Isolation of *Listeria monocytogenes* in lamb meat and determination of the antibiotic resistance[*]

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Summary: In this study the occurrence of *Listeria monocytogenes* and other *Listeria* species were investigated from a total of 120 retail raw lamb meat samples and *L. monocytogenes* isolates were verified by using PCR based on *hylA* gene which is responsible for virulence. All *L. monocytogenes* isolates were analyzed by antimicrobial susceptibility tests. *L. monocytogenes* was isolated from 19 (15.8%) of the samples examined. The other species isolated were *L. innocua, L. weshimeri* and *L. grayi* 26 (21.6%), nine (7.5%) 2 (1.6%) strains respectively. According to the PCR analysis, *hlyA* gene was detected from the 58 of the isolates which were isolated from *L. monocytogenes* positive 19 lamb meat samples. The antibiotic resistance profiles of the 58 isolates to nine antibiotics were detected by disc diffusion method. Five (8.6%) isolates were found to be resistant to antibiotics. Among the five isolates, four (6.9%) displayed multiple resistance, only one isolate was resistant to tetracycline.

Keywords: Antimicrobial resistance, food safety, lamb meat, *Listeria,* PCR.

Kuzu etlerinden *Listeria monocytogenes* izolasyonu ve antibiyotik dirençliliklerinin belirlenmesi

Özet: Bu çalışmada, satışa sunulan toplam 120 çiğ kuzu eti örneklerinde *Listeria monocytogenes* ve diğer *Listeria* türlerinin varlığı araştırılmış ve bulunan *L. monocytogenes* izolatları virülensten sorumlu *hylA* geni esas alınarak PZR ile doğrulanmıştır. Bütün *L. monocytogenes* izolatları antibiyotik duyarlılık testi ile analiz edilmiştir. Test edilen örneklerin 19'undan (15.8%) *L. monocytogenes* izole edilmiştir. İzole edilen diğer türler sırasıyla 26 adet (21.6%) *L. innocua*, dokuz adet (7.5%) *L. weshimeri* ve iki adet (1.6%) *L. grayi*'dir. PZR analizine göre 19 kuzu eti örneğinden izole edilen *L. monocytogenes* pozitif izolatların 58'inde *hlyA* geni belirlenmiştir. 58 izolatın dokuz antibiyotiğe karşı antibiyotik direnç profili disk difüzyon metodu kullanılarak belirlenmiştir. Beş izolatın (8.6%) antibiyotiklere karşı dirençli olduğu bulunmuştur. Bu beş izolat arasında dördü (6.9%) çoklu direnç gösterirken, bir izolat sadece tetrasikline karşı dirençli bulunmuştur.

Anahtar sözcükler: Antimikrobiyal direnç, gıda güvenliği, kuzu eti, *Listeria,* PZR.

Introduction

Listeria monocytogenes (*L. monocytogenes*) a Gram-positive bacteria, is ubiquitous in nature and primarily causes listeriosis in humans through contaminated foods. Foodborne listeriosis may cause serious illness in newborns, abortions in pregnant women, and septicemia, meningitis, and encephalitis in immunocompromised individuals (22). Because of its high mortality, listeriosis ranks among the most frequent causes of death due to foodborne illnesses (10).

A variety of foods including red meat, poultry meat, dairy products, ready-to-eat foods and vegetables have been implicated as vehicles for *L. monocytogenes* transmission (4, 25, 31, 39). Although occurrence of *Listeria* species in lamb meat and lamb meat products has been investigated in several countries (16, 37, 47). However, little has been reported about the incidence of the organism in lamb meat in Turkey that total lamb meat production was 98 977 tons in 2014 (1).

Currently, the treatment for listeriosis is a β-lactam antibiotic (e.g. penicillin or ampicillin), alone or in combination with an aminoglycoside (e.g. gentamicin) in immunocompromised patients. The second choice for treatment is the association of trimethoprim and a sulfonamide (e.g. sulfamethoxazole), especially for patients allergic to β-lactams (11). Vancomycin and erythromycin are also used to treat bacteraemia and pregnant women diagnosed with listeriosis respectively (17).

L. monocytogenes rarely develops acquired resistance to antibiotics. However, some recent studies have reported an increased rate of resistance to one or several clinically relevant antibiotics in environmental isolates and less frequently in clinical strains (36).

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[*] This study was reviewed from the doctorate thesis.

Emergence and dissemination of antibiotic resistance in *L. monocytogenes* may have significant for the future clinical implications of the treatment of listeriosis.

