Original Article

Phylogenetic positioning of *Listeria ivanovii* identified in aborted sheep in Kars Region (Turkey)

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Abstract

This study aimed to investigate *Listeria* species in various samples taken from aborted sheep in Kars province and to determine the position of emerged strains in the phylogenetic tree. Milk (n= 229) and vaginal swab (n= 263) samples from aborted sheep and abomasum contents (n= 46) of aborted sheep fetuses were investigated by the culture and Polymerase Chain Reaction (PCR) methods. For the phylogenetic analysis of isolates, the 16S rRNA gene region was sequenced. In the culture, the *Listeria* agent was not isolated from any of the vaginal swabs or milk samples. Among the 46 fetal abomasum contents, a Listeria suspicious colony was obtained from only one (2.17%) sample which belonged to an enterprise with a history of silage feeding. This isolate was identified as *Listeria ivanovii* by both conventional methods and genus and species-specific PCR. In addition, *Listeria* DNA was detected in a total of 8 (1.48%) samples (in 1 vaginal swab, 1 milk and 6 fetal abomasum samples) by direct genus-specific PCR. However, these samples could not be identified, except for one of the 6 abomasum content samples that was found as *L. ivanovii*. The *L. ivanovii* isolate was double-identified after the amplification of the 16S rRNA gene region followed by sequence analysis. Sequence analysis of the 16S rRNA gene region gave a phylogenetic position to the isolate, *L. ivanovii*, which had been scarcely identified from the abortive specimens.

Keywords: *Listeria ivanovii*, culture, PCR, 16S rRNA sequencing, phylogeny

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Introduction

Listeriosis caused by the *Listeria* species is a zoonotic infection that leads to abortions in ruminants and has been acknowledged to be an important problem to sheep breeding worldwide (Low and Donachie, 1997; Brugère-Picoux, 2008). The fact that the digestive system of mammals and soil are the natural reservoirs of *Listeria* increases the possibility of the contamination of foods and the environment with *Listeria* species. The most pathogenic and prevalent species of the *Listeria* genus is *Listeria monocytogenes (L. monocytogenes)* (Schmid *et al.*, 2005). *L. monocytogenes* has been detected in spoiled silage or feedstuffs (Perry and Donnelly, 1990; Nightingale *et al.*, 2004; Sharifzadeh *et al.*, 2015). In addition, *L. monocytogenes* has been found in the feces, blood, milk and uterine secretions of sick and healthy animals (Gicik *et al.*, 2010; Akca and Sahin, 2011; Abay *et al.*, 2012; Matto *et al.*, 2018). *Listeria ivanovii (L. ivanovii)* represents another species of pathogen within the genus *Listeria*. This species, which was formerly known as *L. monocytogenes* serotype 5, causes abortion, stillbirth and the birth of weak offspring in sheep (Ivanov, 1962; Schmid *et al.*, 2005). Poor quality silage is known to predispose sheep to Listeria infections, resulting in complications such as meningoencephalitis, septicemia, mastitis and keratoconjunctivitis (Pauly and Tham, 2003). Meningitis and encephalitis are rare in *L. ivanovii* infections (Low JC and Donachie, 1997; Sahin and Beytut, 2006).

Listeriosis is diagnosed using conventional techniques in which the agent is isolated and identified; serological tests in which the presence of bacterial antigens or antibodies in the blood and serum is determined; or molecular techniques in which nucleic acid amplification and hybridization are performed *in vitro* (Muz *et al.*, 1999; Akca and Sahin, 2011; Matto *et al.*, 2018). Molecular techniques, which are more powerful than the other diagnostic methods, have been used in the taxonomic and phylogenetic analysis of bacteria in recent years. In this context, the 16S rRNA gene region, which is found in all bacteria and is highly stabile and sufficiently large (~1.500 bp) in terms of informatics, provides useful information for sequence analysis (Patel, 2001).

Using culturing and molecular methods, this study aimed to isolate and identify *Listeria* species from various samples taken from aborted sheep in Kars province (Turkey) and to determine the position of emerged strains in the phylogenetic tree.

