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Korrespondenzadresse:
bkaragulle@firat.edu.tr

[1])Giresun University, Espiye Vocational School, Department of Medical Services and Techniques, Giresun, Turkey; [2])Department of Microbiology, Faculty of Veterinary Medicine, Firat University, Elazig, Turkey; [3])Department of Medical Microbiology, Faculty of Medicine, Inonu University, Malatya, Turkey

# Investigation of prevalence, serotyping, and antibiotic resistance of *Listeria monocytogenes* in samples of meat, milk, and cheese from eastern Turkey

## *Untersuchung der Prävalenz, Serotypisierung, und Antibiotikaresistenz von Listeria monocytogenes in Proben von Fleisch, Milch und Käse aus der Osttürkei*

Tugba Cebeci[1]) (ORCID: 0000-0001-8960-0587), Burcu Karagulle[2]) (ORCID: 0000-0002-6628-4515), Barıs Otlu[3]) (ORCID: 0000-0002-6220-0521)

**Summary**

The aim of the present study was to investigate *Listeria monocytogenes* contamination in ground meat purchased from local butchers and raw cow milk and tulum cheese samples sold in public markets to identify serotypes of the obtained isolates and examine their antibiotic-resistance profiles. We microbiologically tested 300 samples of different origins for the presence of *L. monocytogenes* and confirmed the isolates found using matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF MS) and polymerase chain reaction, (PCR) after which they were serotyped and tested for antibiotic resistance against 8 antimicrobial agents using the disk diffusion method. *L. monocytogenes* was identified in 18 (6%) all of the collected samples. The serotypes of the 18 *L .monocytogenes* isolated in our study were 1/2b (3b) in 9 (50%), 1/2c (3c) in 4 (22.2%), 4b (4e, 4e) in 3 (16.7%), and 1/2a (3a) in 2 (11.1%) of the isolates. All isolates were found to be sensitive to ampicillin, erythromycin, gentamicin, chloramphenicol, penicillin, tetracycline, vancomycin, and sulfamethoxazole/trimethoprim. Although the isolates were sensitive to all commonly used antibiotics and given that *L. monocytogenes* serotypes were isolated in the examined samples, this pathogen should be closely monitored and its prevalence should be controlled using precautionary measures.

**Keywords:** Antibiotic resistance, food, *Listeria monocytogenes,* MALDI-TOF MS, serotyping

## Introduction

*Listeria monocytogenes* is a human pathogen that was discovered ~80 years ago and that causes rarely seen food-borne listeriosis. This pathogen may also cause life threatening infections in some specific groups of people, such as newborns, pregnant women, the elderly, transplant recipients and immunocompromised (1). The name of this intracellular organism refers to the relationship between infection in the host and the development of monocytosis. *L. monocytogenes* is a ubiquitous, Gram positive, nonspore forming, nonencapsulated, facultative anaerobic bacilli, with 13 known serotypes. *L. monocytogenes* infection usually follows the ingestion of contaminated food, and may result in septicemia, encephalitis, or abortion. These organisms may possibly pass into microfold cells in the intestinal Peyer's patches (2).

It has been estimated that 99% of all human listeriosis cases are caused by consuming contaminated food products (3), hence the name "emerging food-borne pathogen" for *L. monocytogenes* (4). According to Mead et al. (5), this pathogen is widely zoonotic and affects mainly cattle, sheep and goats. Human listeriosis has been reported in immunocompromised patients who were hospitalized following consumption of cheese and milk, and infants in the neonatal units (5). In addition, *L. monocytogenes* may be directly transmitted from the mother to the infant (vertical transmission) as well as from contact with animals and nosocomial infections (6). The annual incidence of listeriosis in developed countries is 0.2%–0.8% out of 100,000 persons (7). Although the incidence of listeriosis is low in developed countries, the rate of mortality is nearly 25%, and it is the third most common cause of food-borne mortality in these countries following salmonellosis and toxoplasmosis. However, reports on listeriosis are more limited in developing countries. It is important to investigate the presence and distribution of *L. monocytogenes* serotypes to be able to take the necessary precautions for preventing this type of infection.

