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Biological Properties of a Newly Isolated Bacteriophage (NL1) that Infects *Escherichia coli* O157:H7 Strain

Neslihan MUTLU[1], Evren KOÇ[2], Özkan ÖZDEN[3]

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Öz

Escherichia coli O157: H7 suşu ve daha önce sporadik vakalardan izole edilmiş enteroinvaziv *Escherichia coli* suşları**,** kirli çevresel odaklardan ve hayvansal atıklarla kontamine olmuş sulardan izole edilmiş bakteriyofajların konakçı spektrumunu belirlemek için standart suş olarak kullanılmıştır. NL1 Olarak isimlendirilen, *Escherichia coli* O157:H7'ye spesifik bakteriyofajın fizyolojik özellikleri belirlenmiştir. İzolasyon basamağından sonra bakteyofajlar pürifiye edilmiş ve titreleri artırılmıştır. Konakçıların çapraz antijenik özellikleri *Escherichia* phage NL1 fajı ile araştırılmıştır. Fajların adsorpsiyon süresi, latent periyot, patlama boyutu, enfeksiyon değerinin çokluğu (MOI) gibi tek aşamalı büyüme eğrisi parametreleri belirlenmiştir. Sonuçlar, bakteriyofaj NL1'in *E.coli* O157:H7 (RSKK 09007) ve *E.coli O:164* RSKK 324'ü tamamen lize ettiğini göstermiştir. Bu çalışmada, faj NL1'in sadece *E.coli* O157:H7 üzerindeki etkileri araştırılmıştır. Hesaplanan en yüksek titre, optimal enfeksiyon çokluğu ($2.16x10^8$) olarak belirlenmiştir Latent periyodun 20 dakika sürdüğü ve fajın yaklaşık 90 dakikada patlama boyutuna ulaştığı belirlenmiştir.

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Abstract

Escherichia coli O157: H7 strain was used as the standard strain for bacteriophages isolated from polluted environmental foci and animal waste-contaminated waters, and some of the enteroinvasive *Escherichia coli* strains previously isolated from sporadic cases were used to determine the host diversity of phages. The physiological properties of bacteriophages specific to *Escherichia coli* O157:H7, designated *Escherichiaphage* NL1, were studied. After the isolation step, bacteriophages were purified and their titer was increased. Cross-antigenic properties of the hosts were investigated with *Escherichia* phage NL1 phage. One-step growth curve parameters such as adsorption time of phages, latent period, burst size, multiplicity of infection value (MOI) were determined. Results showed that bacteriophage NL1 completely lysed the *E.coli* O157:H7 (RSKK 09007) and *E.coli* O:164 (RSKK 324). In this study the effects of phage NL1 only on *E.coli* O157:H7 were investigated. The calculated highest titer was determined as optimal multiplycity of infection ($2.16x10^8$). It was determined that the latent period lasted for 20 minutes and the phage was reached the burst size at approximately 90 minutes.

[1] Kafkas University Science and Art Faculty-Kars/Turkey; e-mail: nslhnmtl81@gmail.com; ORCID: 0000-0002-1339-3267 (Corresponding author)
[2] Kafkas University Engineering and Architecture Faculty-Kars/Turkey; e-mail: evrenkoc@hotmail.com ORCID: 0000-0002-0022-9433
[3] Kafkas University Engineering and Architecture Faculty-Kars/Turkey; e-mail: ozzkan1@gmail.com ORCID: 0000-0002-9467-3761

