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ORIGINAL ARTICLE / ÖZGÜN MAKALE

ISOLATION OF NEWLY ISOLATED VB_K1 BACTERIOPHAGE AND INVESTIGATION OF SUSCEPTIBILITY ON ESBL POSITIVE KLEBSIELLA SPP. STRAINS

YENİ İZOLE EDILEN VB_K1 BAKTERİYOFAJININ İZOLASYONU VE ESBL POZİTİF KLEBSİELLA TÜRLERİ ÜZERİNE DUYARLILIĞININ ARAŞTIRILMASI

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ABSTRACT

Objective: *The decrease in the efficacy of antimicrobials in the treatment of Klebsiella-related infections necessitated the search for alternative treatment strategies. This study aims to provide isolation of lytic bacteriophage specific to Klebsiella species and to investigate its potential for use as alternative antimicrobial agent.*

Material and Method: *One Extended spectrum beta lactamase (ESBL) producer Klebsiella strain was used as host bacteria and water samples were collected from river in Ankara for bacteriophage isolation. Spot test method was applied to determine the possible presence of bacteriophage after phage enrichment. To confirm the presence of the lytic bacteriophage, double layer agar method was applied to spot test positive samples. The susceptibility of the bacteriophage was determined using in vitro spot test. 38 clinical ESBL positive Klebsiella spp. strains were used for this analysis.*

Result and Discussion: *In the initial screening, the vB_K1 bacteriophage producing visible plaques with a diameter of 1.00 mm was isolated in the petri dish. The susceptibility of ESBL positive Klebsiella spp. strains to this bacteriophage was determined as 73.7%. It was proved that vB_K1 bacteriophage is very effective to Klebsiella spp. strains. However, in vitro bacteriophage susceptibility of characterized bacteriophage is encouraging development.*

Keywords: *Bacteriophage, resistance, ESBL, Klebsiella spp.*

ÖZ

Amaç: *Klebsiella ile ilişkili enfeksiyonların tedavisinde kullanılan antimikrobiyallerin etkinliğindeki azalma, alternatif tedavi strateji arayışlarını gerektirmiştir. Bu çalışma Klebsiella türlerine özgü litik karakterde bakteriyofaj izolasyonunun sağlanmasını ve alternatif antimikrobiyal ajan olarak kullanım potansiyelinin araştırılmasını amaçlamaktadır.*

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Gereç ve Yöntem: *Ankara deresinden alınan su örneklerinden faj izolasyonu için, Genişlemiş spektrumlu beta laktamaz (GSBL) üreticisi bir adet Klebsiella spp. suşu konak bakteri olarak kullanıldı. Faj zenginleştirme sonrası, olası bakteriyofaj varlığını belirlemek için spot test yöntemi uygulandı. Litik bakteriyofaj varlığı, spot testi pozitif numunelere çift tabaka agar yöntemi uygulanarak doğrulandı. Bakteriyofajın duyarlılığı, 38 klinik GSBL pozitif Klebsiella spp. suşu kullanılarak in vitro spot test ile belirlendi.*

Sonuç ve Tartışma: *İlk taramada petride 1.00 mm çapında görünür plaklar üreten vB_K1 bakteriyofajı izole edildi. GSBL pozitif Klebsiella spp. suşlarının bakteriyofaj duyarlılığı %73.7 olarak belirlendi. vB_K1 bakteriyofajının Klebsiella suşları üzerinde çok etkili olduğu kanıtlanmıştır. Bununla birlikte, karakterize edilen bakteriyofajın in vitro bakteriyofaj duyarlılığı cesaret verici bir gelişmedir.*

Anahtar Kelimeler: *Bakteriyofaj, direnç, ESBL, Klebsiella spp.*

INTRODUCTION

Klebsiella spp. belong to the family Enterobacteriaceae and are found in human microbiota, especially in the gastrointestinal, respiratory tracts and skin. It is an opportunistic pathogen that can cause a wide variety of community-acquired and nosocomial infections, such as urinary tract infections, respiratory tract infections, wound and soft tissue infections [1]. Resistance to antimicrobials is increasing day by day and β-lactamase production is an important resistance mechanism among them. Pathogens that produce extended-spectrum β-lactamases (ESBLs) show resistance not only to third-generation cephalosporins and monobactams, but also to other classes of antibiotics [2]. ESBL resistance genes are located on plasmids and therefore are carried and cause significant infections in other strains. This causes an increase in infection control problems [3].

