

## Isolation, Characterization and Anti-Biofilm Activity of Bacteriophages Against *Acinetobacter baumannii* Biofilm Producer

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### Abstract:

The bacterium *Acinetobacter baumannii* (*A. baumannii*) causes serious human diseases. The emergence of multidrug-resistant (MDR) *A. baumannii* has increased the need for alternative treatments. Bacteriophage therapy offers a promising alternative for MDR *A. baumannii* elimination. This study aimed to isolate, characterize, and identify lytic phage activity. A phage designated RKL was isolated from sewage water of Baghdad Medical City hospital as a possible agent for phage therapy to treat infections caused by *A. baumannii*. Based on transmission electron microscopy, phage RKL is a siphovirus from the *Siphoviridae* family of the *Caudovirales* order. The optimal multiplicity of infection (MOI) was at 10. A total of 54 different clinical isolates and host bacteria were tested by the VITEK®2 system for phenotypic identification and antimicrobial profile. The phage showed a narrow host spectrum by lysing only *A. baumannii* isolates (15/26). The results suggest that RKL phage has a potential impact in inhibiting MDR *A. baumannii* infection.

**Keywords:** *Acinetobacter baumannii*, bacteriophage, *Siphoviridae*, host range

**Note:** The research is based on an MSc thesis.

### 1. Introduction:

The rate of antibiotic resistance is rising rapidly and dramatically worldwide as a result of antibiotic misuse. Healthcare system problems, such as long hospitalization times, reduced recovery time, and high costs, are mainly due to gram-negative bacteria (1). One of the threatening nosocomial bacteria is *A. baumannii*, as most of the strains are becoming resistant to almost all frequently used antibiotics to treat infections. *A. baumannii* is an abundant organism that can live in an environment with diverse conditions. *A.*

*baumannii* is responsible for hospital-acquired infections, including pneumonia, urinary tract infections (UTI), and bloodstream infections, especially in patients in Intensive Care Units (ICUs) (2,3). *A. baumannii* can escape from antibiotic effects by evolving different mechanisms (4). The remarkable ability to survive in strict environments is due to biofilm production (5,6). Bacterial biofilm formation is one of the advantages that enable bacteria to develop antimicrobial resistance. In addition, horizontal gene transfer (HGT) acts as a means of spreading resistance markers via mobile genetic elements (plasmids, transposons, or bacteriophages). Other resistance mechanisms include high expression levels of efflux pumps found in biofilms compared with planktonic cells, leading to reduced efficacy of antimicrobial agents (7).

#### **Previous Studies:**

With the increasing numbers of newly diagnosed cases related to MDR and biofilm producing *A. baumannii* infections and reductions in available antimicrobial alternatives, the focus on finding new alternatives is increasing. Novel strategies are required to control the dissemination of MDR bacteria, including *A. baumannii*. Bacteriophages are attractive options that have brought researchers' attention. Bacteriophages are used in many therapeutic and non-therapeutic applications. For example, bacteriophages are used as a bio-control in agriculture and food safety. Due to their host specificity, bacteriophages are used as a specific marker for bacterial species detection. In therapeutic applications, bacteriophages are used to treat many infections caused by MDR bacteria including biofilm-producing bacteria. A previous study reported that a new lytic phage could inhibit biofilm formation and disrupt pre-formed biofilms by *A. baumannii* isolates (8). A phage cocktail from nine phages was applied to a patient suffering from necrotizing pancreatitis caused by *A. baumannii* infection (9). Different studies have reported the success of phage treatment in eradicating biofilm-producing bacteria (10–12). Recently, a local study showed that a mixture of a phage and colistin could reduce biofilm formation in carbapenem-resistant *A. baumannii* (13). The current study's aim is to isolate phages against multidrug-resistant *A. baumannii* that are able to form biofilm and examine the lytic activity of a bacteriophage against MDR *A. baumannii* clinical isolates. The identified phage will contribute to the phage database and can be employed as a promising antimicrobial agent to reduce the risk of MDR *A. baumannii* infections.