Materials and Methods

Study material: The study was conducted with the approval of the Kafkas University Animal Experiments Local Ethics Committee (KAU-HADYEK – 2015/007). The study was carried out on 6 sheep enterprises in which small-scale family-type livestock farming was carried out semi-extensively and uniform management approaches were conducted, in which abortion cases, coinciding with the last few weeks of gestation, were observed in Kars province, between 2016 and 2019. The history of the feeding of animals with silage that might be a predisposing factor for Listeriosis was recorded in only one of the enterprises (in the Alem Village). The material of the study consisted of milk and vaginal swab samples, taken from 263 aborted sheep. Vaginal samples were sampled from whole aborted sheep whereas only 229 milk samples could achieved. In addition, the abomasum contents of 46 aborted sheep fetuses brought to the Faculty of Veterinary Medicine, Kafkas University were included in the study (Table 2).

Isolation and identification: The isolation of bacteria from the milk and vaginal swab samples was performed according to the FDA method, which was proposed by Lovett and Hitchins (1988) and was later on modified by IDF (Twedt and Hitchins, 1994). Twenty-five ml of the milk sample was added to 225 ml of Listeria enrichment broth and allowed to incubate at 30 °C under microaerobic conditions. Vaginal swabs carried in Tryptose Broth were transferred to 10 ml of Listeria Enrichment Broth (UVM formulation) (Oxoid CM0863) and allowed to incubate at 30 °C under microaerobic conditions. After 24 and 48 hours of incubation, Listeria Selective Agar (Oxoid, CM0856) medium was plated from the Listeria Enrichment Broth and incubated at 30 °C for 24 hours in the same atmospheric conditions. The isolation of *Listeria* species from the aborted fetal abomasum content was adapted from the method reported by McClain and Lee (1988). For this purpose, 2.5 ml abomasum content was inoculated in 22.5 ml pre-enrichment liquid medium (Tryptic Soy Broth (Merck 1.05459) containing 0.6% yeast extract) and incubated for 24 hours at 30 °C under microaerobic conditions. At the end of this period, 1 ml of this pre-growth liquid medium was transferred to 9 ml of Listeria Enrichment Broth and incubated overnight at 30 °C in the same atmosphere. At the end of the period, Listeria Selective Agar (LSA) was inoculated from the enrichment broth and incubated at 30 °C for 24 hours in a microaerobic medium. Following the culture of the samples, the identification of the smooth, round colonies with bright grey-black centres that reproduced in the LSA medium and were suspected to be Listeria was carried out by determining their Gram staining properties, semi-solid media motility at 25 °C, carbonhydrate fermentation reactions (xylose and rhamnose) and CAMP test reactions. In the CAMP assay *Rhodococcus equi* (ATCC-33701) and *Staphylococcus aureus* (ATCC-25923) standard strains were used as control bacteria (FDA, 2015).

Molecular analysis: For the identification of *Listeria* isolates obtained after the culture processes and for direct analysis of the *Listeria* species from the samples, genus and species specific PCRs were performed using primers targeting the amplification of the *iap* gene (invasion associated protein). The PCR technique using universal primers targeting the amplification of the 16S rRNA gene was performed for the purpose of sequencing the isolates obtained by culturing surveys and genus and species specific PCR analyses (shown in Table 1) (Lane, 1991; Bubert *et al.*, 1999). PCRs were performed with minor modifications in the presence of the relevant references. *L. monocytogenes* ATTC-7644 and *L. ivanovii* type 5 SLCC-2379 (from culture collection of the Kafkas University) were used as standard strains in PCRs.

Genus and species-specific PCR reactions in which the total reaction volume was determined as 25 µl for each sample were constructed in accordance with the substances of 2.5 µl PCR buffer (x10), 2.5 µl $MgCl_2$ (10 mM), 0.5 µl dNTP (10 mM), 0.25 µl Primer F (20 pmol), 0.25 µl Primer R (20 pmol), 0.2 µl Taq DNA polymerase, 14.8 µl ddH_2O and 4 µl template DNA. The 16S rRNA PCR reactions in which the total reaction volume was determined as 25 µl for each sample were constructed in accordance with the substances of 2.5 µl PCR buffer (x10), 3 µl $MgCl_2$ (10 mM), 0.5 µl dNTP (10 mM), 1 µl Primer F (20 pmol), 1 µl Primer R (20 pmol), 0.4 µl Taq DNA polymerase, 13.6 µl ddH_2O and 3 µl template DNA.

Table 1 Primers used in the characterisation and sequence analysis of *Listeria* species.

