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Investigation of *Escherichia coli* O157:H7 and *Salmonella* Bacteriophages in Cattle Fecal Sources

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ABSTRACT *S.* Typhimurium and *E. coli* O157:H7 are the most important foodborne pathogens forming bacterial biofilms that contribute to their virulence, antimicrobial resistance, and surface survival, causing severe food poisoning outbreaks worldwide. Bacteriophages are antibacterial agents that are increasingly used to control foodborne pathogens, and they also play a role in the solution against the development of antibiotic resistance. In addition, bacteriophages can be found in wastewater, natural and animal wastes, and foodstuffs. Aim of this study to determine the purification and lytic effects of *Salmonella* spp. and *E. coli* specific phages circulating in our country, which can effectively combat common *Salmonella* spp. and *E. coli* infections in our country and the world by using samples taken from the cowshed. In this study, 3 *S.* Typhimurium and 1 *E. coli* O157:H5 bacteriophages were isolated, and their lytic activities were determined. As a result, it is thought that the lytic activities of *S.* Typhimurium and *E. coli* O157:H7 bacteriophages purified from Aksaray province in this study can shed light on the treatment of *S.* Typhimurium and *E. coli* O157:H7 infections and prevention studies in the food industry.

Keywords: *Bacteriophage, E. coli O157:H7, Fecal, Purification, S.Typhimurium.*

ÖZ

Sığır Fekal Örneklerinden *Salmonella* spp. ve *Escherichia coli* O157:H7'ye Özgü Bakteriyofaj Varlığının Araştırılması

Salmonella Typhimurium *(S.* Typhimurium*) ve Escherichia coli (E. coli)* O157:H7, virülanslarına, antimikrobiyal dirençlerine ve yüzeylerde hayatta kalmalarına katkıda bulunan bakteriyel biyofilmler oluşturan ve dünya çapında ciddi gıda zehirlenmelerine neden olan önemli gıda kaynaklı patojenlerdir. Bakteriyofajlar, gıda kaynaklı patojenleri kontrol etmek adına her geçen gün artarak kullanılan antibakteriyel maddeler olup ayrıca antibiyotik direncin gelişmesine karşı çözümde rol oynamaktadır. Ayrıca bakteriyofajlar atık sularda, doğada ve hayvansal atıklarda olabileceği gibi birçok gıda maddesinde bulunmaktadır. Bu çalışmanın amacı sığır altlıklarından alınan dışkı örnekleri kullanılarak ülkemizde ve dünyada sık rastlanan *Salmonella* türleri. ve *E. coli* infeksiyonları ile mücadele etmede etkili olabilecek, ülkemizde sirküle olan *Salmonella* spp. ve *E. coli* spesifik spot test ile fajların purifikasyonu ve litik etkilerinin belirlenmesi amaçlanmıştır. Bu çalışmada 3 tane *S.* Typhimurium ve 1 tane *E. coli* O157:H7 bakteriyofajları izole edilerek, litik aktiviteleri belirlenmiştir. Sonuç olarak bu çalışmada Aksaray ilinden pürifiye edilen *Salmonella* Typhimurium ve *E. coli* O157:H7 bakteriyofajlarının tespit edilen litik aktiviteleri ile *Salmonella* Typhimurium ve *E. coli* O157:H7 kaynaklı enfeksiyonların tedavisi ve gıda endüstrisinde korunma çalışmalarına ışık tutabileceği düşünülmektedir.

Anahtar Kelimeler: *Bakteriyofaj, E. coli O157:H7, Fekal, Pürifikasyon, S. Typhimurium.*

INTRODUCTION

Salmonella spp. and *E. coli* are zoonotic foodborne pathogens of worldwide importance (EFSA 2021). Salmonella spp. is estimated to cause 93 million enteric infections and 155,000 deaths worldwide yearly (Ritter et al. 2019). Although *E. coli* is found in the gastrointestinal tract of animals and humans, it has many pathogenic serogroups (Wasteson 2001; Zhu et al. 2022). *E. coli* O157:H7 is often isolated in cattle and poultry products (Schoeni and Doyle 1994; Naylor et al. 2005). In addition, it is reported that *Salmonella* and *E. coli* can adhere, colonise and form biofilms on various food surfaces and fresh products, exacerbating their dangers (Zhu et al. 2022).

In the one health context, *Salmonella* spp. and *E. coli* have been reported to have complex routes of transmission

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involving the environment, animal reservoirs and water. Foodstuffs, especially livestock and poultry, are considered the most common sources of *Salmonella* spp. *and E. coli* infections in humans (Yim et al. 2011; Li et al. 2018).