Extended-spectrum β-lactamase (ESBL) producer *Klebsiella spp*. are among the six drug-resistant microorganisms for which new therapies are urgently needed. Furthermore, ESBL positive Klebsiella infections cause serious morbidity and mortality in humans, increasing healthcare costs and treatment burden [4]. Alternative or complementary therapies for these infections are required.

Bacteriophages are defined as bacterial viruses and they show specific effects to their specific target bacteria. The existence of bacteriophages first emerged in 1896 when M. Ernest Hankin observed that people washed by the Ganges river waters of India did not contract cholera. Felix d'Herelle named "invisible microbe of the dysentery bacillus" as bacteriophage in 1917 and was the first to announce the antimicrobial effect of bacteriophages to the world [5]. Bacteriophages are defined as bacterial viruses that target and kill their specific bacteria. It was used clinically for the first time in 1919 by Felix D'Herelle in France [6]. Bacteriophages, which were thought to be used as antibacterial material, were used in the treatment of many diseases such as upper respiratory tract infections, abscesses, burns and inflamed wounds in a few countries such as Georgia, Poland, former Russia and the USA until the 1940s. With the discovery of antibiotics in 1941, bacteriophage therapy somehow fell into the background, but in the Soviet line countries (in the early 1990s) while the civil war continued, it continued to be used in the treatment of soldiers against *Staphylococcus aureus, Pseudomonas aeruginosa, Escherichia coli and Streptococcus pyogenes* bacteria [7].

Bacteriophages can be found wherever bacteria are found. Bacteria can be found in almost any environment, such as seawater, fresh water and soil, so bacteriophages can be found anywhere they host [8]. In addition to their therapeutic usage, they can be used in many areas such as disinfection, bacterial identification, determination of subtypes of bacterial strains, in food and livestock conservation [9].

Bacteriophages basically have two life cycles, in some, both life cycles can be seen. Bacteriophages in the lytic life cycle are called virulent (lytic) bacteriophages, while bacteriophages in the lysogenic life cycle are called lysogenic bacteriophages [10]. In the lytic bacteriophages used in the treatment, the target bacteria are broken down and hundreds of phage progeny are released [11]. In the treatment of bacteriophage, it is important that the bacteriophage has a strong lytic effect and has a high host cell specificity [12].

To develop an effective antimicrobial agent, previously studies have been isolated and characterized bacteriophages specific for *Klebsiella spp*., especially *Klebsiella pneumoniae* (*K. pneumoniae*) [13-16]. The objective of this work was to isolate potentially therapeutic bacteriophage against ESBL positive Klebsiella strains. We also determined the in vitro susceptibility of 38 previously characterized ESBL-producing *Klebsiella spp.* to a newly isolated bacteriophage.

MATERIAL AND METHOD

Bacteriophage Isolation

One ESBL producer *Klebsiella spp.* strain was used as host bacteria and water samples were collected from river in Ankara for bacteriophage isolation. Spot test method was applied to determine the possible presence of bacteriophage after phage enrichment. For this, the water sample was centrifuged and the particles in it were removed, and the supernatant was passed through a 0.22 μm membrane filter. Fresh bacteria culture was cultivated in x2 Luria Bertani Broth (Merck) medium enriched with $CaCl_2$ and $MgSO_4$ and incubated at 37 °C for one night. After incubation, the suspension was centrifuged to remove the cell debris and particles, and bacteria were killed by addition of supernatant to 10% chloroform. For spot test, a strip of fresh bacterial culture was inoculated into petri dishes containing the medium, and 10 μL of the possible bacteriophage suspension obtained was dripped onto these areas. At the end of one-night incubation, the presence of zones in the cultivation areas was evaluated [17].

To confirm the presence of the lytic bacteriophage, a double layer agar method was applied to spot test positive samples. The supernatant was mixed with fresh bacterial culture and soft agar (0.6% agar) and spread on agar plate (1.5% agar). After one-night incubation at 37 °C, the petri dishes were evaluated for the presence of bacteriophage plaques [18, 19].

Single Plaque Isolation

Single plaque isolation was performed from one of the petri dishes in which bacteriophage plaque was detected. The plaque was cut from the area with a sterile pasteur pipette and transferred to 3 mL Luria Bertani broth medium. Fresh bacterial culture was added on it 3 mL more Luria Bertani broth was added on it and incubated at 37 °C. The next day, the bacteriophage suspension was centrifuged at 5000 rpm for 10 minutes and passed through a 0.22 μm membrane filter. The double layer agar method was applied to the dilutions of the bacteriophage. This stage will be repeated at least 5 times [20, 21].

