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RESEARCH

Investigation of *Acinetobacter baumannii*-specific bacteriophage

Acinetobacter baumannii'ye spesifik bakteriyofaj araştırılması

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Abstract

Purpose: In recent years, the increasing resistance to antibiotics observed in many important bacterial groups has led to a growing interest in the literature regarding phage isolation and characterization, and the expanding clinical potential of phages. Considering the antimicrobial resistance profiles, the isolation of phages to be used in the treatment of *Acinetobacter baumannii* infections, determination of their action spectrum and characterization are very important. This study aimed to isolate bacteriophages specific to the target microorganism, *A. baumannii*, from environmental water sources.

Materials and Methods: Sixteen different environmental water samples were investigated as potential sources of phages. Clinical isolates of *A. baumannii* with multi-drug resistant were used as the host bacteria. Phages specific to the target bacteria were isolated using a single plaque isolation method. During in vitro studies, the titers of the isolated phages were increased using the double agar method, and their plaque morphology and host specificity were evaluated.

Results: The phage vB_KlAcineto13 exhibited lytic activity exclusively against the target bacterium and did not infect other bacterial isolates.

Conclusion: Based on the findings of this study, it can be concluded that phage vB_KlAcineto13 has a narrow host range and does not infect other tested bacteria outside the host bacterium. However, characterization studies are likely to provide more detailed information about the phage.

Keywords:. Bacteriophage, phage, phage therapy, *Acinetobacter baumannii*, phage isolation

Öz

Amaç: Son yıllarda antibiyotiklere olan direncin pek çok önemli bakteri grubunda görülmesiyle, literatüründe faj izolasyon ve karakterizasyon çalışmalarına olan ilginin arttığı ve fajların klinik kullanım potansiyellerinin yaygınlaştığı görülmektedir. Antimikrobiyal direnç profilleri göz önüne alındığında *Acinetobacter baumannii* enfeksiyonların tedavisinde kullanılacak fajların izolasyonu ve etki spektrumlarının belirlenmesi, karakterizasyonlarının yapılması oldukça önemlidir. Bu çalışmada çevresel su kaynaklarından toplanan su örneklerinden hedef mikroorganizma olan *A. baumannii*'ye spesifik bakteriyofaj izolasyonu amaçlanmıştır.

Gereç ve Yöntem: Farklı 16 çevresel su örneği faj kaynağı olarak incelenmiştir. Faj konak bakterisi olarak çoklu ilaca dirençli olan *A. baumannii* klinik izolatları kullanılmıştır. Tek plak izolasyon yöntemiyle izole edilen hedef bakteriye spesifik faj; in vitro çalışmalar süresince çift agar yöntemiyle titresi arttırılarak plak morfolojisi ve konak özgüllüğü değerlendirilmiştir.

Bulgular: vB_KlAcineto13 fajının, yalnızca hedef bakteri üzerinde litik aktivite gösterdiği ve diğer bakteri izolatlarını enfekte etmediği tespit edilmiştir.

Sonuç: Bu çalışmanın bulgularına dayanarak, vB_KlAcineto13 fajının dar bir konak aralığına sahip olduğu ve konak bakterisi dışındaki diğer test edilen bakterileri enfekte etmediği söylenebilir. Ancak, karakterizasyon çalışmalarının faj hakkında daha ayrıntılı bilgi sağlaması muhtemeldir.

Anahtar kelimeler: Bakteriyofaj, faj, faj terapi, *Acinetobacter baumannii*, faj izolasyon

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INTRODUCTION

Antibiotic resistance, which has become a global problem worldwide, has reached alarming levels according to statistics shared in recent years. It is reported that factors such as excessive and unnecessary antibiotic use, an increase in the number of individuals with weakened immune systems and bacterial mutations are among the factors causing antibiotic resistance[1]. Bacteriophages (phages) are emerging as an alternative treatment method that is gaining increasing interest due to increasing antibiotic resistance[2-4].

Phages are bacterial viruses that attack and disrupt bacterial metabolism, resulting in bacterial lysis. There are 10^{31} phages on Earth, which constitutes a vast natural antimicrobial reservoir. Phages are found in environments rich in bacteria, such as the digestive system, feces, sewage, lakes, streams, and fertilized soils, and can be easily isolated from these sources. Feces and liquids containing feces are the most commonly preferred samples for phage isolation. Phages and their products, such as endolysins, are attracting attention for their potential use as antimicrobial agents[3,4]. Research has demonstrated the efficacy of phage therapy in treating and preventing infections caused by various multi-drug resistant (MDR) and extreme drug resistance (XDR) bacteria, including *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Escherichia coli*, and *Acinetobacter baumannii*[5-7].

