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Phage Cocktails Against Highly Multi-Drug Resistant *Acinetobacter baumanii*

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Abstract

Background Phage therapy is a potential alternative treatment for infections caused by many bacterial species such as *Acinetobacter baumannii* (*A. baumannii*), a significant nosocomial pathogen, has evolved resistance to almost all conventional antimicrobial drugs in poor hygiene and conflicts areas like Iraq.

Objective Isolate and apply bacteriophages as alternative therapeutic agents against extensively drug-resistant (XDR) and pan-drug-resistant *A. baumannii* and evaluation extracted native endolysin activity.

Methods Twenty-three bacterial samples were collected in Al-Imamein Al-kadhimein Medical City hospital. Phages were isolated from different regions in Baghdad city including (soil, sewage, irrigation channels). Native endolysin was extracted from highly lytic phage that produced halo-like appearance around inhibition zone.

Results Out of 50 isolates, 23 isolates (46%) of XDR, pan-drug resistant (PDR) *A. baumanii* have been isolated from patients with various infections. 136 lytic phages specific to *A. baumanii* were isolated. Each bacterial isolate was sensitive to at least one lytic phage. Phage cocktails were formulated and were shown remarkably minimize the bacterial resistance to individual lytic phages. In addition, the endolysin native activity of lytic phages specific to *A. baumanii* evaluated in this study revealed a potent antibacterial activity (> 1 log) reduction of bacterial density in just one hour of endolysin treatment.

Conclusion Phage therapy assessed in this study was shown ability to efficiently solve the problems of "superbug" bacteria by lysing effectively most XDR, PDR bacteria in vitro. And, phage cocktails were shown to be superior over single-phage preparations in treating *A. baumanii* with much less resistance rate to therapeutic phages. Furthermore, intrinsic activity of native endolysin revealed promising results to tackling superbug pathogens.

Keywords Phage, Phage cocktails, Acinetobacter, native endolysin, extensively drug-resistant (XDR), and pan-drug resistant (PDR)

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List of abbreviations: *A. baumanii* = *Acenitobacter baumanii* CLSI = Clinical and Laboratory Standards Institute, MDR = Multiple drug resistant bacteria, PDR = Pan drug resistant bacteria XDR = Extensive drug resistant bacteria

Introduction

Antibiotic resistance is an emerging global health disaster, resulting from the constant use (and misuse) of antibiotics in healthcare [1,2]. *Acinetobacter baumannii* (*A. baumannii*) is a Gram-negative, capsulated, opportunistic pathogen that is effortlessly spread in hospital intensive care units (ICU) [3]. Most of *A. baumannii* clinical isolates are multi-drug resistant (MDR), extensively drug-resistant (XDR), and pan-drug resistant (PDR) bacteria, which greatly restricts the available treatment choices [4]. To prevent

returning to the dark "post antibiotics" era, there is an urgent need for new therapeutic agents against the MDR, XDR, PDR pathogens. To fight these bacteria, the scientists suggest a number of new therapeutics alternatives or complements to antibiotics against the "superbug" pathogens, of which *A. baumannii*. Interestingly, bacteriophage, or phage, therapy has been placed at the top of table presenting a possible alternative mean to tackle refractory bacterial infections [5].

Phage therapy refers to the utilization of phages to treat bacterial diseases [6]. Phages are very abundant in nature [7] and every bacterium is likely to have their own specific viruses that could be utilized as antibacterial agents [8-10]. The host range of a given phage is often very specific to the sub-species level, which may confer an advantage over antibiotics if infectious bacteria can be targeted without damaging commensal members of the host microbial community.

The formulation of phage cocktail could save lives of uncountable patients suffering from serious and devastating *A. baumanii* infections resistant to the conventional antibiotics. This highlights the importance of using phage cocktails especially in a country like Iraq where *A. baumanii* flourishes in poor hygiene and areas of conflicts [11].

The current study aims at testing the efficacy of phage therapy, via using a single phage and a phage cocktail, to treat infections with MDR *A. baumanii* bacteria in vitro and to extract and determine intrinsic activity of native endolysin.

Methods

Specimen collection and identification

Fifty samples of bacteria were collected in Al-Imamein Al-kadhimein Medical City Hospital in Alkademiya, Baghdad. Bacterial sampling was carried out during the period from September 2016 to November 2016. A total of twenty-three (23) different *A. baumanii* isolates (11 XDR, 12 PDR), belonging to hospitalized patients with various infections including septicemia, skin infection, severe urinary tract infection, pneumonia, and meningitis, were obtained from the Central Laboratory of the hospital. At the same day, samples were transferred to the laboratory of the Medical Microbiology Department in the College of Medicine, Al-Nahrain University to for cultivated bacteria by using nutrient, MacConkey agar and blood agar then incubate at 37 °C for 18-24 h. Next day, all bacterial isolates were subjected to a full set of diagnosis including Gram staining, culture, and bio-chemical tests including Oxidase test, Catalase test, Kligler iron agar (KIA), Indole production test, Motility test, Urease production test, Citrate utilization test, Lactose fermentation test, and growth at 44 °C test [12]. Furthermore, the results of the identification of *A. baumanii* were confirmed by API 20E system.

Antibiotic susceptibility test

Antibiotic susceptibility test was carried out on *A. baumanii* isolates using Kirby-Bauer method [13]. Seventeen types of antibiotic disks were used as following Imipenem (10 μg), Ciprofloxacin (5 μg), Colistin (10 μg), Tigacyclin (15 μg), Gentamicin (10 μg), Cefotaxime (30 µg), Ceftazidime (30 μg), Ceftriaxone (30 μg), Trimethoprim/sulphamethaxazole (10 μg), Cefepime (30 µg), Levofloxacin (10 μg), Piperacillin (100 µg), Tobramycin (10 µg), Amikacin (30 µg), Meropenem (10 µg), Aztreonam (30 µg) and Amoxicillin-clavulanic acid (20 µg). A 0.5 McFarland standards of bacteria was used and inoculated and spread by a sterile swab on Muller-Hinton agar Medium. Antibiotic discs were then placed on inoculated agar plates by forceps. The plates were left in incubator upside down at 37 °C for 18-24 h.

