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Prevalence and Serotype Distribution of *Listeria monocytogenes* Isolated from Retail Raw Meats*

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Summary: *Listeria monocytogenes* is a foodborne pathogen, which has great importance for human and animal health. Although 13 serotypes have been identified for *L. monocytogenes,* four of these serotypes (1/2a, 1/2b, 1/2c and 4b) are significant with regard to human listeriosis. With this study, beef, sheep and chicken meat samples were examined for *L. monocytogenes* and serotype of isolates were detected by molecular methods. A total of 156 marketed meat samples comprising of beef, sheep and chicken were collected from different supermarkets, butchers and retail shops. *L. monocytogenes* contamination rate was found as 12.8% in overall meat samples. In this study *L. monocytogenes* were detected in meat samples of beef, sheep and chicken meat samples as 14.8% (8 of 54), 9.6% (5 of 52), 14% (7 of 50), respectively. Common serotype was belonged to 1/2c in beef meats, 1/2a in chicken meats. *L. monocytogenes* strains of sheep were serotyped first time and dominant serotype was found to be 1/2c in Turkey. The presence of *L. monocytogenes*, contamination level and predominant serotype distribution in diverse foods should be elucidated with comprehensive studies mainly in animal products. Additionally, genetic relationship of human and animal isolates should be revealed and the role of animal products in human listeriosis should be investigated.
Key words: *Listeria monocytogenes,* mPCR, meat, serotype

Satışa Sunulan Etlerde *Listeria monocytogenes* Prevalansı ve Serotip Dağılımının Belirlenmesi

Özet: *Listeria monocytogenes,* gıda kaynaklı patojenlerden biri olup insan ve hayvan sağlığı açısından büyük öneme sahiptir. *L. monocytogenes*'in 13 serotipi bulunmasına rağmen, bunlardan dördü (1/2a, 1/2b, 1/2c ve 4b) insan listeriozisi açısından önemlidir. Bu çalışmada sığır, koyun ve tavuk et örnekleri *L. monocytogenes* yönünden incelenerek, moleküler yöntemlerle serotipleri ortaya kondu. Sığır, koyun ve tavuk etinden oluşan toplam 156 örnek süpermarket, kasap ve perakende satış noktalarından alındı. Tüm et örneklerinin %12.8'i *L. monocytogenes* ile kontamine bulundu. Bu çalışmada, *L. monocytogenes* sığır et örneklerinde %14.8 (8/54), koyun et örneklerinde %9.6 (5/52), tavuk et örneklerinde %14.0 (7/50) oranında tespit edildi. Sığır etinde 1/2c, tavuk etinde ise 1/2a serotipi baskın olarak bulundu. Bu çalışmada, Türkiye'de koyun listeria suşları ilk defa serotiplendirildi ve baskın serotip 1/2c olarak belirlendi. Kapsamlı çalışmalar ile başta hayvansal gıdalar olmak üzere farklı kaynaklarda *L. monocytogenes* varlığı, kontaminasyon düzeyi ve predominant serotip dağılımı belirlenmelidir. Buna ilaveten, insan ve hayvan izolatlarının genetik ilişkileri ortaya konularak insan listeriozisinde hayvansal ürünlerin rolü araştırılmalıdır.
Anahtar kelimeler: Et, *Listeria monocytogenes,* mPCR, serotip

Introduction

A significant rise has been observed in foodborne infections and intoxications throughout the world and one of the bacterial agents responsible for this is *L. monocytogenes*. European Food Safety Authority (EFSA) reports that *L. monocytogenes* ranks fifth among foodborne outbreaks (EFSA, 2015). According to Center for Disease Control and Prevention (CDC) data, 1500 people are being hospitalized and 260 people are losing their lives due to *L. monocytogenes* annually in the USA (CDC, 2016).

It has been reported that vast majority of human listeriosis cases originate from the consumption of ready to eat products and animal foods *L. monocytogenes* isolation was stated in red meat, chicken meat, raw milk and milk products and ready to eat foods (Norton and Braden, 2007).