A. baumannii is a frequently encountered in both natural and clinical settings[8]. It can lead to a range of infections, including meningitis, endocarditis, urinary tract infections, and bacteremia[9]. *A. baumannii* has been listed by the American Society for Infectious Diseases as one of the highest-priority organisms. In a press release from the WHO in February 2017, carbapenem-resistant strains of *A. baumannii* were identified as the top critical pathogens for which antimicrobial development is urgently needed[1]. The significance of *A. baumannii* has increased due to its MDR and XDR. It is anticipated that the global rise of multi-drug-resistant bacteria will continue to escalate[3].

Given the increasing frequency of *A. baumannii* infections, along with their high morbidity and mortality rates, and the antimicrobial resistance profiles, it is crucial to develop phage libraries for the control and treatment of these infections[7,10,11]. Isolating phages from various sources that can be included in phage therapy libraries is a fundamental step. In line with these factors, this study aims to investigate specific bacteriophages for *A. baumannii* from water samples collected from environmental water sources.

MATERIALS AND METHODS

The study included ten different strains of *A. baumannii*. All of clinical *A. baumannii* strains are MDR and were previously identified from a variety of patient samples[12].

Preparation of bacteriophage ısolation sources

A total of 16 samples were obtained from diverse environmental water sources in Kırklareli and surrounding districts. Water from each source was transferred into transport containers and used immediately for phage isolation studies. The water samples were placed into two sterile, labeled 1-liter polyethylene containers. Each container was filled with approximately 800 mL of water, tightly sealed, and cleaned with 70% alcohol before being transported to the laboratory in a cooler with ice packs. 20 mL from each water sample was aliquoted into 50 mL Falcon tubes and stored in 15% glycerol at -80°C. The water samples were allowed to settle, after which the supernatant was centrifuged. The supernatant was then filtered through 0.22 µm filters. The filtered liquid (filtrate) was used as the source of phages[10].

Bacteriophage isolation and propagation

Bacteriophage enrichment was accomplished using the method reported by Badawy and colleagues[10]. A 100 µL sample from the *A. baumannii* culture was divided into two groups. One milliliter of sterile-filtered wastewater was mixed with 9 mL of lysogeny broth (LB) and incubated overnight. 0.2 mL of chloroform was added, and the mixture was agitated for 20 minutes before centrifugation. Then, the supernatants were filtered using 0.45 µm filters (Minisart® Sartorius, Göttingen, Germany).

Bacteriophage titration by droplet and double-agar overlay methods

Bacteriophage titrations were performed using the Droplet and Double-Agar Overlay procedures[10].

Initially, 100 µL of the *A. baumannii* culture was mixed with 3 mL of molten soft agar and put onto Luria agar (LA) plates. The enriched lysates were serially diluted tenfold in saline magnesium (SM) buffer. Five µL drops of both undiluted and diluted solutions were put on solidified soft agar plates and incubated overnight at 37°C. Mix 50 µL of phage aliquots with 100 µL of bacteria in 3 mL of soft agar, then pour onto LA plates. Following an overnight incubation, the number of individual plaques was counted, and the original phage titers (PFU/mL) were calculated using the plaque count and dilution factors. The number of phage particles per millilitre was calculated by counting the phage plaques formed [plaque forming unit/millilitre (PFU/mL) = number of plaques in the agar medium/amount of phage cultured x dilution amount].

Plaque purification

Single plaques from double-agar overlay plates of were used to extract specific phages. A specific plaque was selected and treated with 500 µL of SM buffer and 50 µL of chloroform. The mixture underwent centrifugation. The concentration of phages was then measured[10].

Preparation of phage stocks

The semiconfluent plaque was treated with 3 mL of SM buffer and incubated for 30 minutes. Approximately 200 µL of chloroform was added. The tubes were shaken centrifugation at 5000× g for 10 minutes. The supernatant was filtered and 40% sugar added. The phage stocks were then titrated and kept at 4 °C[10].

