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Isolation and Identification of *Listeria* spp. from White Cheese Samples Presented for Consumption in Istanbul

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Abstract

In this study, 119 feta cheese samples taken from different vendors in Istanbul were examined for the presence of *Listeria monocytogenes*. Isolates were confirmed by the PCR method using *iap* and *hlyA* primers, and the antibiotic susceptibility of the identified strains was performed by the Kirby-Bauer protocol. Seven *Listeria* spp. were isolated from three (2.52%) of 119 cheese samples analyzed. The seven *Listeria* spp. obtained from these three samples were found to contain the *iap* gene region but not the *hlyA* gene region. As a result of the sequence analysis using the 16S rRNA gene region, it was determined that these isolates were *L. seeligeri*. As a result of antibiotic susceptibility tests, it was observed that *L. seeligeri* isolates showed ciprofloxacin (85.71%) and penicillin (42.85%) resistance. All strains were susceptible to amikacin, amoxicillin/clavulanic acid, chloramphenicol, rifampin, gentamicin, cefaclor, ampicillin, trimethoprim-sulfamethoxazole, tetracycline, vancomycin, and clarithromycin antibiotics. The detection of *Listeria spp.* isolates in feta cheese samples made with pasteurized milk revealed that packaging, distribution, and storage practices following the pasteurization process should be followed more strictly. It is recommended to apply controls at each stage to prevent contamination.

Keywords: *L. monocytogenes*, Feta cheese, *iap*, *hlyA*, Antibiotic sensitivity

1. INTRODUCTION

Gram-positive, non-spore-forming, facultative anaerobe, rod-shaped *Listeria* bacteria, due to their tolerance to low pH, low temperature and high salt concentration, can be found in various environments such as water, silage, soil, foods and sewage [1-5] The *Listeria* genus has 20 different species, included *L. innocua*, *L. seeligeri*, *L. ivanovii*, *L. fleischmannii*, *L. welshimeri*, *L. marthii*, *L. grayi*, *L. rocourtiae*, *L. weihenstephanensis*, *L. cornellensis*, *L. floridensis*, *L. riparia*, *L. grandensis*, *L. aquatica*, *L. booriae*, *L. newyorkensis*, *L. costaricensis*, *L. goaensis*, and *L. thailandensis*. Among these bacteria, the *L. monocytogenes* and *L. ivanovii* are pathogenic to animals and humans.

L. monocytogenes, the causative agent of listeriosis, is an important foodborne pathogen [6-

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8] This infection can occur through the consumption of cheese, milk, cooked and ready-to-eat foods, pork, fermented sausage, meat and meat products such as beef, unwashed raw vegetables and fruits contaminated by *L. monocytogenes* [6, 9, 10] Although *L. monocytogenes* infection is less common than other foodborne diseases, it is the bacterium with the second-highest mortality rate among all foodborne pathogens [11, 12] Despite effective antibiotic treatments, Listeriosis, which has a mortality rate of 30%, seems to be a problem that threatens public health [6, 13].

Cheese is a food product that is widely consumed in the world and has many varieties. Feta cheese, in case of ignoring factors such as pasteurization, business hygiene etc. that need to be considered during production, can be a convenient source for the contamination and reproduction of *L. monocytogenes* bacteria. Pathogenic bacteria found in cheese because of contamination can also cause ailments up to food poisoning. Among these pathogenic bacteria, *L. monocytogenes* due to resistance to temperature and pH changes, the ability to growing at high salt concentration, can survive the production and ripening stages of cheese. The growth of this bacterium can be slowed down by lactic starter cultures in cheese milk but is not completely inhibited. Therefore, to avoid outbreaks from *L. monocytogenes*, the strict control of this food from the production stage until it reaches the consumer is important [9, 14].

Examination of cheese, which has an important consumption rate in our country, in terms of *L. monocytogenes* is important for public health. In the present study, we aimed to detect *Listeria* contamination in white cheese, sold in İstanbul, and determine the risks in terms of public health of the contamination.

