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ISOLATION OF PHAGES INFECTING *LISTERIA MONOCYTOGENES*

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ABSTRACT
It was aimed to isolate, purify and determine host ranges of lytic phages that infect *Listeria monocytogenes* in this study. Out of 68 samples screened, 4 positive isolates were recovered from feces, food processing waste water, fisheries waste water, and fish samples. Recovery status of the *L. monocytogenes* phage was found to be 5.88%. To determine host ranges of phages, soft agar overlay plaque assay was used. While eleven *L. monocytogenes* strains showed resistant to all four isolated phages, 24 strains were sensitive. The plaque sizes of the 4 phages against 24 *L. monocytogenes* strains ranged from 0.4 and 2.6 mm. None of the phages had identical host ranges.
Keywords: *Listeria monocytogenes,* Phage, Isolation, Host Range, Susceptibility

LISTERIA MONOCYTOGENES SPESİFİK FAJLARIN İZOLASYONU

ÖZ
Bu çalışmada *Listeria monocytogenes*'i enfekte eden litik fajların izole edilmesi, saflaştırılması ve konakçı etkinliklerinin belirlenmesi amaçlanmıştır. Toplamda 68 örnek taranmış ve dışkı, gıda işleme atık suları, balıkçılık atık suları ve balık örneklerinden 4 pozitif izolat elde edilmiştir. Faj varlığı bakımından taranan örneklerin % 5.88'inde *L. monocytogenes* fajı izole edilmiştir. Faj konakçı etkinliğinin belirlenmesi amacıyla, çift tabaka agar yöntemi kullanılmıştır. 11 *L. monocytogenes* suşu denenen dört faja karşı dirençli bulunurken, 24 suşta ise duyarlılık saptanmıştır. Dört fajın 24 *L. monocytogenes* suşuna karşı oluşturduğu faj plak çapı ise 0.4 ve 2.6 mm arasında değişmiştir. Hiçbir faj birbiri ile aynı konakçı etkinliği göstermemiştir.
Anahtar kelimeler: *Listeria monocytogenes,* Faj, İzolasyon, Konakçı Etkinliği, Duyarlılık

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INTRODUCTION

Listeria monocytogenes is a Gram (+), rod-shaped facultative anaerobic foodborne bacterium. *L. monocytogenes* has been isolated from soil, rivers, water, silage, animal, plants, the intestinal track of many mammals, and food sources (Gutierrez et al., 2017; Yang et al., 2017). This organism has ability to survive and grow in different adverse environmental conditions, such as low temperature, high salt content, low oxygen levels, and acidic conditions (Perera et al., 2015).Foods can be contaminated with *L. monocytogenes* during fermentation, processing, storage, or even packaging of foods. Ready to eat foods (RTE), dairy products, different types of meats, smoked fish, and sea foods are risky foods in terms of *L. monocytogenes* (Carlton et al., 2005; Lacumin et al., 2016).

It has been associated with a number of serious foodborne outbreaks. It has been reported that listeriosis, caused by *L. monocytogenes*, has a low incidence but high morbidity and mortality (up to 40%) (Sadekuzzaman et al., 2017). The disease is most frequently clinically manifested as septicemia, meningitis or meningoencephalitis, central nervous system infection, and feto-maternal infections. Persons over the age of 65, young persons, pregnant women, and immunocompromised individuals account for approximately 75% of these infections (Klumpp and Loessner, 2013; Perera et al., 2015). In 2014, 675 listeriosis cases were reported, caused by543 hospitalizations and 462 deaths (CDC, 2019). Additionally, the last report from European Food Safety Authority (EFSA) informed that the frozen corn outbreak in 2018 caused 47 cases including 9 deaths (EFSA, 2019).

Bacteriophages (also called phage) were first discovered in 1915 by William Twort, and in 1917 by Felix d'Herelle (Coffey et al. 2010; Clokie et al. 2011). Phages can be lytic or lysogenic. The lytic life cycle is where phages are able to infect and rapidly kill their infected host cells without integrating with the host DNA. A large number of progeny phages are released and then, they able to infect neighboring host cells (Soni and Nannapaneni, 2010; Lone et al., 2016). However, lysogenic life cycle in contrast to lytic cycle is where phages integrate into their host genome, or exist as plasmids within their host cell. It has been reported that lysogenic life cycle can be stable for thousands of generations (Sulakvelidze, 2013).

