Isolation and characterization of *Listeria monocytogenes* from the environment of three ewes' milk processing factories in Slovakia

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Summary

The aim of the study was to investigate the occurence and diversity of *Listeria monocytogenes* in three ewes' milk processing factories in Slovakia, and to reveal its sources. A total of 639 samples from the cheese production chain were collected during the years 2011–2014, out of which 20 (3.1%) were found to be positive for *L. monocytogenes*. *L. ivanovii* was detected in two samples and other *Listeria* species in 309 (48.4%) samples. Sporadic *L. monocytogenes* contamination was observed in all three factories with 10% positive samples being the products, while 90% of positive samples were associated with the production environment. By molecular serotyping using multiplex polymerase chain reaction, the *L. monocytogenes* isolates were classified in three serogroups – 80% in serogroup IIa, 10% in serogroup IIc and 10% in serogroup IVb. Testing for markers specific for three epidemic clones (ECI–ECIII) produced negative results. Molecular diversity evaluation of isolated strains by AscI-restriction and pulsed-field gel electrophoresis (PFGE) resulted in strain discrimination into 14 clusters at 100% similarity. The observed occasional incidence of *L. monocytogenes* and high strain diversity indicated the origin of contamination from external sources.

Keywords:

Listeria monocytogenes; molecular typing; serogrouping; pulsed-field gel electrophoresis; ewes' cheese

Listeria monocytogenes is an opportunistic bacterial pathogen that represents an important hazard to human health because it is capable of causing disease, mainly in certain well-defined high-risk groups of newborns, elderly, immunocompromised individuals, and pregnant women [1], with clinical manifestations of meningitis, encephalitis and/or septicemia [2]. Although less frequent in humans compared to e. g. campylobacteriosis or salmonelosis, with a 17.8% fatality rate reported in 2012 [3] listeriosis ranks among the most frequent causes of hospitalization and death due to food-borne illness. Sporadic cases of listeriosis were recently reported also in Slovakia, 31 cases in 2011 [4] and 11 cases in 2012 [3]. In recent years, an increasing rate of listeriosis was reported in several European countries [5].

Ingestion of food contaminated with *L. monocytogenes* is the primary route of transmission of the pathogen to humans [6]. According to the current European regulations on microbiological criteria for foodstuffs, zero tolerance for the presence of *L. monocytogenes* is set for ready-to-eat foods for infants, as well as for other ready-to-eat foods able to support the growth of *L. monocytogenes*, before leaving the immediate producer control. In 2005, the European Union adopted a policy that *L. monocytogenes* may be present at levels up to 100 CFU per gram in the rest of food products placed on the market [7]. This regulation also requires that the producers of ready-to-eat foods examine the processing environment and exclude *L. monocytogenes* contamination.

L. monocytogenes has been isolated from a variety of food products, most foodborne cases of lis-

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teriosis being connected to ready to-eat products that are believed to get contaminated during and after processing [3]. This emphasizes the importance of monitoring the environment of food processing factories, to identify potential contamination sources and transmission routes in the food production chain, particularly regarding persistent *L. monocytogenes* strains. Presence of persistent strains could result from insufficient effectiveness of cleaning and sanitizing procedures, which allow survival and adaptation of *L. monocytogenes* strains [8]. The source of contamination may originate in external environment and, after breaking the hygienic barriers, can cause contamination of the internal environment and, subsequently, contamination of final products. This route has been identified by characterization of *L. monocytogenes* isolates by molecular typing methods with high discriminatory power, such as whole genome DNA restriction coupled to pulsed-field gel electrophoresis (PFGE) [9], amplified fragment length polymorphism (AFLP) or multi-locus sequence typing (MLST) [10].

Ready-to eat foods, smoked fish and other fish products, followed by meat products and cheese have been identified as the foods most frequently associated with listeriosis outbreaks [11]. Cheese has been implicated in sporadic cases and also in major listeriosis outbreaks worldwide. Environmental contamination, in several occasions by persistent strains, has been considered an important source of final product contamination, particularly in raw milk cheeses [12, 13].

Genome DNA restriction coupled to PFGE is currently the preferred technique for molecular typing of *L. monocytogenes* due to its high reproducibility and discriminatory power. Applicability of the method is very high also thanks to availability of an international database of PFGE patterns, PulseNet [14]. PFGE has been successfully used to differentiate *L. monocytogenes* isolates associated with foods and food processing environments, facilitating identification of persistent contamination [8, 15].