The most prevalent pathogen that still poses a significant threat to the public's health by causing gastroenteritis, enteric fever, infectious diarrhea, and sepsis is *S.* Typhimurium (Ao et al. 2015; Jeon and Ahn 2021). The issue is getting national and international due to the rapid appearance and spread of *Salmonella*, which is resistant to the antibiotics. Due to the limited antibiotic regimens, multidrug-resistant *Salmonella* infections can lead to treatment failure (Wang et al. 2019; Wolput et al. 2022). Therefore, the pharmaceutical sector has long focused on creating new antibiotics. The rate at which multidrug-resistant bacteria developed resistance to newly discovered antibiotics, however, was much slower than the rate at which bacteria developed antibiotic resistance (Hede 2014). Therefore, alternative treatments are urgently required to treat and stop infections resistant to several drugs (CDC 2019).

It has been confirmed use of bacteriophages for treatment and prevention in disease struggles for resistant bacteria in drugs used in treatments. Based on the experience and current results of bacteriophage applications against bacterial infections in countries where alternative therapies have been approved, many scientists and companies have come to believe that the use of phages to treat and prevent bacterial diseases will be successful (Kutateladze and Adamia 2010). Felix-O1 (FO1) phage belonging to *Salmonella* spp. was first described in the 1930s (Ata 2018). It has been reported that they can also be found in many natural foodstuffs, especially in animal wastes and wastewater. *Salmonella* spp. and *E. coli* phages can be isolated from poultry extracts, poultry and farm environments, and wastewater treatment plants. Since the bacteriophages are generally non-toxic and bacteria-specific and affect many microorganisms, the recent resistance of bacteria to the antibiotics has brought phages to an important place in this field (Atterbury et al. 2007).

The life cycle of the phage consists of several steps, including attachment, penetration, replication, virion assembly, and release. Phage adsorption to the surface of host cells is an essential step in species-specific interaction (Rakhuba et al. 2010). The phages can nonspecifically recognise receptors on the host cell surface and then specifically bind to them (Guglielmotti et al. 2012). Although there are many studies of *Salmonella* spp. and *E. coli* bacteriophages in many countries, specific phage studies for *Salmonella* spp. and *E. coli* species in Türkiye are very few, and they are generally purified from poultry litter (Ata 2018; Sahin et al. 2020). Therefore, study aimed to determine the purification and lytic effects of phages that may be effective in controlling zoonotic infections, which are essential for human and animal health, from faeces samples taken from litter in cattle farms.

MATERIAL AND METHODS

Bacterial Strain and Culture Conditions

This study was approved by Aksaray University Experimental Animal Practice Center Ethics Committee (Aksaray, Türkiye) (RPN: 2023/5-18).

All litter samples in this study were taken from healthy cattle farms in Aksaray province and delivered to the laboratory within 24 hours at +2 – 8 °C under cold chain conditions. To examine the lytic activity of infective phages against *Salmonella* spp. and *E. coli* O157:H7 taken in this study, a total of 50 stool samples were obtained from litters of 38 cows and 12 calves from 50 different farms with the help of transportable swabs. The samples delivered to the laboratory were diluted 1:10 with physiological saline. After the debris part precipitated, it was centrifuged at 13,000g at 4 °C for 10 minutes, and the supernatant parts were passed through a 0.2 µm filter and used as a phage source (Sambrook et al. 1989; Higgins et al. 2005). *Salmonella and E. coli* strains were obtained from field strains from Aksaray and Yuzuncu Yil University Veterinary Faculty Veterinary Microbiology Culture Collections to determine the lytic activities of phages.

Bacteriophage Isolation

Salmonella spp. phages were purified by direct isolation and modified double layer agarose method by enriching with *Salmonella* Typhimurium, *S.* Dublin, *S. enterica*, *E. coli*, and *E. coli* O157:H7 field isolates. For direct isolation, samples were mixed with a 1:10 magnesium-salt buffer (50 mM Tris-HCl [pH: 7.5], 0.1 M NaCl, eight mM MgSO4) followed by filtration through a 0.45 mm bottle-top filter and a two-step procedure through a 0.2 mm syringe attached filter. After the filtered filtrate was thickened at 55 °C by adding 0.7% Nutrient agar, passages of 4 ml to 1.5% TSA agar were performed and then incubated at 37 °C. For the phage enrichment method, the obtained filtrates used for direct isolation were mixed with Nutrient-broth (Oxoid Ltd., London, England) at a ratio of 1:10 and then 1 mL of bacterial strain was added. After 16 hours of incubation at 37°C, it was subjected to a spot test and modified double-layer agarose method (Switt et al. 2013). For these processes, culture supernatants were obtained by concentrating at 7500 x G for 15 minutes using an ultrafiltration unit with ten kdA Vivaspin® (Sartorius), following manufacturer's recommendations.

