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Isolation and characterization of lytic bacteriophages specific for antibiotic-resistant *Acinetobacter baumannii*

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ABSTRACT

Almost all conventional antibiotics are no longer effective against the common nosocomial infection *Acinetobacter baumannii.* Bacteriophage therapy is an effective alternative for bacterial infections resistant to many drugs. In the present study, a lytic bacteriophage, ΦEAP, which infects *A. baumannii* was chosen for characterization. Phage ΦEAP described in this research has the ability to lysis *A. baumannii* that resists 94 % of the used antibiotics. *A. baumannii* isolated from a resistant wound abscess. When ΦEAP alone incubated at different pH values and different temperatures, the phage was stable over a wide pH range (4 to 9) and at extreme temperatures (between 50°C and 60°C). In addition, ΦEAP tolerates all concentrations of NaCl solutions used so it shows salinity tolerance and resistance to UV rays. ΦEAP has a 94.4 nm icosahedral head and a 302 nm contractile tail. Pure concentrated phage was negatively stained and investigated using transmission electron microscopy (TEM). Electron micrographs showed that the phage belonged to a member of the *Myoviridae* family and the *Caudovirales* order.

1.Introduction

A serious concern to human health is now antimicrobial resistance (AMR) worldwide, due to widespread antibiotic resistance; some microorganisms are on surveillance lists [1, 2]. The world as a whole is transitioning towards the end of the antibiotic period, necessitating the development of new, operational treatments. According to a thorough research of 204 nations on the effects of AMR, 1.27 million of the 4.95 million fatalities globally caused by AMR in 2019 was solely attributed to bacteria [3]. Antibiotic use for secondary bacterial infections has increased, and there has been a delay in taking worldwide action to combat AMR, which can be blamed for the rise in AMR infections. A special report from the Centres for Disease Control (CDC) in 2022 describes a fifteen percent rise in drug-resistant infections in hospitals from 2019 until 2020, illustrating how the COVID-19 pandemic has aggravated the AMR epidemic [4]. As a Category 1 bacterium with MDR outbreaks, the Worldwide Organization of Health [5] has designated *A. baumannii* (a gram-negative bacterium). Between (2019 and 2020), there would be an increase in Acinetobacter infections that are resistant to carbapenem 78% [6]. Due to the substantial amount of innate antibiotic struggle that *Acinetobacter baumannii* carries and its capacity to quickly pick up novel factors of resistance in its environment, the handling of *A. baumannii* infection is made more difficult by the paucity of antibiotics that are effective against this organism [6,7].

Modern healthcare systems have been ravaged by the opportunistic bacteria *A. baumannii*, which is the source of nosocomial infections acquired in hospitals [6]. The management of *A. baumannii* contamination has become a significant clinical problem due to *A. baumannii's* propensity to acquire resistance to a number of disinfectants and antibiotics, and there is an immediate requirement for further investigation of innovative unconventional medicines.

The bactericidal mechanism of phages, which are common in nature and have a distinct structure from that of antibiotics, allows them to successfully control bacterial infections. In order to cure *A. baumannii*, phage therapy is being studied [8]. Therefore, alternative therapies need to be thought about and improved. The clinical application of viruses or bacteriophages, which solely infect bacteria, is a potential therapy for infections that are resistant to antibiotics. Because phages are found everywhere and are only isolated from areas where their host is present, it is very successful to isolate phages that are effective against a variety of opportunistic infections utilizing wastewater. Owing to their widespread prevalence, the very particular, limited

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chance of resistive cross-over with antibiotics, and diminished influence on the protective gut microbiome, phages are seen as a possible therapy option to address the AMR epidemic [9, 10]. Very few antibiotics in the pipeline have reached an advanced stage of clinical testing. To address the AMR dilemma, innovative alternatives to antibiotics—like the therapeutic use of bacteriophages—need to be further studied [10]. Because they may swiftly pick up novel resistance determinants from their environment, phages specific to *A. baumannii* contain several fundamental antibiotic resistance mechanisms. The goal of this study was to identify and describe the phage. Treatment for *A. baumannii* is made more difficult by the lack of medications that are effective against this infection. Additionally, employing bacteriophage to combat the rise in microbial antibiotic resistance is appealing and is already practised in nations like Poland, Russia, and Georgia [11]. Need, increased amount of phage clinical studies. Creating computational algorithms and genomics and bioinformatics analysis to help find suitable phage candidates. The issue of how to prevent or reduce the possibility of the establishment resistance of bacteria and genetic material transfer is also not fully resolved when creating formulations for standardized and clinical usage in the management of bacteria. In order to anticipate the effective application of phage treatment to microorganisms that are considerably more difficult to cultivate at a lab or in which there are no model animals for both acute and chronic bacterial infections illnesses, dependable models, such as non-pathogenic *E. coli*, must be used. In addition, interactions with the human microbiota and immune system are anticipated [12, 13, 14]. Before BPs (Bacteriophages) may be employed in humans, additional research is required to address these issues. Therefore, the aim of this work was - isolation and characterization of lytic bacteriophages specific for antibiotic-resistant *Acinetobacter baumannii*.

2.Materials and methods

2.1 Isolation of bacterial isolates:

Pathogenic bacteria isolates were collected from Fayoum University and Elnabawy Elmohands hospitals, Fayoum Governorate, Egypt. At the same time of collection, many sub-cultures of bacterial isolates were prepared for further investigation. Isolates 1:70 from urine samples, 71: 140 from pus samples