The aims of the present study were to investigate the prevalence of *L. monocytogenes* in lamb meat collected from different butcher-shops and markets in Ankara, to detect *hlyA* gene by PCR for the confirmation of the isolates, and to determine the resistance profiles of isolated strains to 9 antibiotics currently used in veterinary and human therapy.

Materials and Methods

Samples: A total of 120 lamb meat samples were purchased from different markets [77] and butcher-shops [43] in Ankara, Turkey. All samples were collected aseptically and transferred to the laboratory under cold conditions and processed immediately within 2 h were analysed for *Listeria* on the same day.

Isolation and identification of Listeria monocytogenes: Isolation and identification of *Listeria* spp. and *L. monocytogenes* was performed as described by ISO 11290-1 (The International Organization of Standardizations) (29), ISO 11290-1/A1 (30) respectively. Under aseptic conditions, an amount of 25 g from each sample was incubated in 225 mL Half Fraser Broth (Oxoid CM 895, SR 166G, Hampshire, UK) at 30ºC for 24±2 h then 0.1 mL pre-enrichment broth was added to 10 mL of Fraser Broth (Oxoid CM 895, SR 156E) as a second enrichment culture for 48±2 h at 37ºC. Finally, the culture was streaked onto both Oxford agar (Oxoid CM 856, SR 140) and ALOA media (Oxoid CM 1084, SR 226, SR 244), and incubated for 24-48 h at 37ºC. Up to five typical *Listeria* colonies were selected from each of the selective agars and were plated on a separate Tryptic Soy Agar, yeast extract added (Oxoid CM 129, LP 21, LP11) and incubated at 37ºC for 24-48 h. These isolates were used for biochemical tests (Gram staining, catalase test and oxidase test) [Merck 1.11885, Merck 1.08597, Bactident oxidase Merck 1.13300] and motility test by Sulphate Indole Motility medium (Oxoid CM 435). For the identification of the isolates β-hemolysis, carbohydrate utilization (L-rhamnose, D-xylose and D-mannitol), nitrate reduction and CAMP tests were carried out. *L. monocytogenes* isolates were maintained at -80ºC in cryovials until use for PCR analysis. Cryovials contained 1 mL of 24 h incubated Brain-Heart Infusion broth (Oxoid CM 225), *L. monocytogenes* cultures and 0.5 mL of sterile glycerine.

Detection of presence of virulence-related gene: Extraction of DNA from all of the isolates was performed using chelex-100 (Sigma C7901) resin-based technique (19). Special virulence gene (hlyA), which was encoding L. *monocytogenes* listeriolysin, were used for verification of the *L. monocytogenes* isolates by PCR assay. The primer pairs PCRGO: 5'- GAATGTAAACTTCGGCG CAATCAG - 3' and PCRDO: 5'- GCCGTCGATGATT TGAACTTCATC - 3'(IDT Integrated DNA Technologies) were used to amplify a 388 bp DNA fragment of *hly*A (5, 8).

Antimicrobial susceptibility testing: The resistance profiles of the isolates verified by PCR were examined to 9 antibiotics (ampicillin-Oxoid CT0002B, penicillinG-Oxoid CT0152B, gentamicin-Oxoid CT0024B, erythromycin-Oxoid CT0020B, streptomycin-Oxoid CT0047B, tetracycline- Oxoid CT0054B, meropenem-Oxoid CT0774B, trimethoprim-sulfamethoxazole-Oxoid CT0052B, rifampicin-Oxoid CT0207B). Antibiotic susceptibility of all isolates was determined by the disc diffusion method as recommended by the European Committee on Antimicrobial Susceptibility Testing (20) and Clinical and Laboratory Standards Institute (14) in Mueller-Hinton agar supplemented with 5% mechanically defibrinated horse blood and 20 mg/L β-NAD (nicotinamide adenine dinucleotide) (MH-F) and in Mueller-Hinton agar (Oxoid CM337), respectively. The inhibition zone diameters were measured and interpreted in accordance with the breakpoints recommended by EUCAST for *L. monocytogenes* on ampicillin, penicilin G, erythromycin, meropenem, trimethoprim-sulfamethoxazole and interpreted in accordance with the breakpoints recommended by CLSI for other Gram-positive bacteria (15, 21). Because a specific breakpoints of *Listeria* for gentamicin, streptomycin, tetracycline and rifampicin did not exist.

