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ISOLATION AND CHARACTERIZATION OF A NOVEL BACTERIOPHAGE TO CONTROL MULTIDRUG-RESISTANT *ACINETOBACTER BAUMANNII*

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Abstract
In order to tackle the urgent problem of multidrug-resistant (MDR) *Acinetobacter baumannii* infections, this work describes Phage-1, a lytic bacteriophage that has strong anti-bacterial properties. Patients in the intensive care unit with suspicision of septicemia had bacterial samples taken, and antibiotic susceptibility testing was carried out in accordance with CLSI/USA guidelines. Automated VITEK 2 system was used to assess the antibiotic susceptibility of 60 isolates of *A. baumannii*. Phage-1 was isolated from sewage system of a tertiary care hospital in Faisalabad using conventional screening techniques. Centrifugation, binary layer agar identification, and many rounds of isolation were used in the phage extraction and purification procedure. The Phenol Chloroform technique was used to extract DNA from Phage-1. Using a one-step growth curve analysis, the life cycle of Phage-1 was deciphered. This revealed a latent time of 30 minutes, a lysis period of 60 minutes, and a burst size of 8.6 PFU/mL. Phage-1 was shown to be resilient in thermal and pH stability testing, exhibiting substantial activity at 20-40°C and 6-8 pH as well as vulnerability to higher temperatures and severe pH values. Sixty distinct strains of MDR *A. baumannii* were subjected to host range studies, which demonstrated Phage-1's capacity to lyse local strains within a given geographic area. Minimum Inhibotory Concentration (MIC) was assessed using VITEK 2 system, which showed that the mostly isolates were resistant to cephalosprins, carbapenems and aminoglycosides. All strains are sensitive to Polymyxin B. This study adds important new information on how Phage-1 may be used as a therapeutic agent to treat MDR *A. baumannii* infections. The results show its lytic efficiency, stability, and geographic uniqueness, emphasizing the need of taking regional factors into account when developing phage-based therapies to fight bacterial illnesses resistant to antibiotics.

Keywords: Clinical, bacteriophage, host range, resistance, applications

Introduction

An increasing threat, *Acinetobacter baumannii* causes hospital acquired infections including sepsis, lower respiratory tract infections and urinary territory infections as well as wound infections [1]. These hospital acquired infections, specifically by MDR bacteria are challanging and requires innovative treatments. Faliure of treatment by broad spectrum antiobiotics, urges the scientist for some alternative treatment options. Bacteriophages have been used therapeutically since the 1910s, and interest has grown in recent decades. Due to the risk of MDR *A. baumannii* strains, bacteriophage therapy is being considered as a good alternative choice instead of antibiotics [2].

Since most of the *A. baumannii* strains are resistant to β-lactam drugs like cephalosporin, carbapenem and monobactum phage therapy is an effective option. Bacteriophages are becoming popular again to tackle antibiotic-resistant bacteria [3]. Bacteriophage therapy is difficult to apply clinically despite its promise. Medical integration requires addressing health, host-bacteriophage interactions, and host range. In response to the MDR *A. baumannii* epidemic, a countrywide study screened and stored bacteriophages. The findings will aid Phage-1 research and antibiotic-resistant *A. baumannii* treatment. This finding improves bacteriophage treatment of multidrug-resistant bacteria [4].

Intensive care unit of many tertiary care hospitals are facing the threat of hospital acquired infections by highly resistant bacteria including *A. baumannii.* The lytic bacteriophages against these resistant strains would address this situation in a better way. The isolation of required lytic Phage from the sewage of these hospitals expands the therapeutic bacteriophage candidate pool [5]. To define the appropriate use of lytic bacteriophage, its morphology, its stability at different PH and temperature are explored. The page covers Phage-1's non-antimicrobial applications. Phages contain antibacterial potential due to different enzymes or proteins [6]. Phage-derived antimicrobials may augment the arsenal. Phage characterisation is essential for MDR *A. baumannii* therapy. Differnt research findings show that bacteriophage therapy may provide a good alternative approach to tackle the multidrug-resistant bacteria [7].

The current research work was designed to isolate Phage-1, a lytic bacteriophage, from clinical MDR *A. baumannii* isolates and to investigate its environmental conditions including optimum temperature, pH and host range to shed light on its properties and its future applications in therapeutic industry.

