Pakistan Veterinary Journal

ISSN: 0253-8318 (PRINT), 2074-7764 (ONLINE)
DOI: 10.29261/pakvetj/2018.090

RESEARCH ARTICLE

Isolation and Characterisation of *Listeria monocytogenes* Bacteriophages from Environmental Sources in Kars-Turkey

Neslihan Mutlu[1,*] and Mitat Şahin[2]

[1]Department of Biology, Faculty of Arts and Sciences, Kafkas University, Kars, Turkey
[2]Department of Microbiology, Faculty of Veterinary Medicine, Kafkas University, Kars, Turkey
*Corresponding author: nslhnmtl81@gmail.com

ARTICLE HISTORY (18-227)

Received: June 29, 2018
Revised: September 12, 2018
Accepted: September 13, 2018
Published online: September 27, 2018

Key words:
Bacteriophages
Characterization
Isolation
Listeria monocytogenes
RAPD PCR

ABSTRACT

In this study, 45 samples were collected from some commercial businesses and environmental focus located in Kars center. Dirty areas, the area in contact with the sewage and poultry environmental sources were preferred while collecting samples. Again, 3 different silage samples and two samples were suspicious of *Listeria* were evaluated that brought to Kafkas University, Faculty of Veterinary Microbiology Laboratory. Isolation of lytic phages and lysogenic phages with mitomycin-C from total of 50 samples was performed. From 1 out of 12 of these samples lysogenic, from the remaining 11 samples lytic phage isolation was made. Isolation was confirmed by plaque assay. RAPD-PCR method was used for genotyping. The agarose gel images of 3 phages were observed to have been determined as previous study vB_SepiS-philPLA7, vB_SauS-philPLA35 and ΦH5. When band profiles belong to remaining 9 phages were compared, band profiles of 3 phages and the other 5 phages band profiles evaluated to be the same with each other. This study is thought to be precursors for purposes such as the decontamination of silage will be produced in the region and animal food, since *Listeria* infections are important for animal and human health and cause considerable economic losses.

To Cite This Article: Mutlu N and Şahin M, 2018. Isolation and characterisation of *listeria monocytogenes* bacteriophages from environmental sources in Kars-Turkey. Pak Vet J. http://dx.doi.org/10.29261/pakvetj/2018.090

INTRODUCTION

Listeria monocytogenes is commonly found in the environment, in the healthy animal feces and in the soil. Under certain circumstances they are pathogenic and causes serious infections (Picoux, 2008). Until 1981, when it was known that listeriosis was related to foods, it was regarded as an animal disease that rarely appeared in humans. Subsequently emerged outbreaks have made *L. monocytogenes* an important food borne pathogen (Warriner and Namvar, 2009). *L. monocytogenes* has been isolated from various foods such as meat, dairy products, vegetables, poultry, and fish (Parihar, 2008). More than 95% of infections in human are caused by strains 1 / 2a, 1 / 2b and 4b of *L.monocytogenes.* It is noted that most *Listeria* outbreaks are caused by 4b strains (Swaminathan and Gerner-Smidt, 2007). The infectious dose determined in foods that have been related with listeriosis, has typically been greater than 1000 CFU/g (Tompkin, 2002). Because microorganisms can tolerate low temperatures, high pH ratios and high salt concentrations, they can survive in soil, water, sewage, animal feed, and refrigerated foods (Klara, 2009). Human infections are usually caused by the consumption of contaminated foods such as raw milk, cream cheese, cabbage salad, raw vegetables, meat and fish products, frozen foods (Arda, 2000).

