The Prevalence and Molecular Characterization of *Listeria monocytogenes* in Corn Silage, Feces and Bulk Tank Milk Samples in Dairy Cattle Farms in Balikesir, Turkey

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ABSTRACT

The objective of this study was to investigate the prevalence and molecular characterization of *Listeria monocytogenes* in corn silage, feces and bulk tank milk (BTM) samples. The samples (n=150/each; 450 in total) were obtained from dairy cattle farms and analyzed for *Listeria spp*. and *L. monocytogenes*. The isolates were identified by using biochemical tests. Serotyping was done by using Polymerase Chain Reaction (PCR). Also the source and possible contamination routes with *L. monocytogenes* were determined by using Pulsed Field Gel Electrophoresis (PFGE). The percentages of *L. monocytogenes* detected in the silage, feces and milk samples were 4%, 2.4% and 2.4%, respectively. There were 10 different PFGE types and 4 serotypes of the 14 isolates. The isolates of 14 *L. monocytogenes* were distributed into four serogroups as "1/2a, 3a" (n=6), "1/2b, 3b" (n=3) "1/2c, 3c" (n=2), and "4b" (n=3). According to the method of PFGE, *L. monocytogenes* strains obtained from the samples were determined to be related to each other. Although the prevalence of *L. monocytogenes* is low in those samples, it is a serious risk in terms of food safety and public health.

Keywords: Bulk Tank Milk; Feces; *Listeria monocytogenes;* PFGE; Silage.

INTRODUCTION

Listeria monocytogenes causes a range of clinical manifestations including septicemia, meningitis, gastroenteritis, and abortion (1). Recently, it was reported that about 23% of deaths in the humans related to bacterial diseases in the USA was originated from listeriosis (2). In 2015, about 2,200 people were reported to be infected with *Listeria* in the European countries and 12% of those died (3).

The pathogen is commonly found in the soil, silage and water and can easily contaminate meat, raw milk and other dairy products (4). In addition, contamination of raw milk with the bacteria was reported to be mainly by feces, beddings and udder infections (5). Previously, it was reported that about 50% of listeriosis outbreaks in Europe originated from dairy products contaminated with *L. monocytogenes* (6). Also consumption of contaminated milk directly or in cheese poses a severe risk for human health (7). Therefore, the purpose of this study was to investigate the prevalence and molecular characterization of *L. monocytogenes* in corn silage, feces and BTM samples taken from various dairy cattle farms in Balikesir, Turkey.

MATERIALS AND METHODS

Sampling

The silage, feces and BTM samples (n=150/each) were aseptically collected from the different dairy cattle farms (30 dairy herds/farm on average) in Balikesir, Turkey. These samples were carried at +4°C in an icebox, transferred into the laboratory within 2 hours and analyzed for *Listeria* spp. and *L. monocytogenes*.

Microbiological analyses

The presence of *Listeria* spp. and *L. monocytogenes* from the samples were determined according to the standard method described (8). As a primary enrichment; samples (25 g/ml/sample) were added in 225 ml Half Fraser Broth (Oxoid, CM0895, UK), homogenized for 2 min at stomacher (IUL 400) and incubated at 30°C for 24±2 hrs. Then, 0.1 ml of primary enrichment were added into 10 ml of Fraser broth (Oxoid, CM0895, UK) and incubated at 35±2°C for 48±2 hrs. as secondary enrichment. A loopful of selective enrichment broth was streaked on the surface of Oxford Agar (Oxoid, CM0856, UK) and PALCAM agar (Oxoid, CM0877, England) and then were incubated at 35°C for 48 hours. Suspected *Listeria* isolates were firstly grown in Tryptone Soya Yeast Extract Agar (Sigma Aldrich, 93395, India), then they were tested for Gram staining (Oxoid, Basingstoke, UK) and by the Microbact™ *Listeria* 12 L system (Oxoid, MB1128, UK). β-haemolytic activity of the bacteria was tested by using Columbia agar (Thermo Fisher Scientific, Lenexa, Kansas, US) added with 5% sheep blood.

DNA extraction

The reference strain of *L. monocytogenes* (ATCC13932) and 14 isolates were incubated in Brain Heart Infusion Broth (BHI, Merck, 110493) at 37°C for 24 hrs. Then according to manufacturer's instructions, DNA from the isolates was extracted by using a Genomic DNA purification kit (Thermo Scientific K0722, Lithuania).