Materials and methods

Bacterial sample collection and processing:

Clinical samples had been taken from hospitalized patients in Intensive Care Unit (ICU) of a tertiry care hospital. Blood culture of the patients suspected of septicemia had taken. Bact/Alert 3D FN and FA bottles have been used for blood culture. Positive blood culture were sub-cultured on Blood, Chocolate, and MacConkey agars and incubated for 24-48 hours at 35-37 C. For phenotypic identification, Gram staining of bacteral colonies were performed. VITEK2 automated system was used of biochemical identification and Antimicrobial sensitivity testing (AST) of these bacterial strains.

A. baumannii isolates were tested for antibiotic susceptibility (AST) according to the latest CLSI-2022 guidelines. [13].

VITEK2-AST card including the panel of following drugs; Piperacillin, Piperacillin/ tazobactum, cefaperazone/sulbactam, sulfa-methazole, ceftazidime, cefotaxime, imipenem, meropenem, gentamicin, amikacin, ciprofloxacin, and tetracycline. Since this vitek AST card does not include the polymyxin B and E so Kirby baurae disk diffusion method have been used to check the susceptibility of antibiotic.

Molecular confirmation of *A. baumannii* isolates

Using 16S rRNA universal primers, the isolated and pure isolates were further confirmed through PCR. To perform this characterization, the steps described below were followed.

Extraction of DNA

Bacterial DNA was isolated by following steps; the overnight grown bacterial suspension (1.5 mL) was centrifuged at 8000 rpm for five minutes. The pellet was washed by normal saline and centrifuged again at the same previous speed. The pellet was dissolved in 0.5 mL of normal saline followed by addition of 0.5 mL of 1% SDS solution. Then 5 µL of proteinase K was added and the suspension was incubated at 56ºC for 18 hrs. Then 1 mL of phenol chloroform isoamyl alcohol was added and agitated with 1 mL pipette followed by centrifugation at 8000 rpm for 5 minutes. This step was repeated again. Again, this step was repeated using chloroform. Using supernatant, 0.8 volumes of ice chilled isopropanol and 0.2 volumes of sodium acetate was added and incubated at -5ºC for 10 minutes. Then this suspension was centrifuged at 13000 rpm for 15 minutes. After discarding supernatant, the pellet was washed using 70% ethanol. After drying it, it was dissolved in 20 µL of DNAase free molecular biology grade water and saved for next use.

Phage extraction and prepration

Traditional screening was employed to isolate bacteriophages [14]. The Faisalabad Aziz Fatima Hospital sewage management facility was approached for a 500-1000 cc sewage water sample. Bacteriophages were recovered from MDR *A. baumannii* cultured in partially sterile sewage using a 0.22-µm membrane. After reaching room temperature, the substantial was centrifugated at 13,000 × g for 15 minutes. Binary layer agar was employed to recognize bacteriophages [14]. Mix 10 microliters of super-natant with 100 microliters of MDR *A. baumannii* straining and let soak for 10 minutes. They were then assorted with 3 ml liquefied 0.7 percent lax agar. The mix was incubated overnight on LB agar at 37 °C. To suspend pure plaques, 500 µl of fluid LB media was utilized aseptically. This was done three times to isolate the phage. A on its own clone lytic phage plaque was re-suspended in 6ml of SM solution at 180 rpm from the presumably heterogeneous mixture. A 0.22-µm filter was used to transfer the bacteriophage-containing SM supernatant to 4 °C storage. Following the approach described before [15], the lysis straining were excluded from subsequent bacteriophage isolation rounds to prevent overlap.

Suspended phage plaques nurtured instantaneous at 37 °C with 160 rpm shaky generated many Phage-1 particles. The illustration was placed in an ice immersion for one hour after adding 5.84 g/100 ml NaCl and 10% (w/v) Solid PEG 8000 (Oxoid, UK). The pellet was retrieved after 30 minutes of 10,000 Å g centrifugation at 4 °C. The precipitate was suspended by adding 2 ml per 100 ml of the inventive bacterial resolution to the SM solution. After centrifugation at 5000 × g for 10 minutes, add an equal amount of trichloromethane to the SM resolution and shake intended for 30 seconds. The top watery layer was collected. The extraction was repeated with the same chloroform.

