ISOLATION AND CHARACTERIZATION OF A LYTIC *ACINETOBACTER BAUMANNII* BACTERIOPHAGE FROM WASTE WATER IN EGYPT

BY

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ABSTRACT

The spread of multidrug resistant (MDR) *Acinetobacter baumannii* is a global health threat, especially in healthcare associated environments. Over the years, *Acinetobacter baumannii* has acquired resistance to almost all known types of antibiotics. Subsequently, national and international health organizations are calling for the development of new treatments and alternative therapies to control this pathogen. Bacteriophage therapy is an old concept that reemerged with the rising problem of antimicrobial resistance. The aim of our study was to isolate and identify an environmental bacteriophage, specific and active against *A. baumannii* as a trial to introduce new treatment options for this difficult to treat microorganism.

A total of 21 lytic bacteriophages were recovered from activated sludge obtained from the municipal waste water treatment plant (WWTP) of Gabal Al-Asfar, Cairo, Egypt using 15 multidrug resistant *A. baumannii* strains as target hosts. In particular, a bacteriophage ‘‘K4’’ showed the broadest activity against the tested MDR *A. baumannii* strains. One step growth curve of K4 bacteriophage showed a latent period of 20 min followed by 80 min of rise period, reaching 10^8 PFUml^{-1} in 120 min. It also exhibited rapid adsorption where >99% of phages adsorbed on their host surfaces in 5 min. Morphologically, after TEM examination, phage K4 had an icosahedral head, non-enveloped capsid and a contractile tail; therefore, it was tentatively classified as a member of the *Myoviridae* family. The lytic bacteriophage K4 can be considered as a candidate therapeutic agent to combat *A. baumannii*-associated nosocomial infections.

INTRODUCTION

Acinetobacter baumannii is an opportunistic human pathogen that is increasingly isolated from hospital-acquired infections (Harding *et al.*, 2018). Naturally competent, *A. baumannii* is acquiring multiple genetic elements coding for antimicrobial resistance at an unprecedented rate (Pendleton *et al.*, 2013). Although infections caused by *A. baumannii* account for 4% of all healthcare-associated infections in the Middle East, rates as high as 70% of all isolates are found to be multidrug-resistant (Lob *et al.*, 2016). Therefore, the WHO was prompted to classify carbapenem-resistant isolates among bacteria posing a great human health threat requiring public monitoring and prevention measures (Collignon *et al.*, 2016).

In Egypt, carbapenem-hydrolyzing oxacillinase gene "*bla$_{OXA-23}$"* is the most prevalent in *A. baumannii* clinical isolates (Mugnier et al., 2010). However, in a study including three Egyptian hospitals, the spread of *bla$_{NDM-1}$* in addition to the co-occurrence of 16S rRNA methylase *armA* with *bla$_{NDM-1}$* and *bla$_{OXA-23}$* in 27 distinct sequence types was reported (El-Sayed *et al.*, 2015).

The continuous emergence of new strains of MDR *A. baumannii* in Egypt urges the development of alternative treatment strategies. Among the possible therapeutic approaches, phage therapy is regaining attention to overcome these antibiotic-resistant bacterial infections. Phages are specific virus-killing bacteria that have been discovered about 100 years ago (Moelling *et al.*, 2018). These phages could be isolated and developed as personalized therapeutic cocktails to treat MDR infections. In fact, the case of a man who was infected with a MDR *A. baumannii* strain during a trip to Egypt and treated with an intravenous cocktail of phages is a striking proof of the success of such approach to conquer these life-threatening infections (Moelling *et al.*, 2018).

Given that the most suitable phages to kill specific bacterial lineages are those isolated form the same environment, the present study aimed to isolating and characterizing bacteriophages from the Egyptian environment with specific ability to kill *A. baumannii*.

Here, we describe the successful isolation and characterization of a myophage with a wide range of activity on *A. baumannii* strains isolated from Egypt.

MATERIALS AND METHODS

Bacterial Strains

Fifteen MDR *Acinetobacter baumannii* strains were recovered from clinical samples (skin, blood and sputum) obtained from hospitalized patients in Egypt. The recovered strains were identified as *A. baumannii* by amplification of the *oxa $_{51}$* gene intrinsic to this species (Turton *et al.*, 2006). DNA diversity of the isolated strains was detected by PCR-based RAPD fingerprinting. The antibiotic susceptibility of the isolated strains was determined by disc diffusion method against 10 selected antibiotics. A standard strain, *Acinetobacter baumannii* ATCC 19606, was included in the isolation and characterization of lytic bacteriophages.