2. MATERIAL AND METHOD

2.1. Bacteria Strains

In the study, *Staphylococcus aureus* ATCC 25923 and *L. monocytogenes* RSKK 475 were used as positive control.

2.2. Isolation and Identification of *Listeria* spp.

119 white cheeses made from pasteurized milk obtained from different districts in Istanbul province were examined according to FDA (Food and Drug Administration) standard culture method [15]. The culture method was carried out in three stages. For the primary enrichment process, after a 2-minute homogenization step, a 25 g sample in 225 ml Listeria Enrichment Broth (HiMedia, India) was incubated at 30 ° C for 4 h. After the incubation, the selective additive was added to the culture and incubation was continued. Liquid cultures were kept for 1 and 2 weeks in addition to the incubation period recommended in the standard protocol, and at the end of all periods, they were inoculated onto the PALCAM (Polymyxin-Acriflavin Lithium Chloride-Ceftazidime- Aesculin-Mannitol Agar; HiMedia, India) media by streak plate method. After 48 h incubation at 30 °C, five *Listeria* suspicious colonies, which were grey-green colonies surrounding a black halo growing on the medium, were inoculated on the TSA-YE (Trypticase soy agar with yeast extract; HiMedia, India) media for obtaining pure cultures.

Listeria suspicious colonies growing on TSA-YE medium has been evaluated for biochemical tests such as Gram staining, oxidase, catalase, movement in the medium of Sulphate Indole Motility (SIM), hemolysis, nitrate reduction, Methyl Red (MR) -Voges-Proskauer (VP), acid production from sugar (rhamnose, mannitol, ribose, xylose, and α-methyl-D-mannocide), and Christie Atkins and Munch-Petersen (CAMP) factor [16].

2.3. Antibiotic Susceptibility Test

Antibiotic susceptibilities of *Listeria* spp. isolates were investigated by the Kirby Bauer test protocol [17]. In the study, ampicillin (10 µg), amoxicillin-clavulanic acid (30 µg), penicillin (10 units), cefaclor (30 µg), vancomycin (30 µg), gentamicin (10 µg), amikacin (30 µg), clarithromycin (15 µg), ciprofloxacin (5 µg), tetracycline (30 µg), trimetprim-sulfamethoxazole (25 µg), chloramphenicol (30 µg), and rifampin (5 µg), antibiotics were used.

2.4. Verification of isolates by PCR application

2.4.1. DNA Isolation

Chromosomal DNA was extracted from the *Listeria* suspicious isolates using the IDPURE Universal Spin Column Genomic DNA Mini Kit (IDLabs, Canada).

2.4.2. PCR Amplification

Listeria spp. isolates were verified by PCR method using *iap* (IAP1: 5′ACAAGCTGCACCTGTTGCAG3′ and IAP2: 5′TGACAGCGTGTGTAGTAGCA3′) and *hlyA* (PCRGO: 5′GAATGTAAACTTCGGCGCAATCAG3' and PCRDO: 5′GCCGTCGATGATTTGAACTTCATC3′) primers [18, 19]. Primers of the *iap* and *hlyA* genes used in the experiment were prepared following the manufacturer's instructions.

The PCR mix was prepared with MyTaq^{TM} Mix (Bioline, Germany). After 0.5 µl (20 µM) of each reverse and forward primer, 1 µl (10 nmol) template DNA, 5 µl MyTaq Reaction Buffer, 0.5 µl MyTaq DNA polymerase was mixed into 0.2 ml microtubes was added, the final volume was completed to 25 µl with sterile ultrapure water. All procedures were repeated using L. monocytogenes RSKK 475 template DNA and ultrapure water as positive and negative control, respectively.

The reaction was performed in the Thermal Cycler (TC-PLUS, Techne, England) device without losing time. The PCR protocol set up following: 95 °C for 1 min for initial denaturation; 35 cycles, 15 s at 95 °C for denaturation, 15 s annealing at 65 °C or 55 °C for *hlyA* or *iap* primers respectively and 10 s extension at 72 °C. The PCR products obtained were visualized on a 1.2% agarose gel, stained with GelRed (Biotium, Hayward, CA, USA), for 45 minutes at 90 volts for electrophoresis. The band lengths of the PCR products obtained were determined using a 100 bp DNA Ladder (Bioline, Singapore) and imaged in a UV imaging system (Wisedoc, South Korea).