Listeria phages can be isolated from several of sources by the soft agar overlay method. Sewage plants, silage, food processing environments, foods we eat and even from lysogenic strains can be listed as phage isolation materials. Phages are also normal commensals of humans and animals. They are especially abundant in the gastrointestinal tract (Carlton et al., 2005; Soni et al., 2010). The first *L. monocytogenes* specific phage was published in 1945 (Kim et al., 2008). To date, more than 500 listerial phages have been isolated, while only a limited number has been fully characterized on molecular and genomic level (Hagens and Loessner, 2014). Phages have been found in all major *Listeria* species and serovars. However, no phages for *L. monocytogenes* serovar 3 or *L. grayii* strains or the newly proposed species *L. rocourtii* and *L. marthii* have yet been found. All *Listeria* specific phages, found to date, are the members of the *Caudovirale* (tailed phages) which includes *Myoviridae* (long, inflexible contractile tails), *Siphoviridae* (long, flexible non contractile tails), and *Podoviridae* (short non contractile tails). Although members of both the *Siphoviridae* and *Myoviridae* are common, no *Podoviridae* infecting *Listeria* has yet been found and reported (Klumpp and Loessner, 2013). Many lysogenic *Listeria* strains carrying multiple prophages are determined. Exposure to DNA-damaging agents such as UV light or Mitomycin C can induce the lytic cycle, and also lead to production of infective phage. Temperate *Listeria* phages generally display narrow host ranges, infecting only a small percentage of strains. It is also noteworthy that the temperate phages of *Listeria* appear to be largely serovar-specific (Hagens and Loessner, 2014).

Phages can be regarded as natural predators of bacteria (Carlton et al., 2005; Lacumin et al., 2016). Therefore, phages have been used effectively to control several foodborne pathogens, such as *L. monocytogenes*, *Escherichia coli*

O157:H7, *Salmonella* spp., *Staphylococcus aureus, Campylobacter jejuni*, and *Bacillus cereus* (Guenther et al., 2009; Soni et al., 2010; Akhtar et al., 2017). As of now, two *Listeria* specific phage products are commercially available: ListShield™ (Intralytix Inc, USA) and Listex™ P100 (Micreos Food Safety). These commercial phage preparations have been used for direct food application in different countries including the United States of America, Europe, Canada, and Australia (Yang et al. 2017). Listex™ P100 that is a broad host range myovirus was isolated from a sewage effluent sample taken from a dairy plant in Germany (Carlton et al., 2005). This phage was granted Generally Recognized as Safe (GRAS) status by Food and Drug Administration (FDA) and United States Department of Agriculture (USDA) in 2007 for use in all food products such as cheese, ready to eat meats and poultry, fruits, vegetables, and smoked fish. Listex™ P100 can be used either alone or in combination with a growth limiting antimicrobial (Hagens and Loessner, 2014). Another product is termed ListShield™. ListShield™ (formerly known as LMP-102™) was the first commercial phage preparation approved by FDA and the Environmental Protection Agency. It has also been approved in Canada and Israel. This product is composed of a mixture of six natural and safe phages. This phage cocktail can be used on food and surfaces in food production facilities (Gutierrez et al., 2017; Roy et al., 2018).

Several studies (Özkan et al., 2016; Sağlam et al., 2017; Ata, 2018; Uğur and Öner, 2018; Yıldırım et al., 2018) have been conducted to isolate the phage efficacy against foodborne-pathogen in Turkey. To the best of our knowledge, there is no information about listerial phage isolation in Turkey. The objective of the present study, therefore, was to isolate, and purify lytic phages infecting *L. monocytogenes* and determine their host ranges.

MATERIALS AND METHODS

Sampling

A total of 68 samples were collected from different sources from Ankara and Çanakkale. The samples were transported to the laboratory under cold conditions on the sampling day and analyzed immediately.

Bacterial Strains and Culture Conditions

Thirty-five *L. monocytogenes* strains isolated from RTE foods previously described by Şanlıbaba et al. (2018) and the reference strain (*L. monocytogenes* ATCC 7644) were obtained from the culture collection of Food Microbiology Culture Collections, Department of Food Engineering, Engineering Faculty, Ankara University, Ankara, Turkey. Bacteria were routinely culture at 35 °C in Tryptic Soy Broth supplemented with 0.6% of yeast extract (TSB-YE) (Sigma, Germany).The *Listeria* spp. was stored at –20 °C with 30% (v/v) glycerol (Merck, Germany).

