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ORIGINAL ARTICLE / ÖZGÜN MAKALE

THE EFFECT OF *ESCHERICHIA COLI* BACTERIOPHAGE COCKTAIL ON BACTERIAL CONTAMINATION IN WATER

ESCHERICHIA COLI BAKTERİYOFAJ KOKTEYLİNİN SUDAKİ BAKTERİ KONTAMİNASYONU ÜZERİNE ETKİSİ

Hilal Basak EROL[1]*, Aylin KESKİN[2]

[1]Ankara University, Faculty of Pharmacy, Department of Pharmaceutical Microbiology, 06560, Ankara, Türkiye
[2]Ankara University, Faculty of Pharmacy, 06560, Ankara, Türkiye

ABSTRACT

Objective: *Wastewater and environmental water resources are treated to eliminate pathogenic microorganisms but contamination is still a significant problem. In particular, the presence of contamination with Escherichia coli (E. coli) is an important indicator of fecal contamination. Due to increasing antimicrobial resistance and failures of new antimicrobial processes, interest in bacteriophages in pathogen control has increased. Therefore, in our study, phage-based bacteria control in environmental waters was investigated as a natural solution.*
Material and Method: *In our study, E. coli and lytic bacteriophages specific to these E. coli were isolated from environmental water samples in Ankara. The lytic activities of the isolated phages were determined on environmental and clinical extended-spectrum β-lactamases E. coli isolates. Three phages with high lytic activity were selected, and the effectiveness of the single phage and their mixtures on E. coli contamination in water was tested.*
Result and Discussion: *As a result of the study, 17 E. coli strains were isolated from 30 environmental water samples. Lytic bacteriophages in 30 different plaque structures were also isolated from water samples. The isolated phages were found to have lytic activity in the range of 32-70% on the tested bacteria. The effectiveness of three selected phages and their cocktail on E. coli contamination in water was measured at 6th and 24th. As a result, it was observed that the cocktail application reduced the number of host bacteria in the water below detectable limits, also provided a 5-log reduction in non-host test bacteria and maintained its effect for 24 hours. When the results are evaluated, it is thought that cocktail phage application will be an effective method against E. coli contamination in water.*
Keywords: *Escherichia coli, phage application, phage cocktail, water contamination*

ÖZ

Amaç: *Atık su ve çevresel su kaynaklarında patojen mikroorganizmaları ortadan kaldırmak için arıtma yapılsa da bulaş hâlâ önemli bir sorundur. Özellikle Escherichia coli (E. coli) ile kontaminasyonun varlığı dışkı ile kontaminasyonun önemli bir göstergesidir. Artan antimikrobiyal direnç ve yeni antimikrobiyal geliştirme süreçlerindeki başarısızlıklar nedeniyle patojen kontrolünde bakteriyofajlara olan ilgi artmıştır. Bu nedenle çalışmamızda doğal bir çözüm önerisi olarak çevresel sularda faj bazlı bakteri kontrolü araştırılmıştır.*
Gereç ve Yöntem: *Çalışmamızda Ankara ili çevresel su örneklerinden E. coli ve bu bakterilere özgü litik bakteriyofajlar izole edilmiştir. İzole edilen fajların litik etkinlikleri çevresel ve klinik E.*

* **Corresponding Author / Sorumlu Yazar:** Hilal Basak Erol
e-mail / e-posta: hcuhadaroglu@ankara.edu.tr, **Phone / Tel:** +903122033187

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coli izolatları üzerinde belirlenmiştir. Yüksek litik etkinliğe sahip 3 faj seçilerek, fajların tek tek ve karışımlarından hazırlanan kokteylinin sudaki E. coli kontaminasyonu üzerine etkinlikleri test edilmiştir.