At Subsequent day, plates were carefully examined for any inhibition zones along with measuring their diameter in millimeters (mm) using a metric ruler. Then, classified as sensitive, intermediate, or resistant, according to the standardized table supplied by Clinical and Laboratory Standards Institute (CLSI) guidelines [14]. Furthermore, the results of antibiotics susceptibility test were confirmed by VITEK 2 System.

Bacteriophage sampling, isolation

Different crude samples for phage isolation were obtained from different regions in Baghdad city including sewage, farm soil, feces of sheep, chicken litter, and swab from surgical lounge in Al-Imamein Al-kadhimein Medical City Hospital during the period from January 2017 to April 2017. Overnight bacterial broth (100 µl) was mixed with 2-3 ml of crude samples, which were derived from sewage, cattle feces, chicken litter that might contain Acinetobacter-specific phages. Then, 2-3 ml, equal volume, of nutrient broth and 2ml of Lambda buffer were added to the mixture as well. Then, the mixture was incubated overnight at 37 °C with continuous shaking. Next day, supernatant was taken and 1:10 v/v chloroform was added with gentle shaking for 5-7 min at room temperature to lyse the remaining bacteria. Then, centrifugation at 1000 g for 3 min was carried out to produce primary phage suspension. Subsequently, one ml of overnight bacterial broth was poured onto nutrient agar plate and spread by sterile swab in order to make bacterial lawn. After 10-20 min, the lawn should have been dried. Ten (10) µl of primary phage suspension were spotted onto the surface of the bacterial lawn and were allowed to dry before incubating at 37 °C for 18-24 h in inverted state. On the next day, if zone of lysis was developed at the spot of the primary phage suspension, a lytic and specific phage for the target bacteria was identified and picked up the inhibition zone by sterile loop and put into 1 ml of Lambda buffer in 1.5 ml sterile Eppendorf tubes, then 1:10 v/v chloroform was added to the lysate with gentle shaking for 5-7 min at room temperature, then, centrifuged at 1000 g for 3 min and bacterial cell debris were pelleted, and the supernatant containing phages was transferred to 1.5 ml sterile Eppendorf tubes and stored at 4 °C for one month. The supernatant was called transient phage stock suspension [15].

Optimization and characterization of isolated phages

Plaque characteristics were determined using top layer plaque assay and according to the following parameters: a) Diameter (mm) of the plaque. b) Shape of the plaque. c) Depth of the plaque. d) Margin cut. e) Clarity or turbidity of the plaque. Accordingly, the clearest and largest plaques were selected; moreover, small or turbid plaques were subjected to optimization by conducting serial passage in top layer plaque assays; at each run, the best of the best plaques, in terms of the above-mentioned parameters, were selected in order to acquire better virulence characteristics of the isolated lytic phages. It is noteworthy to mention that not all turbid and small plaques were optimized. This depends on the potential of the phage to enhance its virulence characteristics [15]. In this approach, burst size, burst time, and infection percentage are determined according to [15]. One hundred (100) µl of 106 PFU/ml of phage were added to 100 µl of 104 CFU/ml of target bacterial broth culture at MOI equal to 100. This mixture was dispensed into a sterile 1.5 ml Eppendorf tube and then incubated at 37 °C for 5-10 min to allow the phage to enter into bacterial host (phage contact time). Then, the mixture of phage-bacteria was centrifuged at room temperature at 1000 g for 3 min. Subsequently, the pellet was re-suspended in one ml of nutrient broth (this step was repeated 3 times). The aim of this step is to remove all of the extracellular phages and to neglect the supernatant and keep only the bacterial cells infected with specific phages. Afterwards, the re-suspended tube was ten-fold serially diluted (10-1-10--2) by adding 100 µl of the suspension to 900 µl of nutrient broth. Ten µl from each dilution were spotted on target bacterial lawn at timely intervals; 20, 25, 30, 35, 40, and 45 min, then, let the plate get wet and incubated overnight at 37 °C. Next day, the plaques were counted to calculate the infection percentage, burst time, and burst size [15] as follows:

Infective percentage (IP %): refer to the percentage of specific lytic phages that invade the target bacteria. This percentage was calculated by dividing the number of plaques during the pre-burst time over the number of bacteria used in the assay. Burst time (BT): refer to the time required by the infecting

phages to burst and exist from bacterial cells. So, BT is the period before a sharp rise was detected in the number of the progeny phage particles for the certain dilution. Burst size (BS): refer to the number of the new progenies of phage per one cell of target bacteria. BS was calculated by dividing the number of plaques post- burst time over the number of plaques pre-burst time [15].

Testing of bacterial resistance rate of *A. baumanii* to infecting bacteriophages

The resistance rate of bacteria to infecting phages was measured. A piece from the same bacterial lawn of the target bacteria that is equal in diameter to phage lysis spot was cut by a sterile loop and put in 1.5 ml sterile Eppendorf tube containing one ml of normal saline. This approach is to obtain the same number of bacteria that was present in the phage spot lysis zone. Then, the tube was subjected to periodic shaking for 5 min. Then, tubes were centrifuged at 1000 g for 3 min at room temperature. Afterwards, the supernatant was removed and the precipitate was re-suspended in one ml of normal saline [15].

Ten-fold serial dilutions of the resulting bacterial suspension (10-1-10-5) were made. Then, 10 µl drop of the bacterial suspension was spotted on a nutrient agar plate inclined 45 degrees in one direction in order to spread the drop to one direction forming lines at which counting of bacterial colonies becomes much easier. The plates were incubated at 37 °C for 24 h. The bacterial resistance rate was calculated as the following [15]:

Resistance rate = Number of resistant colonies per phage lysis spot / number of bacterial colonies formed from the same size cut of bacterial lawn.