Isolation of *Listeria monocytogenes* Specific Phages

To isolate *L. monocytogenes*-specific phages, 68 samples were collected from Ankara and Çanakkale. Phage isolation samples were divided into two groups in this study. First of all, liquid samples were only centrifuged at 6000 rpm for 10 min (Lee et al., 2017). Feces and soft samples were in the second group. Twenty grams of samples were mixed in 100 mL SM buffer (0.05 M TRIS, 0.1 M NaCl, 0.008 M $MgSO_4$, 0.01% (w/v) gelatin pH 7.5).After chloroform (50 µL/mL) extraction, samples were mixed on a rotary shaker (40 rpm) at room temperature for 15 min. Then, samples were centrifuged at 10000 rpm for 10 min, as described by Bigot et al. (2011). Finally, all of the supernant was filtered using 0.22 µm in pore size (Milipore™, Ireland).

The presence of phage in the samples was determined by soft agar overlay method (Adams, 1953). In short, a 1 in 10 dilution of the filtrates was made in TSB-YE supplemented with 10mM $CaCl_2$ and 0.2 mL of exponential phase *L. monocytogenes* strains was added (Arachchi et al. 2013). Each filtrate was tested in a separate tube with each of the thirty-five indicator strains. After incubation overnight at 35 °C, each culture was centrifuged at 10000 rpm for 10 min and filtered using 0.22 µm in pore size. The supernatant was used in a plaque assay with the same strains. Phage filtrates (150 µL filtrate and 10 mM$CaCl_2$) were

prepared in a sterile test tube. Then, 100 µL of the exponential phase *L. monocytogenes* strains with a concentration of approximately 10^6 CFU/mL was added, separately. After 30 min of incubation at 35 °C, 5 mL of soft TSA-YE (0.45% agar) was poured on the filtrate-bacterium mixture. The resulting mixture was gently vortexed and spotted on the pre-solidified TSA-YE plate containing 10 mM $CaCl_2$. After solidification of agar for one hour at room temperature, the plates were incubated at 35 °C for 20–24 h to determine plaque formation. This part of the study was repeated twice. The presence of phage was identified by the formation of clear plaques. Individual plaques were used in the next round of purification.

Propagating Plaques and Purifying Phages

For the propagation and purifying of phages, the soft agar overlay technique was performed, as previously described by Kim et al. (2008). An overnight culture of *L. monocytogenes* strains was diluted (1:100) into fresh TSB-YE and incubated at 35 °C for 3 h for phage propagation. Then, 200 µL phage suspension and 10 mM$CaCl_2$ were added. After further incubation at 35 °C for 6 h, a phage lysate was obtained by centrifugation (10000 rpm for 10 min at 4 °C) and filtration (0.22 µm filter). Phage titers were determined following infection of host strain and enumeration of plaques. To determine of phage titer, soft agar overlay method was also used as described by Lacumin et al. (2016). In this assay the phage suspension was first serially diluted in sterile physiological saline buffer (PBS). Briefly, 100 µL of each dilution was mixed with 100 µL of any overnight grown cells of *L. monocytogenes* strains. After mixing both suspensions were added to 4 milliliters of sterile soft agar. Then, resulting mixture was vortexed and poured onto a TSA-YE agar plate. The plates were incubated at 35 °C for 24 h for plague formation. The number of visible plaques were counted and multiplied with the dilution factor to quantify the plaque forming units (PFU)/mL. For phage purifying, well isolated unique single plaque from each positive sample was taken and mixed in TSB-YE. Each tube was placed on an orbital shaker (Optima, Tokyo, Japan) at 100 rpm for 3 h and filtered using a sterile 0.22 µm syringe filter. Later, 100 µL of the filter-sterilized sample was mixed with 200 µL of overnight-grown bacterial culture and 10 mM $CaCl_2$. Finally, 4 mL of TSA-YE soft agar were added and the mixtures were poured onto a pre-solidified TSA-YE plate. The plates were incubated overnight 35 °C. Individual plaques were picked. The single plaque isolation was repeated three times and final lysates were used to prepare high titer phage stocks for further analyses. Phage stocks were prepared by adding 15% glycerol to the phage suspensions with 10^7 PFU/mL and above phage titers. The prepared stocks were maintained at three different temperatures as + 4 °C, - 4 °C and - 20 ° C (Loessner and Buse, 1990). Phage lysates containing approximately 10^7 PFU/mL were prepared using *L. monocytogenes* strains as host.