Phage isolation, purification and propagation

Activated sludge from the municipal wastewater treatment plant of Gabal Al-Asfar was used to isolate lytic phages against *A. baumannii*. The sludge was cleared by low-speed centrifugation followed by filtration of the supernatant through a 0.45µm pore size membrane filter to remove bacterial debris.

The presence of lytic phages was tested by the spot method and the plaque assay. Briefly, 0.3ml of the sludge filtrate were mixed with 0.5ml of a fresh culture of *A. baumannii* strains with O.D 0.1 at 600 nm in 50ml of 5X TSB containing $CaCO_3$. After 18h incubation at 30^{o}C and agitation at 180rpm, the culture was centrifuged at

6000 g and - 4°C for 10 min and the supernatant was filtered through a 0.45µm membrane filter (Inal *et al.*, 1990).

For the spot test, an overlay of the indicator *A. baumannii* strain in 0.7% TSA was poured on pre-solidified TSA base plate. An aliquot of 20µl of the filtrate was spotted on the surface and incubated at 30°C for 18h. After incubation, a clear zone indicated the presence of a lytic phage against the indicator strain.

For the plaque assay, 10-fold serial dilutions of each phage lysate were prepared in PBS (phosphate buffer saline). An aliquot of 10µl of each dilution and 0.5ml of the indicator strain were mixed in 3ml of 0.7% TSA and poured on pre-solidified TSA base plate. Phage plaques were counted after incubation of the plates at 37°C for 18h. The phage count was expressed as pfu/ml (Inal *et al.*, 1990).

To propagate a single phage type at a time, single plaques were picked and suspended in 1ml LB broth. Each suspension was shaken for 60 min and centrifuged at 6000 g for 15 min. The supernatant was then filtered through a 0.45µm membrane filter to remove bacterial cells. The filtrate was mixed with 0.5ml of the indicator strain in 50ml TSB and incubated at 37°C for 18h. After incubation, the culture was centrifuged at 6000 g for 15min. The supernatant was then filtered through a 0.22µm membrane filter. Plaque assay was repeated thrice using the filtrate from each single plaque to obtain pure phage stocks.

Host Range Determination

The specificity of each purified phage sample against its host bacterial strain was determined using the double agar layer technique. An overlay of the indicator *A. baumannii* strain in 0.7% TSA was poured on pre-solidified TSA base plate. The plate was divided into equal grids. Aliquots of 20µl of each phage sample were spotted on the surface and plates were incubated at 30°C for 18h. After incubation, a clear zone (plaque) indicated the ability of the phage to lyse the indicator strain.

One Step Growth

Equal volumes of an overnight culture of *A. baumannii* susceptible strain (OD_{600} 0.125) and **K4** phage (MOI 0.001) were mixed and incubated at room temperature for 5 min to allow for the adsorption of phages. After incubation, the mixture was centrifuged at 6000 g for 5 min to remove unadsorbed particles. The resultant pellet was then resuspended in 50ml TSB and incubated at 30°C under agitation of 180 rpm. Samples of 40µl were taken at 5 min intervals for 2h and immediately titrated using the double layer agar technique. The above steps were repeated two times (You *et al.*, 2002).

Phage Adsorption

Equal volumes of an overnight culture of *A. baumannii* strain **K4** (OD_{600} 0.125) and phage **K4** (MOI 0.001) were mixed and incubated at room temperature for 5 min. Volumes of 40µl were taken at 5 min intervals for 30 min and immediately centrifuged. The supernatants were used to determine the unadsorbed phage particles

at different time intervals by the double agar layer technique. The test was repeated two times.

Electron Microscopy

The purified phage preparation was fixed with 1% glutaraldehyde in 0.1 M phosphate buffer (pH 7.0) and placed on transmission EM support grids followed by rinsing with distilled water several times. The grids were examined with a Hitachi H-300TM electron microscope (Japan). The electronic phage images were used for phage morphology determination.

RESULTS

Isolation, purification and propagation of bacteriophages

A total of 21 lytic bacteriophages, against 15 MDR *Acinetobacter baumannii*, were isolated from activated sludge obtained from municipal waste water treatment plant (WWTP) of Gabal Al-Asfar, Cairo, Egypt.