2.2 Antibiotic sensitivity testing

Among 140 bacteria isolates, the MDR only were selected for further examination. The disk diffusion technique [15] for studying the susceptibility of bacterial isolates to antibiotics have been used. Seventeen antibiotics (Ceftazidime (CAZ), 30mg; Cefepime (FEP), 30mg; Cefotaxime (CTX),30mg;Cfixime(CFM),5mg;Cefuroxime(CXM),30mg;Ceftriaxone(CRO),30mg;Doxycycline(DO,DXT),30mg;Minocycline(MN),10mg;Ciprofloxacin(CIP),5mg;Levofloxacin(LVX), 5 mg; Ofloxacin (OFX), 5 mg; Gatifloxacin (GAT), 5 mg; Imipenem (IPM), 10mg; Aztreonam(ATM),30mg; Amikacin(AK), 30mg; Amoxicillin/Clavulanic (AMC),30 mg, and Colistin (CT), 10 mg) have been used. The outermost layer of a single, well-isolated colony was grasped with a sterile loop during the normal inoculation process, and the development process was then introduced into 2 ml of broth. The young culture was then obtained by incubating the culture broth at 37°C for 4 hours. After dipping a sterile cotton swab into the suspension, the surplus broth was drawn out by twisting and squeezing the swab tightly against its interior above the liquid level. To obtain homogenous inoculum, the swab was then equally dispersed around Muller Hinton agar plate's surface. The panels were then allowed to dry for 3 to 5 minutes. Discs impregnated with antibiotics were then applied to the surface of the inoculated plates with sterile forceps. Each disk was gently pressed onto the agar to ensure full contact with the agar surface. Attention was paid to an even distribution of the panes and a minimum distance of 30 mm from center to center . The plates were inverted and incubated within 15 minutes of disc placement at 37°C. After 16 to 18 hours of incubation, the sizes of the inhibitory zones were measured in millimetres after the plates were examined. The resistance ratio was calculated by dividing the sensitive antibiotics to the resistant one. A bacterial strain was referred to as a resistant strain if it was resistant to more than a three of antibiotics chosen from various groups [16].

2.3 Characterization the most resistant bacterial isolates:

The most resistant bacterial isolate was biochemically characterized, and the results were compared to information on recognized microorganisms described in Berge's Manual of Determinative Bacteriology, 9th edition. Estimates were made of bacteria isolates' cell shape and movement using fresh cultures. With the aid of Bergey's Manual, several biochemical assays were carried out [17].

2.4 Identification of the bacterial isolates:

Bacterial isolates were identified using VITEK II automated system [18]. A suitable number of colonies of pure culture were transferred using a sterile swab, and the microorganism was then suspended in 3.0 mL of sterile saline in a test tube made of clear polystyrene measuring 12 x 75 mm. The cloudiness is adjusted with a DensiChek turbidity metre between 0.5 and 0.63. Using integrated vacuum equipment, microorganism suspensions were inoculated onto the appropriate identification cards, either gram-negative or gram-positive. The identification card is inserted into the adjacent slot, the test tube holding the suspension of the microorganism is placed into a particular rack (cassette), and the transfer tube is inserted into the matching suspension tube.. The filled VITEK 2 compact cassette was manually inserted into the vacuum chamber station. The organism suspension was pushed via the transfer tube and into tiny channels that filled each test well once the vacuum had been established and the air was let back into the station. Cards that had been vaccinated were then passed through a device that sealed the card and cut off the transfer tube before placing it into the carousel incubator. At 35.5°C, all card classes were incubated online. Every 15 minutes, each card was automatically taken out of the carousel incubator, brought to the optical device to take reaction assessments, and then put back until the following reading time. Throughout the whole incubation period, data were gathered at 15-minute intervals.

2.5 Phage isolation:

Sewage prepared by mixing SM buffer (NaCl, 5.8 g; $MgSO_4.7H_2O$, 2 g; 1 M Tris-HCl ‘pH 7.5, 50 ml; 2 % gelatin, 5 ml; add d.H2O to 1,000 ml) with sewage sample[19]. Twenty millilitres of Fayoum University Hospital sewage sample were centrifuged 10,000 g for 10 min at 4°C to remove dirt and

germs. The supernatant has been placed in a new tube and maintained at 4°C. To amplify the phage, 20 ml of new LB media (containing 10 mg/l $CaCl_2$) and 20 ml of samples of water were combined with one millilitre of an overnight culture of bacterial suspension. The culture media was then centrifuged at 8,000xg for 10 min at 4°C after having been incubated for 10 h at 37°C with agitation at 160 rpm. The supernatant was filtered through Millipore filters with a pore size of 0.22 lm. Three times this amplification process was carried out. 0.1 ml of final filtered culture media was combined with 0.3 ml of bacterial suspension to test for the presence of phage, which was then incubated at 37°C for 20 min. The mixture was then mixed with 3 ml of LB top agar that had been heated to 47°C (containing 0.4% agar) and spread over a 1.5% LB agar plate. The plate was kept warm for six hours at 37°C, or until lysis zones developed.

2.6 lysate preparation, phage tittering, and single plaque filtration:

A series of concentrations in clean SM buffer (NaCl, 5.8 g; $MgSO_4.7H_2O$, 2 g; 1 M Tris-HCl pH 7.5, 50 ml; 2% gelatin, 5 ml; add d.H2O to 1,000 ml) are necessary. To 3 ml of soft agar that had been pre-warmed at 45°C in a water bath, 0.1 ml of phage and 0.2 ml of fresh microbial suspension were added. A 1.5% LB agar plate was then immediately covered with the mixture. Plaques were counted on each plate after each plate was incubated at 37°C overnight [20].

2.7 Purification and concentration of isolated phages:

Isolated strains of bacteriophage particles were purified and concentrated by ultracentrifugation method as follows; Firstly: phages lysates of different isolated strains phages were centrifuged at low-speed for 15 min at 4°C to remove and slow bacteria and the cell debris. Then, results liquids were centrifuged in Back-man L7-35 at a high speed After chilling for 90 minutes at 30000 rpm, the pellets were carefully re-suspended in the supernatants. A small amount of SM buffer, transferred into sterilized tubes and the process (alternative low and high-speed centrifugation) was repeated two cycles according to [21].

2.8 Characterization of the isolated phages:

2.8.1 Examination of phage morphology by Transmission Electron Microscopy:

The morphology of bacterium isolate phage lysates was examined using TEM. A 20-minute adsorption period was given for a drop of focused phage sample to adhere to a copper grid with a carbon coating of 200 mesh. The filter paper was used to remove the extra liquid. The grids had been negatively dyed with 2% acetate of uranyl (pH 4.5) for the 90s, allowed to dry, and examined under an 80kV electron microscope equipped with a JOEL-JEM 1010 at the Regional Centre for Mycology & Biotechnology of Al-Azhar University in Cairo. The size of viral particles was determined by using the Jimage program [22].