Host Range Determination

Host ranges were determined by soft agar overlay plaque assay as described by Zinno et al. (2014). Each *L. monocytogenes* strains was grown individually to log phase and combined with the individual phages. Briefly, 0.5 mL of *L. monocytogenes* culture at mid exponential growth phase was added to 5 mL of soft agar, mixed gently swirling and then poured on a TSA-YE agar base plate. Ten µL of a phage suspension at 10^7 PFU/mL was spotted onto the bacterial lawn and the plate incubated at 35 °C for 24 h. The lytic activity was checked by the appearance of clear zones. Cleared zones were measured for positive lytic spots against *L. monocytogenes* strains. The control plates were treated with a suspension without phage.

RESULTS AND DISCUSSION

Although there are several studies (Arachchi et al., 2013; Sulakvelidze, 2013; George et al., 2014; Pulido et al., 2016; Lee et al., 2017) on listerial phage isolation from different sources in the world, there is no information about listerial phage isolation in Turkey. Earlier studies in Turkey (Özkan et al., 2016; Sağlam et al., 2017; Ata, 2018; Uğur and Öner, 2018; Yıldırım et al., 2018) were focused on *Escherichia coli* O157:H7, *Salmonella* Enteritidis, *Pseudomonas aeruginosa*, and *S. aureus* specific phage. Therefore, we aimed to

isolate of lytic phages infecting *L. monocytogenes* and determine their host ranges.

Sixty-eight samples were tested for presence of *L. monocytogenes* phage in this study. Of the 68 samples, 5 were taken from soil, 20 from raw foods (milk, fish, chicken meat, red meat, vegetable, and fruit), 15 from feces, 4 from fisheries waste water, 15 from food processing waste water, 5 from sea water and, 4 from river. From these, 4 virulent phages were isolated from chicken feces, food processing waste water, fisheries waste water, and fish samples against four strains of *L. monocytogenes*. The results of phage screening were given in Table 1. All four isolated phages formed clear plaques with the strains used for isolation. Phage titers (PFU/mL) were ranged from 10^8 PFU/mL for ΦPLB33, 10^{10} PFU/mL for ΦPLB39, 10^7 PFU/mL for ΦPLB47, and 10^8 PFU/mL for ΦPLB92. Listerial phages are naturally present in fresh water, silage, marine environments, soil, foods, feces, sewage, humans and animals, chicken processing plants (Arachchi et al., 2013; Sulakvelidze, 2013;George et al., 2014;Pulido et al., 2016; Lee et al., 2017). Kim et al. (2008), Bigot et al. (2011), Arachchi et al. (2013), George et al. (2014), Akhtar et al. (2017), and Lee et al. (2017) were isolated listerial phages from turkey processing plant, sheep feces, seafood waste water, sewage, raw sewage sludge and chicken feces, respectively. The recovery of *L. monocytogenes* phage from isolation samples in this study was found to be 5.88%. In contrast to our study, George et al. (2014) could isolate a total of18listerialphage, with a recovery status of 16.36%.

Table 1. Occurrence of phages in samples

Source	Number of Samples	Number of Positive Samples
Soil	5	-
Raw foods (milk, fish, chicken meat, red meat, vegetable, and fruit)	20	1
Chicken Feces	15	1
Fisheries waste water	4	1
Food processing waste water	15	1
Sea water	5	-
River	4	-
Total	68	4

Each phage was then screened for its ability to lyse *L. monocytogenes* strains as well using soft agar overlay method in this study, as can be seen in Table 2. The four isolated phages infected many of the *L. monocytogenes* strains tested in this study. It was observed that *L. monocytogenes* specific phages do not have strain specificity. Eleven *L. monocytogenes* strains showed resistant to all four isolated phages. Plaque size of the all of the phages varied depending on the isolate. The plaque sizes of the 4 phages against 24 *L. monocytogenes* strains ranged from 0.4 and 2.6 mm. Moreover, the plaque sizes of the all phages against reference strain ranged from 1.2 and 1.7 mm. Phage ΦPLB39 produced the largest plaque with 2.6 mm. None of the phages had identical host ranges. The prevalence of resistance to the phages was found to be between 48.57-57.14% (Table 3). An important finding of this study was the high phage resistant rate. In order to reduce *L. monocytogenes* strains, phage resistant strains are undesirable. These finding also may suggest that