Thermal and pH stability

Various temperature and pH settings were used to examine the stability (titer) of isolated lytic Phage-1, in line with our previously disclosed technique [9]. In a nutshell, the phage that had been purified was left to incubate for 48 hours at different incubation (-80, 4, 20, 60, and 80, -20 °C) and for 1 hour at varied pH points (varying from 1 to 14). After that, every hour, we used the double-layer agar technique to detect the titer of the cultured phages. The pH stability test and the heat stability test were each performed three times.

One-step growth assays

Lytic Phage-1 was used to infect MDR *A. baeumannii* in one-step growth assays; the MOI was 0.1, and the adsorption time was 10 minutes. To get rid of any phages that weren't absorbed from the supernatants, the samples were spun at 13,000 × g for half a minute. This was done twice, each time rinsing the mixture with LB medium. The *A. baumannii* pellets that had Phage-1 adsorbed were then mixed with 4 millilitres of LB fluid media. After that, the broth were incubated at 37 °C with 160 rpm shaky, and samples were taken every 5 or 10 minutes for as long as 100 minutes. The dual layer agar method, which was previously reported, was usage to rapidly determine the quantity of Phage-1 particles. This evaluation was performed on three separate occasions.

Effectiveness of plating and the assessment of the host range
Following earlier instructions, we used a spotting approach to assess Phage-1's host range [16]. For host range testing, a total of sixty different strains of multidrug-resistant Acinetobacter baumannii were considered (Table 1). The overnight incubation of all MDR *A. baumannii* strains with Phage-1 at 37 °C facilitated cell lysis. The transparency and clarity of plaques were used to evaluate their lytic activity. There were three iterations of the host range determination.
Using EOP as a metric, a more thorough evaluation of productive infection was conducted. We repeated the host range determination test three times with each strain using one of four Phage-1 dilutions that had previously been shown to cause lysis. The identical conditions used for the spot tests were also used for this. Diluting the bacteriophage lysating soln from 10^6 to 10^9 times was the process. After leaving the plates to incubate instantaneous at 30 °C, we counted the commemoration forming units (PFU) for each variant. After confirming that the EOP was less than 0.001 with the 10^6 dilution, a lower dilution was tried if plaques were not seen. As a last step, the EOP and standard deviation for all three measures were computed by dividing the usual PFU on aimed bacteria by the typical PFU on host microbes.

Results
Antibiotic Susceptibility of *A. baumannii*
Antibiotic susceptibility pattern of 60 positive isolates of *A. baumannii* was evaluated using VITEK 2. Following CLSI-2022 guidelines, Minimum Inhibitory concentration MIC of following antibiotics have been detected. Figure 1 depicts the antibiotic susceptibility of *A. baumannii* against different commonly used antibiotics. As far as, the results depicts, 69% of the isolates were sensitive to colistin, 31% showed sensitivity to meropenem, 37% were sensitive to piperacillin/tazobactum. Cefotaxime and ceftazidime also showed the poor susceptibility pattern (18% resistance) . Ciprofloxacin and levofloxacin had the lower sensitivity rate at 10%. Gentamicin and Sulfamethoxazole (8% resistance) showed the lowest resistance rates.