Antimicrobial therapy is the mainstay of listeriosis treatment; however, the unusual growth characteristics of this organism and incorrect diagnoses often result in inadequate and failed treatment that may lead to serious outcomes (8). Despite adequate antibiotic therapy, the rate of mortality is high in invasive *L. monocytogenes* infections (9); therefore, it is equally important to investigate the resistance of *L. monocytogenes* isolates from various food samples to antibiotics to improve the effectiveness of treatment in risky patients infected with this pathogen.

The aim of the present study was to investigate *L. monocytogenes* contamination in ground meat samples sold in butcher stores and in raw cow milk and tulum cheese samples sold in public markets and to serotype the obtained isolates and examine their resistance to 8 different antibiotics.

## Materials and methods

We collected 100 ground meat samples from butchers and 100 raw cow-milk and 100 tulum cheese samples from public markets in specific districts of Giresun Province, Turkey, between 2017 and 2018. All samples were brought to the laboratory under cold chain and the investigation of the presence of *L. monocytogenes* was conducted. During the first stage, the International Standards Organization (ISO) 11290-1:2017 (10) and matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS, Vitek MS system, bioMerieux, France) methods were used to isolate and identify *L. monocytogenes*. We determined our sample size using the random sampling method while considering sales rates.

### Sample preparation

The ground meat, tulum cheese, and raw cow milk were weighed and separated into 25 g/mL samples, added to sterile bottles of 225 g/mL half Fraser broth media, and homogenized for 2 min using the Waring blender (New Hartford, CT, USA). The half Fraser broth (LAB211, UK) was then incubated at 30 °C for 24–26 h. After homogenization, dilutions to $10^5$ were prepared from buffered peptone water (LAB204, UK) and the enrichment media was left to incubate (10).

### Isolation of *L. monocytogenes* from the samples

To identify *L. monocytogenes* in the samples following the pre-enrichment stage, 1 mL fluid was taken from each sample, inoculated into tubes containing 9 mL Fraser broth, and incubated at 37 °C for 24 h. After incubating, petri dishes containing Oxford Agar (Himedia, M1145) and Listeria Chromogenic Agar (Lab M Hal010) were inoculated with 0,1 mL fluid taken from each dilution. The inoculated petri dishes were incubated at 37 °C for 24–48 h. After incubating, we observed blackish green colonies with a collapsed center, some of which had black-brown zones on the Oxford agar and blue colonies surrounded by an opaque halo on Listeria Chromogenic Agar. We considered these to be suspicious evidence for the presence of the pathogen (Fig. 1). Following the incubation, five colonies suspected as *L. monocytogenes* were taken from each petri dish and inoculated into Tryptic Soy Agar-Yeast Extract (Himedia, M1214) agar for purification and incubated at 30 °C for 24 h. The colonies were controlled morphologically for purifying using Gram staining. The Gram positive, catalase positive, and oxidase negative colonies that exhibited umbrella-like growth in Sulfide Indole Motility medium were determined to be *Listeria* spp. (10).

### Typing of the isolates with MALDI-TOF MS

Cultures of the suspected isolates were inoculated into Blood Agar Base (Oxoid, CM0055) and incubated at 37 °C for 24 h. One or two colonies from the typical suspected colonies that were isolated from the media were spread into the wells on the slides of the MALDI-TOF MS (Vitek MS system, bioMerieux, France) equipment, after which 1 µL matrix solution (saturated cyano-4-hydroxycinnamic acid solution in 50% acetonitrile and 2.5% trifluoroacetic