Results

In the current study, a total of 120 lamb meat samples were analyzed during 4 months period. 30 samples were collected each month. 44 (36.6 %) of the 120 samples were contaminated with *Listeria* spp. Nineteen (15.8 %) of the samples were contaminated with *L. monocytogenes*, 26 (21.6%) with *L. innocua*, 9 (7.5%) with *L. welshimeri,* 2 (1.6%) with *L. grayi.* Eleven of the 44 samples were found to be mixed contaminated with more than one *Listeria* species (Table 2).

In our study, ALOA and Oxford mediums were used as selective agar for the isolation of *Listeria* spp. and *L. monocytogenes*. From the 19 *L. monocytogenes*, 18 (15%) was found positive in ALOA medium while only 8 (6.7%) in Oxford medium (Table 3).

In order to confirm the isolates, PCR assay was performed. One of the major virulence factors of *L. monocytogenes*, *hlyA* (LLO-listeriolysin O) gene specific primers were used. According to the PCR analysis, *hlyA* gene was detected in 58 *L. monocytogenes* isolates which were isolated from *L. monocytogenes* positive 19 lamb meat samples. The *hlyA* gene was found in all 58 (100%) isolates. As a result, all the isolates were verified by PCR (Figure 1).

Table 1. Breakpoint table for interpretation of zone diameters (15, 21)
Tablo 1. Zon çaplarının yorumlanmasında kullanılan limit değerler tablosu.

Antibiotics	Disk content (µg)	Zone diameter breakpoint (mm)			
		S ≥	I	R <	
Benzylpenicillin	1 unit	13	-	13	EUCAST *L. monocytogenes*
Ampicillin	2	16	-	16	EUCAST *L. monocytogenes*
Meropenem	10	26	-	26	EUCAST *L. monocytogenes*
Erythromycin	15	25	-	25	EUCAST *L. monocytogenes*
Trimethoprim-sulfamethoxazole	1.25-23.75	29	-	29	EUCAST *L. monocytogenes*
Rifampicin	5	20	17-19	≤ 16	CLSI *Staphylococcus* spp.
Gentamicin	10	15	13-14	≤ 12	CLSI *Enterococcus* spp.
Streptomycin	10	15	12-14	≤ 11	CLSI *Enterobacteriaceae*
Tetracycline	30	19	15-18	≤ 14	CLSI *Staphylococcus* spp.

S: Susceptible, I: intermediate, R: Resistant.

Table 2. The occurrence and the numerical distribution of *Listeria* species, in *Listeria* spp. positive samples.
Tablo 2. *Listeria* spp. pozitif örneklerde *Listeria* türlerinin varlığı ve sayısal dağılımı.

Sample	*Listeria* positive samples	*L.monocyto genes*		*L. innocua*		*L. welshimeri*		*L.grayi*		**L.monocytogenes L. innocua*		**L. innocua L.welshimeri*		**L.monocytogenes L. innocua L.welshimeri*	
	n	n	%	n	%	n	%	n	%	n	%	n	%	n	%
Market	35	10	28.6	12	34.2	4	11.4	2	5.7	6	17.1	1	2.9	-	-
Butcher - shop	9	-	-	3	33.3	2	22.2	-	-	2	22.2	1	11.1	1	11.1
Total	44	10	22.7	15	34.1	6	13.6	2	4.5	8	18.2	2	4.5	1	2.3

*Isolated species from the same sample

Table 3. Comparison of ALOA and Oxford Agar for the isolation of *Listeria monocytogenes*.
Tablo 3. *Listeria monocytogenes* izolasyonunda ALOA ve Oxford Agar'ın karşılaştırılması.

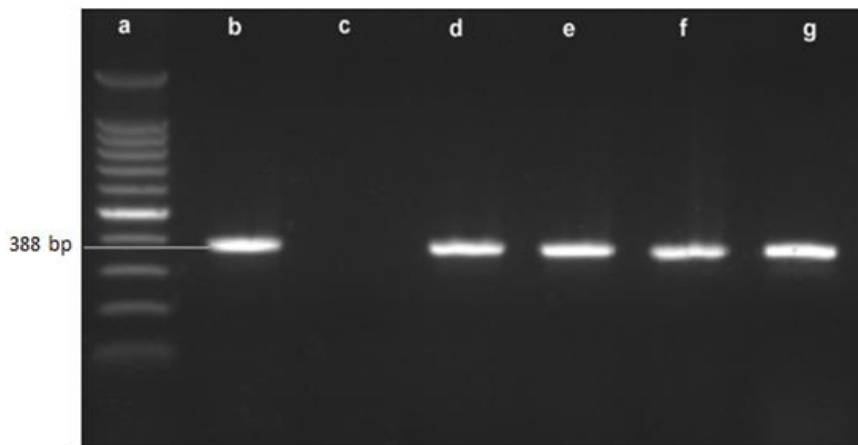| Selective Agar	*L. monocytogenes* positive samples
ALOA	11
Oxford	1
ALOA and Oxford	7
Total	19