1. INTRODUCTION

Enterohemorrhagic *Escherichia coli* (EHEC) is a pathogen that can cause worldwide epidemics in humans and animals. The most important component of their virulence is the production of one or both Shiga-like toxins (Stx I and II) (O'Brien et al., 1992). Strains belonging to the O157 serogroup, specifically O157:H7, have been associated with epidemics and sporadic cases around the world. The Japanese Ministry of Health and Welfare recommends the use of kanamycin, fosfomycin, and a new quinolone antimicrobial agent, norfloxacin (Ministry of Health and Welfare, 1996). However, these various antimicrobial agents, including the novel quinolone, caused the production of Stx under certain conditions (Walterspiel et al., 1992). It has also been shown that, unlike antibiotics, phage therapy does not induce Shiga toxin production. These findings suggest that some newly isolated bacteriophages have biocontrol potential and are therapeutic agents for pathogenic *E. coli* (EHEC and EPEC) strains (Viscardi et al., 2008). Today, the most basic treatment for *E.coli* is oral rehydration (Bhan et al., 1994). This simple and inexpensive measure has saved countless lives, but it does not affect the natural course of the disease and has no antibacterial activity. *E.coli* vaccine studies are still in the early development phase (Savarino et al., 2002). Water and sanitation programs can be improved and drinking water quality can be improved, but they are very expensive methods for developing countries. Some researchers have investigated the use of O157-specific phages in food sanitation (Kudva et al., 1999, O'Flynn et al., 2004). Experimentally, when meat was contaminated with O157 strains, high titers of bacteriophages were required to reduce bacterial density. Similar high phage titers were required to clear chicken skin of Salmonella and Campylobacter contaminations (Goodridge et al., 2003). The biggest obstacle to the practical use of colyphages can be considered as the destruction of the intestinal commensals by phage cocktails, however, in an experiment conducted in mice, it was shown that four orally administered phage cocktails did not affect the amount of intestinal commensals (Chibani-Chennoufi et al., 2004). Similarly, volunteers orally exposed to T4 phage maintained a commensal *E. coli* population (Bruttin and Brüssow, 2005).

The fact that resistance to antibiotics has become an important problem today will lead to a longer interest in bacteriophage studies. In our study, the phage that we predict can be used in the biocontrol of *E.coli* O157: H7, which can also cause food-borne outbreaks, and some properties of this phage have been determined.

2. MATERIALS AND METHODS

Collection of samples and bacterial strains

Liquid samples (sewage and contaminated water) were collected in sterile capped bottles. Samples were taken from 20 different foci in Kars (Turkey).

Bacterial strains were obtained from the Ministry of Health, General Directorate of Public Health, National Type Culture Collection Laboratory (Ankara/Turkey) (Table 1).

Table 1. Bacterial strains

Species	Isolate
Escherichia coli O157:H7	RSKK 09007
Escherichia coli O:28	RSKK 314
Escherichia coli O:164	RSKK 324
Escherichia coli O:112	RSKK 688
Escherichia coli O:124	RSKK 318
Escherichia coli O:143	RSKK 322
Escherichia coli O:152	RSKK 323

Isolation of bacteriophage

After the collected samples were centrifuged at 5000 g for 20 min, the supernatant was filtered through 0.22 millipore membrane filters. 10 ml of the single colony of the *E.coli* strain, which was cultivated the day before, incubated at 37 °C for 3 hours in LB broth, and 10 ml of the filtrate were mixed in the test tube. The mixture was transferred into 100 ml of LB broth supplemented with 0.1 M calcium chloride and 0.1 M magnesium sulfate, and the mixture was incubated at 37 °C for 16 hours. During this process, the number of bacteriophages remaining in incubation with their host was expected to increase. After the incubation period, the mixture was filtered through 0.22 millipore membrane filters by centrifuging again (5000 g, 10 min) to get rid of bacteria (Clokie and Kropinski, 2009)

Spot test

An overnight fresh bacterial culture (*Escherichia coli* O157: H7) incubated on agar medium was added to 4.5 ml of LB broth and vortexed, and 100 µl of the obtained culture was transferred to a sterile tube. Then, 4 ml of soft agar was added to the bacterial culture and

spread on LB agar. 10 µl of bacteriophage suspension was dripped onto the prepared agar preparations and incubated at 37 °C for 18 hours in aerobic environment, and plaques formed on the agar were observed the next day. This procedure was applied separately for all strains. The experiment was repeated until plaque formation was observed (Clokie and Kropinski, 2009)

Purification of bacteriophages

In order to dilute the phage titer of the liquid samples in which the presence of phage was determined, sequential dilutions up to 10-8 were prepared, 1 ml of bacteriophage sample taken from the dilution tubes was mixed with 100 µl of fresh target bacteria culture and kept at room temperature for 10 minutes. Then, using the double layer agar method, 4 ml of soft LB agar was added and mixed, and it was spread on LB agar and incubated at 37 °C for 18 hours. After 18 hours of incubation, plaque formations on petri dishes were examined, bacteriophage plaques from petri dishes with single plaque formation were cut with a sterile pasteur pipette and transferred into LB broth. The cut bacteriophage plates were mixed in the broth medium and the bacteriophages were allowed to diffuse into the medium. This bacteriophage suspension was diluted again and incubated at 37 °C for 18 hours using the double layer agar method. This process was repeated for three days. The final medium phage mixture was used as purified bacteriophage stock.