The problem of antibiotic resistance in bacteria has led the anti-infective models to become more of an issue in modern medicine and biotechnology. Along with the increase in antibiotic-resistant bacteria, the difficulties in developing effective new antibiotics have brought forward the use of phages in the treatment of infections (Sulakvelidze *et al.,* 2001). Decontamination studies with phages and local phages in treatments are highly important as well as increasing phage diversity. As resistance to a phage develops, other phage or phages lyse the target bacterium (Boyd and Brüssow, 2002). About 400 *Listeria* phages have been isolated from environmental sources or from lysogenic strains since the first isolation of *Listeria* specific phages in 1945 to present (Zink and Loessner, 1992). *Listeria* phages are generally strain specific phages. Non-lytic phages display activity in a fairly narrow host range and have an impact

on certain serovar groups of bacteria (Loessner and Busse, 1990, Loessner, 1991). Nevertheless, lytic phages can infect several species and serovar. This is thought to be due to the fact that phages used non-serovar, listerial peptidoglycan as receptors (Wendlinger *et al*., 1996). One of the commercially available products today is preparations for ready to eat meat products and poultry products, consisting of 6 different phage combinations, which are approved by the United States Food and Drug Administration (FDA) in 2006. This product is used in the United States to fight against the *L. monocytogenes* agent, which causes about 2500 cases each year and followed by 20% death (Lang, 2006). Phage preparations for Salmonella species have also been proposed in the meat industry (Atterbury *et al*., 2007).

MATERIALS AND METHODS

Sample collection: In this study, a total of 45 environmental samples from 15 different environmental centers of Kars city center selected by randomly between 11.21.2012 and 07.28.2013 were analyzed in terms of bacteriophage specific to *L. monocytogenes*. Samples were collected from sewage sludge, wastewater polluted with tankage, pastures, animal farms, wastewater of cheese factories and poultry contacted backwater. Between the same dates, 3 silage samples brought to the Microbiology Department of the Veterinary Faculty of the Kafkas University (KAU) and sheep lungs suspected to be *Listeria* were also examined. Environmental samples were taken as a volume of approximately 40 ml into a 50 ml falcon centrifuge tube with a sterile screw cap and they were brought to the the Department of Microbiology of Veterinary Medicine of the KAU for examination on the same day.

Standard bacterial strains: The *L. monocytogenes* ATTC-7644, used to determine the host intolerance of bacteriophages and to conduct PCR analyses, was obtained from Etlik Veterinary Control Central Research Institute. Two *Listeria* spp. bacterial strains used in the study were isolated from *Listeria* suspected samples brought to the KAU Veterinary Faculty.

Phage isolation from the samples: For phage isolation from the samples, modified bacteriophage isolation method of Demirbag and Demir (Demirbağ and Demir, 2011) was used. 45 ml of liquid sample or 5 g of solid sample were added to 5 ml of Listeria enrichment broth base and shaken gently.

This enrichment setting was incubated at 30°C for 24 hours. The 10 ml of culture was transferred to a centrifuge tube and solid matters were removed by centrifuging at 2500 rpm for 10 min. The obtained supernatant was filtered through a membrane filter of 0.22 µm por diameter and poured into a capped sterile tubes.

Phage isolation with mitomycin-C: A 1: 100 dilution was prepared from overnight bacterial culture prepared on BHI broth medium. Mitomycin-C (Sigma Aldrich, USA) was dissolved in distilled water at 2 mg / ml. Mitomycin-C was added at 1 µl / ml to the prepared bacterial culture.The culture removed to 6 h of incubation. After 6 h, culture with mitomycin-C was centrifuged at 2500 rpm. for 15 min. The supernatant was filtered through a membrane filter of 0.22 µm por diameter (Loessner *et al.*, 1991).

Host range test: In order to observe the effectiveness of the lytic phages that we thought isolated directly from the samples or the lysogenic phages that we thought obtained using mitomycin-C, standard strains were streaked on Listeria selective agar base containing Listeria selective supplement. Immediately after the steaking, approximately 15 µl of isolates obtained from the samples and will be tested for bacteriophage availability were dropped into the petri dish and incubated overnight at 30°C.

DNA isolation from bacteriophages: DNA isolation was performed using DNeasy blood and tissue kit (Qiagen, Germany) and DNA isolation kit, and following the protocol as described in the commercial kit.

RAPD-PCR: PCR was carried out with the protocol as described in the commercial kit using Gold Multiplex PCR PreMix (AccuPower, Austria). OPL5, P1, P2 and RAPD5 primers (Methabion, Germany) were used.