The isolated bacteriophages showed clear round plaques on *A. baumannii*-sensitive strains (Figure1). The obtained plaques were repeatedly propagated using the respective bacterial strain to enhance selectivity and then they were kept at -80°C as LB broth suspension.

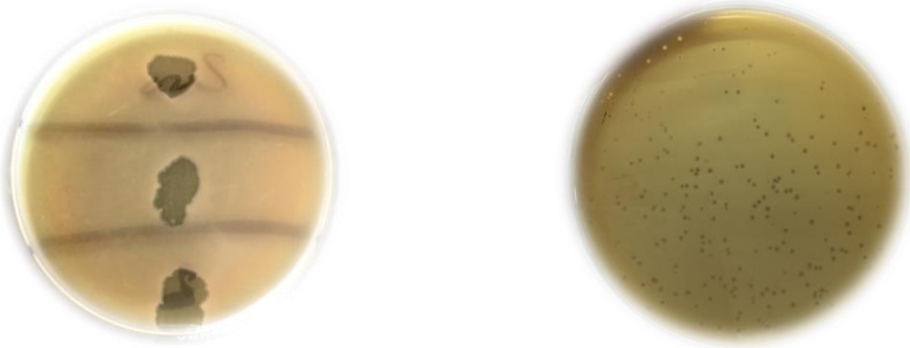

Figure1: Clear plaques obtained by the isolated lytic bacteriophages against *A. baumannii*

One the left-hand side spot test after surface inoculation of phage lysate on the surface of seed inoculated bacterial medium. One the right –hand side plaque assay showing isolated plaques obtained after seed inoculation of both bacterial and phage lysate in the cultivation medium.

Host range analysis

An isolated bacteriophage "K4" showed the broadest activity against the tested MDR *A. baumannii* strains, while the other phages isolate displayed a narrower spectrum of activity as illustrated in table (1). Ten isolates of *A. baumannii* did not show any lytic activities by any of the isolated phages.

Table 1: Host range analysis of the isolated bacteriophages active against MDR *Acinetobacter baumannii* strains

Phage isolates	A1	A2	A3	B1	B2	B3	C1	C3	D1	D2	D3	E1	E3	F1	F3	I1	I2	I3	J1	K1	K4
Strain 1	+					+			+		+		+	+	+	+	+	+	+		+
Strain 2	+	+	+	+	+	+	+	+		+	+	+	+							+	+
Strain 3	+	+	+	+	+	+		+	+	+	+	+	+				+			+	+
Strain 4					+		+	+	+	+	+	+	+		+	+	+		+	+	+
Strain 5		+														+	+	+	+		+

(+) indicates the presence of bacteriophage lytic activity against the tested bacterial strain.

Determination of multiplicity of infection (MOI) of K4 bacteriophage

K4 bacteriophage was chosen as having the broadest spectrum of activity against the tested bacterial strains, its MOI was determined using plaque assay to be equal to 10^8 $PFUml^{-1}$.

Bacteriophage adsorption and one-step growth curve

The one step growth curve trend line showed a latent phase of 20 min. Followed by the rise period where the burst of host cells and the release of bacteriophages were demonstrated. This phase continued for about 80 min after which there was almost no increase in phage propagation number due to decrease in the available host cells. The maximum phage count (~ 10^8 PFU/ml) indicated the burst size of the bacteriophage which represents the total number of virus released per bacterium **(Figure 2).**

Regarding the adsorption of the bacteriophage on the host cell walls, the adsorption reached its maximum value after 5 min of infection. After 5 min, the available unadsorbed phage remained almost constant **(Figure 2).**