Determination of lysis titer of bacteriophage

The bacteriophage stock was diluted to 10^{-8}. Bacterial strains incubated in slant agar aerobically for 24 hours at 37°C, and after incubation, they vortexed with 4.5 ml of LB broth, and 100 µl taken from it and transferred to another sterile tube. 1 ml of bacteriophage dilutions was added to each bacterial culture and 3 ml of soft LB agar was added on them and poured into petri dishes containing LB agar, so the double layer agar method was applied. The number of bacteriophages in the starting stock was determined by calculating pfu/ml on 30-300 plaque-formed petri dishes (Li et al.,2016, Clokie and Kropinski, 2009).

Host range

Strains susceptible to bacteriophages were determined by applying the "spot test" protocol on each of the bacterial strains.

Determination of optimal MOI of bacteriophages

The host bacteria were incubated at 37 ^{0}C for 16 hours and adjusted to 1x10^7 cfu/ml with a spectrometer. 3x10^7 pfu/ml phage solution and *E.coli* were mixed together to the MOI

would be 10, 1, 0.1, 0.01 and 0.001, respectively. Each tubes were kept at 37 ^{0}C for 3 hours and centrifuged at 5,000 g for 20 minutes and the supernatants were collected. They were filtered through 0.22 µm pores and the highest calculated titer was determined as the optimal multiplycity of infection (Li et al.,2016).

Determination of one-step growth parameters

Bacteriophage samples were mixed with *E.coli* strains (3x108 cfu/ml) with a MOI of 1 and kept at 55 ^{0}C for 10 minutes. After centrifugation at 10,000 g for 5 minutes, cells were collected, transferred to 1 ml of fresh medium and mixed. This process was repeated 3 times, so that non-absorbed phage particles were removed. 10 µl of the sample was taken into 100 ml broth medium and incubated at 55 ^{0}C for 100 min. The phage titer in the culture was tested with the double layer agar technique at 10 min intervals (Lin et al., 2011).

3. RESULTS AND DISCUSSION

Isolation and purification

The lytic bacteriophage of the host bacterium was isolated from environmental samples. The plaques observed in the double layer agar method were smooth-edged and 1.00-1.10 mm in diameter.

Determination of bacteriophage titer

Petri dishes consisting of 30 plaques (dilution ratio=10^{-6}) were selected for the *E.coli* O157:H7 (RSKK 09007) phage. It was calculated with formula;

Pfu/ml= Number of plaques / amount of phage stock (ml) x Dilution rate

The stock titer for *E.coli* O157:H7 (RSKK 09007) phage was calculated as 3x10^7 pfu/ml.

Host range

Results showed that bacteriophage NL1 completely lysed the *E.coli* O157:H7 (RSKK 09007) and *E.coli* O:164 RSKK 324. In this study, the effects of phage NL1 only on *E.coli* O157:H7 were investigated.

One-step growth curve

Results showed that the highest phage titer of 2.16x10^8 was obtained when the MOI value was 1 (Table 2).

Table 2: Optimal multiplycity of infection (MOI) of *E.coli* O157:H7 phage

***** The calculated highest titer was determined as Optimal multiplycity of infection

MOI	**Phage concentration**	**Bacteria solution concentration**	**Phage lysis titer after 6 hours**
10	$3x10^7$	$1x10^7$	$4.22x10^7$
1	$3x10^7$	$1x10^7$	***$2.16x10^8$**
0.1	$3x10^7$	$1x10^7$	$4.44x10^7$
0.01	$3x10^7$	$1x10^7$	$2.12x10^7$
0.001	$3x10^7$	$1x10^7$	$1.00x10^7$
0.0001	$3x10^7$	$1x10^7$	$8.00x10^6$

E.coli O157:H7 culture and NL1 phage were mixed at MOI 1 and the titer of NL1 phage was determined every 10 minutes. As seen in **figure 1**, it was determined that the latent period lasted for 20 minutes and the phage was reached the burst size at approximately 90 minutes.