Sonuç ve Tartışma: *Çalışma sonucunda 30 çevresel su örneğinden 17 E. coli suşu izole edilmiştir. Su örneklerinden aynı zamanda 30 farklı plak yapısında litik bakteriyofaj izolasyonu sağlanmıştır. İzole edilen fajların test edilen bakteriler üzerinde %32-70 aralığında litik etkinliğe sahip oldukları bulunmuştur. Seçilen üç fajın ve bunlardan hazırlanan kokteylin sudaki E. coli kontaminasyonu üzerine etkinliği 6 ve 24. saatte ölçülmüştür. Bunun sonucunda kokteyl uygulamasının sudaki konak bakteri sayısını tespit edilebilen sınırların altına düşürdüğü ayrıca konak olmayan test bakterilerinde 5 log azalma sağladığı ve etkisini 24 saat süresince de koruduğu görülmüştür. Sonuçlar değerlendirildiğinde kokteyl faj uygulamasının sudaki E. coli kontaminasyonuna karşı etkin bir yöntem olacağı düşünülmektedir.*

Anahtar Kelimeler: *Escherichia coli, faj kokteyli, faj uygulaması, su kontaminasyonu*

INTRODUCTION

Water that is safe for health and drinkable must have certain properties. These include the absence of pathogenic microorganisms, the absence of toxic or harmful substances within certain limits or at all, clarity, absence of saltiness, absence of offensive odors and tastes, not being hard enough to cause economic damage, and not being corrosive [1]. Quality water for human health is directly related to socio-economic development and the demand for safe drinking water is increasing. However, water resources are limited around the world, resulting in water inequality. To reduce this inequality, effective treatment and reuse of wastewater is important [2]. More than 80% of wastewater produced by society worldwide returns to the ecosystem without treatment or reuse, and as a result, 1.8 billion people use a drinking water source contaminated with feces. It causes approximately 842,000 deaths each year due to unsafe water and hygiene conditions. 663 million people around the world do not have access to reliable water sources [3].

Water-related infections occur as a result of contamination of water sources. Pathogens such as *Escherichia coli* (*E. coli)* are major causes of waterborne diseases, and poor sanitation and storage conditions contribute to the spread of these pathogens [4]. An increase in antibiotic-resistant bacteria may further complicate the problem of microbiological water pollution. *E. coli* is an indicator bacterium that gives clear evidence of fecal contamination. *E. coli* is the most notable example of fecal contaminant bacteria because of the variety of pathogenicity mechanisms and diseases it can cause [5].

These untreated water sources cause global diseases. Although antibacterial agents are widely used to kill microorganisms, they can lead to antibiotic resistance. This is an important public health problem and shows that more precise and efficient methods need to be developed for water pollution control [6]. Bacteriophages play an important role in controlling water pollution. They are preferred due to their short replication times and their lack of harm to non-pathogenic bacteria. Bacteriophages are used in many applications in environmental fields, from water purification systems to monitoring water resources. As a result, water quality is critical for human health and socio-economic development, and the need for new methods for water pollution control is increasing. One of these methods is research on bacteriophages [7-9].

One of the most successful therapeutic interventions in the history of medicine, antibiotics have played an important role in achieving medical breakthroughs such as fighting infections, organ transplants, and even cancer chemotherapy [10]. Antibiotics are used in many medical conditions. Therefore, it is thought that the decrease or loss of their effectiveness will cause a disaster. Unfortunately, we are rapidly entering a period called the "post-antibiotic era" [11]. Although bacteriophage therapy is not a new treatment approach, it is seen as a new hope for resistance to antimicrobials. Approximately a century ago, the first report on the effectiveness of bacteriophage therapy was reported. Bacteriophages or phages are small viruses ranging in size from 20 nm to 200 nm. Their proliferation and dissemination are particularly depend on the biosynthetic pathways of bacteria [12].

Phages play an important role in the ecosystem and were discovered independently by two scientists - Frederick Twort in 1915 and Felix d'Herelle in 1917. Temperature, nutrients, light and other

environmental factors affect the formation of new phages [13]. Their genetic material (DNA or RNA) is encapsulated with capsid proteins [14]. Phages have lytic or lysogenic life cycles. In the lytic cycle, host DNA is degraded and different proteins, such as capsid protein and lysis protein, are formed [15]. Phages are the most widespread viruses on the biosphere. They are easily found and isolated wherever bacteria are found [16]. The most important advantages of bacteriophages are that they are cheap and easy to obtain, as well as protecting the natural microbiota and being non-toxic. In addition, phages can go to where they are needed and multiply, regardless of the application method, and they act and show activity regardless of the antibiotic resistance in that area [17]. In order for phages to be used therapeutically, they must (1) preferably be lytic, (2) have a wide host range, and (3) be fully characterized without side effects. Considering these features, the development of therapeutic phages requires the coordinated work of multiple stakeholders [18]. Currently, great progress has been made in bacteriophage research. The potential application of phages as therapeutic agents in different hospitals, clinics and food industries in different parts of the world, especially in western countries, has increased due to the increase in antimicrobial resistance of different bacterial pathogens. In our study, *E. coli* and lytic phages specific to them was isolated from environmental water samples. We also investigated phage-based bacteria control in water contaminated with *E. coli*.