Lytic Activity Test

The lysis activity of each of the four bacteriophage isolates was evaluated in the specific host and other hosts. The cross-lytic activities of the phages were determined by assuming that the structure of the isolates could vary depending on the serotype, origin, region, and year differences. First, the lysis activities of bacteriophages were standardized in their hosts, and then their activities in other hosts were determined. For this purpose, spot test and double layer agarose method were used (Holmfeldt et al. 2007).

In the spot agar method, the target bacteria were adjusted to $2x10^4$ cfu/bacteria and spread on Nutrient Agar, and 15 µl phage dilutions were dropped on it. The numbers are determined according to the McFarland method (McFarland 1907). After the plates were left to dry for 30 minutes at room temperature, they were incubated at 37 °C for 18 hours, and the diameters of the lytic activities were evaluated. Lytic activity after various phage dilutions; was recorded using a scale ranging from no fragmentation (-), blurred point (+), clear point (++), and diffuse clear point (+++) (D'Andrea et al. 2020).

In the double-layered agarose method, 100 µl of phage and 300 µl of target bacterial culture were mixed in a sterile bottle, added to 4ml molten LB agar at 44-47 °C, and poured onto the plates.

After incubation, plaque formation and lytic activity were evaluated (Sahin et al. 2020).

RESULTS

In this study, 50 samples, 38 of which were collected from cows and 12 from calf litters, were used to examine the lytic activity of infective phages against *Salmonella* spp. and *E. coli* O157:H7. Of these, bacteriophage activity was determined against *S.* Typhimurium in only three samples and *E. coli* O157:H7 in 1 sample. Two of the samples showing lytic activity with *S.* Typhimurim host bacteria were from calves, and one from cows; the sample showing lytic activity with *E. coli* O157:H7 was obtained from samples taken from cows (Table 1). No lytic activity was detected in *S.* Dublin and *S. enterica* strains. While determining the lytic activity of bacteriophages, McFarland, and *E. coli* O157:H7 and *S.* Typhimurium waters were adjusted to 2×10^4 and controlled on Nutrient Agar. *E. coli* O157:H7 10^{-6}; phages were obtained, in which lytic activity continued until the dilutions of *S.* Typhimurium 10^{-8}, *S.* Typhimurium 10^{-4}, and *S.* Typhimurium 10^{-4}.

Table 2 shows the lytic activities of four bacteriophages in their possible hosts. Accordingly, it was determined that Phage-1 (*E. coli* O157:H7) showed lytic activity in *the E. coli* O157:H7 strain as expected and showed the highest lytic activity with +++ at 10^{-2}. It was determined that it did not give any action in hosts belonging to *Salmonella* species. In other phage's (Phage-2, Phage-3 and Phage-4) (*S.* Typhimurium), it was determined that it gave lytic activity in its born host, *S.* Typhimurium, but did not show lytic activity in *S.* Dublin, *S. enterica* and *E. coli* hosts. The lytic activities of phage-2 were determined as +++ in 10^{-2} and 10^{-4}, ++ in 10^{-6} and + in 10^{-8}. The lytic activities of Phage-3 and Phage-4 were determined with ++ at 10^{-2}.

Dilutions of 10^0, 10^{-2}, 10^{-4}, 10^{-6} and 10^{-8} of bacteriophages were evaluated by spot test using 10 µl by double seeding. It was determined that they gave the best lytic activity in 10^{-2} and $10^{-4,}$ shown (Figure 1).

Table 1: Farm, source, year and regions of the four isolated bacteriophages.

	Farm	Source	Year	Region
Phage 1	3	Dairy cow	2023	Central Anatolia
Phage 2	5	Dairy cow	2023	Central Anatolia
Phage 3	7	Calf	2023	Central Anatolia;
Phage 4	8	Calf	2023	Central Anatolia;

Table 2: Lytic activities of four isolated bacteriophages in *S.* Typhimurium, *E. coli* O157:H7, *S.* Dublin, *S.* enterica, *E. coli* (Field strain) bacteria.

	S. Typhimurium				*E. coli* O157:H7				*S. Dublin*	*S. Enteritica*	*E. coli* (Field strain)			
	10^{-2}	10^{-4}	10^{-6}	10^{-8}	10^{-2}	10^{-4}	10^{-6}	10^{-8}			10^{-2}	10^{-4}	10^{-6}	10^{-8}
Phage 1	-	-	-	-	+++	++	+	-	-	-	+	-	-	-
Phage 2	+++	+++	++	+	-	-	-	-	-	-	-	-	-	-
Phage 3	++	+	-	-	-	-	-	-	-	-	-	-	-	-
Phage 4	++	+	-	-	-	-	-	-	-	-	-	-	-	-