Another typing scheme for *L. monocytogenes* is serogrouping, which reflects to a certain extent the virulence potential of individual strains. It is known that serovars 1/2a, 1/2b and 4b are responsible for about 90% of human infections [16]. Thirteen serovars described for *L. monocytogenes* are distinguishable by classical agglutination serotyping method using antisera [17]. This method can be effectively replaced by a more convenient multiplex polymerase chain reaction (PCR) method originally developed by Doumith et al. [18] and improved by Kérouanton et al. [19]. The method enables to cluster *L. monocytogenes* strains into five molecular serogroups IIa, IIb, IIc, IVa and IVb in agreement with the most commonly encountered serotypes 1/2a (together with 3a), 1/2b (3b, 7), 1/2c (3c), 4a (4c) and 4b (4ab, 4d, 4e), respectively. Another PCR, targeted to *fla*A gene, facilitates specific identification of serogroup IIa.

L. monocytogenes strains are divided to four genetic lineages (I, II, III, and IV), for which the different pathogenic potential is assumed. Most *L. monocytogenes* isolates belong to lineages I and II. Lineage I strains, in particular serovar 4b, are associated with most human listeriosis outbreaks. Lineage II strains are common in foods, natural and farm environments, commonly isolated from animal listeriosis cases and from sporadic human clinical cases [20].

Typing of *L. monocytogenes* isolates from listeriosis outbreaks has suggested that many outbreaks were caused by a small number of so called epidemic clones (EC). These are closely related groups of isolates that evolved clonally. Seven *L. monocytogenes* epidemic clones (ECI to ECVII) have been reported up to date. Epidemic clones were implicated in several outbreaks and sporadic cases of listeriosis worldwide [12, 21]. They are considered more virulent than other strains and/or have a better ability to persist in different environments and/or multiply faster in foods [22].

Cheese products belong to important vehicles of *L. monocytogenes*. In this study, contamination by *L. monocytogenes* of three small or medium-sized factories producing bryndza cheese was investigated. This type of cheese is a raw ewes' milk-based cheese very popular in Slovakia [23]. All three production factories were well equipped, having most food-contact surfaces and equipment made from stainless steel. Appropriate procedures of cleaning and sanitation were performed on a systematic basis according to the good hygienic and sanitation practice. From these premises, *L. monocytogenes* was isolated and characterized by molecular serogrouping and PFGE, with the aim to reveal sources and routes of contamination by the pathogen.

MATERIALS AND METHODS

Sample collection

Samples were collected from three ewes' milk processing factories in Slovakia, quarterly from May 2011 to December 2014 in two factories (A and B), and from January 2013 to December 2014 in the third factory (C). Two of them (A and C) represented factories with their own

Tab. 1. Samples analysed and detected positive for *L. monocytogenes*.

	2011		2012		2013		2014		TOTAL	
	Number of samples									
	Analysed	Positive	Analysed	Positive	Analysed	Positive	Analysed	Positive	Analysed	Positive
Environmental samples										
Factory A	34	0	107	1	46	6	31	1	218	8
Factory B	23	1	90	0	55	5	37	1	205	7
Factory C	–	–	–	–	39	2	37	1	76	3
Subtotal	57	1	197	1	140	13	105	3	499	18
Samples from products										
Factory A	20	0	17	0	13	0	8	0	58	0
Factory B	21	1	35	1	9	0	6	0	71	2
Factory C	–	–	–	–	6	0	5	0	11	0
Subtotal	41	1	52	1	28	0	19	0	140	2
TOTAL	98	2	249	2	168	13	124	3	639	20

ewes' milk production, and one (B) was a production factory processing ewes' lump cheeses purchased from several sheepfarms in the region. The factories were situated in central Slovakia with a minimum distance from each other of approx. 100 km, and were independent on each other. The samplings were performed 2 h after the end of a production run. From a total of 639 samples (Tab. 1), 499 environmental samples were collected from different places along the production chain, 216 from food-contact areas and 283 from non-food-contact areas and equipment (including cleaning rags, gloves, walls, floors, fly papers and feed), and 140 food samples of milk, raw materials, semi-products (ewes' lump cheese) and final products were analysed. The sampling programme included sites of difficult sanitation and was focused on sites where *L. monocytogenes* was previously detected. Environmental swabs were collected using a sponge sampling kit (3M, St. Paul, Minnesota, United States) containing a sterile sponge moistened with buffered peptone water. The sponges were dragged back and forth in order to cover the area of 0.3 m^2. To analyse raw materials, semi-products, final products, feed or water and brine samples, amounts of 25 g or 25 ml were collected. Disposable equipment, such as gloves or rags, were collected whole. Immediately after sampling, each sponge and samples were placed into individual sterile bags, refrigerated and analysed within 24 h.