2.4.3. Molecular Identification

The phylogenetic analysis of the isolates was performed using bacterial universal primers, 1492R (5'-GGYTACCTTGTTACGACTT-3') and 27F (5'-AGAGTTTG ATCMTGGCTCAG-3') described by Lane (1991) using a thermal cycler (TC-PLUS, Techne, England) under the following conditions: 95 °C for 1 min initial denaturation; 35 cycles, at 95 °C for 15 s denaturation, 15 s annealing at 60 °C, 10 s extension at 72 °C.

The reaction mixture (25 µl) contained 0.5 µl (20 µM) of each reverse and forward primer, 1 µl (10 ng) template DNA, 5 µl MyTaq Reaction Buffer, 0.5µl MyTaq DNA polymerase. The PCR products obtained were carried out on a 1.2% agarose gel for 45 minutes at 90 volts for electrophoresis. The band lengths of the PCR products obtained were determined using a 100 bp DNA Ladder (Bioline, Singapore) and imaged in a UV imaging system (Wisedoc, South Korea).

Sanger sequence analyzes of the PCR products were made by BM Laboratory Systems (Ankara, Turkey). The 16S rRNA sequences obtained were aligned against the GenBank database using the BLAST program of the National Centre for Biotechnology Information.

3. RESULTS AND DISCUSSION

In the current study, using the culture method recommended by the FDA, 119 feta cheese samples were analyzed. The pure cultures of suspicious colonies, which appeared grey-green colored with a black halo on PALCAM medium, were obtained by inoculation into TSA-YE medium. According to the phenotypical examinations, 19 isolates obtained from eight cheese samples, which were Gram-positive rod or coccobacillus, catalase positive and oxidase reaction negative, were accepted as *Listeria* spp. suspicious.

19 *Listeria* spp. suspect isolates were examined in terms of motility, hemolysis, nitrate reduction, MR-VP, carbohydrate fermentation and CAMP tests, and 7 of them were found to have similar

biochemical results, except carbohydrate fermentation, with *L. monocytogenes* in all tests (Table 1). Seven *Listeria* spp. were isolated from three (2.52%) cheese samples by the traditional culture method.

Table 1. Biochemical test results of *Listeria* spp. suspicious isolates

	Biochemical Tests										
Isolate	**MR**	**VP**	**Nitrate Reduction**	**Motility**	**Rhamnose**	**α-methyl-D-**	**Mannitol**	**Ribose**	**Xylose**	**CAMP**	**β -hemolysis**
N1	-	-	-	-	-	-	-	-	-	-	-
N2	-	-	-	-	-	-	-	-	-	-	-
N3	-	-	-	-	-	-	-	-	-	-	-
N4	-	-	-	-	-	-	-	-	-	-	-
N5	-	-	-	-	-	-	-	-	-	-	-
N6	-	-	-	-	-	-	-	-	-	-	-
N7	-	-	-	-	-	-	-	-	-	-	-
N8	-	-	-	-	-	-	-	-	-	-	-
N9	-	-	-	-	-	-	-	-	-	-	-
N10	-	-	-	-	-	-	-	-	-	-	-
N11	-	-	-	-	-	-	-	-	-	-	-
N12	-	-	+	-	-	-	-	-	-	-	-
N13	+	+	-	+	-	-	-	-	+	+	+
N14	+	+	-	+	-	-	-	-	+	+	+
N15	+	+	-	+	-	-	-	-	+	+	+
N16	+	+	-	+	-	-	-	-	+	+	+
N17	+	+	-	+	-	-	-	-	+	+	+
N18	+	+	-	+	-	-	-	-	+	+	+
N19	+	+	-	+	-	-	-	-	+	+	+
S.B.	+	+	-	+	+	+	-	-	-	+	+

S.B.: *L. monocytogenes* RSKK 475

As a result of the PCR experiment, it was determined that the 7 strains isolated by the culture method only have the *iap* gene region which was specific for the *Listeria* genus, but not the *hlyA* gene that was specific for *L. monocytogenes* (Figure 1).