MATERIAL AND METHOD

Isolation of Bacteria

Water samples were taken into sterile bottles from 0.3-0.6 meters below the surface of Ankara environmental water resources (Cubuk Stream, Mogan Lake, Eymir Lake) and transported to the laboratory in an ice box and studied within 4 h. Water samples taken for bacterial isolation were added to tubes containing 3 ml of Tryptic Soy Broth (Merck, Germany) liquid medium and incubated at 37°C in an oven overnight. The next day, the samples were inoculated into the CHROMagar *E. coli* selective medium and the isolates giving blue colonies were selected as *E. coli* [19]. The isolated bacteria were stored in 20% glycerol at -20°C.

Isolation of Bacteriophages

For bacteriophage isolation, phage enrichment was first performed [20]. For this purpose, the environmental water samples taken were first centrifuged at 10000 rpm and passed through a 0.22 µm membrane filter. Water filtrates were incubated overnight with fresh bacterial cultures in x2 Luria Bertani (LB) broth (Merck, Germany) medium enriched with $CaCl_2$ and $MgSO_4$ at 37 °C for one-night. The following day, the suspension was centrifuged at 10000 rpm for 10 min. The double layer agar method was used to determine the presence of phages. Host *E. coli* bacteria in log phase were added to the phage suspension and waited for 10 min. Simultaneously, the soft agar (0.6% agar) was heated and cooled to 45 °C. 3750 µl of soft agar was added to this mixture and poured onto LB agar medium. After overnight incubation, the petri dishes were evaluated for the presence of phage plaques. A single plaque was isolated for purification in petri dishes showing bacteriophage plaque. After the phage plaques were selected with a Pasteur pipette, they were placed in tubes containing 4000 µl LB broth. Then 100 µl bacteria were added. Then, 4000 µl of LB broth was added to the tubes. This process was repeated at least five times until a uniform plaque was seen in the petri dish.

Determination of Host Ranges

Concentrations of isolated and purified bacteriophages were calculated as plaque-forming units (PFU/ml). The host range of phages was determined on a total of 37 bacteria using our own newly isolated *E. coli* isolates (n:17) and clinical *E. coli* isolates (n:20) from the culture collection of Ankara University, Department of Pharmaceutical Microbiology.

In test procedure, a suspension of 10^8 CFU/ml concentration of each test bacteria were prepared from fresh cultures and strips were sown on the agar plate, and 10 µl of 10^8 PFU/ml concentration of bacteriophage suspensions were dropped onto these areas. After one-night incubation, the inoculation areas were evaluated for bacterial growth [21].

Effect of Bacteriophages on Water Contamination

Water biocontrol with bacteriophages was carried out according to Kauppen et al. method with some modifications [22]. The environmental water sample was autoclaved prior to tests. Phage biocontrol tests were carried out with the Ea1, Ea3 used for host bacteria (used in phage production) test and Ea6, Ea7, and Ea8 used for non-host bacteria (outside the range of phage effect) test. Bacterial concentration of sterile environmental water samples was adjusted to McFarland 0.5 turbidity with fresh bacterial cultures mix.

Five aliquots of 10 ml of water sample with bacteria were collected in sterile tubes. The first sample is considered as a control (not added phage). The second, third and the fourth samples were inoculated respectively with 2 ml volume of F4, F14, and F23 phage suspension at titter of 10^8 PFU/ml. The last tubes were inoculated with 2 ml volume of phage cocktail (F4+F14+F23). The samples were incubated at 37°C for 24 h. Triplicate 100 ml samples were taken after 6 and 24 h. At the end of the period, samples were taken from each tube, diluted and 20 µl inoculated on LB agar. All petri dishes were incubated overnight at 37 °C. The following day, the number of viable bacteria in each petri dish was calculated. Phage biocontrol test without the host were also performed for each phage.