PCR amplification and electrophoresis

The *Listeria* primers (5'-biotin-ATCATCGACG-GCAACCTCGGAGAC-3' and 5'-biotin-CAC-CATTCCCAAGCTAAACCAGTGC-3') are specific for the hlyA gene of *L. monocytogenes* (9). The amplification of these genes was performed using a thermal cycler (Thermo Scientific, Finland). Total reaction volumes (25 µL) in which PCR was carried out contained 12.5 µL Master Mix (Thermo Scientific, K0171), 0.25 µM of each primer, 1 µl DNA and 11 µL DNase free water. Conditions of thermo cycling were an initial hold of 94°C (10 min), a denaturation step at 94°C (30s), annealing at 68°C (60s) and extension at 72°C (90s; 35 cycles). A final hold at 4°C followed a final extension at 72°C for 10 min. The PCR products were analyzed by using agarose gel electrophoresis after staining with 1% ethidium bromide and visualized under UV illumination (Vilber Lourmat, France).

Serotyping

L. monocytogenes isolates were serotyped as described in Table 1.

Pulsed Field Gel Electrophoresis (PFGE)

PFGE was performed according to the standard PulseNet protocol (13). In this study, the PFGE profiles were scanned. Also the computerized data of these profiles were analyzed by the Gel logic 2200 imaging system (Kodak Company, USA). Similarities among the profiles were obtained from Dice coefficient (position tolerance and optimization value

Table 1: PCR primers used for serotype *L. monocytogenes* strains

Gene	Primers	PCR (bp)	Serotype	Reference
FlaA	TTACTAGATCAAACTGCTCC AAGAAAAGCCCCTCGTCC	538	Serotype 1/2a or 3a	10
GLT	AAAGTGAGTTCTTACGAGATTT AATTAGGAAATCGACCTTCT	483	Serotype 1/2b or 3b	10
lmo1118	AGGGGTCTTAAATCCTGGAA CGGCTTGTTCGGCATACTTA	906	Serotype 1/2c or 3c	11
ORF0799	5'-GCTGGGTTTCTTACGA-3' 5'-CAACCGTTCATTTAGCTCAT-3'	83	Serotype 4b	12

Table 2: Prevalence of *Listeria* spp. and *L. monocytogenes* in silage, feces and bulk tank milk samples

Samples	No. Samples	*Listeria* spp. (Positive No. Samples and %)	*L. monocytogenes* (Positive No. Samples and %)
Silage	150	27 (18)	6 (4)
Feces	150	21 (14)	4 (2.6)
Bulk tank milk (BTM)	150	17 (11)	4 (2.6)
Total	450	65 (14.4)	14 (3.1)

1.5%). *L. monocytogenes* strains were clustered using the unweighted pair group method with arithmetic averages (14).

RESULTS

In this study, the prevalence and molecular characterization of *Listeria* spp. and *L. monocytogenes* were investigated in the silage, feces and BTM samples. Of the 450 samples collected, 14.4% and 3.1% were observed to be contaminated with *Listeria* spp. and *L. monocytogenes*, respectively. *Listeria* spp. was detected as 18%, 14% and 11% in the silage, feces and BTM samples, respectively. *L. monocytogenes* was isolated from 4%, 2.6% and 2.6% of the silage, feces and BTM samples, respectively (Table 2).

Table 3: Serogroup of *L. monocytogenes* in silage, feces and bulk tank milk samples

Samples	No. serogroup isolates (%)				Total (%)
	"1/2a, 3a"	"1/2b, 3b"	"1/2c, 3c"	"4b"	
Silage	2	3	1	–	6 (42.8)
Feces	–	–	1	3	4 (28.6)
Bulk tank milk	4	–	–	–	4 (28.6)
Total (%)	6 (42.8)	3 (21.4)	2 (14.3)	3 (21.4)	14 (100)

Table 3 represents the serotypes of *L. monocytogenes* in the silage, feces and BTM samples. According to PCR serotyping, the isolates of 14 *L. monocytogenes* were distributed into four serogroups as "1/2a, 3a", "1/2b, 3b", "1/2c, 3c" and "4b". In the present study, "1/2a, 3a" was found to be the predominant serogroup of *L. monocytogenes*.