Determination of the coverage rate of bacteriophage cocktails to *A. baumanii*

In this approach, after mixed numerous phages in one suspension, randomly sampled 10 *A. baumanii* isolates were collected from patients in Al-Imamein Al-kadhimein Medical City Hospital. Ten (10) µl of bacteriophage cocktails suspension were spotted on to the surface of the overnight bacterial lawn and were allowed to dry before incubating at 37 °C for 24 h. On the next day, if a zone of lysis was developed at the spot where the phages suspension was applied, a susceptible bacterial isolate to phage cocktail was found. Then, the coverage rate of the formed bacteriophage cocktails was measured using this formula:

Coverage rate = (number of bacteria lysed by cocktails / total number of bacteria) x 100%.

The assessment of the activity of phage Endolysin on A.r baumanii bacteria

Extraction of Endolysin

About 100 ml of broth of *A. baumanii* bacteria were incubated for 18-24 h at 37 °C. Next day, 250 ml of broth medium were added to the bacterial broth and incubated for another 3 hours at titer 1×10^9 CFU/ml. Up to 10 ml of phage at titer 1×10^{11} PFU/ml (1:100 MOI) were mixed with bacteria for 20 minutes and then put them directly in ice. Centrifugation at 10,000 g for 20 minutes and take the sediment. The sediment was suspended in 10 ml of 0.05 M phosphate buffer + 5 mg deoxyribonuclease and incubated for 60min at 37 °C. And 0.005 M EDTA was added and centrifugation at 10,000 g for 1 h and then the supernatant was taken. Disodium tetrathionate (0.3 M) was added and mixed for one hour at 4ºC. Ammonium sulfate was added to 85% saturation and incubated for 18-24 h at 4 °C. Next day, centrifugation at 10,000g for 1h and resuspended in 5 ml of 0.05 M phosphate buffer saline (PH 7.5). Dialysis against 200ml of the buffer at 4 °C was conducted. The resultant solution was added to column chromatography sephadex G.100 in 0.1 M phosphate buffer saline PH 7.5, in 18×0.5 cm column. Each one ml of the resultant filtrate was collected in Eppendorf tubes. From each Eppendorf tube, 10 µl of the filtrate were dropped by automatic pipette onto *A. baumanii* bacterial lawns of the specific bacteria to see which Eppendorf tube contains the lytic and native activity of endolysin.

Measurement of the native activity of Endolysin on A. baumanii bacteria

Upon using sephadex G100 chromatography, the used phage (AB3P5) gave 10 Eppendorf tubes of one ml eluted fluid; in case endolysin was extracted, at least one of these tubes must show a lytic activity against the corresponding *A. baumanii* isolate. The endolysin activity was first checked by lysis on bacterial lawn and second by decreasing the optical density of the bacterial broth when measured by a spectrophotometer. *A. baumanii* broth was composed of bacterial cells growing at mid-log phase (OD600 = 0.6) and were centrifuged (4000 g, 30 min, 4 °C) and then re-suspended in a phosphate-buffered saline (PBS) at PH (7.5). After assigning the tube that showed lysis in the bacterial lawn assay, 30 µl of this supposed-to-be endolysin-containing elute were added to 270 µl of the prepared bacterial broth at room temperature. Then, the optical density was measured spectrophotometrically every ten minutes for 1 h at 600 nm [16].

Results

Characteristics of the isolates of *A. baumanii*

The characteristic features of the bacterial isolates used were the site of infection, patient's sex, patient's age, and the disease or lesion from which bacterial isolates were taken. As shown in table 1, the patients were infected with virulent bacteria causing serious and life-threatening diseases including urinary tract infection, septicemia, wound infection, pneumonia, and meningitis. A total of 23 *A. baumanii* isolates were collected. The specimens from which *A. baumanii* were isolated are as follows: blood 7/23 (30.4%), urine 2/23 (9%), wound swab 7/23 (30.4%), diabetic foot 3/23 (13%), sputum 2/23 (9%), and C.S.F 2/23 (9%). The diseases from which *A. baumanii* bacteria were isolated wound infection 10/23 (43.4%), urinary tract infection 2/23 (9%), septicemia 9/23 (39.1%), pneumonia 2/23 (9%), and meningitis 2/23 (9%) (Tables 1 & 2). Hence, the most prevalent disease related to *A. baumanii* was wound infection followed by septicemia. The age of patients ranged from 1 day to 70 years and male to female ratio was 2.3:1.

All bacterial isolates appeared as Gram-negative coccobacilli and occasionally arranged in diplococci. All of the isolates were tested for biochemical tests and *A. baumanii* showed negative results for oxidase, motility, indole production and urease production tests, and positive results to catalase and citrate utilization tests; Kligler iron agar test developed an alkaline slant, no change at bottom, H2S negative without gas production. Also, when *A. baumanii* isolates were cultured on MacConkey agar, they appeared as small, pale and lactose non-fermenter colonies, while on blood agar they appeared as opaque, creamy and non-hemolytic colonies. Growth at 44ºC was positive for all *A. baumanii* isolates which showed the ability to grow at this temperature degree. This test was used to distinguish *A. baumanii* (which was able to grow at this temperature) from other Acinetobacter species which are unable to grow at this temperature degree.

Antibiotic sensitivity test

The results showed that different *A. baumanii* isolates had different antibiotic sensitivity profiles; of 23 isolates included in the current study, 11 were XDR and 12 were PDR. As shown in Figure 1.

The characteristic features of the isolated and optimized phages

The characteristics of plaque assay of the isolated phages showed that plaques clarity (clear, semi-clear, turbid, semi-turbid), plaques size was varied and ranged between 0.5 mm to 6.5 mm, margin cut (regular and irregular), and plaques shape (oval and circular).