Statistical Analysis

Each experiment was repeated three times. The results were presented as mean values and standard deviation values of the mean. One-way Anova Kruskal Wallis test ($p < 0.05$; GraphPad Prism version 5) was used to determine statistically significant differences between the treatment and control groups.

RESULT AND DISCUSSION

E. coli is an important cause of urinary tract and gastrointestinal infections in humans and is an indicator of wastewater contamination of water, food and agricultural products [23]. Pathogens such as *E. coli, Staphylococcus aureus and Campylobacter jejuni* are generally detected in biological wastewater treatment systems [24]. These pathogens are adsorbed by activated sludge and, although they can be removed with more sludge, the presence of pathogens can often be detected in wastewater and pose potential health risks to consumers or environmental water supplies [25]. Therefore, it is necessary to remove as many pathogens as possible during the biological wastewater treatment process. Compared to physicochemical treatment methods, the use of pathogen-specific phage control systems may offer an effective solution [26,27].

In our study, 30 water samples were taken from Ankara province between April to October, 2023. From the water samples taken, 17 bacteria were isolated and purified in CHROMagar *E. coli* selective medium. Location and time information of the isolated bacteria are given in Table 1.

Table 1. Location and time information of the isolated bacteria

Water Number	*E. coli* isolates	Time (month)	Location
1	Ea1	April	Golbası (Mogan lake)
2	Ea2	April	Golbası (Mogan lake)
3	-	April	Golbası (Mogan lake)
4	-	April	Golbası (Mogan lake)
5	-	April	Golbası (Mogan lake)
6	Ea3	May	Cubuk Stream (Etimesgut)
7	Ea4	May	Cubuk Stream (Etimesgut)
8	Ea5	May	Cubuk Stream (Etimesgut)
9	-	May	Cubuk Stream (Etimesgut)
10	Ea6	May	Cubuk Stream (Akköprü)
11	Ea7	May	Çubuk Stream (Akköprü)
12	Ea8	May	Çubuk Stream (Akköprü)
13	-	June	Ankara University Faculty of Science Artificial Lake
14	Ea10	June	Ankara University Faculty of Science Artificial Lake

Table 1 (*continue*). Location and time information of the isolated bacteria

Water Number	*E. coli* isolates	Time (month)	Location
15	-	June	Ankara University Faculty of Science Artificial Lake
16	Ea9	June	Cubuk Stream (Gumusdere)
17	-	August	Cubuk Stream (Gumusdere)
18	-	August	Cubuk Stream (Gumusdere)
19	Ea11	August	Cubuk Stream (Gumusdere)
20	-	August	Cubuk Stream (Gumusdere)
21	Ea12	August	Cubuk Stream (Gumusdere)
22	-	August	Cubuk Stream (Gumusdere)
23	Ea13	August	Ankara University Faculty of Science Artificial Lake
24	Ea14	August	Ankara University Faculty of Science Artificial Lake
25	Ea15	August	Cubuk Stream (Gumusdere)
26	-	August	Cubuk Stream (Gumusdere)
27	Ea16	September	Cubuk Stream (Gumusdere)
28	-	September	Eymir lake
29	-	October	Eymir lake
30	Ea17	October	Eymir lake

For phage isolation, 4 water samples and 8 bacterial isolates (host bacteria) were used. In our study, 30 different plaques were selected from the water samples studied and purified, and their concentrations were calculated as plaque-forming units (PFU/ml). The host bacteria of the phages, the water samples they were isolated from, and their concentrations are given in Table 2. Some plaque images of isolated and uniformly purified phages are seen in Figure 1.

Adhesion of phage to bacteria depends on the relationship between host cell surface receptors and phage binding structures [26]. Phages are assumed to have a narrow host range by nature, which is one of the main issues limiting their use in therapy. However, studies report that some phages are effective on different serotypes of the tested host bacteria and even on different types of bacteria [28]. Yamaki et al. reported that the EscoHU1 phage they characterized was effective against different serotypes of *E. coli*, Salmonella, Citrobacter and Shigella and had a wide host range [29]. As a result of the host range test of 30 phages isolated in the study, it was found that the phages had lytic activity in the range of 32-70%. The table containing the host ranges of the phages is given in supplementary file. The host ranges of the phages isolated in our study were tested not only on environmental *E. coli* isolates but also on clinical extended-spectrum β-lactamases *E. coli* isolates. It has been observed that environmental phage isolates also show high lytic activity on clinical strains.