Microbiological detection and isolation

The detection of *L. monocytogenes* was performed using a conventional culture-based method according to ISO 11290-1:1996/Amd 1:2004 [24]. The method involved a 24 h pre-enrichment in half-Fraser broth (Merck, Darmstadt, Germany) at 30 °C , 24 h and 48 h enrichment in Fraser broth (Merck) at 37 °C, and streaking of samples on chromogenic Agar Listeria according to Ottaviani and Agosti (ALOA) medium (Merck) in order to obtain well isolated colonies. From the positive samples, up to five presumptive *L. monocytogenes* typical blue-green colonies with opaque haloes were streaked for isolation on Tryptose Soy Yeast Extract Agar (TSYEA; Merck) plates. In the case of growth of a mixture of colonies with massive numbers of blue-green colonies without haloes (predominantly of other *Listeria* spp.), the step of isolation on ALOA was repeated. All strains were maintained in 20% glycerol solution and freeze-dried for long-term storage at –18 °C.

Real-time PCR identification

To confirm *L. monocytogenes* identity, species-specific real-time PCR targeting *act*A gene [25] was used. DNA was extracted from 18 h culture in Tryptose Soy Yeast Extract Broth (TSYEB; Merck) by using QIAamp DNA MiniKit (Qiagen, Hilden, Germany). Typical colonies with halo, which were negative by PCR for *act*A and *prf*A genes [26], were tested by PCR specific for *L. ivanovii* [27]. Isolates identified as *L. monocytogenes* were subjected to molecular characterization by molecular serogrouping, PFGE and detection of EC markers.

Molecular serogrouping

Multiplex gel-based PCR, originally developed by Doumith et al. [18], targeting serotype-specific marker genes, modified and improved by

KÉROUANTON et al. [19], was used in order to cluster *L. monocytogenes* strains into five molecular serogroups IIa, IIb, IIc, IVa and IVb in agreement with serotypes 1/2a (3a), 1/2b (3b, 7), 1/2c (3c), 4a (4c) and 4b (4ab, 4d, 4e), respectively. Detection of *fla*A gene complemented the method and facilitated resolution of IIa and IIc serogroups. The protocol was modified by using LIP1 and LIP2 primers for *prf*A gene marker [26] specific for *L. monocytogenes* instead of *prs* gene marker for all *Listeria* spp. strains. DNA for the analyses was prepared from overnight cultures in TSYEB using InstaGene Matrix (BioRad, Hercules, California, USA) according to instructions for use of the manufacturer. PCR products were analysed by electrophoresis in 1.5% agarose gel stained with GelRed (Biotium, Hayward, California, USA).

Pulsed-field gel electrophoresis

Isolates were analysed using PFGE protocol with AscI restriction enzyme [28] with modifications according to the last updated Standard Operating Procedure for PulseNet PFGE of *Listeria monocytogenes* [29]. After DNA extraction according to the protocol, the prepared agarose plug slices were digested by restriction enzyme AscI (New England BioLabs, Ipswich, Massachusetts, USA). Electrophoresis was performed in 1.5% SeaKem Gold agarose (Lonza, Rockland, Maine, USA) in CHEF Mapper III (BioRad) with *Salmonella* ser. Braenderup H9812 digested by XbaI as a size reference standard. Running parameters were as follows: voltage gradient 6 $V \cdot cm^{-1}$, angle 120°, temperature 14 °C, initial switch 4 s, final switch 40 s, duration 18.5 h. Gels were stained in 0.01% ethidium bromide (Serva, Heidelberg, Germany) solution and visualized by UV transillumination. Up to five *L. monocytogenes* colonies from individual samples were analysed in order to check their identity and, in case of identity, one representative was selected. In the case that more than one PFGE profile of isolates from the same independent sample were obtained, all individual profiles were involved in further investigation. The PFGE AscI-patterns of the selected isolates were analysed by BioNumerics software (Applied Maths, Kortrijk, Belgium). The levels of similarity between AscI patterns were calculated using Dice's coefficient with a band tolerance of 1.0%. Cluster analysis was performed by the unweighted pair group method with average linkages (UPGMA).