Figure 1. *hlyA* gene detected from *L. monocytogenes* isolates by PCR (a: 100-bp DNA marker; b: Positive control, *L. monocytogenes* ATCC 7644; c: Negative control; d-g: *hlyA* positive *L. monocytogenes* isolates).
Şekil 1. PZR ile *L. monocytogenes* izolatlarından saptanan *hlyA* geni (a: 100-bp DNA marker; b: Pozitif kontrol, *L. monocytogenes* ATCC 7644; c: Negatif kontrol; d-g: *hlyA* pozitif *L. monocytogenes* izolatları).

Table 4. The antimicrobial susceptibility profiles of the isolated strains.
Tablo 4. İzole edilen suşların antimikrobiyal duyarlılık profilleri.

Antibiotics	Concentration µg/disc	Number of isolates (%)		
		Susceptible	Intermediate	Resistant
Ampicillin	2	58 (100)	-	-
Penicillin G	1 U	55 (94.8)	-	3 (5.2)
Gentamicin	10	54 (93.1)	1 (1.7)	3 (5.2)
Erythromycin	15	56 (96.6)	-	2 (3.4)
Streptomycin	10	54 (93.1)	-	4 (6.9)
Tetracycline	30	56 (96.6)	-	2 (3.4)
Meropenem	10	54 (93.1)	-	4 (6.9)
Trimethoprim/Sulfamethoxazole	1,25 – 23,75	54 (93.1)	-	4 (6.9)
Rifampicin	5	55 (94.8)	-	3 (5.2)

According to the disc diffusion test, five (8.6%) of the 58 isolates displayed resistant to at least one antibiotic and among this isolates four were found multi-resistant. Other 53 isolates were sensitive to nine antibiotics. In particular, multiple resistance was more common than resistance to one antibiotic, i.e., one isolates were resistant to one antibiotic, one to six antibiotic, one to seven antibiotic, one to eight antibiotic. The last isolate was resistant to streptomycin, meropenem, trimethoprim/sulfamethoxazole and was intermediate to gentamicin. Resistance to streptomycin, meropenem, trimethoprim/sulfamethoxazole was most common with 4 isolates showing resistance, then gentamicin (3 resistant isolates and 1 intermediate), penicillin G and rifampicin [3], and finally, erythromycin and tetracycline [2]. All isolates were sensitive to ampicillin (Table 4).

Discussion and Conclusion

L. monocytogenes has been frequently isolated from various foods of animal origin throughout the globe (7, 13, 34, 47). In the present study, 19 (15.8%) of a total of 120 samples examined were positive for *L. monocytogenes*. Compatible results were reported by Gökmen and Alişarlı (24), they determined 11 *L. monocytogenes* from 100 lamb minced meat from supermarkets. Indrawattana et al. (28) collected 104 meat samples from open markets and reported 16 *L. monocytogenes.* In a study in China, Wang et al. (43) isolated *L. monocytogenes* from 4.8% of raw mutton samples.

Zarei et al. (47) reported 4.3%, Bhat et al. (7) 5% *L. monocytogenes* from lamb meat. Philips et al. (37) detected microbiological quality of 613 lamb and sheep meat, and found only in one sample *Listeria* spp., also they didn't report any *Listeria* spp. from the frozen boneless samples. On the other hand, Cohen et al. (16) not detected any *L. monocytogenes* from 52 lamb sample purchased from supermarkets, slaughterhouses and butchers in Morocco. Similarly, Gupta et al. (26) not found any *L.*

monocytogenes from 50 lamb meat and edible offal. Likewise, Wang et al. (42) reported no *L. monocytogenes* in 14 lamb meat. The difference between the results may be due to the sources of meat, country, slaughtering conditions, isolation and identification methods, market hygiene, sample number and part of the animal.

In our study we found that beside the 19 (15.8%) lamb meat sample containing *L. monocytogenes*, 25 samples (30%) were contaminated with different *Listeria* species. In total, 44 (36.6%) of the 120 tested samples were contaminated with *Listeria* spp. Although the presence of other *Listeria* spp. in food are not of direct pathogenic significance, these organisms can be considered as useful indicators of a deterioration in hygiene or process conditions, leading to an increased risk of contamination with pathogenic *Listeria* species. Chen et al. (13) reported the prevalence in China as 14.7% in lamb meat, Gökmen and Alişarlı (24) as 63% in minced lamb meat, Gupta et al. (26) as 2% in lamb meat.