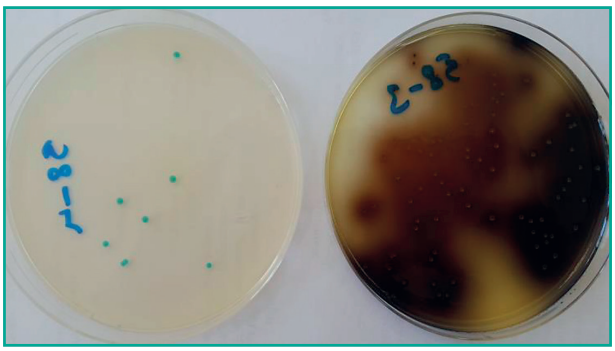

**FIGURE 1:** *Listeria monocytogenes growth on Oxford and Chromogenic agar.*

Journal of Food Safety and Food Quality 73, Heft 5 (2022), Seiten 139–168



acid) was pipetted into the wells and kept at room temperature until drying. The slide was then inserted into the equipment cassette and loaded into MALDI-TOF MS (11).

### Confirmation of *L. monocytogenes* with PCR

All *L. monocytogenes* isolates identified using the MALDI-TOF MS method were confirmed by polymerase chain reaction (PCR) using specific primers for *hlyA* and *iap*. Primer pairs 5'-GAA TGTAAACTTCGGCGCAATCAG-3' and 5'-GCCGTC GATGATTTGAACTTCATC-3' specific for *hlyA* (12) and 5'- ACAAGCTGCACCTG-TTGCAG -3' and 5'- TGACAGCGTGTGTAGTAGCA -3' specific for *iap* (13) of *L. monocytogenes* were used by optimizing the different conditions. DNA was extracted from the suspected isolates using the boiling method. For *hlyA* and *iap,* the PCR mixture was prepared according to the method applied by Bouayad et al (14). *hlyA* and *iap* were amplified as the first denaturation at 94 °C for 4 min, at 94 °C for 5 min in the thermal cycler, denaturation 35 cycles at 94 °C for 15 sec and 94 °C for 30 min, primer binding at 55 °C for 15 sec and 55 °C for 45 sec, primer elongation at 72 °C for 30 sec and 72 °C for 45 sec, and the last elongation at 72 °C for 8 min and 72 °C for 5 min, respectively. The amplicons obtained were run in 2% agarose at 100 V for electrophoresis, after which *hlyA* and *iap* were displayed on the UV transilluminator.

### Serotyping of *L. monocytogenes* isolates

Imo1118, Imo0737, ORF2110, ORF2819 and prs primers adapted by Doumith et al. (2004) (15) were used in serotyping the *L. monocytogenes* isolates. For serotyping, the PCR mixture was prepared as mentioned above. For serotyping the isolates, the amplification conditions in the thermal cycler were set at 94 °C for 4 min, 94 °C for 30 sec, 50 °C for 15 sec, and 72 °C for 60 sec for 35 cycles, after which the final elongation was programmed at 72 °C for 8 min. Primer binding temperatures were set at 50 °C for prs, ORF2819, ORF2110, Imo0737, and Imo1118. The obtained amplicons were run in 2% agarose at 80 V for electrophoresis, after which an isolate was evaluated as 1/2a or (3a) if only Imo0737 was positive, 1/2c or (3c) if both Imo0737 and Imo1118 were positive, 1/2b or (3b) if only ORF2819 was positive, and 4b or (4e, 4d) if both ORF2819 and ORF2110 were positive. *L. monocytogenes* ATCC 7644 (serotype 1/2c), RSKK 472 (serotype 1/2b), RSKK 471 (serotype 1/2a) RSKK 475 (serotype 4b) were used as the quality control strain.