Primer sequence (5′—3′)	Gene	Band size (bp)	Reference
Genus specific PCR			
Lis1A; 5′- ATGAATATGAAAAAAGCAAC -3′	*iap*	1600	Bubert *et al.* 1999
Lis1B; 5′- TTATACGCGACCGAAGCCAAC -3′			
Species specific PCR			
MonoA; 5′- CAAACTGCTAACACAGCTACT -3′ (*L. monocytogenes*)	*iap*	660	Bubert *et al.* 1999
Iva1; 5′- CTACTCAAGCGCAAGCGGCAC -3′ (*L. ivanovii*)		1100	
Lis1B; 5′- TTATACGCGACCGAAGCCAAC -3′ (common for both species)			
16S rRNA PCR			
27F; 5′- AGAGTTTGATCCTGGCTCAG -3′	16S rRNA	1492	Lane, 1991
1492R; 5′- GGTTACCTTGTTACGACTT -3′			

Genus and species specific PCR thermal cycling was programmed with the initial denaturation at 95 °C for 5 mins, followed by 30 cycles of denaturation at 95 °C for 15 secs, annealing at 58 °C for 30 secs, extension at 72 °C for 50 secs and a final extension at 72 °C for 10 mins. The PCR analysis of the 16S rRNA gene region was programmed with the initial denaturation at 95 °C for 5 mins, followed by 30 cycles of denaturation at 94 °C for 15 secs, annealing at 59 °C for 30 secs, extension at 72 °C for 45 secs and a final extension at 72 °C for 5 mins. Electrophoretic analysis of the amplified products obtained by PCR were run on 1% horizontal agarose gel.

The 16S rRNA region was sequenced on the ABI 3500 Genetic Analyzer using the BigDye Terminator v3.1 Cycle Sequencing Kit. The primers used in the sequence are given in Table 1. Sequence analysis was performed with the CLC Main Workbench 7.7.3 (Qiagen) program and the data was compared with *Listeria* species in NCBI GenBank. *Listeria* species names were identified according to BLAST analysis and similarity scores obtained from NCBI GenBank (CLSI, 2008).

Results

In this study, the Listeria agent was not isolated from any of the 263 vaginal swabs and 229 milk samples cultured. Of the 46 fetal abomasum contents, which were analyzed by culturing methods, one (2.17%) was identified as *Listeria* spp. with a smooth, round and central bright gray-black colony structure on LSA medium. The isolate was determined as *L. ivanovii* based on microscopic findings (0.4-0.5 µm wide and 1-2 µm long, non-spore forming Gram-positive bacillus), motility in semi-solid medium, positive reaction with xylose and its positive CAMP reaction with *R. equi*.

The isolate obtained from the fetal abomasum after the culturing processes was identified as *Listeria* spp. by genus specific PCR analysis. Listeria specific DNA was detected in a total of 8 (1.48%) samples, that is in 1 (0.38%) of 263 vaginal swab samples, in 1 (0.43%) of 229 milk samples and in 6 (13.33%) of 45 fetal abomasum samples by direct genus specific PCR analysis (in Table 2).

One isolate, obtained from fetal abomasum content after culturing procedures and determined to be *Listeria* spp. by genus specific PCR, was identified as *L. ivanovii* as a result of species specific PCR. One vaginal swab and 1 milk sample which were found to be *Listeria* spp. in the direct genus specific PCR analysis, could not be identified in the species specific PCR. One of the 6 abomasum content samples found positive with genus specific PCR was identified as *L. ivanovii* but the other 5 samples could not be classified at the species level (in Table 2). A 1492 bp long amplified product was obtained by PCR analysis of the isolate that had been identified as *L. ivanovii* using universal primers targeting the 16S rRNA gene.

After the amplification of the 16S rRNA gene region followed by sequence analysis, the isolate was identified as *L. ivanovii*. The partial sequence of the 16S rRNA gene region of the isolate was recorded in the GenBank database with the name LIVANOVII1 and access number MK789474.1.

The *L. ivanovii* isolate identified in this study was found to be in the same cluster as the *L. ivanovii* strain isolated from a sheep fetus in Spain in 1997, which showed the serotype 5 antigenic character and was encoded ATCC-BAA-678 (JF967631). This isolate was positioned in the same branch with sheep isolates

which were defined as *L. ivanovii* subp. *ivanovii* by Seeliger *et al*. (1984) and encoded as NCTC11846 (LT906478) and NCTC11007 (LT906466) (shown on Figure 1).