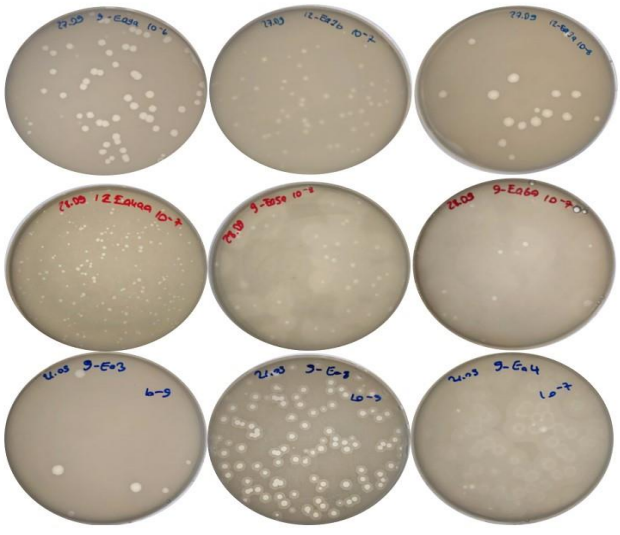

Figure 1. Different plaque images of isolated phages

Table 2. List of isolated bacteriophages

Phage No	Host bacteria	PFU/ml
F1	Ea4	$4.4x10^{9}$
F2	Ea4	$6.8x10^{8}$
F3	Ea1	$0.3x10^{8}$
F4	Ea1	$0.5x10^{8}$
F5	Ea2	$1.1x10^{3}$
F6	Ea2	$2.3x10^{5}$
F7	Ea2	$1.6x10^{10}$
F8	Ea3	$0.1x10^{1}$
F9	Ea3	$0.3x10^{8}$
F10	Ea3	$0.6x10^{6}$
F11	Ea3	$2.2x10^{8}$
F12	Ea5	$0.9x10^{8}$
F13	Ea2	$0.3x10^{8}$
F14	Ea3	$6.5x10^{9}$
F15	Ea4	$4.4x10^{9}$
F16	Ea5	$4.4x10^{10}$
F17	Ea6	$1.3x10^{9}$
F18	Ea7	$0.3x10^{6}$
F19	Ea8	$4.7x10^{10}$
F20	Ea9	$0.3x10^{8}$
F21	Ea1	$2.2x10^{12}$
F22	Ea2	$0.3x10^{8}$
F23	Ea3	$1.6x10^{10}$
F24	Ea3	$6.4x10^{9}$
F25	Ea4	$2.09x10^{10}$
F26	Ea4	$6.5x10^{9}$
F27	Ea4	$1.96x10^{10}$
F28	Ea5	$0.3x10^{8}$
F29	Ea6	$0.5x10^{8}$
F30	Ea7	$2.2x10^{12}$

In this study, the activities of F4, F14, and F23 phages, selected for their high lytic activities, on *E. coli* in water, alone and with a cocktail prepared from a mixture of three phages, were investigated. Two-time parameters (6 h and 24 h) were tested in the study. The bacterial concentration of the water sample was increased with the host bacteria Ea1 and Ea3 of the selected phages and the number of bacteria was calculated after phage treatment. At the same time, the test was repeated with environmental *E. coli* isolates (Ea6, Ea7, Ea8), which were not host bacteria of the phages and at least one phage was effective.

In the test protocol using host bacteria (Figure 2A), it was observed that *E. coli* was completely eliminated in the water in the samples treated with F4 and phage cocktail as a result of the first 6 h. Here, it is thought that the F4 phage's complete destruction of bacteria is due to its ability to completely lysis both bacteria. It is observed that F14 and F23 phages cause a 1-2 log decrease in bacterial concentration compared to the control, but cannot completely destroy the bacteria. In addition, it is seen that the effects of phages decrease over time and the effect almost completely disappears after 24 h. Since F4 and phage cocktail completely eliminated the bacteria in the first 6 h, no bacterial growth was observed in 24 h.