Phage host range determination

The specificity of bacteriophages for their host organisms was assessed using the droplet titration technique on ten strains of *A. baumannii*. Positive results from the droplet tests were verified with the double-layer method employing suitably diluted phage preparations[10].

RESULTS

A spot test method was used to determine the potential presence of bacteriophages. Using a fresh bacterial culture, a streak inoculation was performed, and 10 µL of phage suspension was added to the inoculated areas. After overnight incubation at 37°C, the presence of zones at the inoculation sites was assessed. 15 petri dishes inoculated with water from various sources were found to be negative, as no zones of lysis were observed. However, one petri dish inoculated with water from a single source was found to be positive due to the presence of a zone of lysis (Figure 1).

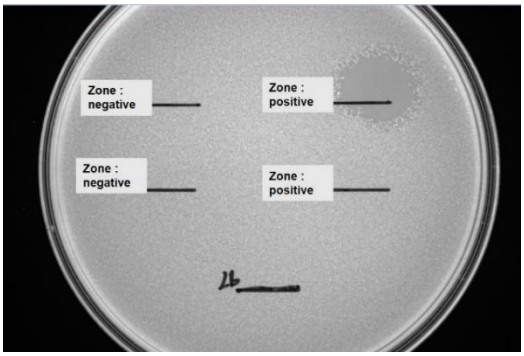

Figure 1. Agar spot test results for phage source.

Phages specific to *A. baumannii* were detected in only 1 out of 16 water samples. It was determined that the water sample formed strong lytic phage plaques against *A. baumannii* in the double layer agar. The plaque morphology of the phages was shown in Figure 2. The phage produced clear, circular plaques with diameters of 0.5 cm, each encircled by halos (zones of clearance surrounding each plaque).

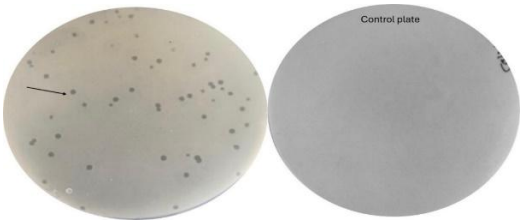

Figure 2. Bacteriophage isolation; arrow line exhibits the formation of plaques formed by phages.

For the single plaque obtained using the double-layer agar method, plaque purification was used to ensure the purity of the culture. The phage concentration was determined by titration, and to ensure the phage's purity, single plaque isolation from new double-layer agar plates was repeated at least five times.

The ability of the isolated *A. baumannii* phage, which formed a single type of plaque on the petri dish, to lyse its host bacterium was assessed by the double-layer agar method to calculate the phage titer. For this

purpose, single-type plaques were first selected, combined with the host bacterium at the most suitable dilution, and their concentrations were increased. Lysis areas and plaque counts were observed at dilutions. Stock concentration determinations were performed periodically in triplicate. The stock (PFU/mL) was $5.9x10^{12}$ PFU/mL. The names of the stock concentrations determined for the bacteriophage isolates were coded as follows: v: virus, B: Bacteriophage, Water sample location: KL, Host strain: Acineto, and Water sample number: 13 (vB_KlAcineto13: virusBacteriophage_KırklareliAcinetobacter no.13).

The vB_KlAcineto13 phage isolated from this study was determined to exhibit lytic activity only on #AB-2221.

Table 1. The host spectrum of phage vB_KlAcineto13 on *Acinetobacter baumannii* strains.

***A. baumannii* strains**	**Source**	**Antibiotic sensitivity**	**Acinetobacter Phage (vB_KlAcineto13)**
AB-2212	Blood	MDR	-*
AB-2213	Blood	MDR	-*
AB-2214	Blood	MDR	-*
AB-2215	Blood	MDR	-*
AB-2216	Blood	MDR	-*
AB-2217	Blood	MDR	-*
AB-2218	Blood	MDR	-*
AB-2219	Blood	MDR	-*
AB-2220	Blood	MDR	-*
AB-2221	Blood	MDR	+*

*(+: Phage lysis, -: no lysis, MDR: multidrug resistant)

DISCUSSION

Phages are known to have significant biological efficacy due to their high genetic diversity and potential for transduction. In recent years, the use of phages as an alternative to antibiotics in the treatment of infectious diseases has gained renewed popularity due to the increasing rise in antibiotic resistance, owing to their lytic activities against bacteria[13].