2.8.2 Studying the physical characters:

Like pH phages stability, the effect of different NaCl concentrations on isolated phages, Thermal inactivation point on isolated phages, and dilution endpoint on isolated phages (DEP).

2.8.3 One-step growth curve:

ΦEAP was grown at 37° C in N.B. To determine the mid-exponential phases, the optical density value at 600 nm (OD600) was measured (OD600=0.3). After being centrifuged for one minute at 6000 rpm to remove one millilitre of the culture, 100 L of phage lysate was added. After 15 minutes, the phage bacteria complex was separated by centrifugation and reconstituted in 10 mL N.B. to facilitate phage adsorption to the bacteria (A.baumannii). After that, samples were collected every five minutes for 60 minutes while the resuspension was incubated at 37°C. After 24 hours at 37°C, plaques were collected and represented as PFU per infected cell [23].

2.8 Statistical analysis:

The findings of each experiment were performed in triplicate and are shown as mean values. The analysis of variance in two ways was conducted by means of the statistical InfoStat (version 2019) software bundle [24]. The multiple range test of Duncan was used to distinguish the mean value of treatments when significant differences at $p \leq 0.05$ were detected [25].

3 . Results and discussion

3.1 Collection of bacterial isolates.

There were 140 bacterial isolates collected from Fayoum University Hospital and Elnabawy Elmohands general hospital, Fayoum Governorate, Egypt. At February 2021 to October 2022. The isolates were grouped as follows: seventy bacterial from urine samples with codes EU1-EU70; seventy isolates from pus samples with codes EP71-EP140.

3.2 Antibiotic sensitivity of the bacterial isolates.

Based on their antibiotic resistance only 30 isolates were chosen out of the 140 bacterial isolates. Fifteen bacterial isolates of urine samples codes EU1-EU15, and fifteen bacterial isolates of pus samples with codes EP1-EP15. The inhibition zone of antibiotics obtained from 30 bacterial isolates was given in (Table 1). The isolate EP15 showed resistance for all antibiotics except Colistin (CT) with a ratio of 94 %(16/17) (Table 2).

3.3 Phenotypic and biochemical characterization of the most resistant pathogenic bacteria:

To gain a thorough understanding of the traits of the chosen bacterial isolate as shown in Tables (3), numerous biochemical and morphological investigations were conducted. Isolates EP15, dealing with them, were gram-negative, cocobacillary: rod becomes spherical at the stationary phase of growth. In addition, these strains demonstrated positive results with enzymes: Arginine hydrolase, β-galactosidase & catalase. At the same time, these isolates showed an ability to utilize glucose, xylose, mannose, and rhamnose.

Table 1: Results in Table 1 revealed that the bacterial isolates across the different sources significantly differed regarding resistance ratios. The isolate of EP15, which was identified as *A. baumannii* exhibited the best resistance ratio of 0.90. Also, show antibiotic sensitivity test for screening the most multi-drug resistant isolate, showing the mean of inhibition zone ratios. A, b, c, d, f and g: statically analysis. E: Essam, P: pus, and U: urine.

Code number of bacterial Isolates	Source	Means of resistance ratio
EU١	Urine	0.63^{c}
EU٢	Urine	0.57^{cde}
EU٣	Urine	0.61^{cd}
EU٤	Urine	0.49^{fgh}
EU٥	Urine	0.6^{3c}
EU٦	Urine	0.57^{def}
EU٧	Urine	0.49^{fgh}
EU٨	Urine	0.43^{h}
EU٩	Urine	0.49^{fgh}
EU١٠	Urine	0.63^{c}
EU١١	Urine	0.61^{cd}
EU١٢	Urine	0.49^{fgh}
EU١٣	Urine	0.57^{def}
EU١٤	Urine	0.49^{fgh}
EU١٥	Urine	0.49^{fgh}
EP١	Pus	0.63^{c}
EP٢	Pus	0.57^{def}
EP٣	Pus	0.51^{efg}
EP٤	Pus	0.49^{fgh}
EP٥	Pus	0.80^{b}
EP٦	Pus	0.57^{cde}
EP٧	Pus	0.51^{efg}
EP٨	Pus	0.43^{h}
EP٩	Pus	0.49^{fgh}
EP١٠	Pus	0.51^{efg}
EP١١	Pus	0.84^{b}
EP١٢	Pus	0.57^{def}
EP١٣	Pus	0.49^{fgh}
EP١٤	Pus	0.61^{cd}
EP ١٥	Pus	0.90^{a}

Table 2: *A. baumannii* (EP15) Inhibition Zone (mm) by disc diffusion method (CLSI, 2018) including comprised of interpretative Categories, Zone Diameter Breakpoints, and nearest full millimetres(mm). S: susceptible, I: intermediate, R: resistant, the isolate sensitive only to Colistin (CT).

Antibiotic class	Antimicrobial agent	Interpretive Categories and Zone Diameter Breakpoints, nearest whole mm			*Acinetobacter*(mm)
		S	I	R	
Cephalosporins	Ceftazidime(CAZ)	≥18	15 - 17	≤ 14	0
	Cefepime(FEP)	≥18	13 - 16	≤ 12	0
	Cefotaxime(CTX)	≥23	15 - 22	≤ 14	0
	Cefixime(CFM)	≥18	15 - 17	≤ 14	0
	Cefuroxime(CXM)	≥23	15 - 22	≤ 14	0
	Ceftriaxone(CRO)	≥21	14 - 20	≤ 13	0
Carbapenems	Imipenem(IPM)	≥22	19 - 21	≤ 18	0
	Aztreonam(ATM)	≥23	13 - 17	≤ 12	0
Tetracycline	Doxycycline(DO,DXT)	≥13	10. - 12	≤ 9	8 mm
	Minocycline(MN)	≥16	13 -15	≤ 11	0
Fluoroquinolones	Ciprofloxacin(CIP)	≥21	16 – 20	≤ 15	0
	Levofloxacin(LVX)	≥21	16 - 20	≤ 15	0
	Ofloxacin(OFX)	≥21	16 – 20	≤ 15	0
	Gatifloxacin(GAT)	≥18	15 - 17	≤ 14	0
Aminoglycosides	Amikacin(AK)	≥17	15 - 16	≤ 14	0
Penicillins	Amoxicillin/Clavulanic(AMC)	≥18	14 - 17	≤ 13	0
LIPOPEPTIDE	Colistin(CT)	≥13	10. - 12	≤ 9	14 mm