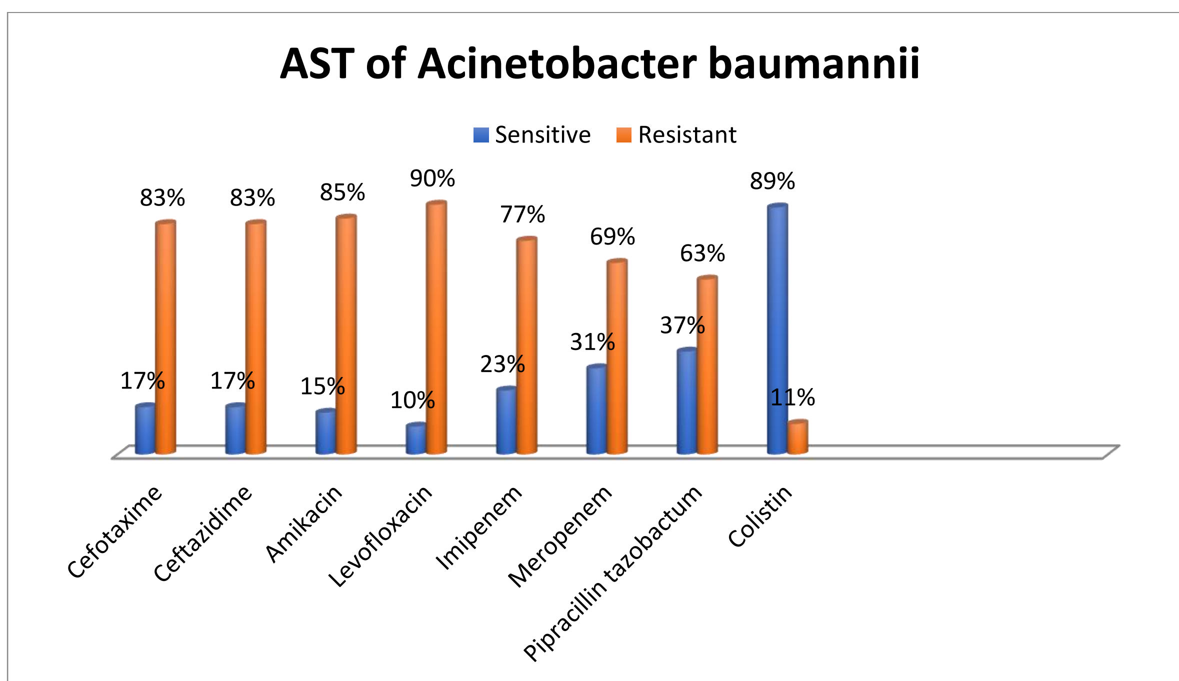

Figure 1. Antibiotic susceptibility pattern of *A. baumannii*-positive isolates

Isolation of Bacteriophages
The agar overlay approach was used to successfully isolate one phage, which was then selected for further study. There are $5x10^{12}$ Phage-1 titers. Figure 2 shows that when tested *A. baumannii* isolates were cultured on petri plates, and large to medium size plaques (5 mm) were found.

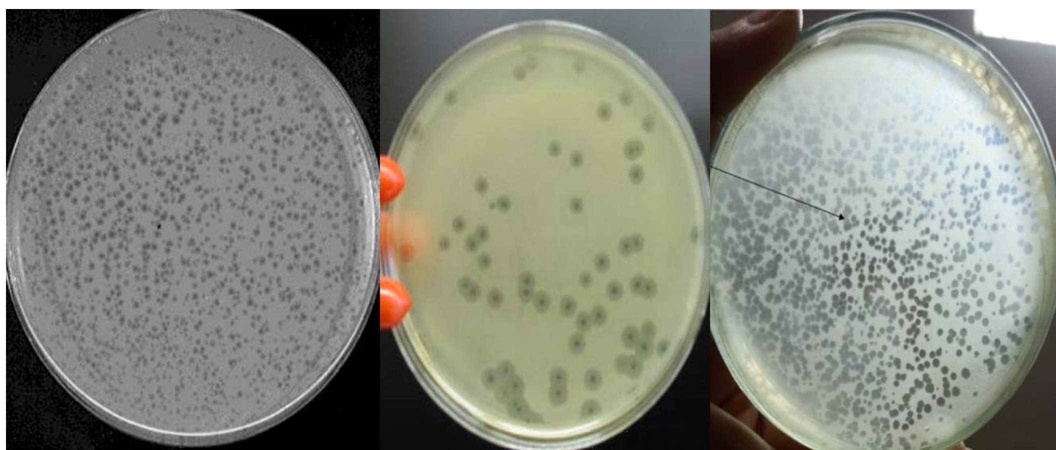

Figure 2. Bacteriophage isolation; arrow line exhibits the formation of plaques formed by Phages.

The life cycle of Phage-1

A single step growing experiment was shown to explore Phage-1 life cycle. Following different time period of 90 min, the magnitude of the spurt was 8.6 PFU/mL (Figure 3). Figure 3 also depicts that at 0-30 min phage particles are in latent phase and at 30-60 min phage particles are in lysis phase and afterwards (60-90 min) in host cell burst phase.