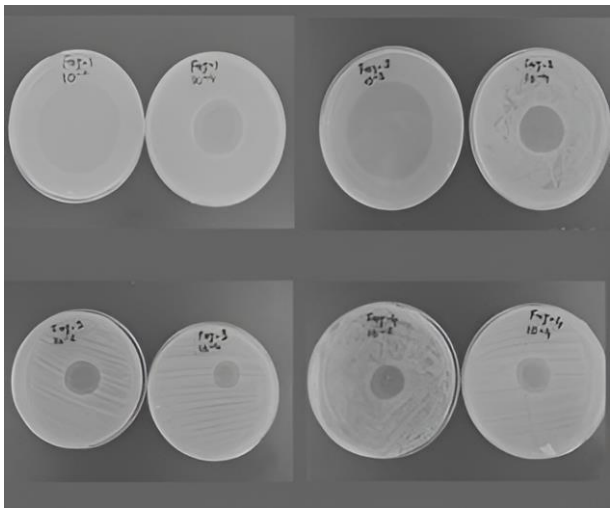

Figure 1: Spot test of lytic activities of isolated *E. coli* O157:H7(Phage-1) and *S.* Typhimurium (Phage-2, Phage-3, Phage-4) bacteriophages.

DISCUSSION AND CONCLUSION

Salmonella spp. and *E. coli* O157:H7 are zoonotic agents that can cause essential epidemics in human and animal health. Especially in recent years, due to the increase in multi-antibiotic resistance, alternative treatment strategies need to be developed (Banin et al. 2017). Recently, the phages have gained renewed attention as potential therapeutic tools due to their specificity (Garrido-Maestu et al. 2019; LeLièvre et al. 2019; Thung et al. 2019). It is vital that phage can be used as an alternative treatment because it is host specific and has less toxicity (Bourdin et al. 2014). It is very promising because it is less economical and because the bacteria rarely develop resistance to phages. It has been reported that bacteriophages cause protection and therapeutic effect in the study of infections caused by many bacteria (Petsong et al. 2019).

The bacteriophages are frequently isolated from poultry farms, poultry meat and faecal samples. Studies in cattle

holdings are rare. Phages can be found in various hosts, and each bacteriophage may have different lytic activity. Bacteriophages isolated from multiple sources are very important in terms of diversity. It has been reported that other *Salmonella* phages were isolated in 45 of 160 stool samples collected from healthy and sick cattle (*S.* Enteritidis bacteriophage 64.4%, *S.* Typhimurium 8.9%). Still, lytic activity did not occur in *E. coli* (Dueñas et al. 2017). Similarly, there are other isolated and purified studies against *Salmonella enterica* serovars (McLaughlin et al. 2006; Akhtar et al. 2014; Duc et al. 2020; Lu et al. 2022; Olsen et al. 2022).

E. coli O157:H7 is a flora bacterium found in the gastrointestinal tract of animals is abundant in faeces. Therefore, it easily contaminates sewage, wastewater, soil, and food. As a result, various wastewater, such as sewage, is known to be the best source for isolating phage that naturally infects *E. coli* O157:H7 (Viazis et al. 2011). Some studies reported that *E. coli* O157:H7 was isolated and purified similarly (Viazis et al. 2011; Litt and Jaroni 2017; Duc et al. 2020).

In a study conducted in the provinces of Nigde, Aksaray, Ankara and Kayseri in Türkiye, it was reported that *S.* Typhimurium was isolated and purified in 33 of 92 samples, and *S.* Enteritidis was isolated in 56 of them (Yildirim et al. 2018a). In another study from the same region, *E. coli* O157:H7 was isolated and purified in 37 of 92 samples (Yildirim et al. 2018b). It was emphasized that phages purified specifically against *S.* Typhimurium and *S.* Enteritidis, and *E. coli* O157:H7, which show lytic activity against *Salmonella* species from foodborne pathogens, can be used as a possible alternative to chemical antimicrobials against these pathogenic bacteria. In another study, it was reported that 13 *S.* Typhimurium and 22 *S.* Enteritidis bacteriophages were isolated and purified in Istanbul (Ang-Kucuker et al. 2000).

In conclusion, this study evaluated *Salmonella* species and bacteriophage specific to *E. coli* O157:H7 strains from litter samples collected from Aksaray province. The collected samples detected phages with lytic activity in 6% *S.* Typhimurium and 2% *E. coli* O157:H7 ratio. Faeces collected from the litters were used as a source for obtaining bacteriophages. These results might lead to phage therapy, food industry, and prevention studies for *Salmonella* species and *E. coli* O157: H7.

CONFLICTS OF INTEREST

The authors report no conflicts of interest.

AUTHOR CONTRIBUTIONS

Idea / Concept: GSG, KA
Supervision / Consultancy: GSG, KA
Data Collection and / or Processing: GSG, KA
Analysis and / or Interpretation: GSG, KA
Writing the Article: KA, GSG
Critical Review: KA, GSG

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