## 2. Methodology

### 2.1 Bacteriophage and host isolation

The sewage water sample was collected from Baghdad Medical City hospitals' sewage. In the laboratory, 10 ml was centrifuged at  $4000\times g$  for 20 min, and the supernatant was cultured in fresh HiChrom Acinetobacter Agar Base medium (Himedia, India) and Eosin Methylene Blue (E.M.B.) Levine agar (Liofilchem, Italy) and incubated overnight at  $37^{\circ} C$  for host isolation. The supernatant of the sewage sample was then filtered first through  $0.45 \mu m$  filters and then through  $0.22 \mu m$  filters (Chm, Spain) for phage isolation and stored in the fridge ( $4^{\circ} C$ ). The next day, the colonies that showed *A. baumannii* characteristics were cultured in fresh HiChrom agar medium for purification and preserved in tryptic soy broth (TSB) supplemented with glycerol (20%) and stored at  $-80^{\circ}C$ . For bacteriophage host isolation, all *A. baumannii* isolates from the sewage water sample were identified by VITEK®2 Compact systems and grown in TSB for 24hr. The optical density (600nm) was measured via spectrophotometer and 0.1 ml from each isolate was mixed with 100  $\mu l$  of water filtrate, left at room temperature (10 min), and 4 ml of soft agar (0.7% agar) was added and overlaid on the top of a fresh nutrient agar plate. A single plaque was selected from the plates by micropipette tips, and each plaque was re-suspended in phosphate-buffered saline. The single plaque isolation cycle was repeated four times to achieve pure phage (14). The host used in phage isolation was resistant to Cefazolin and Ceftriaxone.

### 2.2 Bacteriophage titration

To determine phage titer, the double layer overlay method was performed. The host bacterium was grown overnight in TSB and 1ml was measured using spectrophotometer at OD600. Ten-fold dilutions of phage were conducted with phosphate buffer saline (PBS); 100  $\mu l$  of each dilution was taken and mixed with 100  $\mu l$  of host bacteria. After 10 min, which is the time required for phage adsorption, the mixture was suffused with 4 ml of (0.7%) soft agar and poured onto a fresh nutrient agar plate. After solidification at room temperature for 10 min, plates were incubated at  $37^{\circ} C$  for 24 hr. The titer was calculated using  $\text{pfu/ml} = \text{average of plaques (30-300)} / \text{dilution factor} * \text{volume of diluted virus (15)}$ .

### 2.3 Transmission Electron Microscopy (TEM)

A phage lysate titer was prepared in PBS before negative staining for transmission electron microscopy (TEM) examination. Briefly, a drop of phage lysate ( $10^{10}$  pfu/ml) was placed on a copper grid and stained with 2% uranyl acetate and lead citrate drops. The stained grid was dried with filter paper and examined under a TEM (ZEISS FESEM Supra) at 55 kV (16).

### 2.4 Multiplicity of infection (MOI)

The host bacterium from the overnight nutrient broth culture was measured at OD600, which is equivalent to  $1 \times 10^8$  cfu/ml, and the pfu/ml for the phage stock were measured. The MOI was calculated by dividing the number of phage by the number of bacteria. Various MOIs (0.001, 0.01, 0.1, 1, and 10) of phage and host were prepared in different tubes and left at room temperature for 10 min to permit phage adsorption. For each MOI, 4 ml soft agar was added, and the mixture of phage, host and soft agar was overlaid on the top of a nutrient agar plate. After incubation for 24 hr at 37°C, the titer was determined for each MOI as pfu/ml, and the optimal MOI was calculated as the optimal ratio of the phage particles divided by the number of host cells that is required to reach the highest phage titer (17).

### 2.5 Host range determination

All strains used to determine host range were collected from different clinical sources from the laboratory of Ghazi Al-Hariri Hospital in Baghdad City from the period October 2023 to February 2024, including 26 *A. baumannii*, 8 *E. coli*, 5 *Klebsiella pneumoniae*, 5 *Pseudomonas aeruginosa*, 3 *Serratia marcescens*, 2 *Enterococcus faecalis*, and 5 *Staphylococcus aureus*. All gram-negative bacteria were cultured on MacConkey agar and gram-positive bacteria on Mannitol salt agar (MSA) for colony morphological characteristics (18). The VITEK® 2 (bioMérieux, France) Compact System was performed for species identification and antimicrobial profile in accordance with the manufacturer's instructions, and the data was analyzed using software. A drop test was used for phage host range determination. Briefly, All isolates were grown in TSB for 24 h at 37°C. The next day, the OD600 was measured for each isolate. A total of 100 µl of each specific bacterial isolate was mixed with 4 ml of melted agar (0.7%). The mixture was poured onto a separate nutrient agar plate. Each plate was left for 10 min for agar overlay solidification, and 5 µl of phage high titer stock was spotted onto the agar overlay. After incubation for 24 hr, a clear zone was observed, indicating bacterial sensitivity to the isolated phage (19). This experiment was repeated twice independently.