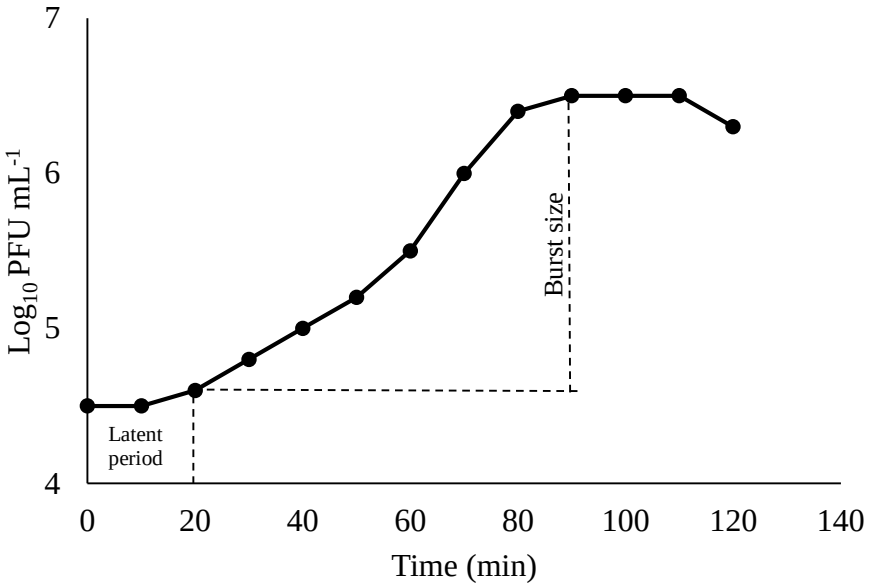

Figure 1. One-step growth curve of NL1

The increasing importance of the resistance problem in bacteria has led to the importance of anti-infective models in modern medicine and biotechnology. There can be resistance to bacteriophages, but this has been poorly documented in the scientific literature to date. The increase in antibiotic-resistant bacteria, as well as the shortcomings in the development of effective new classes of antibiotics, brought up phage's use in the treatment

of infections (Sulakvelidze et al., 2001). As decontamination with phages and local phages are important in treatments, phage diversity is also very important. While developing resistance to a phage, the other phage or phages destroy the target bacteria. Therefore, local phages to be isolated against a bacterium is important (Boyd and Brüssow, 2002).

Various preparations have been prepared and used for phage therapy since the discovery of phages. United States The Food and Drug Administration (US FDA) confirmed the use of listeriaphages on the surfaces of meat and poultry products in 2006 (Kim and Kathariou, 2009). Today, experimental studies are being carried out to protect dental health (Chkonia I. et al., 2012), fight against mastitis (Donjacour A. and Paros M., 2012), meningitis and sepsis (Pouillot F. and Gabard J., 2012), to ensure milk hygiene (Endersen E. et al., 2012).

In our study, the characterized *Escherichia* phage NL1 was found to have broad host range activity infecting *E. coli* isolates that belonged to two different pathotypes of EIEC and EHEC. In some studies, it has been reported that isolated phages show a wide host spectrum. In a study, it was shown that the phage acting on *Klebsiella* is effective on different serotypes. In their study, Monahar et al. reported that applying bacteriophage cocktail on bacterial strains of different species significantly reduced the bacterial load within 24 hours (Monahar et al., 2018). In another study, it was determined that the absorption of T4 phage into the polycaprolactone (PCL) film used packaging raw beef contaminated with *E. coli* O157:H7 showed approximately 30 times more inhibitory effect (Choi et al.2021). In some studies, it has been stated that phages inhibit *E.coli* biofilm formation (Ribeiro et al. 2018, Alves et al. 2014, Dorlan 2009).

It has been shown that the use of antibiotics and phages together may have a synergistic effect and it has been stated that it can prevent antibiotic resistance that will occur with the use of antibiotics or phages alone (Valério et al. 2017, Zhang 2012, Kirby 2012).

In our study, the phage we named NL1 showed lytic effect in *E.coli* O157:H7 (RSKK 09007) and *E.coli* O:164 RSKK 324 strains. *E.coli* O157:H7 is known to cause foodborne outbreaks and it was thought that NL1 could be used in the treatment of E. coli. Since it is known that bacteriophage cocktails are more effective with a synergistic effect, it has been evaluated that NL1 can also be used in phage cocktails. Again, it is thought that it may have a synergistic effect when used with antibiotics. Based on the lytic effect of NL1 on both *E.coli* O157:H7 (RSKK 09007) and *E.coli* O:164 (RSKK 324) strains, cross-antigenic structures between the two strains can be investigated and this new phage can be used for typing.

4. CONCLUSION

In this study, properties different from the physiological properties of previously isolated phages specific to *E. coli* O157:H7 strain were determined. The results of this study need to be expanded and advanced for the biocontrol of *E. coli* O157:H7, one of the important pathogens that can cause epidemics. Determining the identity of the isolated NL1 phage by molecular characterization methods will guide other studies.

Conflict of Interest

The authors wish to declare that they have no conflict of interest

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Authors contribution

The authors contributed equally to the study.

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