L. monocytogenes has 13 serotypes (1/2a, 1/2b, 1/2c, 3a, 3b, 3c, 4a, 4ab, 4b, 4c, 4d, 4e and 7) on the basis of somatic (O factor) and flagellar (H factor) antigens. Four of these serotypes, (1/2a, 1/2b, 1/2c and 4b) are identified mostly in human listeriosis (Doumith et al., 2004). It has been stated that isolates obtained from human cases were mostly 4b serotype, and secondly common serotype was 1/2a in the USA (CDC, 2016). EFSA notified that 1/2a (57.5%) serotype was the most common isolate and followed by 4b, 1/2b, 1/2c, 3a and 3b serotypes for human listeri-

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osis (EFSA, 2015).

Rapid detection of agent and accurate determination of serotypes are crucial for struggling with disease. Consequently, agglutination, ELISA and PCR assays (Doumith et al., 2004; Kerouanton et al., 2010) are widely employed for serotyping. But agglutination method has several limitations such as high-cost antisera, being time-consuming, requirement of experience and low discrimination capacity (Kerouanton et al., 2010). In recent years, specific, rapid, reliable and cost-effective Multiplex Polymerase Chain Reaction (mPCR) method was replaced by conventional serotyping methods, for identification and serotyping of *L. monocytogenes* (Doumith et al., 2004).

The current study aimed to reveal the occurrence of *L. monocytogenes* and to determine the distribution of serotypes by molecular methods in meats samples that belong to different animal species (beef, sheep and chicken).

Materials and Methods

Food samples: A total of 156 marketed fresh meat samples comprising of beef (n=54), sheep (n=52) and chicken (n=50) were collected randomly from 55 different markets, butchers and retail shops between April and October 2016. Samples were brought to the laboratory in cold chain, immediately followed by isolation and identification processes.

Isolation and identification of L. monocytogenes: EN ISO 11290-1 culture technique was utilized for isolation of *L. monocytogenes.* A 225 ml Half Fraser Broth (Merck 1.10398.0500) was added onto 25 g of each sample, and samples were homogenized for 2-3 minutes in the stomacher (Interscience BagMixer 400 cc, France). The suspension was incubated at 30 °C for 24 hours for pre-enrichment. After the pre-enrichment, 0.1 ml was taken and transferred to 10 ml Fraser Broth (Merck 1.10398.0500) and incubated at 37 °C for 24 hours. One loopful of broth was streaked on PALCAM agar (Polymyxin Acriflavine Lithium Chloride Ceftazidime Aesculin Mannitol Oxoid CM0877-SR0150E) and plates were incubated at 37 °C for 48 hours. Presumptive colonies (black-centered, gray-black in color with a black halo) were subcultured onto TSA-YE (Tryptone Soy Agar-Yeast Extract, Oxoid CM0131) and incubated at 37 °C for 24 hours. Colonies grown on TSA-YE were transferred to TSB (Tryptone Soy Broth Oxoid CM0129) with 20% glycerol for further analysis and preserved at -20 °C.

DNA extraction and PCR: Isolates stored in TSB were thawed and 500 µl of broth was transferred to microcentrifuge tubes. Tubes were centrifuged at 10.000 rpm for 5 minutes (VMR International Galaxy 16DH) and the supernatant was decanted. Pellet was suspended in 300 µl of sterile distilled water and 300 µl of K buffer (20 mM Tris, 150 mM NaCl, 10 mM EDTA, 0,2 % Sodium Dodecyl Sulfate-SDS) and 5 µl of proteinase K (20 mg/ml) (Vivantis PC0712-100mg) were added. After the incubation of tubes at 56 °C for 2 hours, samples were heated in water bath at 100 ° C for 10 minutes to inactivate Proteinase K. Tubes were centrifuged at 13.000 rpm for 10 minutes and the supernatant was discarded. Then 100 µl of sterile distilled water was added and used as template DNA in PCR assays.

Initially, DNA samples were analyzed for *Listeria* spp. with the *prs* gene specific primer pair. *Listeria* spp. positive samples were examined for *L. monocytogenes* with *prfA* gene specific primers. Then, serotypes of *L. monocytogenes* were determined by multiplex PCR using specific primer pairs (Table1).