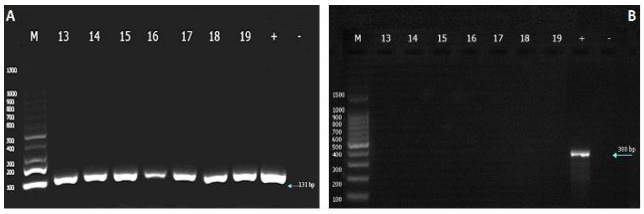

Figure 1. Gel electrophoresis images of the *iap* (A) and *hlyA* (B) gene carried by *Listeria* bacteria isolated from cheese samples [M: 100 bp DNA ladder 13-19: *Listeria* suspected bacteria (N13- N19). +: Positive control -: Negative control]

3.1. Identification of Isolates by Phylogenetic Analysis

It was confirmed that 7 isolates obtained according to the sequence analysis were *L. seeligeri*. The 16S rRNA sequences of the samples obtained from this study were recorded in GenBank under the accession numbers in Table 2. It was determined that the sequence similarity for 16S rRNA was 95-99%. Considering the sequence similarity, the seven isolates obtained were found to be closely related to *L. seeligeri*.

Table 2. Accession numbers of strains obtained from cheese samples

Sample No	Isolate	Similarity	Accession No
	N17	% 95	MK490993
P51	N18	% 98	MK490994
	N19	% 99	MK490995
P53	N15	% 99	MK490991
	N13	% 99	MK490989
P54	N14	% 99	MK490990
	N16	% 97	MK490992

3.2. Determination of Antibiotic Sensitivity of *Listeria* Bacteria

Six (85.71%) of seven *Listeria spp.* isolates obtained from white cheese were found to be resistant to ciprofloxacin and three (42.85%) to penicillin. Besides, 28.57% of the isolates were resistant to both ciprofloxacin and penicillin. All the isolates were sensitive to cefaclor, ampicillin, amikacin, chloramphenicol, rifampin, gentamicin, trimethoprim-sulfamethoxazole, tetracycline, amoxicillin/clavulanic acid, vancomycin, and clarithromycin antibiotics (Table 3).

Table 3. Antibiotic susceptibilities of *Listeria* bacteria

Antibiotic	Isolates						
	N13	N14	N15	N16	N17	N18	N19
Ciprofloxacin	R	I	R	R	R	R	R
Amikacin	S	S	I	I	S	S	S
Penicillin	R	R	S	S	R	S	S
Chloramphenicol	S	S	S	S	S	S	S
Rifampin	S	S	I	I	S	S	S
Gentamicin	S	S	S	S	S	S	S
Cefaclor	S	S	S	I	S	S	S
Ampicillin	S	S	S	S	S	S	S
Amoxicillin/ clavulanic acid	S	S	S	S	S	S	S
Trimethoprim-sulfamethoxazole	S	S	I	I	S	S	S
Tetracycline	S	S	S	S	S	S	S
Clarithromycin	S	S	S	S	S	S	S
Vancomycin	S	S	S	S	S	S	S

L. monocytogenes was not detected in our study, in which 2.52% (3/119) of *L. seeligeri* was detected in the white cheeses examined within the scope. Similar to the results we obtained, Elmas [20] indicated in her study that only one *L. seeligeri* was detected. In many studies [21, 22, 23] reporting that they did not encounter *L. monocytogenes* bacteria in white cheese samples are consistent with our findings.

Failure to report a foodborne listeriosis outbreak in Turkey until today and the infrequency of foodborne *Listeria* infection rate is in line with our findings.