The electrophoresis of PCR products: The agarose gel was run in order to determine the length of the gene regions amplified in the PCR.1xTBE (Tris-Boric Acid-EDTA) was used as tank buffer for the preparation of the gel. For agarose gel (1%), 0.4 g agarose (Prona Basica Le, PL 100 g) was weighed into a volumetric flask containing 40 ml 1xTBE. It was heated in the microwave until it became homogenous and 4 µl of ethidium bromide was added after the steam exhaust stopped. 6.5 µl of the reaction mixture was loaded without stirring agarose gel and was run at 80 volts for 1 hour. DNA fragment sizes were visualized on a UV illuminator (UVP / LMS-20E).

RESULTS

Isolation findings: *Listeria monocytogenes* ATTC 7644 in the 12 samples of a total of 50 and bacteriophages acting on the laboratory-isolated Listeria spp. were obtained. Following incubation of bacteriophages distilled on Listeria spp. at 30ºC overnight, the plate appearance of the positive results is as follows (Fig. 1).

RAPD-PCR findings: For the experimental identification of 12 bacteriophages used in the study, RAPD-PCR method was used. Genomic DNAs previously produced from bacteriophages were used as template DNA. The PCR result is shown in Fig. 2-3. In the study conducted, phage typing of samples 2, 4 and 9 according to the PCR results was defined as vB_SauS-philPLA35, vB_SepiS-philPLA7 and ΦH5, respectively. Since 12 and 7, 11 and 8, and 1,3,5 and 10 have the same gel images, they are considered to be the same strains. However, according to literature review, there were no samples possess the same gel image obtained using these primers.

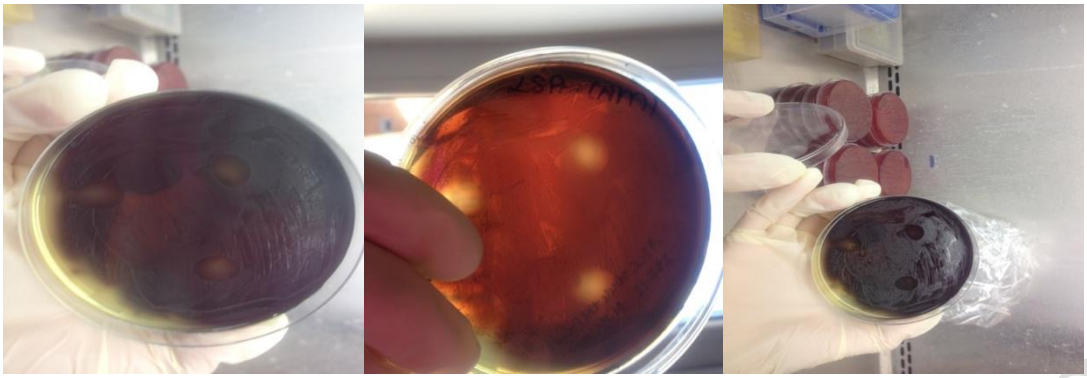

Fig. 1: Bacteriophage plate appearances.