Table 2 Distribution of samples used in the study and the identification results.

Location	Sample type	Number of sample	Culture results	Direct PCR results	
				Genus specific PCR	Species specific PCR
Kars Center Maksutcuk	Vaginal swab	43	-	-	-
	Milk	43	-	*Listeria* spp. (1)	-
Kars Center Kumbetli	Vaginal swab	18	-	-	-
	Milk	18	-	-	-
Arpacay Center	Vaginal swab	38	-	*Listeria* spp. (1)	-
	Milk	36	-	-	-
Arpacay Kockoy	Vaginal swab	41	-	-	-
	Milk	38	-	-	-
Susuz Doyumlu	Vaginal swab	81	-	-	-
	Milk	65	-	-	-
Susuz Center	Vaginal swab	42	-	-	-
	Milk	29	-	-	-
Microbiology Laboratory	Fetus	46	*Listeria ivanovii* (1)	*Listeria* spp. (6)	*Listeria ivanovii* (1)
Total	Vaginal swab	263		*Listeria* spp. (1)	-
	Milk	229		*Listeria* spp. (1)	-
	Fetus	46	*Listeria ivanovii* (1)	*Listeria* spp. (6)	*Listeria ivanovii* (1)

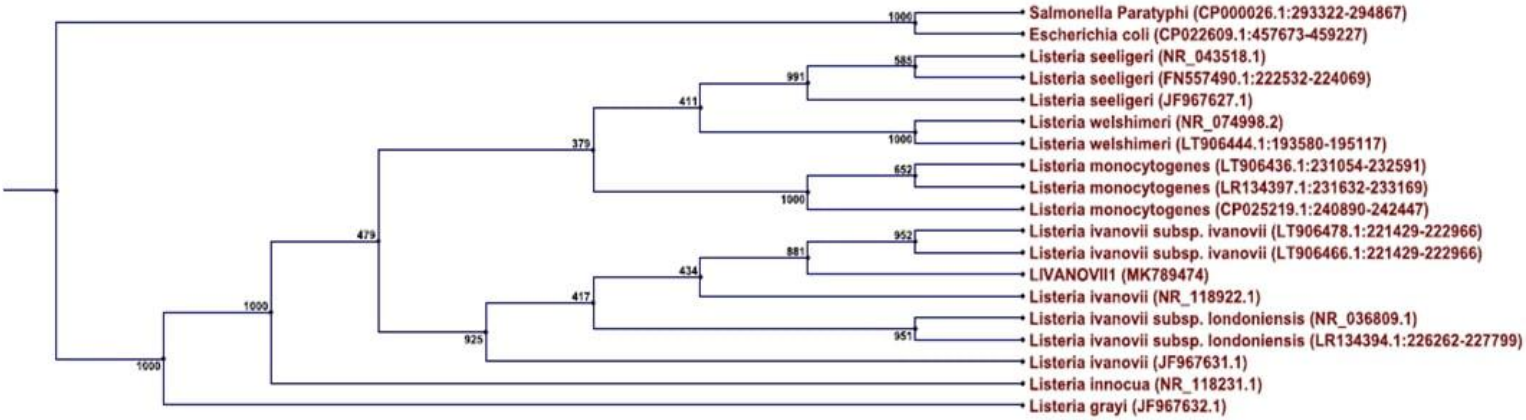

Figure 1 The dendrogram generated by the neighboring joining method for the *L. ivanovii* isolate analyzed according to the sequence of the 16S rRNA gene region and the evolutionary position of the isolate. The GenBank access number of the isolate is given in parentheses.

Discussion

Sheep rearing is an important livestock sector that contributes to the national economy with its products such as meat, milk, wool and leather. Reproductive diseases, abortions and neonatal lamb deaths due to infectious agents are important problems in sheep breeding worldwide. The agents leading to abortion include bacterial species such as *Brucella*, *Campylobacter*, *Chlamydophila*, *Salmonella* and *Listeria*, and some viral and parasitic agents (Djønne, 2007; Ali *et al*., 2019). *Listeria* species play a significant role in abortions in livestock, although the incidence varies due to factors such as climatic conditions, care and feeding and animal movements (Dhama *et al*., 2015). In particular, poor quality silage fodder is known to be a predisposing factor for Listeria infections (Pauly and Tham, 2003). One of the strains identified as *L. ivanovii* was the isolate obtained from the abomasum content of an aborted fetus from a flock of 150 sheep fed by silage in Alem Village in the Digor district of Kars, where about 80 abortions were observed within a period of 1 month. The high abortion rate recorded in the above flock may be explained by the fact that poor quality silage fodder is a known risk factor in the epidemiology of listeriosis. Similar epidemiological data is not available for the other herds sampled in this study in which Listeria was identified, however, besides the stress associated with advanced pregnancy, nutritional challenges and unhygienic environmental conditions have been assumed as potential risk factors for these outbreaks.