Table 1. Primer pairs used for *Listeria* spp., *L. monocytogenes* and serotyping

Target Gene	Primer sequence (5'-3')		Sizes (bp)	Reference
prs	GCTGAAGAGATTGCGAAAGAAG CAAAGAAACCTTGGATTTGCGG	All *Listeria* spp.	370 bp	(Doumith et al., 2004)
prfA	GATACAGAAACATCGGTTGGC GTGTAATCTTGATGCCATCAGG	*L. monocytogenes*	274 bp	(D'Agostino et al., 2004)
lmo0737	AGGGCTTCAAGGACTTACCC ACGATTTCTGCTTGCCATTC	1/ 2a, 1/2 c, 3a and 3c	691bp	(Doumith et al., 2004)
lmo1118	AGGGGTCTTAAATCCTGGAA CGGCTTGTTCGGCATACTTA	1/2 c and 3c	906 bp	(Doumith et al., 2004)
orf2819	AGCAAAATGCCAAAACTCGT CATCACTAAAGCCTCCCATTG	1/ 2 b, 3b, 4b, 4d and 4e	471 bp	(Doumith et al., 2004)
orf2110	AGTGGACAATTGATTGGTGAA CATCCATCCCTTACTTTGGAC	4b, 4d and 4e	597 bp	(Doumith et al., 2004)

Listeria spp., and *L. monocytogenes* PCR reactions were performed in a final volume of 50 µl including 5 µl 10X PCR Buffer (500 mM KCl, 100 mM Tris-HCl (pH 9.1) and 0.1% Triton X-100) (Vivantis,ViBufferA), 5 µl 25 mM $MgCl_2$ (Vivantis, 50 mM), 250 µM each

deoxynucleotide triphosphate (Vivantis, NP2406 100mM), 20 pmol each primer (Biomatik, Canada), 1.25 U Taq DNA polymerase enzyme (Vivantis, PL1202 500 U) and 5 µl (25 ng) template DNA. Serotyping of *L. monocytogenes* was performed by mPCR described by Doumith et al. (2004). All PCR tests were carried out in Biorad-T100 thermal cycler (Biorad, USA). PCR amplification conditions were as follows: an initial denaturation at 94 °C for 3 min. 35 cycles consisting of denaturation at 94 °C for 40s, annealing at 53 °C for 75 s and hybridization at 72 °C for 75 s; final extension at 72 °C for 7 min. PCR products on gel were stained with Ethidium bromide (10 mg/ml) (Merck, 1.11608.0030), after electrophoresis at 110 V for 120 min. and visualized by ultraviolet transilluminator (Vilber Lourmat Quantum ST4) imaging system. For serotype definition was as such; if an isolate was determined as *lmo737* positive serotype 1/2a (or 3a), isolate was determined both *lmo737* and *lmo1118* positive determined as serotype 1/2c (or 3c), isolate was determined as *orf2819* positive determined as serotype 1/2b (or 3b), isolate was determined both *orf2819* and o*rf2110* positive determined as serotype 4b (or 4d, 4e) (Figure 1). Reference strains of *L. monocytogenes* RSKK 471 (serotype 1/2a), *L. monocytogenes* RSKK 472 (serotype 1/2b), *L. monocytogenes* ATCC 7644 (serotype 1/2c) and *L. monocytogenes* RSKK 475 (serotype 4b) were used as positive control, as negative control sterile distilled water was used.

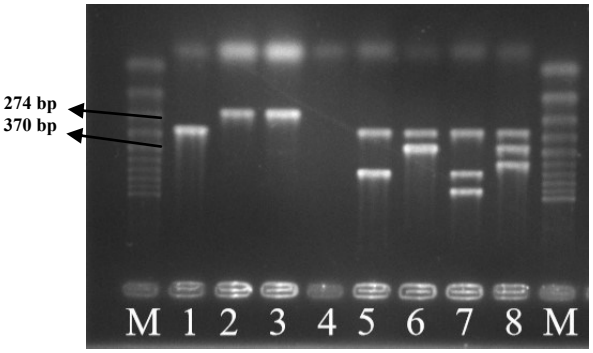

Figure 1. Serotype identification of *L. monocytogenes* isolates from chicken meat products by multiplex PCR
M: 100 bp DNA marker. Lane 1: *prs* gene positive *Listeria* sp. (370 bp) Lane 2: *prfA* gene positive *L. monocytogenes* (274 bp) Lane 3: Positive control (*L. monocytogenes* ATCC 7644) Lane 4: Negative control. Lane 5: *L. monocytogenes* serotype 1/2a (or 3a) (*Lmo0737* 691 bp) Lane 6: *L. monocytogenes* serotype 1/2b (or 3b) (*Orf2819* 471 bp). Lane 7: *L. monocytogenes* serotype 1/2c (or 3c) *(Lmo0737* 691 and *Lmo1118* 906 bp) Lane 8: *L. monocytogenes* serotype 4b (or 4d, 4e) (*Orf 2819* 471 and *Orf 2110* 597 bp)