Unlike our results, Çetin et al. [24] did not find *Listeria* spp. in any of the 40 white cheese samples. Although Atıl et al. [25] isolated *L. seeligeri* (0.19%) and *L. innocua* (0.19%) in 106 milk samples, they could not isolate *Listeria* spp. in 28 cheese samples. In the other study, Rahimi et al [26] found the rate of *Listeria spp*. as 7.2% in their study with dairy products in Iran. The researchers stated that the species they obtained were *L. innocua*, *L. monocytogenes*, *L. murrayi*, and *L. seeligeri* (66.7, 23.8, 4.8, and 4.8%, respectively). In a study conducted with 100 white cheeses purchased from neighbourhood markets, 6% of the cheeses were found to be contaminated with *L. monocytogenes* [27]. Aygün and Pehlivanlar [28] found that 8.23% of samples contained *Listeria* spp. In the mentioned study, *L. seeligeri* (2.35%), *L. ivanovii* (3.52%), *L. monocytogenes* (2.35%), *L. innocua* (3.52%) species were detected in white cheese samples. Ceylan and Demirkaya [29] indicated that *L. monocytogenes* was isolated from one (3.45%) white cheese sample. Elshinaway et al. [30], in their study with 240 dairy products, determined that the presence of *Listeria* spp. in kareish cheeses and buttered milk cheeses were 20% and 12.5%, respectively. *Listeria* spp. distribution in butter milk cheese was five (5%) *L. monocytogenes*, one (2.5%) *L. welshimeri*, two (5%) *L. grayii*, while in Kareish cheese was one (2.5%) *L. welshimeri*, one (2.5%) *L. monocytogenes* three (7.5%) *L. grayii*. In a study, investigating to the *L. monocytogenes* and *Listeria* spp. contamination of the 142 homemade cheese samples, conducted in Bolu [31] the presence of bacteria was reported as 9.2% and 33.1%, respectively. Kevenk and Gülel [32], in their study with 210 milk and dairy samples, indicated that *Listeria* spp. were detected in the raw milk samples (12%) and the white cheese samples (25%), and 5% of them were harboured *L. monocytogenes*.

When the results obtained in the current study were compared with the results obtained by other researchers, it was determined that isolation and identification rates of *Listeria* species in cheese samples vary differ. These differences are thought to be caused by the methods used for cheese production, the contamination rates of the milk, the environmental differences and geographical conditions, and also the insufficient hygiene rules. The presence of *L. seeligeri* in the white cheese samples we examined indicates possible contamination after pasteurization. Failure to comply with hygiene rules during the production, distribution and sale of cheese and cross-contamination during the sale and storage of these products are considered as other sources of contamination.

Within the scope of our study, the susceptibility of *L. seeligeri* isolates was determined by the Kirby-Bauer method. In 1988, the first multi-resistant *L. monocytogenes* was discovered in France [33]. After this date, many *Listeria* spp.

strains isolated from different environments have been determined to be resistant to one or more antibiotics [34-40]. It was determined that six (85.71%) of the seven *L. seeligeri* bacteria were resistant to ciprofloxacin and three (42.85%) to penicillin. At the same time, it was detected that five (71.42%) of the bacteria were resistant to one antibiotic and two (28.57%) to two antibiotics. All strains were found to be amikacin, chloramphenicol, rifampin, gentamicin, cefaclor, ampicillin, amoxicillin/ clavulanic acid, trimethoprim-sulfamethoxazole, tetracycline, clarithromycin and vancomycin sensitive. Our results on penicillin and ciprofloxacin resistance are similar to the study of Rahimi et al. [26]. In a study about *Listeria* spp. isolated from dairy products in Iran, it was detected that 28.6% of the isolates were resistant to one antibiotic and 23.8% to two antibiotics. At the same time, they found that 66.7% of them were resistant to nalidixic acid, 42.9% to penicillin, 23.8% to ciprofloxacin and 19% to tetracycline antibiotics. The resistance of the isolates we obtained from our study against penicillin antibiotics is in line with the findings of other researchers. Arslan and Özdemir [31] reported that similar to our study, 47 *Listeria spp.* isolates from white cheese showed resistance to penicillin (12.8%), at the same time these isolates had cefaclor (2.1%), tetracycline (2.1%), amikacin (4.3%), clarithromycin (6.4%), and chloramphenicol (8.5%) resistance.