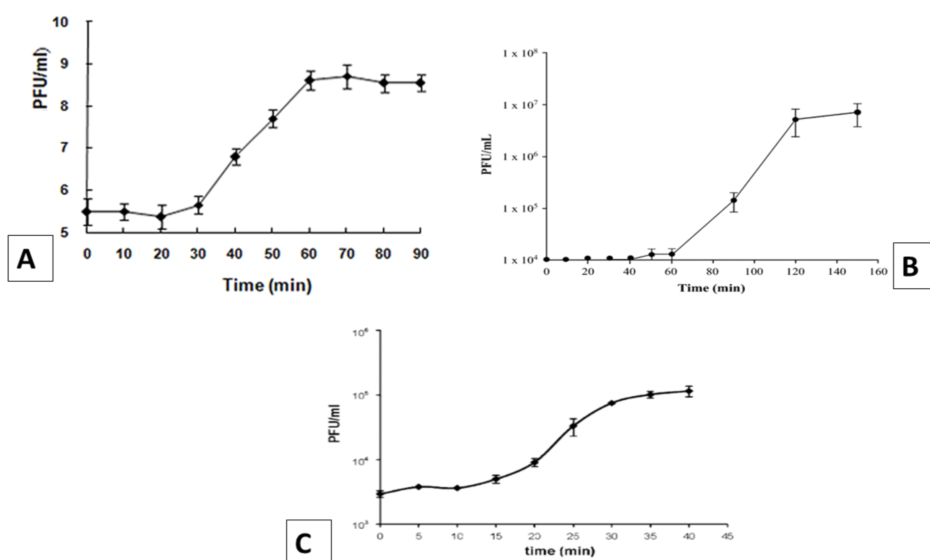

Figure 3: One Step Growth curve of *A. baumannii*

Thermal and pH stability

Phage-1 activity persisted at the same high level at 20-30 °C and completely dissipated after 20 minutes at 70 °C (Figure 4). Phage-1 activity was higher at between pH 6-8 and rapidly decreased at lower and higher pH levels and was comparatively steady at pH 7 (Figure 5).

Figure 4: Temperature stability graph of *A. baumannii*

Figure 5: Ph stability graph of *A. baumannii*

Host range

To govern the lytic assortment of Phage-1, twenty multidrug-resistant Acinetobacter baumannii strains from various regions were chosen for a bacteriophage congregation range assessment. Grounded on the results shown in Table 1, it can be determined that the phage Phage-1 can lyse local

strains of *Acinetobacter baumannii*, however, this ability is only seen in a particular geographic region.

Table 1. List of host range of various *A. baumannii* isolates against the Phage-1. (Colored boxes show Phage lysis and white boxes show no lysis)

A. baumannii **strains**	**Bacteriophages**
	Phage-1
AB-01	
AB-02	
AB-03	
AB-04	
AB-05	
AB-06	
AB-07	
AB-08	
AB-09	
AB-10	
AB-11	
AB-12	
AB-13	
AB-14	
AB-15	
AB-16	
AB-17	
AB-18	
AB-19	
AB-20	

Discussion

Multidrug-resistant (MDR) *Acinetobacter baumannii* strains are a serious challenge to healthcare systems worldwide, and their development and spread call for creative solutions [14]. The objective of this work was to investigate the therapeutic potential of Phage-1, a lytic bacteriophage isolated from clinical MDR *A. baumannii* isolates. The conversation explores several facets of the research, such as host range, stability, life cycle dynamics, phage separation, antibiotic susceptibility, and the consequences of the results. The assessment of *A. baumannii* isolates for antibiotic susceptibility shed light on the resistance patterns often seen in clinical settings. The isolates' notable differences in antibiotic susceptibility highlight the need of individualized and tailored treatment plans. The isolates' high sensitivity to colistin is consistent with earlier findings, indicating the potential effectiveness of these antibiotics against MDR *A. baumannii*. Notable patterns of sensitivity and resistance among the *A. baumannii* isolates were found by the antibiotic susceptibility testing carried out in this investigation. The intricacy of antibiotic resistance in clinical settings is highlighted by the variation in susceptibility to various drugs. The isolates' poor sensitivity to cephalosprins and aminoglycosides highlights the limited effectiveness of certain antibiotics against MDR *A. baumannii*. This highlights the necessity for a diversified strategy that takes into account both conventional antibiotics and non-traditional treatments like bacteriophage therapy [15].