Fighting against antibiotic resistance, the discovery of new phages that can be incorporated into phage libraries is highly valuable, especially given the promising developments in phage therapy. The process of discovering new phages can sometimes be lengthy, but it can also be relatively rapid. Identifying and screening phage sources is considered a fundamental step in phage discovery. It has been reported that sources such as sewage, environmental water sources, food products, and soil can be utilized for phage isolation[7]. literature includes studies on the isolation of *A. baumannii* phages from various water sources. Yang and their colleagues isolated a virulent bacteriophage targeting a clinical strain of *A. baumannii* from a marine sediment sample[14]. In a study, researcher utilized 250 water samples from 22 different locations identified in wastewater treatment facilities and campus environmental water sources. Among these 250 water samples, 12 were found to contain phages with potential lytic activity against *A. baumannii*[15]. Ghajavand and their colleagues isolated two lytic phages targeting multidrug-resistant *A. baumannii* from hospital wastewater[16]. Badawy and their colleagues isolated *A. baumannii*-infecting phages from a sewage water sample[10]. In other study, researcher isolated *A. baumannii* phages from 13 out of 31 water samples collected from different environmental water sources[17]. Khorshidtalab and their colleagues isolated phages from wastewater collected from the municipal sewer system[18]. Kolsi and their colleagues isolated three phages from hospital wastewater that infected clinical *A. baumannii* strains[11]. Choi and their colleagues isolated 21 phages from 20 hospital sewage samples[13]. In this study, a phage exhibiting lytic activity against the target microorganism was identified from 1 of the 16 environmental water samples. The phage, which demonstrated a strong lytic effect specific to *A. baumannii*, was named vB_KlAcineto13.

A key characteristic that must be present for a phage to be eligible for phage therapy is its specificity for a particular host. Phages, especially those of clinical significance, should possess as broad a host range as possible. A broad host range for a bacteriophage indicates its ability to infect and eliminate several

species within a single bacterial genus or even multiple genera. The term "broad host range," often referred to as polyvalency, does not have a definitive criterion in the literature specifying whether it should refer to the same or different species within a genus or to bacteria from different genera[19]. Furthermore, studies have demonstrated that a bacteriophage can infect a variety of genera within a bacterial family, highlighting its broad host range[20]. Popova and their colleagues isolated bacteriophage lytic for *A. baumannii* and researchers determined that the bacteriophage lyse 68% (89 of 130) genotype-varying multidrug-resistant clinical *A. baumannii* strains[21]. Choi and their colleagues determined that of the 21 isolated *A. baumannii* phages, 11 of phages have potent lytic activities against carbapenem-resistant *A. baumannii*[13]. Ghajavand and their colleagues reported that phage IsfAB78 and phage IsfAB39 possess a limited host range and were unable to infect both related and unrelated bacteria[16]. Badawy and colleagues evaluated the host ranges of phages fEg-Abo01, fLi-Aba02, and fLi-Aba03 against 91 strains from 14 different species. They reported that these phages infected solely the original *A. baumannii* host strain #6597, as well as a recent multidrug-resistant *A. baumannii* isolate, strain #6898[10]. Similarly, Kolsi and their team isolated three phages and discovered they had a very limited host range. They discovered that phage fBenAci001 exclusively infected its original host, whereas phages fBenAci002 and fBenAci003 may infect one more *A. baumannii* strain[11]. In this study, the titer of pure *A. baumannii* phage obtained by single plate isolation method was increased by double agar method during in vitro studies and host specificity was studied. It was determined that phage vB_KlAcineto13 specifically infected and lysed only its original isolation host (#AB-2221) multidrug-resistant clinical *A. baumannii* strain by creating clear zones.

The isolation of *A. baumannii*-specific phages, which are listed among critical microorganisms by the World Health Organization, represents an important step in expanding phage libraries and supporting phage therapy. In this regard, water sources such as environmental water samples, wastewater, and hospital sewage should be investigated as potential sources of phages. This study aimed to isolate phages exhibiting lytic activity against MDR *A. baumannii* from environmental water samples. Based on the findings of this study, it can be stated that phage vB_KlAcineto13 has a narrow host range and does not infect other tested bacteria outside of the host bacterium. However, further characterization studies are likely to provide more detailed information about the phage.

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