The subtyping of *L. monocytogenes* isolates by PFGE detected 10 PFGE types (A, B, C, D, E, F, G, H, I, K) with 5 PFGE clusters (I, II, III, IV, V). It was determined that 4 sets of strains obtained were 100% similar (1-6; 7-10; 3-4-5; 9-11). Similar pulsotypes from different samples indicated that *L. monocytogenes* strains may be spread in the farm environments and raw milk through silage and feces. As shown in Figure 1, isolates 1 and 6 were obtained from milk and

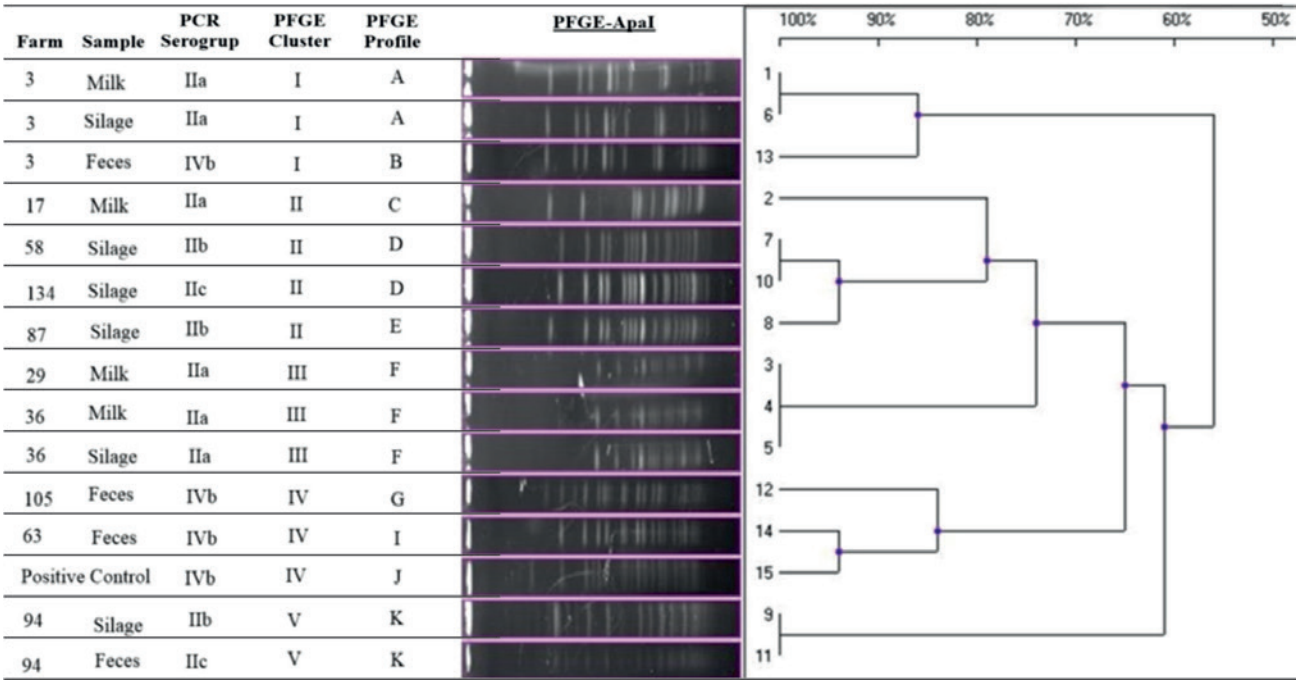

Figure 1: PFGE profiles of *L. monocytogenes* isolates obtained with restriction enzyme ApaI, and information of sources and serogroups (1-14: *L. monocytogenes*; 15: Positive Control)

silage samples of farm 3 and isolates 13 were obtained from feces samples taken from the same farm and all belonged to the same cluster (Cluster I). The present study showed that there were similar strains in the feces and milk of cattle consuming contaminated silage. The isolate number 2 was taken from the milk sample of farm 17; isolates 7, 8 and 10 were obtained from silages from farms 58, 87 and 134 respectively, and they all belonging to cluster II. Isolates 4 and 5 were obtained from the milk and silage samples taken from the farms 36 and isolate 3 and was derived from the milk sample from the farm 29 with all having the same PFGE profile. It is noteworthy that these farms were located in the same area and they collected silages from the same producer. Isolates 9 and 11 were isolated from silage and feces samples of farm 94 and have the same PFGE profile. Isolates 12 and 14 were obtained from feces samples taken from farms 105 and 63 respectively and were in the same cluster (Cluster IV) (Fig 1).