After being effectively separated from sewage water, Phage-1 showed a strong capacity to lyse strains of MDR *A. baumannii* [16]. Traditional screening was used in the isolation process, and the resultant phage showed significant titers. DNA extraction phage-1 showed the confirmation of the phage and it was ready to use for further characterization. The examination of Phage-1's life cycle demonstrated a brief latent phase, an extended lysis phase, and a sizable burst size which is consistence with previous research work [17]. These features highlight how well the phage replicates and infects the bacterial host, which is essential for any prospective therapeutic uses.

Crucial details on Phage-1's resistance to various environmental conditions were revealed by the heat tests. At lower temperatures (20-40 °C), the phage demonstrated stability; nevertheless, it was susceptible to higher temperatures (50-80 °C) and severe pH values. A thorough understanding of these stability criteria is essential for the development of therapeutics based on phages, given the variety of conditions seen in the human body [18]. The environmental resistance of Phage-1 was also investigated by pH stability experiments, which revealed that although it is stable at 6-8 pH and is is susceptible to lower and high pH values. Understanding the circumstances in which the phage stays active is essential for its possible in vivo use, and our discoveries play a significant role in that regard. The information gathered adds to the expanding body of knowledge on phage treatment and is consistent with other studies on the stability of bacteriophages [19].
The host range evaluations indicated that Phage-1 is exclusive to a certain geographic area and that it may lyse local strains of MDR *A. baumannii*. The phage's lytic activity was thoroughly assessed for each strain using the spotting method and the plating efficiency evaluation. The observed geographic specificity of Phage-1 highlights the need for region-specific phage libraries to address the genetic variety of bacterial strains and raises questions about the possibility of its therapeutic use [20]. Even though the study has shed light on Phage-1's possible therapeutic use against MDR *A. baumannii*, there are still a number of obstacles to overcome and directions for further investigation. The broad clinical use of phage treatment is hampered by its limited host range and the need for area-specific phage collections. To increase the viability of bacteriophage treatment in treating antibiotic-resistant bacterial illnesses, further research should be done on broadening the host range, improving phage formulations, and resolving safety issues. As a result, the results of this investigation add to the expanding corpus of information about bacteriophage treatment and highlight Phage-1's potential as a viable option for more research and development [21]. The host range evaluations demonstrated that Phage-1 is capable of lysing strains of MDR *A. baumannii* that are exclusive to a certain area, highlighting its selectivity. The investigation used a spotting methodology and evaluated the effectiveness of plating (EOP) in order to thoroughly examine the lytic activity of the phage across several strains. Phage-1's regional uniqueness highlights the need for region-specific phage libraries and raises questions about possible therapeutic applications [22].
The results of the research provide important new information on how Phage-1 may be used therapeutically to treat MDR *A. baumannii* infections. Phage-1 is a viable option for more research due to its strong lytic efficiency, stability across a range of circumstances, and specificity within a certain geographic area [19]. The difficulty of incorporating phage treatment into clinical practice is highlighted by issues like the limited host range and the need for region-specific phage collections. To improve Phage-1 for possible clinical applications and solve the difficulties in using bacteriophage treatment to treat illnesses caused by bacteria resistant to antibiotics, further study and development work is necessary.

Conclusion

Effective lytic bacteriophage Phage-1 destroys clinically multidrug-resistant (MDR) *A. baumannii* strains. Phage-1 is a good target for antibiotic-resistant infections because it breaks down cells, reproduces well, and infects numerous species. Phage-1 can adjust to pH and heat despite its small burst size. These data suggest Phage-1 may fight MDR *A. baumannii*. This discovery optimizes Phage-1 use to locate antibiotic-resistant microorganisms. As research progresses, the insights gained from this study pave the way for the targeted and optimized use of Phage-1, marking a significant stride in the ongoing quest for alternative treatments for antibiotic-resistant bacterial infections.

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