One hundred and thirty-six (136) phages specific for 23 *A. baumanii* bacteria were isolated. The specimens were obtained mainly from sewage and also from irrigation channels, then from waste water, soil, feces of sheep, chicken litter and swab from lounge. However, most of the isolated phages were highly lytic

and produced obvious inhibition zone on target *A. baumanii* bacteria where plaque size was higher than 3mm with full clarity of plaques; therefore, further optimization was not needed save for 25 phages which required further optimization in order to increase their lytic characteristics, (Table 1). The titer of the specific lytic phages isolated and optimized to the bacterial isolates were amplified and measured by using top layer plaque assay. Most phages reached high titers ranging between 108-1011 PFU/ml using top layer plaque assay. The optimized specific and lytic phages were shown to be able to completely lyse the bacterial host in whatever manner of application of phages as demonstrated in figures 2 and 3.

The characteristics of the isolated and optimized phages in terms of biokinetic assay

In the current study, 10 bacteriophages to different bacterial isolates were randomly selected to give representative values of biokinetic characteristics. The results in this study showed that the average burst time (BT) was 73.5 min ranging between 30 to 45 min. The maximum burst size (BS) of the randomly selected phages to *A. baumanii* was 245 progenies, while the minimum BS was 130 progenies and the average BS was 187.5 progeny. The average infective percentage (IP %) was 85.45% ranging between 74.4% and 94.5%, as shown in table (3).

Formation of phage cocktail to *Acinetobacter baumanii*

A phage cocktail was formed by mixing 64 phages specific for 23 *A. baumanii* isolates (AB1-AB23). One hundred, 100 µl of 10^6 PFU/ml of each phage were collected in one tube to form a bacteriophage stock containing a wide range of isolated phages. All bacterial isolate, except AB2 and AB8, were targeted by more than one phage; the most targeted isolate was AB3 where 6 different phages shared the same specificity towards this isolate.

Bacterial resistance to a single phage versus phage cocktail

Up to 18/23 (78.3%) of *A. baumanii* bacteria were completely sensitive to the applied lytic phages with zero resistant bacterial colonies. So, only 5 out of 23 isolates (21.7%) of *A. baumanii* were shown to develop some level of resistant colonies in the inhibition zone at the spot of lytic phage application. On other hand, the formed phage cocktail was shown to remarkably minimize the number of the resistant bacterial colonies appeared to individual phages. The results revealed that once *A. baumanii* isolate develops resistance to one member of phage cocktails, the bacterial isolate was still sensitive and lysed by other phage members in the cocktails as shown in figure 4.

The coverage rate of the formed phage cocktail to *A. baumanii* bacteria

Ten (10) *A. baumanii* isolates were collected from patients resided in Al-Imamein Al-kadhimein Medical City Hospital. The collected specimens were not biased towards particular disease, site of infection, or patients' age or sex. Full sets of identification were performed, then the results of identification of *A. baumanii* confirmed by API 20E system.

The formed phage cocktail was able to form a clear inhibition zone on the most tested bacterial lawns. The coverage rate of the formed phage cocktail was calculated. The phage cocktail was shown to be able to lyse 7/10 (70%) of *A. baumanii* bacteria and thus the coverage rate was 70%.

Table 1. The characteristic features of *A. baumanii* bacteria to which the lytic and specific phages were isolated

Bacterial isolate	Specimen	Age of patient	Sex of patient	Disease
AB1	Wound swab	30 years	Male	Wound infection
AB2	Urine	40 years	Female	Urinary tract infection
AB3	Blood	7 years	Female	Septicemia
AB4	Throat swab	4 days	Female	Pneumonia
AB5	Wound swab	6 years	Male	Wound infection
AB6	Wound swab	37 years	Male	Wound infection
AB7	Blood	35 years	Male	Septicemia
AB8	Blood	1 day	Male	Septicemia
AB9	Blood	5 days	Male	Septicemia
AB10	Blood	3 days	Male	Septicemia
AB11	Wound swab	2 months	Male	Wound infection
AB12	Wound swab	33 years	Male	Wound infection
AB13	Sputum	38 years	Male	Pneumonia
AB14	Diabetic foot	40 years	Female	Wound infection
AB15	Wound swab	70 years	Male	Wound infection
AB16	CSF	43 years	Female	Meningitis
AB17	Blood	2 years	Female	Septicemia
AB18	Wound swab	12 years	Male	Wound infection
AB19	Diabetic foot	41 years	Male	Wound infection
AB20	Blood	55 years	Male	Septicemia
AB21	Diabetic foot	2 years	Female	Wound infection
AB22	Urine	67 years	Male	Urinary tract infection
AB23	CSF	14 days	Male	Meningitis