Results

In PCR analysis, 60 isolates were found to contain *prs* genes and determined as *Listeria* spp. *Listeria* spp. detection rates were 24.1%, 23% and 70% in beef, sheep and chicken meat samples, respectively. Twenty out of 60 *Listeria* spp. isolates were identified as *L. monocytogenes. L. monocytogenes* contamination rate was found as 12.8% in overall meat samples. Contamination levels of *L. monocytogenes* were estimated as 14.8%, 9.6% and 14% in beef, sheep and chicken meat samples, respectively (Table 2).

In mPCR assays, four different serotypes (1/2a, 1/2b, 1/2c and 4b) were observed among the 20 *L. monocytogenes* isolates. In this study, predominant serotype was 1/2c (55%), and followed 1/2a (25%), 4b (15%) and 1/2b (5%) in *L. monocytogenes* isolates, respectively (Table 3).

Table 2. Distribution of *Listeria* spp. and *L. monocytogenes* in meat samples (%)

Meat samples	No. of samples (n)	*Listeria* spp.	*L. monocytogenes*
Beef	54	24.1% (13/54)	14.8% (8/54)
Sheep	52	23.0% (12/52)	9.6% (5/52)
Chicken	50	70.0 %(35/50)	14.0% (7/50)
Total	**156**	**38.5% (60/156)**	**12.8% (20/156)**

Table 3. Serotype distribution of *L. monocytogenes* in meat samples

Meat samples	Number of serotype positive isolates (%)				Total isolates
	1/2a	1/2b	1/2c	4b	
Beef	0	0	6 (75)	2 (25)	8
Sheep	0	0	4 (80)	1 (20)	5
Chicken	5 (71.4)	1(14.3)	1 (14.3)	0	7
Total	**5 (25)**	**1 (5)**	**11 (55)**	**3 (15)**	**20**

Discussion and Conclusion

L. monocytogenes was detected in the meats of three animal species. Contamination rate was calculated as 12.8% in overall meat samples. The results obtained from this research (14.8%) are consistent with those of the studies conducted in other countries, although it was higher than the previous study carried out in Turkey. *L. monocytogenes* occurrence has been reported at 4.3-75% in beef meats especially 4.3% in Korea (Baek et al., 2000), 15.5% in Japan (Ochiai et al., 2010), 24.4% in Italy (Pesavento et al., 2010) and 75% in Iran (Mashak et al., 2015). The occurrence rate has been found to be between 0.9 and 6.32 % in Turkey (Cetinkaya et al., 2014; Dogruer et al., 2015).

L. monocytogenes has been isolated from various food sources mainly in meat and meat products (Norton and Braden, 2007). *L. monocytogenes* may contaminate in processes of animal production, slaughtering, selling or preparation of foods steps (Bouayad et al., 2015; Dogruer et al., 2015; Mead, 2004).

L. monocytogenes prevalence in chicken meats was reported as 14.3% in South Africa (Van Nierop et al., 2005), 20.2% in Turkey (Ayaz et al., 2009), 24.5% in Italy (Pesavento et al., 2010), and 30.2% in Korea (Baek et al., 2000). The prevalence of the agent revealed in this study (14%) was lower than those of the aforementioned studies. Interestingly, *L. monocytogenes* was detected in chicken meats by 14%, although isolation rate of *Listeria* spp. was 70%, higher *Listeria* spp. presence in chicken meats than red meat; might have resulted from cross contamination that happened during slaughtering excessive number of chicken per hour, bleeding, removal of feathers, evisceration and packaging steps (Mead, 2004).