In the test protocol with non-host bacteria (Figure 2B), it was observed that all single phage applications showed low levels of effect at 6 and 24 h, as it is known that phages show low sensitivity to a single bacterium. However, it was found remarkable that in the sample where phage cocktail was applied, the cocktail reduced the bacterial density by ~5 log and this effect continued at the 24 h.

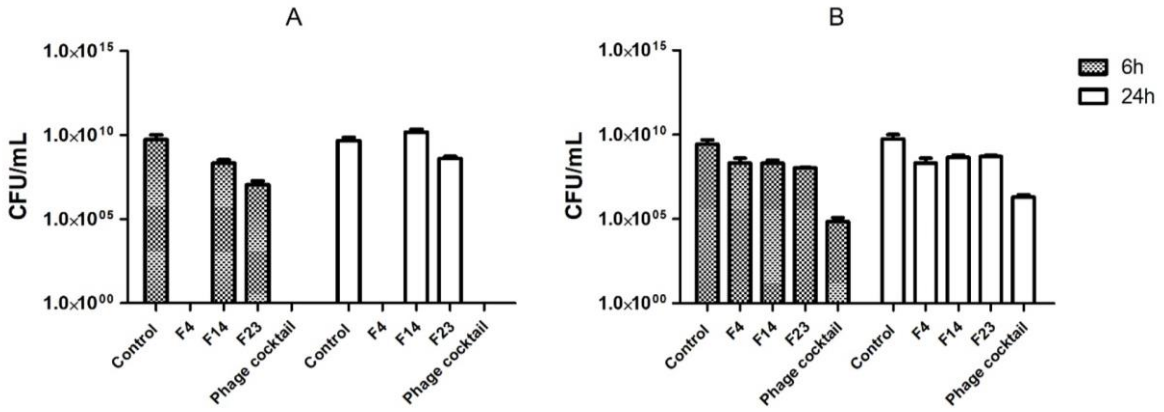

Figure 2. The effect of phages and phage cocktail on contaminated water with *E. coli* (**A.** Host bacteria, **B.** Non-host bacteria)

In literature, mixing phages and using them as cocktails provides advantages in limiting the formation of phage resistance and increasing the effective range of the phage [30]. Turki et al. tested single, double and triple use of phage on Salmonella in wastewater and reported that the cocktail consisting of three phages showed the best removal effect against bacteria [31]. Yu et al. reported that phage cocktails with a wide range of activity were more effective than phage cocktails with a narrow host range in suppressing multidrug-resistant *E. coli* NDM-1 in activated sludge systems [32].

Phage studies specific to the pathogens (*E. coli, Staphylococus aureus, Salmonella spp., Pseudomonas aeruginosa, Acinetobacter baumannii* and *Klebsiella pneumoniae)* in water can be found in the literature. Dhevagi and Anusuya reported that the addition of *E. coli* and Salmonella phages reduced the number of pathogens in sewage sludge [33]. Maal et al. reported that they reduced the coliform value of municipal sewage by 22-fold (from 2400 to 110) using the most probable number method after two hours of incubation with the coliphage mixture they isolated from [34].

In conclusion, the present study *E. coli*-specific lytic bacteriophages were isolated from environmental water samples. The isolated phages showed high lytic activity on environmental and clinical *E. coli* strains. The effectiveness of the cocktail prepared from 3 phages, selected due to their high activity, on *E. coli* contamination in water was tested. As a result, it was observed that the cocktail application reduced the number of host bacteria in the water below detectable limits and also provided a 5-log reduction in non-host test bacteria. It is thought that phage cocktail application will be an effective method against *E. coli* contamination in water.

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AUTHOR CONTRIBUTIONS

Concept: H.B.E., A.K.; Design: H.B.E.; Sources: H.B.E., A.K.; Materials: H.B.E., A.K.; Data Collection and/or Processing: H.B.E., A.K.; Analysis and/or Interpretation: H.B.E., A.K.; Manuscript Writing: H.B.E., A.K.; Critical Review: H.B.E.; Other: -

CONFLICT OF INTEREST

The authors declare that there is no real, potential, or perceived conflict of interest for this article.

ETHICS COMMITTEE APPROVAL

The authors declare that the ethics committee approval is not required for this study.

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