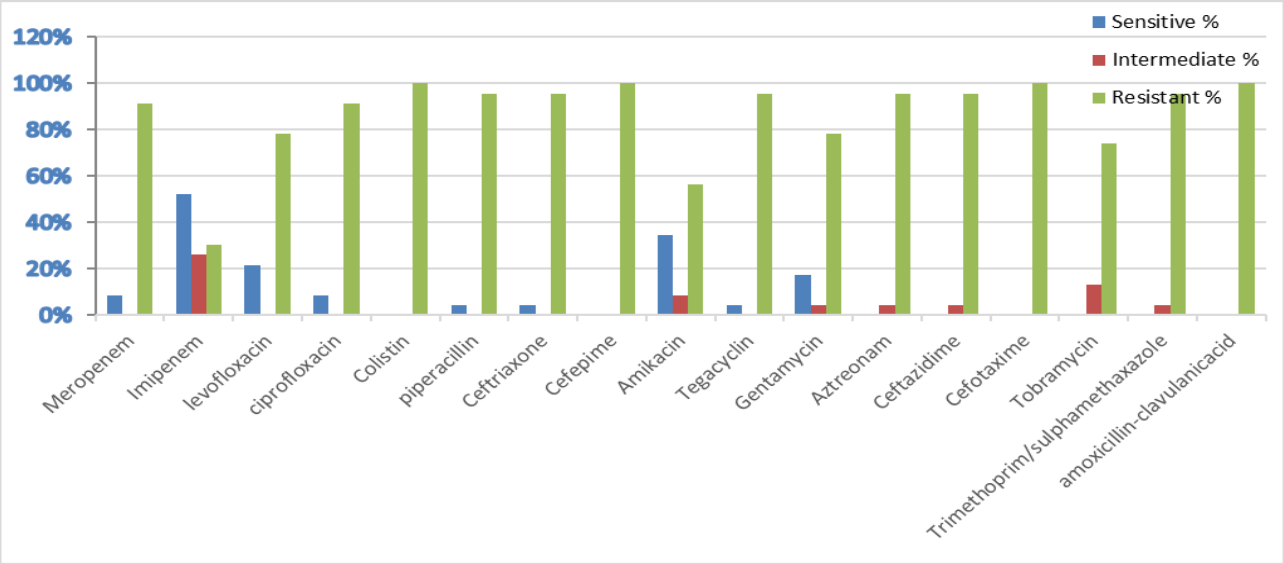

Figure 1. The rate of antibiotic sensitivity/ resistance of 23 *A. baumanii* isolates to a panel of 17 antibiotic disks commonly used in Iraq

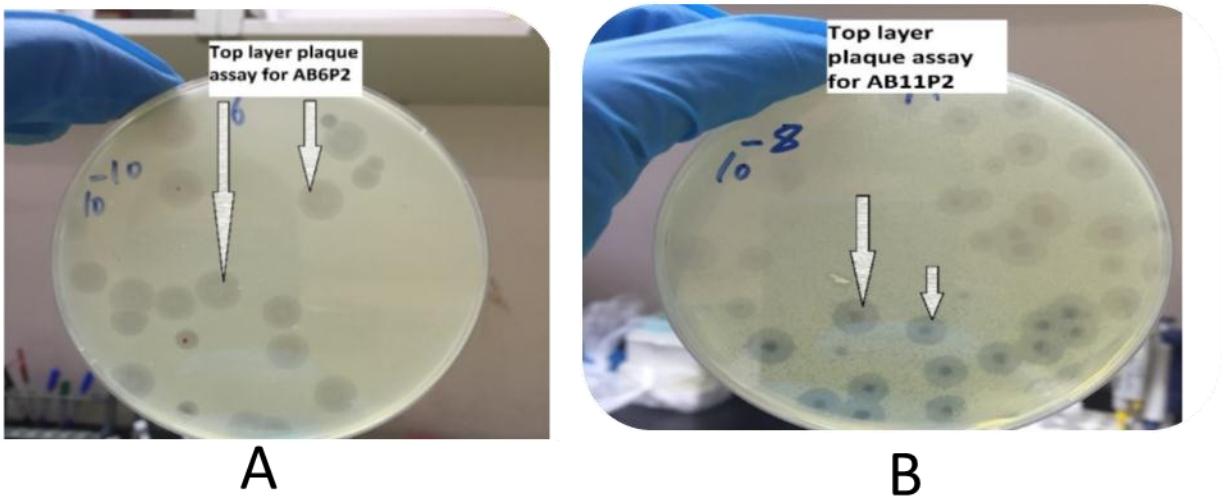

Figure 2. A) plaques produced by of AB6P2 via top-layer plaque assay B) plaques produced by AB11P2 via top-layer plaque assay

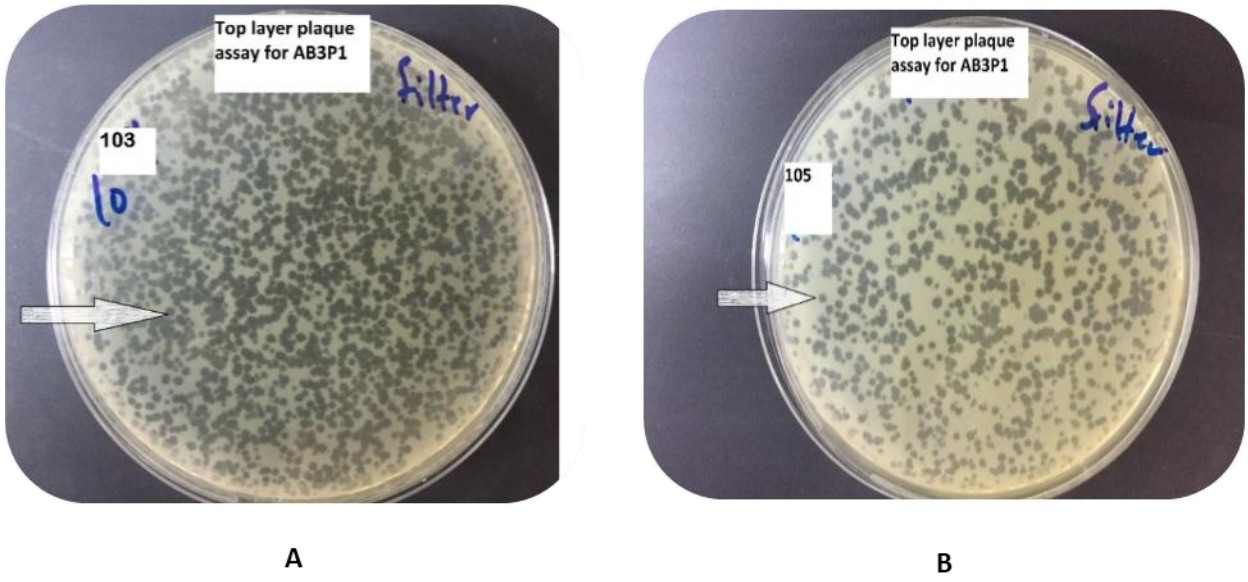

Figure 3. Top layer plaque assay for AB3P1 with different concentrations A) AB3P1 with 103 PFU/ml B) AB3P1 with 105 PFU/ml