### Antibiotic resistance tests of *L. monocytogenes* isolates

To determine antibiotic resistance in the isolates, ampicillin (AM), erythromycin (E), gentamicin (CN), chloramphenicol (C), penicillin G (P), tetracycline (TE), vancomycin (VA) and sulfamethoxazole / trimethoprim (SXT) antibiotic disks (Oxoid, UK) were used. Antibiotic resistance of *L. monocytogenes* isolates was investigated using disk diffusion method according to the rules of European Committee on Antimicrobial Susceptibility Testing (EUCAST) (17). The diameters of the inhibition zones formed around the disks of penicillin G, ampicillin, erythromycin and sulfamethoxazole / trimethoprim were classified as susceptible, intermediate or resistant according to the clinical breakpoints available in EUCAST documents (17). For the remaining antimicrobial agents, due to lack of standardization in the susceptibility criteria for *L. monocytogenes,* the critical values were used for *Staphylococcus* spp. and *Enterococcus* spp. described by the CLSI (16), based on the description of Andriyanov et al. (18).

### Statistical analyses

The data on the mean, percentage, and frequency were analyzed using the SPSS ver. 22 (IBM Inc., Chicago, IL, USA).

**TABLE 1:** *Serotype distribution of L. monocytogenes isolates identified in the collected raw cow milk, tulum cheese and ground meat samples.*

| Districts | Raw cow milk | | | Tulum cheese | | | Ground meat | | |
|---|---|---|---|---|---|---|---|---|---|
| | No of samples | No of *L. monocytogenes* | Serotype | No of samples | No of *L. monocytogenes* | Serotype | No of samples | No of *L. monocytogenes* | Serotype |
| Bulancak | 20 | – | – | 20 | – | – | 12 | – | – |
| Dereli | – | – | – | – | – | – | 3 | – | – |
| Doğankent | – | – | – | – | – | – | 4 | – | – |
| Espiye | 15 | 2 | 4b (4e,4d)<br>1/2b(3b) | 20 | – | – | 10 | 1 | 1/2c(3c) |
| Eynesil | 11 | – | – | 6 | – | – | 7 | 1 | 4b(4e,4d) |
| Görele | 12 | 1 | 1/2b(3b) | 15 | 5 | 4×1/2b(3b)<br>1×4b(4e,4d) | 6 | – | – |
| Güce | 4 | 1 | 1/2b(3b) | 3 | – | – | 2 | – | – |
| Keşap | 10 | 1 | 1/2b(3b) | 10 | – | – | 7 | – | – |
| Merkez | – | – | – | – | – | – | 30 | 3 | 1×1/2c(3c)<br>2×1/2a(3a) |
| Piraziz | 7 | – | – | 10 | – | – | 7 | 1 | 1/2c(3c) |
| Tirebolu | 10 | – | – | 10 | 1 | 1/2c(3c) | 8 | 1 | 1/2b(3b) |
| Yağlıdere | 11 | – | – | 6 | – | – | 4 | – | – |
| TOTAL | 100 | 5 | 4×1/2b(3b)<br>1×4b(4e,4d) | 100 | 6 | 4×1/2b(3b)<br>1×4b(4e,4d)<br>1×1/2c(3c) | 100 | 7 | 1×4b(4e,4d)<br>3×1/2c(3c)<br>2×1/2a(3a)<br>1×1/2b(3b) |

## Results

Of the raw cow milk samples collected for analyses, 20% were obtained from marketplaces in Bulancak, 15% from those in Espiye, and 12% from those in Görele districts. Among the collected tulum cheese samples, 20% were obtained from the public markets in Espiye, 20% from those in Bulancak, and 15% from those in Görele districts. In this study, 30% of the ground meat samples were collected from the local butchers in the city center, 12% from those in the Bulancak district, and 10% from those in the Espiye district. *L. monocytogenes* were identified in 18 of the collected ground meat, raw cow milk, and tulum cheese samples (Table 1).