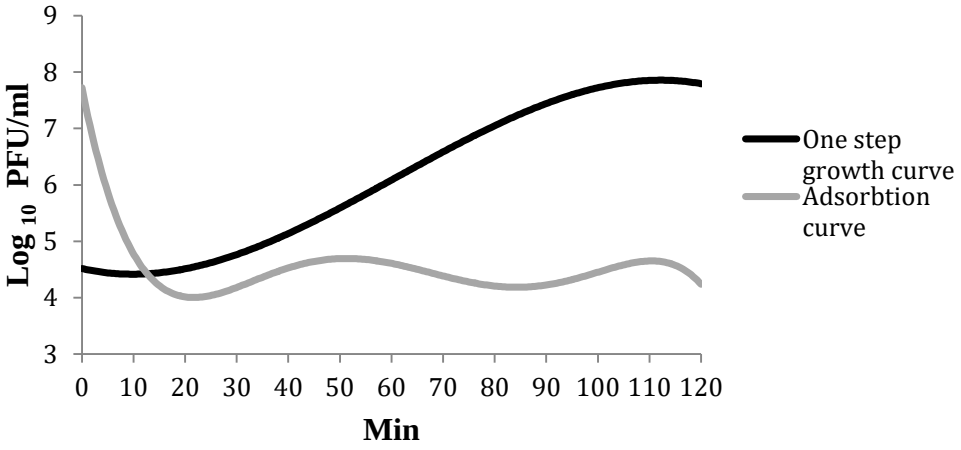

Figure (2): Trend line graph of one step growth curve and adsorbtion curve of K4 bacteriophage

Identification of *A. baumannii* bacteriophage using transmission electron microscope (TEM)

Transmission electron micrographs of bacteriophage suspension showed the presence of non-enveloped capsid and contractile tail bacteriophage of *Myoviridae* family. The phage "**K4**" has an icosahedral head of 90 – 100 nm in diameter and a contractile tail of 60-70 nm in length **(Figure 3).**

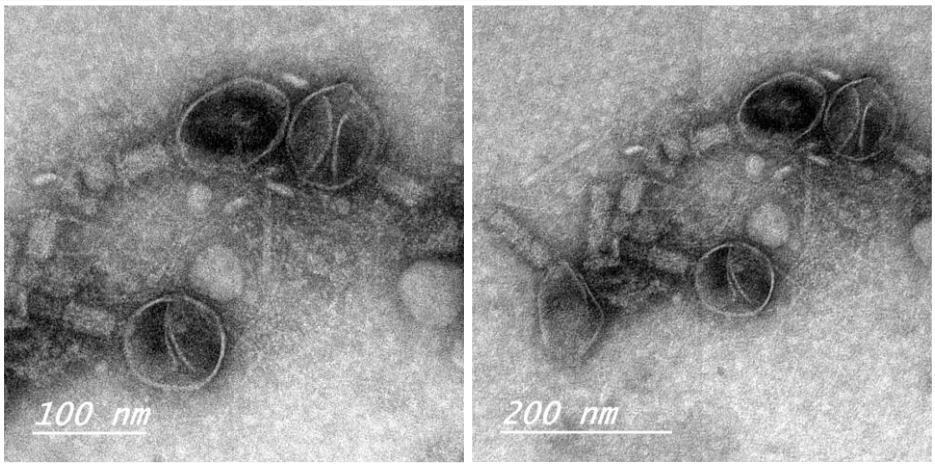

Figure 3: Transmission electron micrographs of K4 bacteriophage with two magnification powers, 100 nm at the left-hand side and 200 nm at the right-hand side.

DISCUSSION

The search for alternative therapeutic tools to combat MDR bacteria is driven by the alarming spread of antibiotic resistance especially in healthcare-associated environments. One of the promising treatment options that demonstrated efficiency and safety is phage therapy (Wittebole *et al.*, 2014) and (Moelling *et al.*, 2018). Phages are considered good candidates for targeted personalized antibacterial therapeutics. Phage cocktails were reported to achieve therapeutic efficacy in a mouse infected wound model by lowering the bio-burden in the wound, halting the spread of

infection, and decreasing morbidity (Regeimbal *et al.*, 2016). Furthermore in 2017, intravenous injection of a phage cocktail saved a patient with severe disseminated resistant *A. baumannii* infection (Schooley *et al.*, 2017). These experimental treatments highlight the potential of bacteriophage-based therapeutics to overcome antibiotic resistance, notably in organisms that survive and spread in multiple habitats, such as *A. baumannii*.

Acinetobacter spp. are ubiquitous microorganisms that can be easily isolated from environmental sources such as soil, surface water and sewage. On the other hand, phages are found wherever their host exists (Chibani-Chennoufi *et al.*, 2004). Accordingly, we thought to explore multiple Egyptian environments for phages that are specific for this opportunistic pathogen notably that Egypt was reported as a source for one of the most severe untreatable *A. baumannii* infections (Moelling *et al.*, 2018). In this study, we successfully isolated and purified 21 environmental lytic phages from local sewage wastewater sludge, active against 15 MDR *A. baumannii* strains isolated from Egyptian patients.