DISCUSSION

L. monocytogenes may be found in dairy cattle farms (15). Fecal contamination with this bacterium was reported to range from 14% to 50% in the farm environment (16). The bacteria surviving in the gastrointestinal tract of animals may easily be spread into BTM (15). In this study, 14.4% and 3.1% of the samples were determined as positive with *Listeria* spp. and *L. monocytogenes*, respectively (Table 2).

Previous research has indicated that the rates of *L. monocytogenes* prevalence ranged from 2% to 9% in the silage samples (17, 18). In the present study, 18% and 4% of the silage samples were determined to be contaminated with *Listeria* spp. and *L. monocytogenes*, respectively. However, another study conducted by Ho *et al.* (19) showed that 38% of 66 silage samples were contaminated with *L. monocytogenes.* They also reported that *L. monocytogenes* ribotypes isolated from the silage samples were similar to those isolated from cow feces (19). Sharifzadeh *et al.* (18) showed that the pH of silage greater than 5.5 was ideal for *Listeria* growth. Therefore, feeding those silages may be a potential risk for dairy animals. Recently, corn silage has been widely used in the dairy rations especially in the region of Balikesir, Turkey. *L. monocytogenes* in the silage or feces may contaminate cow milk and may carry a risk of transmission to humans. Environmental conditions such as temperature, humidity and weather were reported to be important factors in the contamination of silages with *Listeria* spp. and *L. monocytogenes* in the dairy farms (18). Growth of the bacteria in the silages may be prevented by using proper fermentation conditions (20). On the other hand, if silage is naturally contaminated with *L. monocytogenes*, the bacteria may survive as long as 4-6 years (21).

Feces is another source of contamination of BTM with *L. monocytogenes* (19). Many researchers (17, 19, 22, 23) reported that contamination of the cattle feces with *Listeria* spp. and *L. monocytogenes* ranged from 4.61% to 41.2% and 1.53 to 31%, respectively. In the current study, the percentage of the contaminated samples with *Listeria* spp. was 14%. Also 2.6% of the samples were positive with *L. monocytogenes*. A study conducted in the USA (15) reported that *Listeria* spp. and *L. monocytogenes* were isolated from 25% and 7.1% of the 303 fecal samples of dairy cattle, respectively. *Listeria* spp. including *L. monocytogenes* was reported to be widespread in the environment including soil, water, dairy farms and food processing facilities (24, 25). Also, manure spread into agricultural soil was reported to increase risk of bacterial transmission into the dairy products (21).

Detection of *L. monocytogenes* in BTM has previously been demonstrated. Lattore *et al.* (15) reported that 23% and 19.7% of the 172 BTM samples in the USA were contaminated with *Listeria* spp. and *L. monocytogenes*, respectively. It is known that healthy cows may carry the bacteria in the gastrointestinal tract and contaminate the farm environment (15). The current study is in agreement with results of several studies conducted about prevalence of *Listeria* spp. and *L. monocytogenes* in the raw milk. According to the results of studies conducted in Turkey (26, 27), India (28, 29), Iran (30,31) and Italy (32) *Listeria* spp. and *L. monocytogenes* isolated from the raw milk samples were ranged from 0.57% to 6.57 % and 2.12% to 18.6%, respectively. Contamination of raw milk with *L. monocytogenes* was reported to occur either directly by udder infections or contaminated feed and feces (5). Oliver *et al.* (33) reported that the prevalence of milk pathogens was affected by many factors such as farm management systems, sampling method, farm hygienic conditions, sample variability and geographical location.

CONCLUSIONS

Dairy farms are major source of contamination for *Listeria* spp. and *L. monocytogenes*. It is very important to take mea-

sures to reduce the zoonotic bacteria for food safety and public health. It is suggested that implementation of farm hygiene and milking hygiene, separation of milk from mastitis infected animals, preparation and storage of silages under proper conditions must be carried out in order to prevent cross-contamination of the *Listeria* spp. and *L. monocytogenes*.

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