Acinetobacter baumanni inhibition zone (mm) by disc diffusion method (CLSI,2018) including interpretive categories and zone diameter breakpoints, nearest whole mm

Table 3: Characterization of the EP15 bacterial strains' morphology and biochemistry:

Acinetobacter baumannii			
Morphological characters		Fermentation of sugars	
Gram staining	—	Glucose	+
Motility	—	Sucrose	—
Cell shape	coccobacilli	Mannitol	—
Endospore formation	—	Xylose	+
Biochemical characters		Mannose	+
“Enzyme profile”		Rhamnose	+
β-galactosidase	+	Lactate	Variable (+/-)
Arginine dihydrolase	+	Lactose	Variable (+/-)
Lysine decarbolase	Variable (+/-)	Arabinose	+
Orenthine decarbolase	—	Starch	+
Urease	—	Citrate utilization	+
Tryptophane deaminase	—	Other tests	
Gelatenase	—	H2S production	—
Catalase	+	Acetone production	+
Phosphatase	—	l-histidine assimilation	+
Lipase	—		
Oxidase	—		
Nitrate reduction:			
nitrite	—		
Indole production	—		

+: positive result, ±: doubt result, _: negative result

3.4 Confirm identification using the VITEK II system:

The best isolate in resistance to antibiotics (EP15) was identified by using the VITEK II automated system, which confirmed with a probability of 99% that it was Acinetobacter baumannii (Table 4).

Table 4: Biochemical identification of the bacterial strains EP15 using the VITEK II system

Organism Name			Acinetobacter baumannii		
Probability %			99%		
Confidence			Excellent identification		
33	fermentation/glucose	+	3	adonitol	+
10	beta-glucosidase	–	39	L-Pyrolysis-arylamidase	–
18	d-mannitol	–	25	L-arabitol	–
13	beta-xylosidase	–	16	d-cellobiose	+
8	beta-alanine arylamidase pNA	–	9	beta-galactosidase	–
36	l-proline arylamidase	–	23	H2S production	–
28	lipase	–	12	beta-N-acetyl-glucosaminidase	–
35	palatinose	–	5	glutamyl arylamidasep-nitroanilide	+
37	tyrosine arylamidase	+	17	d-glucose	+
7	Ala-Phe-Pro-arylamidase	–	22	gamma-glutamyl-transferase	–
19	d-tagatose	–	34	phosphatase	–
20	d-trehalose	–	32	ornithine decarboxylase	–
14	citrate (sodium)	+	27	lysine decarboxylase	–
29	malonate	+	24	l-histidine assimilation	+
1	5-keto-d-gluconate	–	15	courmarate	+
26	I-lactate alkalinisation	+	11	beta-glucuronidase	–
6	alpha-glucosidase	–	31	O/129 resistance	+
40	succinate alkalinisation	+	21	Glu-Gly-Arg-arylamidase	–
30	beta-N-acetylgalactosaminidase	–	2	L-MALATE assimilation	+
4	alpha-galactosidase	–	38	saccharose/sucrose	–

3.5 Isolation and morphology of phage:

Fig. (1) represents the distinct morphological phage, plaques were isolated from different collected sewage water. Isolated one plaque was repeated three times to obtain biologically purified phages. The plaques produced by the phage ΦEAP, which attacks *Acinetobacter baumannii*, were Circular, clear, without a centre, without hallo and the plaque diameter(mm) is 2.2 - 3.2mm.

4.6 Phage titer of isolated phage:

The isolation of one plaque has been repeated three times to obtain biologically purified phages. After phage propagation, the final concentration was 2.1×106 PFU/mL ΦEAP.

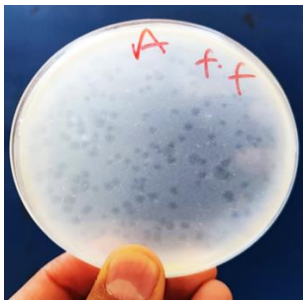

Fig.1: Lytic activity by double agar layer of phage ΦEAP against *A.baumannii*, the host bacterium of the phage ΦEAP showing phage plaque.

3.7 Examination of phage morphology by electron microscope:

Phage lysate in pure suspension was negatively stained for transmission electron microscopy (TEM) examination. Electron micrographs (Fig. 2) showed tailed phage related to three families , in which ΦEAP belonged to Myoviridae. Head with size 94.4 (nm) icosahedral shape and tail with size 302(nm) contractile shape.

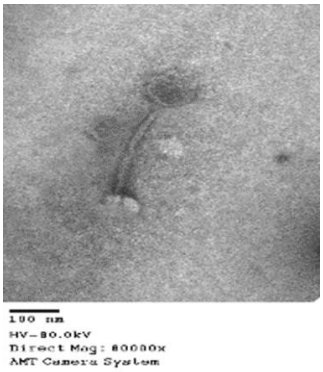

Fig.2: Transmission electron micrographs of bacteriophages, used in this study phage ΦEAP.

3.8. Stability of isolated phages under different pH values:

After an overnight incubation at 28° C, the phage optimal pH value was determined by phage titer to be pH 7; ΦEAP was demonstrated that the phage could lyse its bacterial target inside of a pH range (4 - 11). (Table 5).

Table 5: Effect of different pH concentrations on the phage

pH	4	5	6	7	8	9	10	11	12
ΦEAP	+	+	+	+	+	+	+	+	-

+ = Lysis & — = No lysis

4.9. Stability under different NaCl concentrations:

Four different concentrations of NaCl solution (5%, 10%, 15% and 20%) were exposed to isolated phage ΦEAP. The results were stable up to In addition, the lysis effect appeared on all plates (Table 6).