### 3. Results and discussion:

In this study, different wastewater samples were supplied from the sewage of Baghdad Medical City in Baghdad. As shown in Figure 1, several types of bacteria were obtained, indicating pollution of the water sample with different types of bacteria. A wide range of contaminants is discharged into the river water, including hospital wastewater, domestic waste, and irrigation water, which threaten human health and the aquatic environment (20–22).

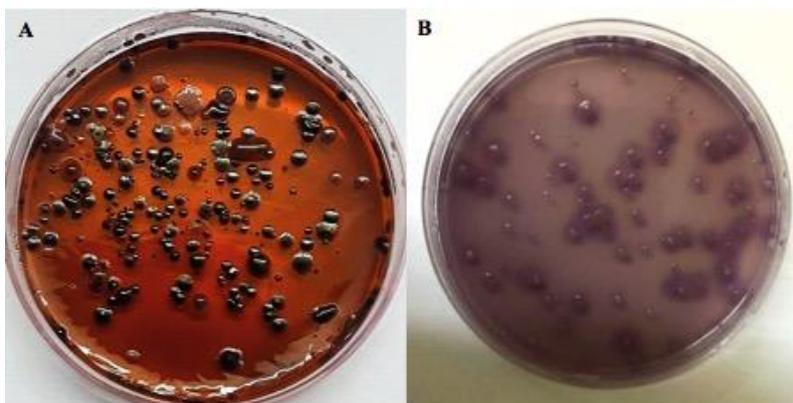
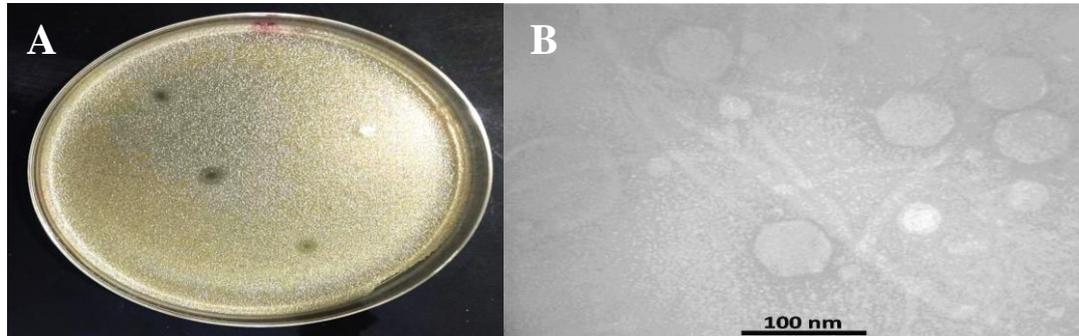


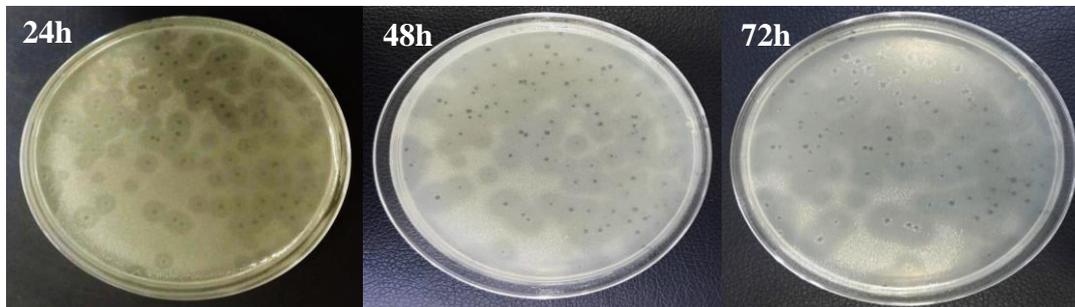
Figure1. Diverse bacterial species from sewage water sample. A: Different bacterial isolates on EMB agar; B: Different bacterial isolates on HiChrom Acinetobacter Agar Base medium.