According to the results of the analyses, 7 (7%) of 100 ground meat samples, 5 (5%) of raw cow milk samples and 6 (6%) of tulum cheese samples were contaminated with *L. monocytogenes.* This contamination was confirmed using PCR. Serotyping showed that four of five isolates obtained from the raw milk samples were 1/2b (3b); the remaining isolate was 4b (4e,4d). The six isolates of tulum cheese samples were analyzed as follows: four 1/2b (3b), one 4b (4e,4e), and one 1/2c (3c). Of the ground meat isolates, seven were 1/2a (3a), three were 1/2c (3c), one was 1/2b (3b), and one was 4b (4e,4d). Of the 18 *L. monocytogenes* samples isolated in our study, 9 (50%) were 1/2b (3b), 4 (22.2%) were 1/2c (3c), 3 (16.7%) were 4b (4e,4e), and 2 (11.1%) were 1/2a (3a).

Of the 18 *L. monocytogenes* samples isolated in our study, 1/2b (3b) serotypes were found in 9 (50%), 1/2c (3c) in 4 (22.2%), 4b (4e,4e) in 3 (16.7%), and 1/2a (3a) in 2 (11.1%) of the isolates (Fig. 2).

All isolates included *hlyA* and *iap.* When the status of antibiotic resistance in the isolates was analyzed using the disk diffusion method, all *L. monocytogenes* isolates were found to be sensitive to AM, E, GN, C, P, TE, VA, and SXT (Fig. 3).

## Discussion

*L. monocytogenes* can contaminate fresh meat, milk and other dairy products, fruits, vegetables and other foods in many different ways because of the ubiquitous trait of the pathogen (19). *L. monocytogenes* has often been isolated throughout the world as coming from foods of animal origin (20, 21). In the present study, *L. monocytogenes* was isolated in 7% of the ground meat samples collected from butchers and in 5% of the raw cow milk and 6% of the tulum cheese samples obtained from public markets in specific districts of Giresun province, Turkey. According to the literature review, in a study conducted in Algeria, Bouayad et al. (14) have found that *L. monocytogenes* was detected in 3% of dairy products and 2.6% of meat products. In a study conducted in Ethiopia, Derra et al. (22) have found that *L. monocytogenes* was isolated in 6.8% of raw meat samples, 3.4% of raw milk samples, and 5.1% of cheese samples. In their study of samples in Morocco, Marnissi et al. (23) have found that *L. monocytogenes* was isolated in 8.33% of the raw milk samples and 4.16% of the cheese samples. In another study conducted in Ethiopia, Garedew et al. (24) have isolated *L. monocytogenes* in 6.66% of the raw meat samples; however, Gupta et al. (25) have identified no *L. monocytogenes* in 50 meat samples. Different results among different studies might have resulted from various factors such as the isolation methods, sources of samples, country of origin, and number of samples.

Although the prevalence of *L. monocytogenes* serotypes may differ among different countries, 1/2a, 1/2b, 1/2c and 4b are the serotypes most often associated with human listeriosis. In addition, 1/2a, 1/2b and 1/2c serotypes have been associated with sporadic cases, while 4b serotype has been found in the patients during listeriosis epidemics. Previous studies have reported that meat is contaminated with mainly 1/2a, 1/2b and 1/2c serotypes (26-28). In our study, 15 (85.7%) of the 18 *L. monocytogenes* isolates were contaminated with 1/2a (3a), 1/2b (3b) and 1/2c (3c) serotypes. Similarly, Hadjilouka et al. (30) have found 1/2a, 1/2b and 1/2c *L. monocytogenes* serotypes in 82.8% of the food samples. Another study from South Africa reported 14.7% of *L. monocytogenes* isolates belonged to the following three serogroups: 1/2a (3a) (45.5%) was the most prevalent, followed by 4b (4d,4e) (24.2%), and 1/2c (3c) (15.2%) (31). One study in Turkey reported that the isolates belonged to the following four serogroups: 1/2a (or 3a) (38.4%), 1/2b (or 3b) (30.7%), 4b (or 4d, 4e) (23%), and 1/2c (or 3c) (7.6%) (32).