Table 6: Effect of different NaCl concentrations on isolated phage

NaCl %	5%	10%	15%	20%
ΦEAP	+	+	+	+

+ = Lysis & — = No lysis

3.10. Stability of isolated phage under different Temperature:

The effect of temperature degrees on the phage activity showed that phages ΦEAP was active over-temperature until 50oC i.e. the thermal inactivation point (TIP) was 55^{o}C for the phages ΦEAP (Table 7).

Table 7: The effect of different temperature degrees on phage activity showed the thermal inactivation point (TIP) for each phage. It was 50 o C for ΦEAP.

Temperature	20oC	30oC	35oC	37oC	38oC	39oC	40oC	45oC	50oC	60oC	70oC
ΦEAP	+	+	+	+	+	+	+	+	+	-	-

+ = Lysis & — = No lysis

3.11. Stability of isolated phage under different UV light:

Phage was stable to UV light up to 40 cm (lamp distance) for various times (5, 10, 15, and 20 minutes). The results were positive for all periods of exposure (Table 8).

Table 8: UV stability detection of isolate phage

UV exposure	5 min	10 min	15min	20min
ΦEAP	+	+	+	+

+ = Lysis & — = No lysis

3.12 Dilution endpoint (DEP):

Spot test was done within dilution from (10-1 to 10-10) to distinguish DEP. It was found that it was 10-7 for ΦEAP phage (Table 9).

Table 9: Dilution endpoint of isolated phages

DEP	10-1	10-2	10-3	10-4	10-5	10-6	10-7	10-8	10-9	10-10
ΦEAP	+	+	+	+	+	+	+	-	-	-

3.13. One-step growth curve:

The phage (ΦEAP) one-step growth curve showed that the respective latent times during infection were 10 minutes; with average burst sizes of 53.5 virions for each infected bacterial cell. (Fig. 3). The complete infection cycles of isolated phage was 35 min.

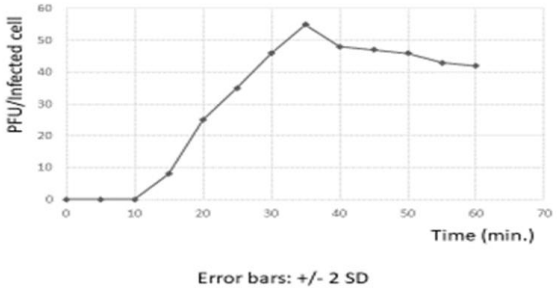

Fig.3: the complete infection cycle of ΦEAP Error bars represents the three replicates' average mean's standard deviation (SD).

4. Discussion

Antibiotics have an important role in preventing bacterial infections during surgery. In the age of antibiotics, it is simple to presume that these medications will always be available in the medicine supply. Sadly, the swift development of antibiotic-resistant bacteria is linked to the improper or excessive usage of antibiotics [26, 27, 28, 29]. Bacteria not only acquired resistant genes to overcome antibiotics but also transfer these resistant genes to neighbouring, Transformation, transduction, and conjugation are frequent methods of gene transfer [30].According to our findings, an organism's inherent capacity to lessen an antibiotic's efficiency through fundamental structural or functional characteristics is referred to as intrinsic antibiotic resistance. High levels of intrinsic resistance to the majority of antibiotics have been observed in *A. baumannii* due to constrained outer membrane permeability, efflux structures that pump antibiotics out of the cell, and the generation of antibiotic-inactivating enzymes like lactamases [31].

According to Mutina and Arias [32], bacteria can develop antibiotic resistance by mutational changes or by gaining genes for resistance through horizontal gene transfer, or transformation. Along with the high intrinsic antibiotic resistance of isolated bacteria in our work (A. baumannii). The multidrug-resistant strains that result from the acquired resistance make the eradication of this bacterium more challenging and increase the incidence of chronic infection [33]. The increased production of efflux pumps & antimicrobial-inactivating enzymes, as well as decreased antibiotic uptake due to mutational alterations, all help bacteria thrive in the presence of antimicrobial compounds [32].

Phage is capable of being genetically modified to transport antimicrobial drugs to bacteria, enhancing Although the treatment with phages has been used for many years in Eastern Europe, it is not widely accepted in the West. In addition to phage therapy's many benefits, other benefits include replication at the site of infection, a high degree of specificity for battling bacteria without influencing commensal flora (microbiome), less side effects compared to other treatments, bactericidal activity towards antibiotic-resistant bacteria, and ease of administration [34].

Previous studies have been showing that researchers had isolated phages from sewages aim to control bacterial infections [35,36]. Our study describes the characterization of a bacteriophage from sewage that showed a lytic ability against multidrug-resistant bacteria i.e. Acinetobacter baumannii. Several sewage samples from agricultural, active sludge, industrial, healthy, and seawater were cheked for the appearance of novel bacteriophages against tested bacteria. The isolated bacteriophage was chosen for further characterization based on its ability to produce clear zones of lytic activity in the plaque assay with tested bacteria. A lytic (virulent) phage penetrates a bacterial cell and multiplies there by using the cell's own resources. The cell lyses and numerous copies that contain the original phage are released once these processes have been used up [37, 38] . Temperate bacteriophages have the ability to integrate their genome into the host genome, which can lead to the parallel transfer of genes such as those involved in toxin output, virulence, and resistance to antibiotics [39]. These phages do not continuously lyse their host cells. Phage treatment cannot be performed on them. In bacteriophage therapy, lytic bacteriophages that kill bacteria are the recommended targets [40].