When Aksoy et al. [41] examined the 15 *L. monocytogenes* isolates in their study, it was found that 26.7% of the isolates were resistant to at least one antibiotic. At the same time, they found that 6.7% of the isolates had multiple antibiotic resistance, and the most resistance was shown against trimethoprim-sulfamethoxazole (26.7% of the isolates). It was determined that 6.7% of isolates were resistant to meropenem, vancomycin, penicillin G, and amikacin at the same rate. In the other study, it was found that most of the isolates were resistant to ampicillin, rifampicin, and florfenicol (92%, 84%, and 66% respectively) and some of them were resistant to tetracycline, penicillin G, and chloramphenicol (45%, 40%, and 32% respectively) [42]. Harakeh et al. [43] when investigated antibiotic profile of *L. monocytogenes* isolated from soft white cheese varieties it was determined that the most resistance against oxacillin, penicillin, and ampicillin (93%, 90%, and 60% respectively). Jamali et al. [44] reported that 83 isolates obtained from raw milk in Malaysia were tetracycline and penicillin G resistance.

Resistance to ciprofloxacin we observed in the study was also reported in different studies. Conter et al. [45], in their study with *L. monocytogenes* strains which were obtained from food and food production areas, found that 10 (8.3%) isolates were resistant to one, three (2.5%) isolates to two and one (0.8%) isolates to five antibiotics. It was determined that the highest antibiotic resistance was against clindamycin and linezolid antibiotics with a ratio of 3.2%. In Ireland, 207 *Listeria* spp. isolates were found to be resistant to gentamicin (5%), sulfamethoxazole-trimethoprim (2%), ciprofloxacin and tetracycline (1.5%) [46]. Unlike the results we obtained, when 1001 *Listeria* spp. strains isolated from food (549 *L. innocua*, 39 *L. welshimeri*, 351 *L. monocytogenes*, 62 *L. seeligeri*) were examined in terms of antibiotic sensitivity, it was reported that *L. seeligeri* bacteria were not resistant to any antibiotics [47].

4. CONCLUSION

It is a known fact that redundant or excessive antibiotic usage in animals and humans also increases the resistance of bacteria to antibiotics. Development of antibiotic resistance not only in terms of pathogenic species of *Listeria* but also in non-pathogenic species, due to the transfer of resistance genes between *Listeria* species, constitutes risks to human health. Although the antibiotics recommended for the treatment of listeriosis are still effective, it is known that the isolated *Listeria* strains exhibit an increasing resistance day by day. On the other hand, resistance to broad-spectrum antibiotics may lead to rapid depletion of antibiotic alternatives, such as penicillin, ampicillin, tetracycline and gentamicin, that can be used for treatment in the future [45]. The increased resistance of *L. monocytogenes* to penicillin and ampicillin is apprehension for public health due to using these antibiotics in the treatment of listeriosis.

It is known that *L. seeligeri* is not a pathogenic *Listeria* species, but there is a case of meningitis due to *L. seeligeri* infection in the literature [48]. Although no other cases have been encountered, these bacteria may also pose a public health risk. For this reason, it is an essential precaution to control the steps such as inadequate pasteurization or post-pasteurization packaging/ distribution and the entire process. It is recommended that strict follow-ups are carried out to take food safety measures and implement HACCP guidelines in food establishments, also local producers do not use non-pasteurized milk.

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The Declaration of Conflict of Interest/ Common Interest

No conflict of interest or common interest has been declared by the authors.

Authors' Contribution

Under this heading, "The authors contributed equally to the study" or "The first author contributed 60%, the second author 40%." expressions such as should be included.

The Declaration of Ethics Committee Approval

This study does not require ethics committee permission or any special permission.

The Declaration of Research and Publication Ethics

The authors of the paper declare that they comply with the scientific, ethical and quotation rules of SAUJS in all processes of the paper and that they do not make any falsification on the data collected. In addition, they declare that Sakarya University Journal of Science and its editorial board have no responsibility for any ethical violations that may be encountered, and that this study has not been evaluated in any academic publication environment other than Sakarya University Journal of Science.

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