However, sewage water is also a common source for the isolation of bacteriophage infecting pathogenic bacteria, including *A. baumannii*, which was isolated from wastewater in order to offer an alternate approach against antibiotic resistance developed by *A. baumannii*. A phage named RKL was isolated and characterized. The phage name was in accordance with the recommended nomenclature procedure (23). Besides that, the host bacterium was isolated along with the bacteriophage. In a double-layer plate, plaques with a central clear zone and a turbid surrounding zone were formed (**Figure 2A**). The TEM examination was performed to provide a better understanding of the phage morphology; the analysis of phage RKL revealed an icosahedral capsid and a non-contractile tail, indicating the phages as siphovirus within the *Caudovirales* order in accordance with the International Committee on Taxonomy of Viruses (ICTV) classification (24) **Figure 2B**. According to published studies, the *Siphoviridae* family is characterized by an icosahedral head (55–86 nm) width, (55–106 nm) length, and a non-contractile tail (79.1–215 nm) (12,25,26).



**Figure 2.** Morphology of phage RKL. (A) Plaque morphology. (B) Transmission electron micrographs of phage particles with icosahedral head and non-contractile tail. Scale bar represents 100 nm.

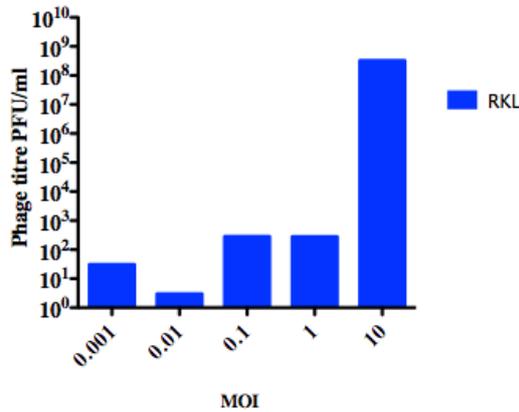
Furthermore, prolonged incubation showed an increase in plaque halo diameter with the remains of the clear center the same size as presented in **Figure 3**. The presence of translucent halos indicates carbohydrate-active enzymes like endolysins, polysaccharide depolymerases, and virion-associated lysins, which are involved in the early attachment to the bacterial cell wall by degrading the exopolymeric matrix of biofilm and the final lysis of the bacterial peptidoglycan cell wall (27).



**Figure 3.** Plaques formed by phage RKL. The infected host was incubated for 24,48 and 72 hours and plaques and halos were observed.

#### MOI

Various MOIs were tested in order to select the optimal MOI that can reduce the viability of the host. The results showed that 10 produced the highest titer and are considered the optimal MOI (**Figure 4**).



**Figure 4.** The optimal multiplicity of infection for RKL phage on *A. baumannii* host.

The optimal MOI is known as the lowest concentration at which a phage can inhibit host cells effectively. Our results agree with a study that showed 10 was the optimal MOI for phage from the *Siphoviridae* family (28). However, another study indicated that the optimal MOI for bacteriophage AB7-IBB1 from the *Siphoviridae* family was 0.1 (8). In addition, another study showed that the best MOI was 1 for siphovirus (25). Changes in environmental factors could affect MOI efficiency and therefore affect infection effectiveness (29).

#### Host range

Further information was obtained through a host range assay, providing evidence that phage RKL inhibits only *A. baumannii* with no inhibitory effect on other species. The results showed that phage RKL exhibited antibacterial activity against 15 out of 26 *A. baumannii* clinical isolates, with no effect against other tested species, suggesting a narrow host range, which is desirable for therapeutic applications. The phages with a narrow host range indicate strain specificity, and that is due to the tail spike protein being highly specific (30). Broad host range phages, co-administration of phage and antibiotics, or phage cocktails are some of the strategies to overcome the limitations of a narrow host range (31–34). Phage therapy offers different mechanisms to control and eradicate MDR *A. baumannii* infection. For example, a phage cocktail from nine phages was applied to a patient suffering from necrotizing pancreatitis caused by *A. baumannii*, resulting in the clearness of infection (9). Enzymes derived from phages, such as endolysin and depolymerase, are another attractive approach. A study on mammalian

cells reported that endolysin enzyme derived from phage could act as an antimicrobial agent with rapid action and low cytotoxicity effects (35). A previous study showed successful results in the inhibition of *A. baumannii* biofilm produced *in-vitro* by using capsular polysaccharide (CPS) depolymerase derived from AB6 phage tail spike protein (36).