Table 2. Morphological features of the isolated phages to *A. baumanii* bacteria before and after optimization via top layer plaque assay

Phage symbol	Plaque size (mm)		Plaque clarity		Plaque shape		Margin cut	
	Before	After	Before	After	Before	After	Before	After
AB1P1	0.5	1.5	Turbid	Semi-clear	Round	Round	Un-obvious	Regular
AB1P2	0.3	1	Semi-turbid	Clear	Round	Round	Irregular	Irregular
AB2P1	2	2.5	Semi-clear	Semi-clear	Round	Round	Regular	Regular
AB3P1	0.8	1.5	Semi-turbid	Semi-clear	Oval	Oval	Irregular	Irregular
AB3P2	0.5	0.5	Semi-clear	Clear	Oval	Oval	Regular	Regular
AB3P3	2.5	4	Semi-Clear	Clear	Round	Round	Irregular	Irregular
AB3P4	3.5	3.5	Semi-Clear	Clear	Oval	Oval	Irregular	Irregular
AB4P1	3.5	7	Clear	Clear	Round	Round	Irregular	Irregular
AB5P1	1	1.5	Turbid	Clear	Oval	Oval	Un-obvious	Irregular
AB6P1	2	2	Semi-turbid	Semi-clear	Semi-round	Round	Irregular	Regular
AB6P2	2	3.5	Semi-clear	Semi-clear	Oval	Oval	Irregular	Irregular
AB9P1	0.5	0.5	Semi-Clear	Clear	Round	Round	Regular	Regular
AB10P1	1.2	2.5	Clear	Clear	Round	Round	Regular	Regular
AB10P2	1.9	5.5	Semi-turbid	Clear	Round	Round	Regular	Regular
AB12P1	1	1	Semi-turbid	Semi-turbid	Oval	Oval	Irregular	Irregular
AB15P1	3.5	5.5	Turbid	Clear	Round	Round	Un-obvious	Regular
AB15P2	0.8	1	Semi-turbid	Clear	Round	Round	Regular	Regular
AB17P1	1.7	3.5	Semi-Clear	Clear	Oval	Oval	Irregular	Irregular
AB19P1	0.5	3	Semi-turbid	Semi-turbid	Round	Round	Un-obvious	Irregular
AB19P2	0.8	1.5	Turbid	Clear	Round	Round	Un-obvious	Regular
AB20P1	0.5	2	Semi-Clear	Clear	Oval	Oval	Regular	Regular
AB21P1	1.5	6.5	Turbid	Semi-clear	Oval	Oval	Regular	Regular
AB21P2	1	2	Semi-turbid	Semi-clear	Round	Round	Irregular	Irregular
AB22P1	0.5	1.5	Turbid	Turbid	Oval	Oval	Un-obvious	Regular
AB22P2	2.3	4.5	Semi-turbid	Semi-turbid	Oval	Oval	Irregular	Irregular

Table 3. Shown the biokinetics: Infective percentage (IP %), Burst time (BT) in minutes, and Burst size (BS) in number of progenies of the randomly selected bacteriophages to *A. baumanii* bacteria

	IP %	BT	BS
AB1P2	91.3	40	170
AB3P4	94.5	45	245
AB5P1	76.4	35	220
AB7P3	85.2	30	160
AB9P1	88	45	210
AB10P2	86.6	45	145
AB14P1	77.5	40	190
AB15P3	82	30	200
AB17P2	80	40	130
AB20P1	79	35	185

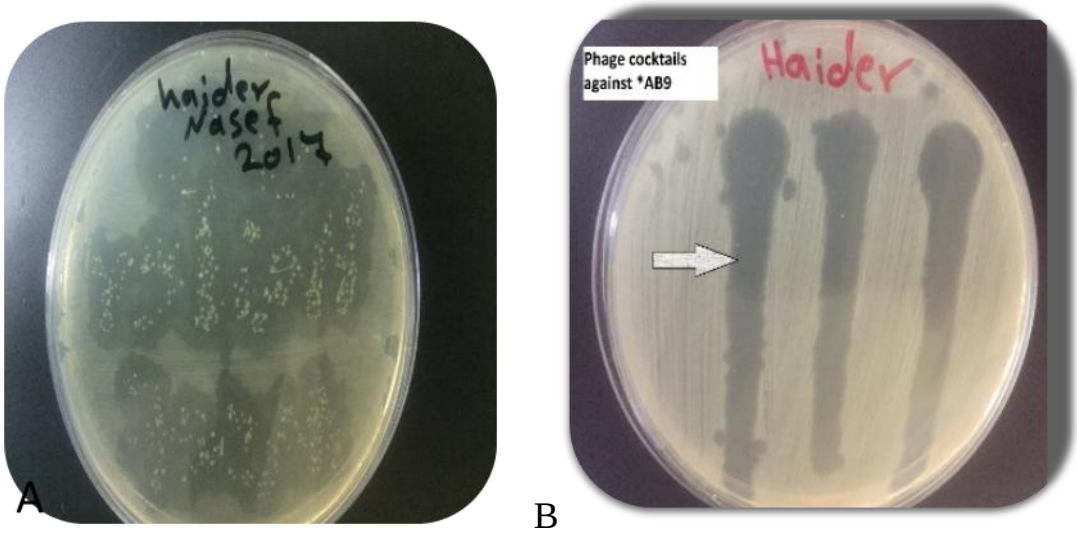

Figure 4. (A) resistant *A. baumanii* bacteria (AB3) to a single specific phage (AB3P1) (B) the phage cocktail completely lysed *A. baumanii* (AB3) bacteria without development of any resistant colonies

Determination of the native activity of phage Endolysin on *A. baumanii* bacteria