L. monocytogenes occurrence in sheep meats were declared as 2% in India (Vinay Kumar et al., 2016), 8% in Iran (Mashak et al., 2015) and 16% in Romania (Carp-Carare et al., 2013), beside it was reported to be between 0-50 % in Turkey (Abay et al., 2012; Kahraman et al., 2005; Kocaman and Sarımehmetoglu, 2017). Reported studies in Turkey are Kocaman and Sarımehmetoğlu (2017) found that *L. monocytogenes* were in lamb meat samples of 15.8%, Abay et al. (2012) isolated *L. monocytogenes* in 50% of minced sheep meat samples. However, Kahraman et al. (2005) reported that they were not able to detect *L. monocytogenes* in lamb carcasses. In this study, *L. monocytogenes* frequency was defined to be 9.6% in sheep meat samples. The difference in prevalence of *L. monocytogenes* could be due to the regional difference, slaughter hygiene, sample types and counts and isolation methods.

L. monocytogenes causes aborts, septicemia and central nervous system infections in humans. It was reported that among the food pathogens, *L. monocytogenes* has the highest mortality rate with 30%.

Consumption of foods contaminated with *L. monocytogenes* causes infection in humans (Schlech, 2000). So, the presence of *L. monocytogenes* on foods is limited via legislations in many countries. Zero tolerance policy on struggling with *L. monocytogenes* is applied in Turkey and many other countries such as the USA and European Union. According to this, 25 grams of ready-to-eat foods should not contain any *L. monocytogenes* agent (EFSA, 2015; Shank et al., 1996). However, no limit has been imposed on minced meat, red meats, chicken meats and meat preparations in Turkey (TFC, 2011). Determination of microbiological threats in meat products is substantial for taking precautionary measures against the risks that threaten human health.

In the current study, predominant serotype was 1/2c (55%), followed by 1/2a, 4b and 1/2b in *L. monocytogenes* isolates, respectively. Even 1/2c and 4b serotypes were isolated in beef meat samples in this study. Another study conducted in Estonian by Kramarenko et al. (2013) reports that common serotype was 1/2a, followed by 1/2c, 1/2b and 4b, respectively. Cetinkaya et al. (2014) demonstrated that common serotype in beef meats was 1/2a.

Although various studies have been conducted on the occurrence of *L. monocytogenes* in sheep meats, no data has been observed in literature upon serotype distribution in Turkey. To our knowledge, this is the first study to investigate the serotypes of *L. monocytogenes* from sheep meats. Most common observed serotypes were 1/2c (80%), secondly 4b (20%). This result is in agreement with result of a study in Romania (Carp-Carare et al., 2013).

Predominant serotypes in chicken meats vary from country to country. While Zhang et al. (2007) reported that the most prevalent serotype was 1/2b followed by 4b, Fallah et al. (2012) reported that the most common serotype was 4b and followed by 1/2a. The most dominant serotype was reported to be 1/2a in Turkey, Portugal and Estonia (Cetinkaya et al., 2014; Erol and Sireli, 1999; Guerra et al., 2001; Praakle-Amin et al., 2006; Siriken et al., 2014). This study revealed that the most predominant serotype was 1/2a (71.4%), followed by 1/2b and 1/2c in chicken meats. When overall the samples were evaluated, only 1/2c serotype was present in the meats of three animal species. Serotype 4b (15%) was indicated mostly in beef and sheep meat samples. Considering the serotype 4b, which is responsible for most human listeriosis cases, it can be asserted that beef and sheep meats may be riskier.

Rapid detection and accurate serotyping in Listeriosis cases are first steps of subtyping and revealing the transmission source and route of infection. Among the 14 serotypes of *L. monocytogenes* 1/2a, 1/2b, 1/2c and 4b serotypes are accounted for 95-98% of human listeriosis (CDC, 2016; Doumith et al., 2004; EFSA, 2015). Almost no serotype data has been introduced in human listeriosis cases in Turkey. Only 4b serotypes was reported in humans (Tekay et al., 2014).

Consequently, contamination of meats to different animal species by *L. monocytogenes* was found to be 12.8% and predominant serotype was 1/2c, followed by 1/2a. Serotype distribution of the pathogen in sheep meat samples was indicated for the first time in Turkey and most common serotype was detected as 1/2c. Comprehensive studies should be conducted on different sources mainly on animal foods to elucidate the presence of *L. monocytogenes,* contamination level and predominant serotype distribution. Also, the role of animal products in human listeriosis should be investigated by clarifying genetic relationships of human and animal isolates. To prevent listeriosis cases caused by meat samples, preventive measures in general hygiene and disinfection applications should be taken throughout the production line and during the cooking process.

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