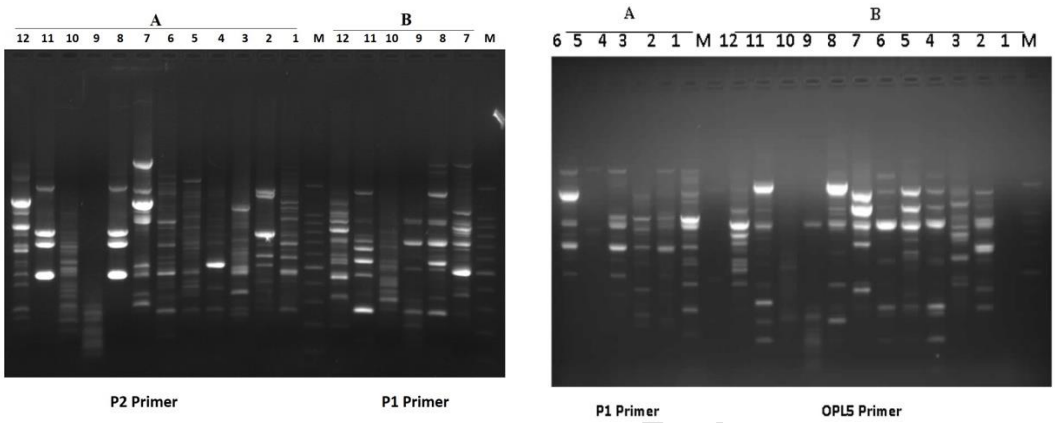

Fig. 2: Agarose gel image of the PCR obtained using OPL5 and P1 primers. A) The PCR image of 6 samples obtained using primer P1. B) The PCR image of samples obtained using the OPL5 primer (loaded with 100 bp markers to 0.8% agarose gel and was run at 80 V for 60 min).

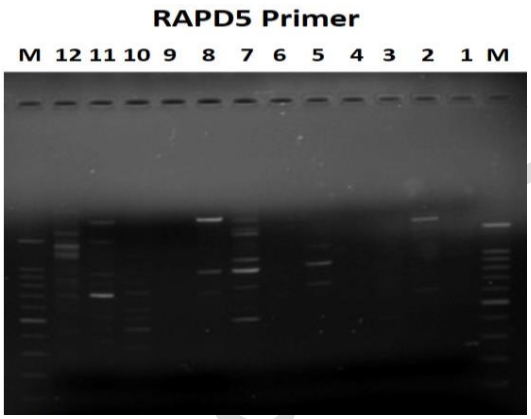

Fig. 3: Agarose gel image of the PCR obtained using PRAPD5 primer

DISCUSSION

Listeria specific phages were first described about 70 years ago. Various methods have been developed that were used to identify *L. monocytogenes* bacteriophages until today. However, standardized methods for obtaining improved results and ensuring progress of typing methods are not sufficient (Loessner and Busse, 1990).

Although many *Listeria* phages are examined electron microscopically and in terms of protein profiles, there are not sufficient studies on their genomes. (Zink and Loessner, 1992). Until today, genomes of PSA and A118 phages which are lysogenic and belong to the *Siphoviridae* family have been sequenced and analyzed (Loessner, 2000, Zimmer, 2003). It has been noted that the present genomic sequencing work on *Listeria* phages is related to virulent Myovirus and the P100 phage showing a broad range of host preference interval (Carlton *et al*., 2005).

Listeria phages have been shown in a variety of studies that can be isolated from sources such as sewage, silage and food production environments and from lysogenic strains (Loessner and Busse, 1990). Approximately 500 listeria phages have been isolated and it has been determined that some phages have the ability to infect different strains. These studies show us that there is quite a broad range between *Listeria* phages (Hagens and Loessner, 2014).

Most of the bacteriophages identified are lysogenic phage and are integrated with the host genome. As in other bacteria, the lytic cycle can be induced with agents that cause DNA damage, such as UV light and mitomycin-C, leading to the formation of infective phages (Loessner, 1991).

Isolated *Listeria*-specific phages are usually used for bacterial typing. This method is quite cheap, simple and fast. Bacteriophages can also be used to transfer genetic traits from one strain to another. These studies allow genetic manipulation and phenotype studies. Lauer *et al*. (2002) reported that there are phages which are able to transfer between *E. coli and Listeria* and are able to integrate into the *Listeria* genome after the transformation.

In their study, Loessner and Busse (1990) performed typing using 16 phage isolates on 57 *Listeria* strains isolated and identified from dairy products and various

foods. In the study they achieved 84.5% success in typing with the 100X routine phage test dilution and revealed that the repeatability of the typing was even higher after a few weeks.