phage resistant may be an important component of the ecology of *L. monocytogenes* strains. Several phage resistant mechanisms have been identified (Kim et al., 2008). Further studies are needed to determine which mechanisms are being responsible for phage resistance in such strains. Genetic nature of the determined phage resistance system observed in this study should be also important to account for their powerful antiphage characters. In this study, twenty-four strains were susceptible to phages. While phage ΦPLB33 was susceptible to 15 *L. monocytogenes* strains, ΦPLB47 was susceptible to 17 strains. Moreover, in contrast, ΦPLB39 and ΦPLB92 were susceptible to 18 and 16 strains, respectively. Only six tested strains (L38, L39, L48, L70, L96, and L97) were susceptible to all four phages. Among all phages, ΦPLB39 was found to be the most virulent phages infecting 51.42% of the tested strains.

Table 2. Phage susceptibility of *Listeria monocytogenes* strains

Listeria monocytogenes strains	ΦPLB33		ΦPLB39		ΦPLB47		ΦPLB92	
	Susceptibility	Phage Plaque Diameter (mm)	Susceptibility	Phage Plaque Diameter (mm)	Susceptibility	Phage Plaque Diameter (mm)	Susceptibility	Phage Plaque Diameter (mm)
L9	-	-	-	-	-	-	-	-
L15	-	-	-	-	-	-	-	-
L16	-	-	-	-	-	-	-	-
L17	-	-	-	-	-	-	-	-
L22	+	1.1	+	0.9	-	-	+	1.2
L26	+	1.5	+	1.6	-	-	+	1.3
L33	HOST	2.4	-	2.0	-	-	-	-
L35	-	-	-	-	+	1.2	+	1.5
L37	-	-	-	-	-	-	-	-
L38	+	1.5	+	0.8	+	0.4	+	0.7
L39	+	1.7	HOST	2.6	+	1.2	+	1.2
L41	+	2.0	-	-	+	0.9	+	1.0
L47	+	1.9	-	-	HOST	2.4	+	0.6
L48	+	1.5	+	1.1	+	2.0	+	2.0
L49	-	-	+	1.6	+	1.9	-	-
L53	-	-	+	1.4	+	1.5	-	-
L54	-	-	-	-	-	-	-	-
L55	-	-	-	-	-	-	-	-
L56	+	0.5	-	-	+	0.9	-	-
L61	+	0.9	-	-	+	1.0	-	-
L62	+	1.1	+	0.9	-	-	-	-
L65	-	-	+	0.7	-	-	-	-
L67	+	0.6	+	1.0	-	-	-	-
L70	+	1.6	+	1.1	+	1.5	+	1.7
L72	-	-	+	0.9	+	1.3	+	1.4
L74	-	-	-	-	+	0.9	+	0.7
L80	-	-	+	0.7	+	1.1	+	1.3
L83	-	-	-	-	-	-	-	-
L86	+	1.4	+	1.2	-	-	+	1.5
L87	-	-	-	-	-	-	-	-
L88	-	-	-	-	-	-	-	-
L91	-	-	-	-	-	-	-	-
L92	-	-	+	1.5	+	1.4	HOST	2.1
L96	+	1.5	+	1.3	+	1.2	+	1.0
L97	+	1.3	+	0.6	+	0.8	+	0.9
ATCC7644	+	1.2	+	1.4	+	1.5	+	1.7

+ : Susceptible

- : Plaque not formed

Table 3. Prevalence of phage resistant strains

Phage	Number of strains resistant to phage (%)
ΦPLB33	57.14
ΦPLB39	48.57
ΦPLB47	51.42
ΦPLB92	54.28

The work presented here is novel, as it presents the first *L. monocytogenes* phage isolation in Turkey. This makes comparisons with studies on similar research difficult to make in Turkey. Further studies are needed to isolate and characterize of *L. monocytogenes* specific phages.

CONCLUSIONS

L. monocytogenes is an important food-borne pathogen. Phages are known as the natural enemies of bacteria. Nowadays listerial phages have again moved into focus of research interest in the world. Several hundred *Listeria* specific phages have been described until today. Our research is novel as we have isolated the *Listeria* genus- specific phage. These isolated *L. monocytogenes* specific phage can be used as a possible alternative to chemical antimicrobials against *L. monocytogenes*.

CONFLICT OF INTEREST

The authors express no conflict of interest associated with this work.

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