Preparation of Concentrated Bacteriophage Suspension

In order to obtain the concentrated bacteriophage suspension, double layer agar was applied to the dilution where the phage and the host achieved the most appropriate growth together. The next day, the soft agar portion was taken with a Drigalski spatula, filtered through 0.22 μm membrane filter. The bacteriophage titer was determined as 'plaque forming unit' (PFU) using the double layer agar method by making 10-fold serial dilutions.

The Susceptibility of Bacteriophage

The susceptibility of the bacteriophage was determined using in vitro spot test. 38 clinical ESBL positive *Klebsiella spp*. strains were used for this analysis. All strains were spread evenly on the LB agar plate. After drying, 10 μL of the phage culture of 10^8 PFU/mL was dropped onto the overlaid top agar. After culturing for 18 h at 37 °C, the presence or absence of a lysis zone was evaluated [22].

RESULT AND DISCUSSION

Klebsiella is a clinically important pathogen causing a variety of antibiotic resistant infections. The fact that ESBL-producing *K. pneumoniae* strains even become resistant to carbapenems increases the incidence of infections caused by these strains. As well as the increase in Klebsiella infections in community and hospital settings, there are many studies on bacteriophages, which are seen as an alternative to treatment [23]. There are a number of considerations in the selection of bacteriophages as therapeutic antimicrobial agents. Firstly, the bacteriophages must be effective at killing Klebsiella. Bacteriophages produce large, clear plaques containing large numbers of phage particles, allowing the bacteria to break down rapidly. In addition, bacteriophages with a wide host range are considered to be more beneficial than those with a narrow host range. Second, compared to lysogenic bacteriophages, which integrate their genetic information into the host genome and remain dormant for an indefinite period, lytic bacteriophages clear bacteria quickly and efficiently [24]. This demonstrates the necessity of characterizing bacteriophages before selecting them for therapeutic use.

In our study, one *Klebsiella spp.* isolate was used to find lytic bacteriophage from the collection of water samples obtained from the river water. Among all, one filtrate produced clear zones against the host

bacteria was selected. A newly isolated bacteriophage was named vB_K1. The vB_K1 bacteriophage produced visible plaques on the bacterial lawns 1.00 mm diameter in the initial screening using plaque assay (Figure 1). The susceptibility to vB_K1 bacteriophage was determined on 38 previously described as ESBL- producing *Klebsiella spp.* isolates. Ten of these were *K. pneumoniae*. Among the 38 Klebsiella spp. strains 29/38 (73.7%) were susceptible to vB_K1 bacteriophage, but 9/38 (26.3%) strains were resistant to vB_K1 bacteriophage. The effect of phage on K. pneumoniae strains were 76.9%. The phage was considered to have high activity against *Klebsiella spp.*. The results are given in Table 1.

Townsend et al. isolated and characterized 30 Klebsiella bacteriophages, distinct lineages, using multiple water samples from different environments and a number of different *Klebsiella spp.*. They reported in this study that bacteriophages were included in 5 phylogenetic groups and the broadest spectrum vB_KoM-MeTiny bacteriophage had 79% lytic activity [25]. In another study, Wintachai et al. reported that KP1801 bacteriophage isolated from hospital wastewater lysed 50% (10/20) of clinical strains [16]. Similarly, Karumidze et al. reported that one or more of the six phages was capable of efficiently lysing 63% of Klebsiella strains comprising a collection of 123 clinical isolates from Georgia and the United Kingdom [26].

Karamoddini et al. isolate bacteriophages against *Klebsiella spp.* and evaluate their efficacy against 72 antibiotic-resistant isolates. At the same time, they reported that the use of bacteriophages in high concentration (over 10^7 PFU/mL) increased the effect [27]. Domingo-Calap et al. characterized two newly isolated *K. pneumoniae* bacteriophages, πVLC5 and πVLC6, from environmental samples as possible therapeutic agents by phenotypic and genomic tests [13]. In another study, Kawa et al. was found that 42 of 101 *K. pneumoniae* isolates (41.6%) were susceptible to newly isolate bacteriophage KP34. They also was found that 47.1% of ESBL (+) and 36% of ESBL (−) strains were susceptible to KP34 phage [28].