Arachchi *et al.* (2014) have examined in their study the susceptibility of Listeria species previously isolated from sea products to unexplored bacteriophages. In a total of 50 Listeria strains, 42 strains were shown to be susceptible to phages. In another study, the same investigators researched the effect of bacteriophages isolated from sea products on Listeria strains and reported that bacteriophages have a very broad host spectrum. In the study, it was suggested that bacteriophages could be used as preservatives in Listeria contamination of seafood (Arachchi *et al.*, 2013). Reserchers showed that lytic phage mix LP1 lysed a mixture of *L. monocytogenes* strains at different temperatures (Akhtar *et al*., 2017). Vongkamjan *et al*. (2012), isolated bacteriophages and *L. monocytogenes* from the silages they got from two separate companies. It was observed that 4.5% of the samples were positive for L. monocytogenes and 47.8% were positive for listeriaphage. In phage activity experiments on a total of 13 reference L. monocytogenes strains, they noted that phages were found to have a broad spectrum. Another study showed that isolation of highly selective phage-displayed oligopeptide probes for detection of *listeria monocytogenes* in ready-to-eat food is possible (Chen *et al*., 2018).

Diana Gutiérrez *et al*. (2011) have suggested that cocktails composed of phages with different genetic characteristics are more effective in phage therapy. They have shown that band patterns obtained by RAPD-PCR are an easy and reproducible method for bacteriophage typing (30). Recent years researchers characterize the receptor binding proteins of Listeria monocytogenes bacteriophages A118 and P35 (Bielmann *et al.*, 2015) and binding and catalytic mechanisms of the *Listeria monocytogenes* bacteriophages (Romero *et al.*, 2018)

Based on the knowledge that bacteriophages are generic, it was thought that different bacteriophage species could be found in the phage suspension isolated from environmental samples and that this mixture (bacteriophage cocktail) could be effective on different species. The presence of other types of bacteriophages other than the primers used and these bacteriophages of which presence are shown can also be demonstrated by various primers.

In this study, phage typing of samples 2, 4 and 9 according to PCR results was defined as vB_SauS-philPLA35, vB_SepiS-philPLA7 and ΦH5, respectively. In the literature we reviewed, we observed bacteriophages undetectable to which bacteriophage specy it belongs but possess the same band pattern. 5 of these and other 3 are found to have the same band images.

By sequencing these obtained bands, and by comparing with the registered bacteriophage genomic sequence we can understand what type of bacteriophage is it and RAPD-PCR band pattern of the same bacteriophage will also be determined by our study.

Conclusions: The presence of listeriaphages in the environmental samples was detected by using RAPD-PCR method. The method and primers used are a quick and easy method for detecting listeriaphages. The listeria bacteriophages detected in the environmental samples can be used for biocontrol of listeriosis. It was thought that the obtained phages could be an effective product for decontamination of Listeria, which may be present in the silage produced in the region

Authors contribution: MŞ planned and followed up the studies. NM collected samples and carried out laboratory studies.

REFERENCES

Akhtar M, Viazis S, Christensen K, *et al*., 2017. Isolation, characterization and evaluation of virulent bacteriophages against *Listeria monocytogenes*. Food Control 75:108-15.

Arachchi GJ, Cruz CD, Dias-Wanigasekera BM, *et al*., 2014. Host range and in vitro lysis of *Listeria monocytogenes* seafood isolates by bacteriophages. Food Sci Technol Int 20:591-603.

Arachchi GJ, Mutukumira AN, Dias-Wanigasekera BM, *et al*., 2013. Characteristics of three listeriaphages isolated from New Zealand seafood environments. J Appl Microbiol 115:1427-38.

Arda M, 2000. Temel mikrobiyoloji ders kitabı. Medisan yayınları. 2nd ed. Ankara, Turkey.

Atterbury RJ, Van Bergen MAP, Ortiz F, *et al*., 2007. Bacteriophage therapy to reduce Salmonella colonization of broiler chickens. Appl Environ Microbiol 73:4543-9.

Bielmann R, Habann M, Eugster M, *et al*., 2015. Receptor binding proteins of *Listeria monocytogenes* bacteriophages A118 and P35 recognize serovar-specific teichoic acids. Virology 477:110-18.

Boyd EF and Brüssow H, 2002. Common themes among bacteriophage-encoded virulence factors and diversity among the bacteriophages involved. Trends Microbiol 10:521-9.

Carlton RM, Noordman WH, Biswas B, *et al*., 2005. Bacteriophage P100 for control of Listeria monocytogenes in foods: genome sequence, bioinformatic analyses, oral toxicity study, and application. Regul Toxicol Pharmacol 43:301-12.