The species *L. monocytogenes* and *L. ivanovii* are responsible for Listeria abortions in sheep. *L. monocytogenes* is important because it is widespread and also because it has zoonotic properties. In natural or experimental infections, Listeria agents are widely distributed through the secretions of the genital system, milk and abortion materials, and these are the sources of infection for new cases (Sahin and Beytut, 2006; Akca and Sahin, 2011; Durmaz *et al*., 2015). Such

materials also represent valuable samples for use in the diagnosis of Listeria infections. In this study, 229 milk and 263 vaginal swab samples taken from sheep in the village of Kars, its center and its districts were examined by culturing methods and no Listeria was isolated. Listeria isolation was achieved from 1 (2.17%) of the 46 fetal abomasum contents and this isolate was identified as *L. ivanovii* by species specific PCR. *L. ivanovii* DNA was also detected in 1 (2.22%) of the fetal abomasum contents. In summary, *Listeria* was identified by PCR analysis in 9 (1.67%) out of 538 samples, while only 2 (0.37%) of them were identified as *L. ivanovii* (shown in Table 2). This indicates that the PCR method, which can detect nucleic acids of both dead and live microorganisms, has a diagnostic superiority over culture methods. Due to its sporadic nature, the isolation rate of pathogenic *Listeria* species in this study is similar to others (Kirkbride, 1993; Muz *et al*., 1999). In this study, *L. ivanovii* is the only *Listeria* species identified from aborted sheep fetuses and the isolation rate remained very low (0.37%), similar to the previous study (Sahin and Beytut, 2006) from sheep abortions in the Kars region. After partial sequence analysis of the 16S rRNA gene region of the isolate identified in this study, it was confirmed as *L. ivanovii*. This isolate was found in the same cluster as the *L. ivanovii* strain (ATCC-BAA-678 (JF967631)) which was isolated from a sheep fetus in Spain in 1997 and positioned in the same branch as the sheep isolates (NCTC11846 (LT906478) and NCTC11007 (LT906466)) which were defined as *L. ivanovii* subp. *ivanovii* by Seeliger *et al*. (1984). Due to the lack of sequence analyses of local *Listeria* strains isolated from our country or to the fact that they have only recently started to be documented, it is not surprising that the isolates obtained in this study coincided with and showed similarities to the isolates in the already classified basic phylogenetic clusters, in which the isolates of countries which are not in close geographical proximity to Turkey are to be found. When country specific clusters have been defined based on new bacterial genome sequencing and bacterial genealogies have been revised, it will become possible to reinterpret these similarities.

In the study, the 7 samples which were identified as *Listeria* spp. by genus specific PCR (5 from fetal abomasum content, 1 from milk and 1 vaginal swab sample) were found to be negative by species specific PCR and not have been cultured successfully. The *Listeria* spp. identified are thought to be non-pathogenic *Listeria* species that are likely to have been spread by milk and vaginal discharge (Akca and Sahin 2011; Botsaris *et al.,* 2016; Sanlıbaba *et al.,* 2018). The presence of Listeria in milk and vaginal swab samples was interpreted as contamination. The pathogenic potential of the Listeria which were found in the abomasum content is open to interpretation and, in the light of recent researches (Favaro *et al.,* 2014; Abay *et al.,* 2019), there is a need for further analysis of these species.

In conclusion, isolation of the pathogen Listeria could not be achieved from milk and vaginal swab samples taken from sheep abortions in the Kars region, whereas *L. ivanovii* from the abomasum contents of aborted fetuses was defined by culturing and molecular methods. Sequence analysis of the 16S rRNA gene region gave a phylogenetic position to the *L. ivanovii* strain which has been scarcely identified from sheep abortions.

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