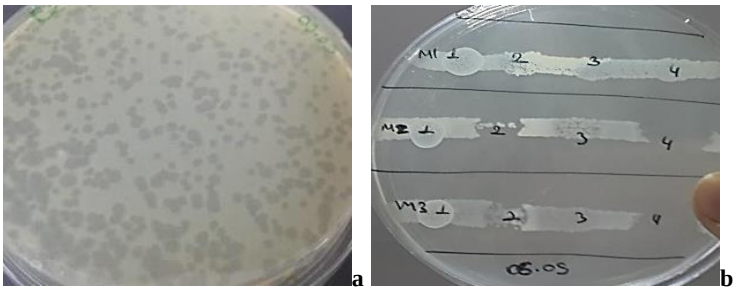

Figure 1. (a) vB_K1 bacteriophage plaque, (b) Spot test results.

Bacteriophage therapy holds promise as a potential response to inhibit the development and spread of MDR Klebsiella species. In vitro and in vivo studies have confirmed the potential use of bacteriophages individually, as bacteriophage cocktails, and in combination with existing antimicrobial chemotherapeutic drugs. In addition, the routine use of bacteriophage therapy in Eastern Europe and the

results of several clinical studies in the West indicate that bacteriophages are considered safe for use in humans. Eventually, it was proved that isolation and in vitro phage susceptibility results of vB_K1 bacteriophage are promising for Klebsiella species for which there are no ready to use phage preparations, but more detailed characterization studies are needed.

Table 1. Bacteriophage susceptibilities of *Klebsiella spp.* strains

Number	**Strain No**		**vB_K1 bacteriophage**
1	384	ESBL *Klebsiella spp.*	-
2	379	ESBL *K. pneumoniae*	+++
3	364 (host)	ESBL *Klebsiella spp.*	+++
4	285	ESBL *Klebsiella spp.*	-
5	205	ESBL *Klebsiella spp.*	+
6	378	ESBL *K. pneumoniae*	+++
7	382	ESBL *K. pneumoniae*	-
8	240	ESBL *Klebsiella spp.*	++
9	405	ESBL *K. pneumoniae*	+++
10	206	ESBL *Klebsiella spp.*	-
11	305	ESBL *Klebsiella spp.*	+++
12	333	ESBL *K. pneumoniae*	+
13	166	ESBL *Klebsiella spp.*	+
14	172	ESBL *Klebsiella spp.*	+
15	200	ESBL *Klebsiella spp.*	-
16	434	ESBL *Klebsiella spp.*	-
17	455	ESBL *Klebsiella spp.*	++
18	338	ESBL *Klebsiella spp.*	+++
19	171	ESBL *Klebsiella spp.*	++
20	119	ESBL *Klebsiella spp.*	++
21	82	ESBL *Klebsiella spp.*	+
22	176	ESBL *Klebsiella spp.*	+++
23	227	ESBL *K. pneumoniae*	+++
24	454	ESBL *Klebsiella spp.*	+++
25	305	ESBL *Klebsiella spp.*	++
26	373	ESBL *K. pneumoniae*	-
27	379	ESBL *Klebsiella spp.*	+++
28	207	ESBL *K. pneumoniae*	+++
29	205	ESBL *Klebsiella spp.*	+
30	378	ESBL *K. pneumoniae*	+++
31	382	ESBL *K. pneumoniae*	-
32	240	ESBL *Klebsiella spp.*	++
33	405	ESBL *K. pneumoniae*	+++
34	206	ESBL *Klebsiella spp.*	-
35	305	ESBL *Klebsiella spp.*	+++
36	333	ESBL *K. pneumoniae*	+
37	201	ESBL *K. pneumoniae*	+++
38	178	ESBL *Klebsiella spp.*	+
Lytic Effect%			73.7

+++: CL (Clear Lysis), ++: SCL (Semi-Clear Lysis), +: OL (Opaque Lysis), -: No lysis [22].

AUTHOR CONTRIBUTIONS

Concept: *H.E, B.K.;* Design: *H.E, B.K.;* Supervision*: H.E, B.K.;* Resources*: H.E, B.K.;* Materials: *H.E, B.K.;* Data Collection and/or Processing*: H.E, B.K. ;* Analysis and/or Interpretation*: H.E, B.K.;* Literature Search: *H.E, B.K.;* Writing*: H.E, B.K.;* Critical Reviews*: H.E, B.K.*

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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