In this study, within the *Listeria* positive samples, 11 (9.1%) was found to be contaminated with more than one *Listeria* species. From these, in 1 sample three different *Listeria* species was detected as a mix. Also in many another studies, authors reported that some non-pathogen *Listeria* species can be with the pathogenic ones (38). It is interesting to note that *L. innocua* was isolated predominantly among *Listeria* species in our study. Other studies also indicated that *L. innocua* is the most prevalent *Listeria* species found in food products (27, 35, 46). This may be related to differential selection during enrichment and recovery procedures, or because *L. innocua* is simply more common in the environment than *L. monocytogenes* (12, 41). According to the studies *L. innocua* grows faster than the pathogenic species in enrichment broths therefore with the *L. monocytogenes* both species are present almost the same levels (35). Also, differences in resistance among *Listeria* spp. were displayed and *L. innocua* was found more resistant than other *Listeria* species (32).

In Oxford medium, *Listeria* species are not differentiate from each other (33, 44). So, it's not possible for every time to detect *L. monocytogenes* with Oxford medium by taking 5 different colonies, through the samples are contaminated with the pathogen. Also, because it chromogenic property and by the production of zones, colonies formed more distinctively in ALOA medium. In compliance with our results, in the study of Vlaemyek et al. (40), 36 *L. monocytogenes* detected from the 208 samples, and from these 31 (86.1%) was found positive by ALOA medium and only 22 (61.1%) with Oxford and PALCAM mediums. In this study 1 sample was identified as *L. monocytogenes* by Oxford medium, but not confirmed in ALOA medium by the color change and zone production. This can be the result of the change of phospholipase C activity. It was reported that because of the mutation in the gene that coded PI-PLC, caused the deactivation of phospholipase C (18). Also, according to Camillia et al. (9) there can be some non-virulent *L. monocytogenes* species that have not PI-PLC production property. According to our findings, it will be suitable to use two or more different mediums for the isolation.

Similarly to our results, Awaishch (3) reported that all the *L. monocytogenes* strains identified by ISO method, can be confirmed 100% by PCR. In contrast with this, Aurora et al. (2) was found 93%, Gouws and Liedemann (23) 84% correlation between chromogenic agar and the PCR results.

The majority of the strains isolated in this study are susceptible to the antibiotics commonly used. However, the results of the present study provide further evidence of the emergence of multi-resistant strains in nature, representing a potential threat to human health. Evidence of the emergence of multiresistant *L. monocytogenes* strains from various sources has also been reported. Wang et al. (43) in China and Wong et al. (45) in Malaysia have been reported high multiple resistances 72.3% and 46%, respectively.

Similar to the present study Barbosa et al. (6), reported that all the *L. monocytogenes* isolates recovered from foods (n=353) and from clinical cases of human listeriosis (n=95) in Portugal were sensitive to ampicillin. Walch et al. (41) investigated the resistance of *L. monocytogenes* isolated from 351 different food like sliced meat, frozen burgers, fish products and minced meat, to 8 antibiotics and reported that 2 (0.2%) of them were resistant and none had multi-resistance. Zhang et al. (48), 27 (3%) *L. monocytogenes* detected from the 902 ready to eat food obtained from China and 39 *L. monocytogenes* isolates were recovered from positive samples and in this isolates, 21 of them showed resistance at least one antibiotic. The isolates displayed resistance most frequently to oxacillin (18 isolates, 46.2%), followed by tetracycline. All the isolates showed susceptibility or

intermediate resistance to the gentamicin, ampicillin, ciprofloxacin and amikacin. Variation in antibiotic susceptibility pattern of *L. monocytogenes* to different antibiotics could be due to strain variation and/or as a result of drug resistance due to indiscriminate use of antibiotics in veterinary and human practise in different geographical areas.

In summary, lamb meats may serve as potential vehicles for transmission of virulent *L. monocytogenes*. The present study showed the prevalence of antimicrobial resistance in *L. monocytogenes* isolates from lamb meat. These data can be helpful in improving background data on antibiotic resistance of strains isolated from food, food environment and for epidemiological and public health studies of *L. monocytogenes*. A continued surveillance of emerging antimicrobial resistance of this pathogen is important to ensure effective treatment of human listeriosis. There is great need for a surveillance programs in Turkey to monitor epidemiological information on *L. monocytogenes* in different sources.

Acknowledgements

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