to more than three classes of antibiotics. Whereas, only one bacterial isolate was resistant to one class of antibiotics. According to the European Centre for Disease Prevention and Control (ECDC) the bacterial isolate that is resistant to three or more antibiotic classes is considered as Multidrug resistant Bacteria (MDR)[18].

For phage isolation, sewage sample collected from ecosystem which contains all types of wastes like hospital effluents and other wastes. The results showed that the sample was positive for the presence of different types of phages which is in accordance with previous research reporting the presence of bacteriophages in sewage water[19-21].This might be due to the fact that waste water is rich in bacterial contaminants that came from the hospital waste waters, which provides wide host range for all types of phages. In several previous studies, researchers were able to isolate phages from waste water samples[19, 20, 22]. Interestingly, the current study revealed the presence of various bacteriophages specific for all types of the tested bacterial isolates (*s aureus, Klebsiella, Pseudomonas, E coli*) and unfortunately MDR. This result is agreed with previous study in which the authors isolated different bacteriophages against multidrug resistant bacteria[23].

Additionally, phages isolation was done using two simple methods in order to clarify the simplest way that could result in phage isolation and at the same time would not require highly trained personnel or specialized instruments which are not available in all laboratories. lytic activities represented by plaque formation were seen in both protocols and for all types of the tested isolates as shown in Figure (1, 2). It is usually assumed that each plaque on plates is initiated by a single virus particle, although not all virus particles in the sample can initiate infections[24].The typical morphology of a circular plaque is simply reflecting cycles of infection of the embedded host cells by the numerous phage progeny spreading in all directions from the original focus of infection[25]. In the present study, Plaques were of two types, namely, clear and turbid. A similar morphology of plaques has been reported previously [21]. However, the plaque size was different between the two methods. Within the first method, the size of plaques was larger than that seen in the second methods for each tested bacteria. The diameter of the plaques seen in the first protocols were more than 4 cm whereas the plaques recovered from the second protocols were ranged from 1-6 mm in diameter. Regarding the number of plaques that seen in the first method, there were single large plaque and numerous small plaques, whereas the number of plaques in the second protocols proportional with the dilution of the phage as shown in table (3). This might be due to overnight incubation of the phages and the tested bacteria that results in propagation of the viral particles within the first protocol. Whereas, in the second method, this step was omitted. Furthermore, It is assumed that the phage with a higher diffusivity would have a larger plaque size; specifically the size would be a quadratic function of the diffusivity[26]. Based on these results, the first protocol had a good opportunity for phage isolation and subsequently, exhibited good lytic activity against bacteria.

## Conclusion

Bacteriophage could be isolated using very simple methods. Different types of phages were isolated with efficient lytic activity against different types of bacterial isolates including MDR. Further studies required for purification and titration of the isolated phage.

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