To maintain the phage survival and population, the presence of MDR strains in wastewater or other bacterial hosts must be present in wastewater. In either case, the co-existence of phages and their respective hosts in this natural environment helps in controlling bacterial populations. This mechanism is especially important in low-income countries where sewage treatment is not optimized (Segura *et al.*, 2015).

The main goal of this study was to isolate and characterize a lytic phage for possible therapeutic use. The isolated phage (K4) demonstrated lytic antibacterial activity against five of the 15 (33%) genetically distinct MDR *A. baumannii* clinical isolates. It formed clear plaques on the lawns of 5/15 isolates, indicating a relatively broad host range infectivity. Usually, isolated *Acinetobacter* phages have a narrow host range, typically infecting 2% of the screened bacterial hosts. In some cases, *Acinetobacter* phages are only capable of infecting the initial bacterial strain in which they were propagated (Jansen *et al.*, 2018).

So far, most of the isolated *Acinetobacter* phages are tailed viruses with double-stranded DNA genomes. They are classified into three families *Myoviridae*, *Podoviridae*, and *Siphoviridae* of the order *Caudovirales (Ackermann et al*., 1994). Morphologically, phage K4 exhibited an icosahedral head and contractile tail structures so it was tentatively classified as a member of the *Myoviridae* family.

The adsorption and one-step growth curves are parameters used to determine the efficacy of phage infectivity on host bacteria (Hyman and Abedon, 2009). Phage "K4" had a short latent period (20 min), which is an additional indication of its lytic nature followed by a rise period of 80 min. In addition, it adsorbed rapidly on the surface of its host cells (>99 % adsorbed in 5 min). Hence, phage "K4" can be considered as a candidate for phage therapy, as it exhibited rapid adsorption rate, lytic activity and a relatively wide host range.

CONCLUSION

The lytic bacteriophage K4, a member of the *Myoviridae* family, was isolated and characterized. Bacteriophage K4 specific against *A. baumannii* exhibited rapid adsorption and relatively short latent period. Thus, it can be considered as a candidate therapeutic agent to combat *A. baumannii*-associated nosocomial infections.

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الملخص العربي

عزل وتوصيف باكتريوفاج تحللي ل ***Acinetobacter baumannii*** من مياه الصرف الصحي في مصر

ريهام سمير, مها إسماعيل ومروة الركاييي

قسم الميكروبيولوجي و المناعة كلية الصيدلة ، جامعة القاهرة ، القاهرة ، مصر.

تعد *Acinetobacter baumannii* نوع من بكتيريات المستشفيات المسؤولة عن وفاة نسبة كبيرة من مرضى وحدات العناية المركزة. ويعزى ذلك إلى قدرتها الهائلة على اكتساب المقاومة لجميع أنواع المضادات الحيوية المعروفة تقريبًا. أصبحت في الآونة الأخيرة الدعوات لطرق جديدة للعلاج والعلاج البديل حاجة ملحة. يعد علاج الباكتريوفاج طريقة قديمة عادت إلى الأضواء مع المشكلة المتزايدة المتمثلة في مقاومة البكتريا للمضادات الميكروبية. الهدف من دراستنا هو عزل وتحديد البكتيريوفاج الذي يكون محدد وفعال ضد *A. baumannii* عن طريق إدخال خيارات علاجية جديدة لهذا الكائن الحي الدقيق صعب العلاج.

تم عزل 21 من بكتيريوفاج ، باستخدام *A. baumannii* 15 ، من الحمأة المنشطة التي تم الحصول عليها من محطة الجبل الأصفر ، القاهرة ، مصر. البكتيريوفاج " K4" أوسع نشاط ضد سلالات *A. baumannii* المختبرة. وأظهر منحنى نمو خطوة واحدة من K4 فترة كمون لمدة عشرون دقيقة تليها فترة صعود 80 دقيقة تصل إلى 10^8 $PFUml^{-1}$ في 120 دقيقة. استغرق الحد الأقصى لامتصاص البكتيريوفاج على مضيفه 5 دقائق فقط. أخيرًا ، تم إجراء التصنيف المورفولوجلي لبكتيريوفاج (K4) باستخدام مجهر الإرسال الإلكتروني (TEM). واوضحت صورة الرأس الغير المغلف والذيل المنقبض للبكتيريوفاج انه ينتمي الي عائلة *Myoviridae*. يعتبر الباكتريوفاج K4 احد الطرق المحتملة لعلاج *Acinetobacter baumannii* التي لا تستجيب للعلاج.