Acinetobacter baumannii is a well-known multidrug-resistant bacterium that causes numerous incapacitating diseases and infections worldwide [41]. According to Murray et al. [42], *Acinetobacter baumannii* is one of the most prevalent six bacterial infections that cause the majority of antibiotic resistance-related fatalities worldwide. Antibiotic resistance is regarded by the World Health Organisation (WHO) as one of the top ten global health threats in 2019. In the era of coronavirus disease (COVID-19), A. baumannii could lead to secondary bacterial infections, leading to an increased mortality rate in at-risk patients according to the US CDC (United States Centers for Disease Control and Prevention) [43]. There are no effective reports in Egypt, as hospitals do not give numbers of patients with covid-19 or the mortality rate. Lytic phages (phages), which exclusively infect and kill bacteria, have been reexamined recently for use in curing multidrug-resistant infections that are otherwise incurable and have showed potential as therapeutic substitutes or supplements to antibiotic treatments [44, 45, 46,47]. *A. baumannii* of this study resist 16 antibiotics from 17 that have been used so all *A. baumannii* species together pose a major threat to the well-being and lives of people everywhere.

To find a solution to this dilemma, we have carried out research focused at isolating and characterising phages particular to these risky drug-resistant strains. The majority of the bacterial strains included in the study were isolated from patients who had wounds that were resistant to some of the most popular antibiotics at the time. The Acinetobacter calcoaceticus-baumannii (Acb) complex includes A. baumannii strains. This complex includes the species *A. baumannii, A. Pitti, A. nosocomial, A. Seifert, A. dijkshoorniae*, and *A. calcoaceticus*, five of which are pathogenic and one of which is not. These species exhibit similarities in their genotypes and phenotypes [48]. The characterization of *A. baumannii* bacteriophage is increasing due to the increasing popularity of this treatment method [10]. According to Tipton et al. [49], the encapsulating bacterium A. baumannii can transition from an aggressive transparent (AV-T) to an aggressive opaque (VIR-O) phase. Galleria mellonella and mice pulmonary infection models show that the VIR-O phase possesses a thicker capsular coating and is more virulent. [49, 50]. Additionally, mutants deficient in capsules can frequently be recovered from overnight cultures of stationary *A. baumannii*. Due to variation in capsule thickness that might affect phage infection, phase variations and mutant capsules can complicate phage infection research. Some phages can be avoided thanks to the thick VIR-O capsule [51]. According to Timoshina et al. [52], the loss of a branching sugar K3-type encapsulating carbohydrate caused by frameshifts in the gtr6glycosyltransferase gene makes an organism susceptible to phage. Our study concurrs with [52, 51, 53]. *A. baumannii* of our study is stable across the temperatures tested in this study, with varying temperature effects observed at degrees 60 and 70, the exhibited maximum stability at 20o C, 37o, this agrees with Enwuru et al [53]. The evaluation of phage stability in environmental stress showed that they are often more stable at alkaline pH than acidic pH and comparatively stable at high temperatures. Every phage has an optimal pH for survival and biological activity. Thus, all the phages showed optimal viability at pH 6. When the pH was increased to 7.0, the phage titer dropped as in acidic pH. From the pH and high-temperature stability, UV, and salinity data, bacteriophage infectivity remained intact at temperature and pH within and outside of the physiological range of the human body. This ought to be a beneficial quality for phages meant for medicinal uses.

5.Conclusion

A greater number of clinical trials on phages are required, nevertheless. The creation of computer algorithms, genomic, and bioinformatics analyses to find viable phage candidates. The creation and manufacture of formulas for standardized and medical use in bacterial control, as well as the avoidance or reduction of danger of occurrence the issue of bacterial genetic material transfer and resistance has not yet been fully resolved, and it is unknown how bacteriophages and bacteria coevolve. The effectiveness of phage treatments for infections that are considerably more challenging to culture in the lab or for which we lack animal models of acute and chronic bacterial illnesses can be predicted using trustworthy models like non-pathogenic E. coli. More studies specifically addressing these issues are needed before bacteriophages can be used in humans.

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Author Contributions

All authors contributed to this work. Essam Saad Mahmoud prepared the samples, completed the experimental measurements, statistics, write and submission the manuiscript. Reda M. Taha, Rasha H. Bassyouni, Fatma A. Ahmed and Farag A.Samhan contribute in the revision of the manuscript for publication.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

References

[1] Majumder, Maimuna S., and Kenneth D. Mandl. "Early in the epidemic: impact of preprints on global discourse about COVID-19 transmissibility." The Lancet Global Health 8, no. 5 (2020): e627-e630.

[2] Jones, Joshua D., Clare Trippett, Mehrunisha Suleman, Martha RJ Clokie, and Jason R. Clark. "The Future of Clinical Phage Therapy in the United Kingdom." Viruses 15, no. 3 (2023): 721.

[3] Murray, Christopher JL, Kevin Shunji Ikuta, Fablina Sharara, Lucien Swetschinski, Gisela Robles Aguilar, Authia Gray, Chieh Han et al. "Global burden of bacterial antimicrobial resistance in 2019: a systematic analysis." The Lancet 399, no. 10325 (2022): 629-655.

[4] COVID, CDC. "19: US Impact on Antimicrobial Resistance, Special Report 2022." Atlanta, GA: US Department of Health and Human Services, CDC (2022).

[5] World Health Organization. "Global antimicrobial resistance and use surveillance system (GLASS) report: 2021." (2021).

[6] Lee, Chang-Ro, Jung Hun Lee, Moonhee Park, Kwang Seung Park, Il Kwon Bae, Young Bae Kim, Chang-Jun Cha, Byeong Chul Jeong, and Sang Hee Lee. "Biology of Acinetobacter baumannii: pathogenesis, antibiotic resistance mechanisms, and prospective treatment options." Frontiers in cellular and infection microbiology 7 (2017): 55.

[7] Ayoub Moubareck, Carole, and Dalal Hammoudi Halat. "Insights into Acinetobacter baumannii: a review of microbiological, virulence, and resistance traits in a threatening nosocomial pathogen." Antibiotics 9, no. 3 (2020): 119.

[8] Han, Ke, Yinuo Zhu, Fei Li, Mengzhe Li, Xiaoping An, Lihua Song, Huahao Fan, and Yigang Tong. "Genomic analysis of Acinetobacter phage BUCT629, a newly isolated member of the genus Obolenskvirus." Archives of Virology 167, no. 4 (2022): 1197-1199.

[9] Kakasis, Athanasios, and Gerasimia Panitsa. "Bacteriophage therapy as an alternative treatment for human infections. A comprehensive review." International journal of antimicrobial agents 53, no. 1 (2019): 16-21.