During the isolation and optimization of different phages against *A. baumanii* isolates; some phages were found to produce a halo-like appearance around the inhibition zone produced by some lytic phages as shown in figure 6. This halo-like appearance suggested a native endolysin production from phage. The findings of this study revealed that a specific phage endolysin to *A. baumanii* was extracted successfully by using sephadex G100 column chromatography. The Eppendorf tube number two showed positive results for phage

endolysin. The optical density of *A. baumanii* broth was measured initially at zero time, just before the addition of the corresponding endolysin, then it was measured every ten minutes for complete one hour and it showed obvious decline in optical density of bacterial broth with time. According to t-distribution test, there is a significant difference between the test groups, bacteria treated with endolysin and control group, bacteria alone with PBS, (P = 0.00134). Moreover, the overall enzymatic activity of extracted native endolysin was quantified by turbidometric reduction analysis, 270 µl of exponentially growing *A. baumanii* (AB3) cultures (1.4 x 108 CFU/ml) were challenged to 30 µl of extracted native endolysin at room temperature. *A. baumanii* optical density and viability counts were reduced from 0.585 to 0.031 after one hour of treatment, compared with the untreated control group that continued to grow (from 577 to 624 after one hour). It was shown to be -0.0092 ΔOD/min. By using standard curve measurements to interpolate OD values to bacterial count, it was shown that the endolysin native activity surpassed 1.4 log reduction threshold after one hour of treatment as shown in figure 5.

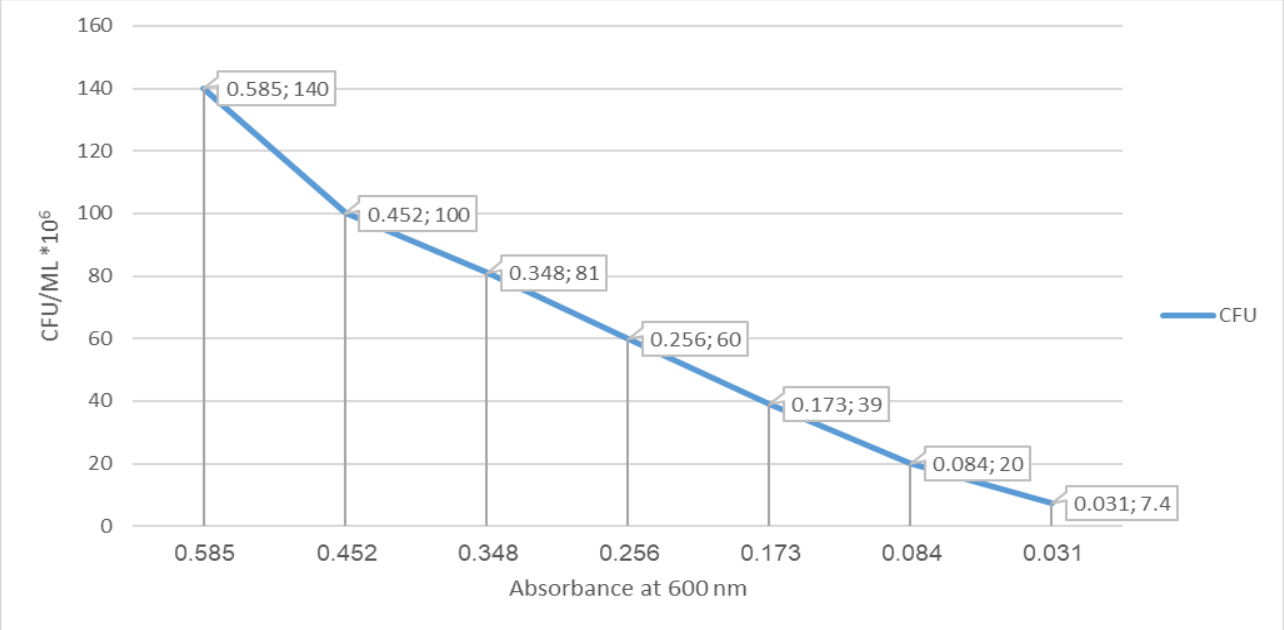

Figure 5. Interpolation of bacterial count in CFU/ml with the optical density (600nm) of *A. baumanii* broth treated with AB3 phage endolysin

Discussion

The results in the current study revealed different antibiotic resistant profiles by different *A. baumanii* isolates. All of the isolates of *A. baumanii* were shown to be fully resistant to several antibiotics tested in the current study and as follows: Cefepime, Cefotaxime, Colistin, and Amoxicillin-clavulanic acid. However, the resistance rate to other antibiotics was less than 100% and ranged from 95.65% to 30.43%. The MDR status reported in current study agrees with the findings of other recent studies carried in Iraq [17-19] but this study disagrees with a study carried out in USA which reported that approximately 50% of patients are with colistin-resistant *A. baumannii* [20]. The variation in the results may be due to sample size, sampling procedure, differences in the time of the studies, or differences in the geographic areas. This study

agrees with study in Iraq which found that, *A. baumanni* clinical isolates developed 97.3% of resistance to Aztreonam and Ceftriaxone, 89.5% to Ceftazidime, and 58.2% to Imipenem [21]. Moreover, this study agrees with another local study conducted in Iraq which found that, a high level of *A. baumanii* resistance, 88.2% to Meropenem and a lower resistance rate 52.9% to Imipenem while complete resistance was reported, 100%, to Cefepime, Azteronam, and Ceftriaxone [22]. The complete resistance of *A. baumanii* isolates collected in this study to colistin might be attributed to the major mechanism of colistin resistance in *A. baumannii*, namely modification of lipopolysaccharide (LPS) outer membrane via adding phosphor ethanol amine to the hepta - acylated lipid A structure [23,24].

The presence of β-lactamases, which are the backbone of the most principal mechanism of β-lactam resistance. These enzymes, at least in part, hydrolyze carbapenems along with other β-lactams [25]. Recently a new extended spectrum AmpC enzyme was identified in *A. baumannii* bacteria; this enzyme has been shown to be able to hydrolyze Ceftazidime, Cefepime and Aztroenam [26].