Chen I, Xi J, Liu Y, *et al*., 2018. Isolation of highly selective phage-displayed oligopeptide probes for detection of listeria monocytogenes in ready-to-eat food. Proc. SPIE 10665, Sensing for Agriculture and Food Quality and Safety X 106650N.

Demirbağ Z and Demir İ, 2011. Genel Mikrobiyoloji Laboratuarı Ders Kitabı. 4th ed.: Esen Ofset; Trabzon, Turkey.

Gutiérrez D, Martín-Platero AM, Rodríguez A, *et al*., 2011. Typing of bacteriophages by randomly amplified polymorphic DNA (RAPD)-PCR to assess genetic diversity. FEMS Microbiol Lett 322:90-7.

Hagens S and Loessner MJ, 2014. Phages of Listeria offer novel tools for diagnostics and biocontrol. Front Microbiol 5:1-6.

Klara M, Posfay B and Ellen RW, 2009. Listeriosis. Seminars in Fetal & Neonatal Medicine 14:228-33.

Lang LH, 2006. FDA approves use of bacteriophages to be added to meat and poultry products. Gastroenterology 131:1370.

Lauer P, Chow MY, Loessner MJ, *et al*., 2002. Construction, characterization and use of two Listeria monocytogenes site specific phage integration vectors. J Bacteriol 184:4177-86.

Loessner MJ, 1991. Improved procedure for bacteriophage typing of Listeria strains and evaluation of new phages. Appl Environ Microbiol 57:882-4.

Loessner MJ and Busse M, 1990. Bacteriophage typing of Listeria species. Appl Environ Microbiol 56:1912-8.

Loessner MJ, Goeppl S and Busse M, 1991. Comparative inducibility of bacteriophage in naturally lysogenic and lysogenized strains of Listeria spp. By UV light and Mitomycin C Lett Appl Microbiol 12:196-9.

Loessner MJ, Inman RB, Lauer P, *et al*., 2000. Complete nucleotide sequence, molecular analysis and genome structure of bacteriophage A118 of Listeria monocytogenes: implications for phage evolution. Mol Microbiol 35:324-40.

Parihar VS, Barbuddhe SB, Danielsson-Tham ML, *et al*., 2008. Isolation and characterization of Listeria species from tropical seafoods. Food Control 19:566-9.

Picoux JB, 2008. Ovine listeriosis. Small Ruminant Research 76:12-20.

Romero P, Bartual SG, Schmelcher M, *et al*., 2018. Structural insights into the binding and catalytic mechanisms of the Listeria monocytogenes bacteriophage glycosyl hydrolase PlyP40. Mol Microbiol 108:128-42.

Sulakvelidze A, Alavidze Z and Morris JG, 2001. Bacteriophage therapy. Antimicrob Agents Chemotether 45:649-59.

Swaminathan B and Gerner-Smidt P, 2007. The epidemiology of human listeriosis. Microb Inf 9:1236-43.

Tompkin RB, 2002. Control of Listeria monocytogenes in the food-processing environment. J Food Prot 65:709-25.

Vongkamjan K, Switt AM, Den Bakker HC, *et al*., 2012. Silage collected from dairy farms harbors an abundance of listeriaphages with considerable host range and genome size diversity. Appl Environ Microbiol 78:8666-75.

Warriner K and Namvar A, 2009. What is the hysteria with Listeria? Trends Food Sci Technol 20:245-54.

Wendlinger G, Loessner MJ and Scherer S, 1996. Bacteriophage receptors on Listeria monocytogenes cells are the N-acetylglucosamine and rhamnose substituents of teichoic acids or the peptidoglycan itself. Microbiology 142:985-92.

Zimmer M, Sattelberger E, Inman RB, *et al*., 2003. Genome and proteome of Listeria monocytogenes phage PSA: an unusual case for programmed +1 translational frameshifting in structural protein synthesis. Mol Microbiol 50:303-17.

Zink R and Loessner MJ, 1992. Classification of virulent and temperate bacteriophages of Listeria spp. on the basis of morphology and protein analysis. Appl Environ Microbiol 58:296-302.