[10] Peters, Danielle L., Carly M. Davis, Greg Harris, Hongyan Zhou, Philip N. Rather, Sabahudin Hrapovic, Edmond Lam, Jonathan J. Dennis, and Wangxue Chen. "Characterization of Virulent T4-Like Acinetobacter baumannii Bacteriophages DLP1 and DLP2." Viruses 15, no. 3 (2023): 739.

[11] Bull, James J., and Jason J. Gill. "The habits of highly effective phages: population dynamics as a framework for identifying therapeutic phages." Frontiers in microbiology 5 (2014): 618.

[12] Torres-Barceló, Clara, and Michael E. Hochberg. "Evolutionary rationale for phages as complements of antibiotics." Trends in microbiology 24, no. 4 (2016): 249-256.

[13] Leung, Chung Yin Joey, and Joshua S. Weitz. "Modeling the synergistic elimination of bacteria by phage and the innate immune system." Journal of theoretical biology 429 (2017): 241-252.

[14] Roach, Dwayne R., Chung Yin Leung, Marine Henry, Eric Morello, Devika Singh, James P. Di Santo, Joshua S. Weitz, and Laurent Debarbieux. "Synergy between the host immune system and bacteriophage is essential for successful phage therapy against an acute respiratory pathogen." Cell host & microbe 22, no. 1 (2017): 38-47.

[15] Bauer, A. W., W. M. M. Kirby, John C. Sherris, and Marvin Turck. "Antibiotic susceptibility testing by a standardized single disk method." American journal of clinical pathology 45, no. 4_ts (1966): 493-496..

[16] Oteo, Jesús, Edurne Lázaro, Francisco J. de Abajo, Fernando Baquero, José Campos, and Spanish members of EARSS. "Antimicrobial-resistant invasive Escherichia coli, Spain." Emerging Infectious Diseases 11, no. 4 (2005): 546.

[17] Holt, J. G., N. R. Krieg, P. H. A. Sneath, J. F. Stanley, and S. T. Williams. "Bergey's Manual of Determinative." Philadelphia: A Wolters Kluwer Company (1994)..

[18] Funke, Guido, Dominique Monnet, Chiara deBernardis, Alexander von Graevenitz, and Jean Freney. "Evaluation of the VITEK 2 system for rapid identification of medically relevant gram-negative rods." Journal of clinical microbiology 36, no. 7 (1998): 1948-1952.

[19] Cerveny, Karen E., Angelo DePaola, Donna H. Duckworth, and Paul A. Gulig. "Phage therapy of local and systemic disease caused by Vibrio vulnificus in iron-dextran-treated mice." Infection and immunity 70, no. 11 (2002): 6251-6262.

[20] Adams, Me He. "Bacteriophages. Interscience Publ." Inc., New York (1959): 110.

[21] Figurski, David H., and J. R. Christensen. "Functional characterization of the genes of bacteriophage T1." Virology 59, no. 2 (1974): 397-402.

[22] Ackermann, Hans-W. "Bacteriophage electron microscopy." Advances in virus research 82 (2012): 1-32.

[23] Lee, Young-Duck, and Jong-Hyun Park. "Characterization and application of phages isolated from sewage for reduction of Escherichia coli O157: H7 in biofilm." LWT-Food Science and Technology 60, no. 1 (2015): 571-577.

[24] Di Rienzo, J. A. "InfoStat versión 2009. Grupo InfoStat, FCA, Universidad Nacional de Córdoba, Argentina." http://www. infostat. com. ar (2009).

[25] Snedecor, George W., and Witiiam G. Cochran. "Statistical methods, 8thEdn." Ames: Iowa State Univ. Press Iowa 54 (1989): 71-82.

[26] Fogel, Nicole. "Tuberculosis: a disease without boundaries." Tuberculosis 95, no. 5 (2015): 527-531.

[27] Li, Bingyun, and Thomas J. Webster. "Bacteria antibiotic resistance: New challenges and opportunities for implant-associated orthopedic infections." Journal of Orthopaedic Research® 36, no. 1 (2018): 22-32.

[28] Zhou, Jiarong, Ashley V. Kroll, Maya Holay, Ronnie H. Fang, and Liangfang Zhang. "Biomimetic nanotechnology toward personalized vaccines." Advanced materials 32, no. 13 (2020): 1901255.

[29] Mostafavi, Ebrahim, Ankit Kumar Dubey, Bogdan Walkowiak, Ajeet Kaushik, Seeram Ramakrishna, and Laura Teodori. "Antimicrobial surfaces for implantable cardiovascular devices." Current Opinion in Biomedical Engineering (2022): 100406.

[30] Arber, Werner. "Horizontal gene transfer among bacteria and its role in biological evolution." Life 4, no. 2 (2014): 217-224..

[31] Breidenstein, Elena BM, César de la Fuente-Núñez, and Robert EW Hancock. "Pseudomonas aeruginosa: all roads lead to resistance." Trends in microbiology 19, no. 8 (2011): 419-426.

[32] Munita, Jose M., and Cesar A. Arias. "Mechanisms of antibiotic resistance." Virulence mechanisms of bacterial pathogens (2016): 481-511.