### Conclusions

Antimicrobial resistance poses a serious threat to human health and is responsible for the death of millions of people annually. MDR *A. baumannii* infections are an ongoing problem in many hospitals. In this study, phage RKL against MDR *A. baumannii* clinical isolates was successfully isolated from sewage water and characterized. MOI analysis showed that 10 were the optimal for the phage. Additionally, the phage RKL exhibited a narrow host range. Our findings propose that RKL may act as a safe therapeutic alternative to available drugs. Further work is needed to reveal the phage genome, determine the optimal dosage to use, and combine phage with antibiotics or use phage cocktails before reaching the point of use on humans. The ability of the host immune system to develop resistance against proposed phage should be investigated.

### Ethical approval

This study does not contain any contact with patients and all sample were taken from laboratory, therefore there was no need for ethical approval.

### Credit authorship contribution statement

Rasha K. Jabbar: Samples collection, Methodology, Writing. Ban O. Abdulsattar: Supervision, formal analysis, investigation, writing, review, editing. Susan A. Ibrahim: Supervision, formal analysis, investigation, writing, review, editing.

### Declaration of competing interest

The authors declare no competing of interests.

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Not applicable

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## عزل وتوصيف ونشاط عاثيات البكتريا المضادة للأغشية الحيوية ضد بكتريا

### المنتجة للأغشية الحيوية *Acinetobacter baumannii*

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### مستخلص البحث:

تسبب بكتريا *Acinetobacter baumannii* في أمراض بشرية خطيرة. وقد أدى ظهور المقاومة المتعددة للأدوية وبالأخص لهذه البكتريا الى زيادة الحاجة الى توفير علاجات بديلة. يوفر العلاج باستخدام العاثيات بديلاً واعداً للقضاء على بكتريا *A. baumannii* التي توصف بانها من اكثر واشد أنواع البكتريا المعروفة بمقاومتها المتعددة للأدوية (MDR). هدفت هذه الدراسة الى عزل وتوصيف وتحديد نشاط العاثي ذو الانزيمات المحللة، حيث تم عزل العاثي المسمى (RKJ) من مياه الصرف الصحي في مستشفى مدينة بغداد الطبية وتم عزلها و استخدامها كعامل محتمل للعلاج بالعاثيات وذلك لعلاج الالتهابات التي تسببها بكتريا *A. baumannii* بواسطة الفحص بواسطة المجهر الالكتروني النافذ، تم تشخيص ان العاثي (RKJ) هو فايروس Siphovirus يعود الى عائلة ال *Siphoviridae* والى رتبة ال *Caudovirales*. وكان التعداد الأمثل للعدوى (MOI) عند 10. استنادا الى نظام VITEK®2 المستخدم لتحديد الأنماط الظاهرية للميكروبات وكذلك الأنماط لمضادات الميكروبات، تم اختيار 54 عزلة بكتيرية من العزلات السريرية المختلفة وكذلك الحال بالنسبة للبكتريا المضيفة. اشارت نتائج هذه الدراسة الى أن العاثي كان له تأثير مباشر من خلال تحلل عزلات ال *A. baumannii* فقط بمعدل (15/26). استنادا الى هذه النتائج، تم الاستنتاج الى أن العاثي (RKJ) له تأثير محتمل في تثبيط عدوى الإصابة بالبكتريا *A. baumannii* ذات القابلية لمقاومة الادوية المتعددة MDR.

الكلمات الافتتاحية: *Acinetobacter baumannii*، عاثيات البكتريا، *Siphoviridae*، نطاق المضيف

ملاحظة: البحث مستل من رسالة ماجستير.