In this regard, bacteriophage or phage therapy could offer one of the best applicable solutions to overwhelm the problem of antibiotics resistance of bacteria in Iraq and in the world [27]. One of the striking merits of using bacteriophages over antibiotics in a country like Iraq is the fact that phages are self-amplifying in the site of infection so phages can be given to patients in a single dose, therefore, unnecessary to repeat doses of phages; hence, incompliance of patients will not affect the success of the course of therapy. In this study, the lytic and specific phages to *A. baumanii* were isolated from various environmental sources; the main source was sewage; this finding is in line with other studies [28,29]. Another main source of phages in this study was waste water [30]. The current study revealed that sewage was the best source to isolate highly lytic and specific phages to *A. baumanii* [31]. Moreover, phages from sewage showed good clarity and size of plaques; this might be credited to the fact that phages in sewage tolerate drastic environment which favors the induction of temperate phages, residing in high number in Acenitobacter bacteria, to lytic ones.

The current study showed successful in vitro use of both single phage and phage cocktail to lyse *A. baumanii* XDR or PDR isolates. Nevertheless, this study revealed a superiority of the phage cocktail over the single phage in lysing *A. baumanii* bacteria without development of resistant colonies to phage therapy. Consequently, such phage cocktails are powerfully supposed to prevent the emergence of phage-resistant mutants [16,32]. The results of the current study highlighted the fact that using phage cocktails provides several advantages. Firstly, phage cocktails broaden the strain-specific range of infective phages. This permits effective therapy of a broader spectrum of bacteria within the same *A. baumanii* species [33,34]. Secondly, phage cocktails solve the serious obstacle of the development of *A. baumanii* resistance to attacking phages. It was stated that using phage cocktails is the finest choice for effective phage therapy without suspicions of rapid emergence of bacterial resistance [33]. The phage cocktail used in this study ensured two important goals, covering as much as possible different strains of *A. baumanii*, and the second goal is that each bacterial isolate was recognized by multiple different phages, a necessary step to combat bacterial resistance to phage therapy. Each *A. baumanii* isolate might have more than one receptor and each receptor is recognized by a different phage to attach and invade [35]. This explains why each bacterial isolate was invaded by more than one different phage. Therefore, when a bacterial isolate develops resistance to one phage in the phage cocktail, it is still sensitive to other phages in the same phage cocktail. From the findings of the top layer plaque assay of this study, each member of the phage cocktail was different from each other, and from the findings of the bacterial resistance rate to the single phage versus the phage cocktail, the phages used in this study seem to target different receptors on the cell wall of *A. baumanii* bacteria. This provides evidence on

the preferred use of phage cocktails in the phage therapy of superbugs like *A. baumanii* XDR and PDR bacteria tested in this study.

The coverage rate of the formed bacteriophage cocktail in this study was shown to be very high, up to 70%. Such high coverage paves the road to successful and ready-to-use therapy of serious and life-threatening infections of *A. baumanii*. Nevertheless, in this study, it was proven that in few months and by a single researcher, a phage cocktail of 64 *anti-A. baumanii* specific and lytic phages was formed. The formed phage cocktail could save lives of uncountable patients suffering from serious and devastating *A. baumanii* infections resistant to the conventional antibiotics. This highlights the importance of using phage cocktails especially in a country like Iraq where *A. baumanii* flourishes in poor hygiene and areas of conflicts [36,37].

The results of the native endolysin activity in the current study are in a harmony with few studies examined the native activity of endolysin produced from bacteriophages that infect gram-negative bacteria such as *A. baumanii*, *P. aeruginosa* and *E. coli* [38-42]. The current study highlights the intrinsic antimicrobial activity of native endolysin produced from phages against G-ve bacterial pathogens. Native endolysin activity is a good candidate for the therapeutic/disinfectant endeavor to control nosocomial infections caused by multiple drug-resistant bacteria, particularly MDR *A. baumanii* bacteria [38]. The intrinsic antibacterial activity of endolysin against G-ve needs the ability of endolysin to get through the outer membrane pf these bacteria. This might explain why endolysins from phages infecting Gram-negative hosts are mostly small single-domain globular proteins (molecular mass between 15 and 20 kDa), and usually without a specific CBD module [39]. These lysins likely better fulfill the catalytic role of classical enzymes (aiding multiple catalytic reactions during cell lysis), as opposed to their Gram-positive counterparts, which are proposed to bind to one site and have a very low off-rate [43,44].

In Acinetobacter, it seems rather unusual that the lysogens would evolve a large diverse group of lysins for the sole purpose of releasing their phage progeny. Researchers guess that these lysins might in some manner be harnessed by the Acenitobacter bacteria to control their environment and fight back competing bacteria of other species [45]. Being a soil organism, *A. baumannii* shares a highly competitive niche with further bacteria, including Pseudomonas and Bacillus which have an advantage over *A. baumannii* with their capability of producing several bacteriocin molecules used to kill bacteria in proximity [46,47].

Taken together, the findings of this study indicate that *A. baumanii* bacteria in Iraq are mostly XDR and PDR bacteria; such abnormally high rate of multiple drug resistance necessities novel methods to tackle this impeding health risk on community. Therefore, the phage therapy assessed in this study was shown to be able to efficiently solve the problems of superbug resistant bacteria by lysing effectively most XDR and PDR bacteria in vitro. And, phage cocktails were shown to be superior over single-phage preparations in treating *A. baumanii* with much less rate of resistance to therapeutic phages. In addition, the endolysin native activity of lytic phages specific to *A. baumanii* evaluated in this study revealed a potent antibacterial activity (> 1 log) reduction of bacterial density in just one hour of endolysin treatment; this provided promising results to tackle Gram negative bacteria by using low molecular weight endolysins which are of high level of native antibacterial activity.

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Authors contribution

Dr. Abdel-Ameer: designed the research and conducted study analysis. Jasim: conducted the research.

Conflict of interest

There is no conflict of interest among authors of this manuscript.

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