[33] Henrichfreise, Beate, Irith Wiegand, Wolfgang Pfister, and Bärbel Wiedemann. "Resistance mechanisms of multiresistant Pseudomonas aeruginosa strains from Germany and correlation with hypermutation." Antimicrobial agents and chemotherapy 51, no. 11 (2007): 4062-4070..
[34] Ly-Chatain, Mai Huong. "The factors affecting effectiveness of treatment in phages therapy." Frontiers in microbiology 5 (2014): 51.
[35] Wills, Quintin F., Claire Kerrigan, and James S. Soothill. "Experimental bacteriophage protection against Staphylococcus aureus abscesses in a rabbit model." Antimicrobial agents and chemotherapy 49, no. 3 (2005): 1220-1221.
[36] Gill, J. J., J. C. Pacan, M. E. Carson, K. E. Leslie, M. W. Griffiths, and P. M. Sabour. "Efficacy and pharmacokinetics of bacteriophage therapy in treatment of subclinical Staphylococcus aureus mastitis in lactating dairy cattle." Antimicrobial agents and chemotherapy 50, no. 9 (2006): 2912-2918. py, 50(9), 2912-2918.
[37] Doyle, Michael P., and Marilyn C. Erickson. "Reducing the carriage of foodborne pathogens in livestock and poultry." Poultry science 85, no. 6 (2006): 960-973.3.
[38] Hanlon, Geoffrey William. "Bacteriophages: an appraisal of their role in the treatment of bacterial infections." International journal of antimicrobial agents 30, no. 2 (2007): 118-128.128.
[39] Brabban, A. D., E. Hite, and T. R. Callaway. "Evolution of foodborne pathogens via temperate bacteriophage-mediated gene transfer." Foodbourne Pathogens & Disease 2, no. 4 (2005): 287-303.
[40] Hendrix, Roger W. "Bacteriophage genomics." Current opinion in microbiology 6, no. 5 (2003): 506-511..
[41] Xie, Ruiqiang, Xiaohua Douglas Zhang, Qi Zhao, Bo Peng, and Jun Zheng. "Analysis of global prevalence of antibiotic resistance in Acinetobacter baumannii infections disclosed a faster increase in OECD countries." Emerging microbes & infections 7, no. 1 (2018): 1-10.
[42] Murray, Christopher JL, Kevin Shunji Ikuta, Fablina Sharara, Lucien Swetschinski, Gisela Robles Aguilar, Authia Gray, Chieh Han et al. "Global burden of bacterial antimicrobial resistance in 2019: a systematic analysis." The Lancet 399, no. 10325 (2022): 629-655.
[43] Rangel, Karyne, Thiago Pavoni Gomes Chagas, and Salvatore Giovanni De-Simone. "Acinetobacter baumannii infections in times of COVID-19 pandemic." Pathogens 10, no. 8 (2021): 1006.
[44] Schooley, Robert T., Biswajit Biswas, Jason J. Gill, Adriana Hernandez-Morales, Jacob Lancaster, Lauren Lessor, Jeremy J. Barr et al. "Development and use of personalized bacteriophage-based therapeutic cocktails to treat a patient with a disseminated resistant Acinetobacter baumannii infection." Antimicrobial agents and chemotherapy 61, no. 10 (2017): 10-1128.
[45] Wu, Nannan, Jia Dai, Mingquan Guo, Jianhui Li, Xin Zhou, Feng Li, Yuan Gao et al. "Pre-optimized phage therapy on secondary Acinetobacter baumannii infection in four critical COVID-19 patients." Emerging microbes & infections 10, no. 1 (2021): 612-618.
[46] Blasco, Lucía, Inés Bleriot, Manuel González de Aledo, Laura Fernández-García, Olga Pacios, Hugo Oliveira, M. López et al. "Development of an anti-Acinetobacter baumannii biofilm phage cocktail: genomic adaptation to the host." Antimicrobial Agents and Chemotherapy 66, no. 3 (2022): e01923-21.1.
[47] Rao, Sohail, Monica Betancourt-Garcia, Yetunde O. Kare-Opaneye, Brett E. Swierczewski, Jason W. Bennett, Bri'Anna Horne, Joseph Fackler, Lia Patricia Suazo Hernandez, and Michael J. Brownstein. "Critically ill patient with multidrug-resistant Acinetobacter baumannii respiratory infection successfully treated with intravenous and nebulized bacteriophage therapy." Antimicrobial Agents and Chemotherapy 66, no. 1 (2022): e00824-21.
[48] Nemec, Alexandr, Lenka Krizova, Martina Maixnerova, Tanny JK van der Reijden, Pieter Deschaght, Virginie Passet, Mario Vaneechoutte, Sylvain Brisse, and Lenie Dijkshoorn. "Genotypic and phenotypic characterization of the Acinetobacter calcoaceticus–Acinetobacter baumannii complex with the proposal of Acinetobacter pittii sp. nov.(formerly Acinetobacter genomic species 3) and Acinetobacter nosocomialis sp. nov.(formerly Acinetobacter genomic species 13TU)." Research in microbiology 162, no. 4 (2011): 393-404.
[49] Tipton, Kyle A., Daniela Dimitrova, and Philip N. Rather. "Phase-variable control of multiple phenotypes in Acinetobacter baumannii strain AB5075." Journal of bacteriology 197, no. 15 (2015): 2593-2599.
[50] Chin, Chui Yoke, Kyle A. Tipton, Marjan Farokhyfar, Eileen M. Burd, David S. Weiss, and Philip N. Rather. "A high-frequency phenotypic switch links bacterial virulence and environmental survival in Acinetobacter baumannii." Nature microbiology 3, no. 5 (2018): 563-569.
[51] Gordillo Altamirano, Fernando, John H. Forsyth, Ruzeen Patwa, Xenia Kostoulias, Michael Trim, Dinesh Subedi, Stuart K. Archer et al. "Bacteriophage-resistant Acinetobacter baumannii are resensitized to antimicrobials." Nature microbiology 6, no. 2 (2021): 157-161.2.
[52] Timoshina, Olga Y., Anastasiya A. Kasimova, Mikhail M. Shneider, Nikolay P. Arbatsky, Alexander S. Shashkov, Andrey A. Shelenkov, Yuliya V. Mikhailova et al. "Loss of a Branch Sugar in the Acinetobacter baumannii K3-Type Capsular Polysaccharide Due To Frameshifts in the gtr6 Glycosyltransferase Gene Leads To Susceptibility To Phage APK37. 1." Microbiology Spectrum 11, no. 1 (2023): e03631-22.
[53] Enwuru, Nkechi V., Jachike T. Nzekwe, Bisola O. Ojo, Abdul-Rahman Usman, and Folake R. Peters. "Isolation and Characterization of Novel Cocktail Phages of Multidrug-Resistant (MDR) Acinetobacter Baumannii: https://doi. org/10.51412/psnnjp. 2023.5." The Nigerian Journal of Pharmacy 57, no. 1 (2023): 435-446. (1), 435-446.