Given the increase in the worldwide number of antibiotic-resistant *L. monocytogenes* isolates, the specific antibiotic-resistance gene patterns of this pathogen, and its ability to acquire resistance from other bacteria species, it is clear that we must better understand the extent of this foodborne issue. Most of the commensal organisms found in the gastrointestinal system are obligate anaerobic species that may be resistant to antibiotics. Specific pH conditions within the bowel ranging from acidic to mild alkaline provide environments that may cause variations in the effectiveness of antibiotics on these pathogens, which enables these commensals and other pathogens to become antibiotic resistant (33). The relevant literature states an increa-

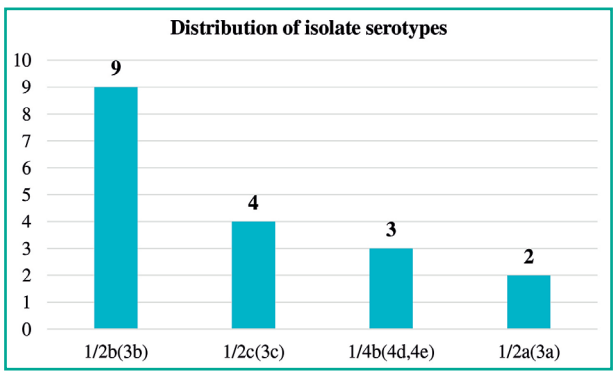


**FIGURE 2:** *Distribution of the collected Listeria monocytogenes isolates.*

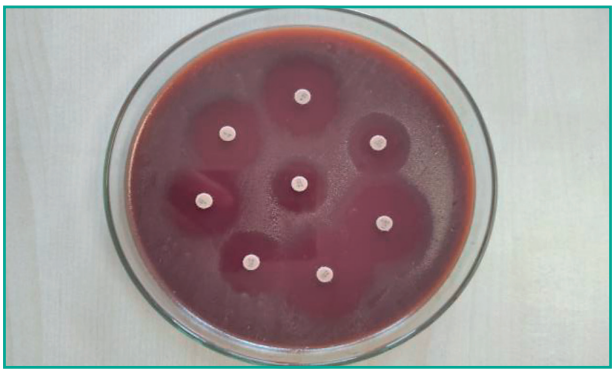

**FIGURE 3:** *Antibiogram of Listeria monocytogenes.*

Journal of Food Safety and Food Quality 73, Heft 5 (2022), Seiten 139–168



sing resistance pattern in the species isolated from food, the environment and clinical cases. Reducing antibiotic use both in agriculture and in the treatment of human diseases will decrease the emergence of more antibiotic-resistant bacteria, but eliminating the already resistant bacteria is a slower process than the emergence of new antibiotic-resistant species in nature (29,34). In the present study, all *L. monocytogenes* isolates identified in ground meat, raw cow milk and tulum cheese samples were sensitive to AM, E, CN, C, P, TE, VA and SXT. Similary, Yucel et al. (35) have demonstrated that *L. monocytogenes* that were found to be sensitive to kanamycin (K), C, and TE were isolated from meat products. Harakeh et al. (36) have stated that *L. monocytogenes* found sensitive to CN, SXT, TE, and E, were isolated from raw milk samples. These fluctuations can be expected to vary among countries because of their different protocols for antibiotic use.

## Conclusion

Meat, milk, and other dairy products are potential sources of *L. monocytogenes* transmission, and we have demonstrated the prevalence of this pathogen in our samples of raw ground meat, raw milk, and Tulum cheese. Also, we found that all of the isolated serotypes were sensitive to commonly used antibiotics; however, given that *L. monocytogenes* was isolated in the examined samples, this pathogen should be closely monitored and its prevalence should be kept under control. The results of this study could be used as a guide for additional epidemiological and public health studies to be conducted in different regions.

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**Ethical Statement**
This study does not present any ethical concerns.

## Conflict of interest

The authors declare no conflict of interest.

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**Address of corresponding author:**
Burcu Karagülle
Department of Microbiology
Faculty of Veterinary Medicine
Fırat University
Elazıg
Turkey
bkaragulle@firat.edu.tr