Detection of epidemic clones

Detection of genetic markers for ECI, ECII and ECIII was performed by multiplex PCR previously described by CHEN and KNABEL [30]. Two isolates belonging to molecular serogroup IVb were screened for ECI and ECII (associated with serotype 4b), while 16 isolates belonging to molecular serogroup IIa were screened for ECIII (associated with serotype 1/2a).

RESULTS AND DISCUSSION

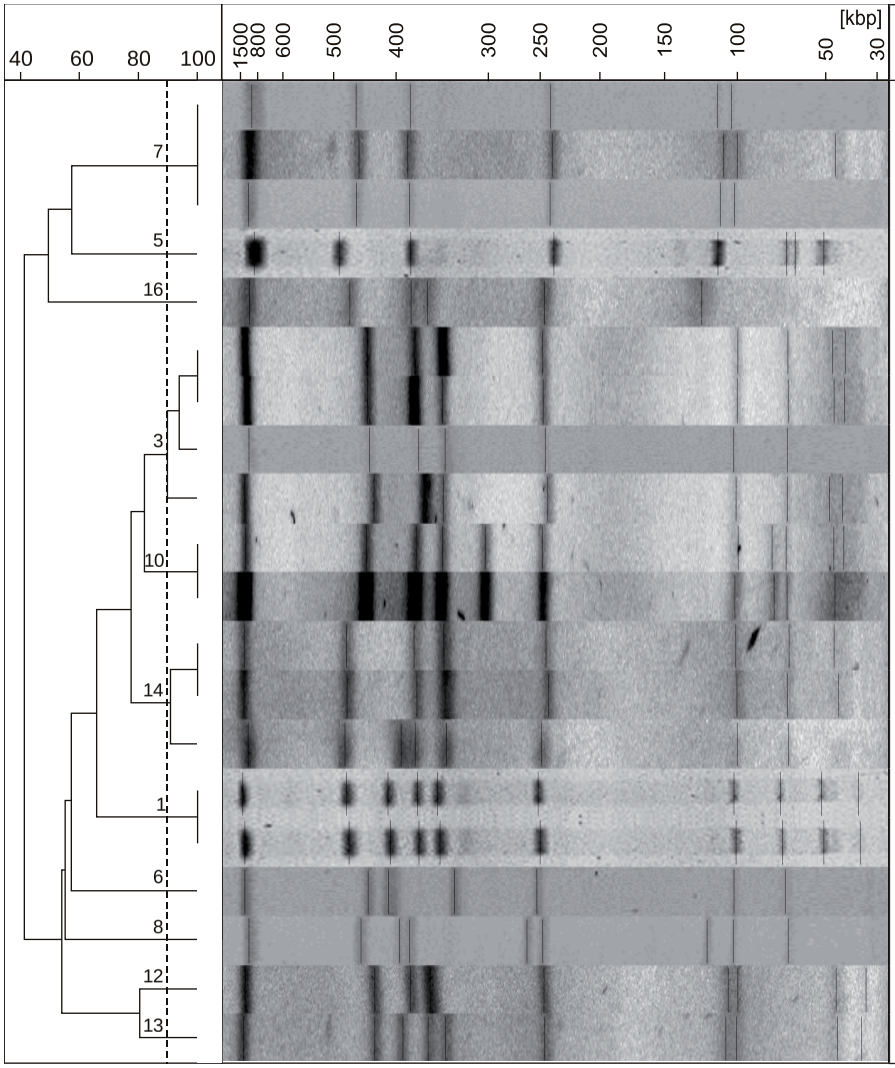
Detection and prevalence of *L. monocytogenes*

The panel of 639 collected and analysed samples was represented by 499 (78%) environmental samples and 140 (22%) food product samples. From the environmental samples, 216 (42%) were collected from food-contact surfaces and 283 (58%) from non-food-contact surfaces and equipment. The prevalence of *L. monocytogenes* in the samples from the three ewes' milk processing factories A, B (from 14 samplings each) and C (from 8 samplings) is summarized in Tab. 1.

Out of all analysed samples, 20 (3.1%) were found to be positive for *L. monocytogenes*, in most samples being in mixture with other, predominant *Listeria* species. Potentially pathogenic *L. ivanovii* was detected in two samples, while other *Listeria* spp. in 309 (48.4%) samples. In some cases, positive *L. monocytogenes* detection by PCR from enrichment in Fraser broth were in discrepancy with negative detection of typical colonies on ALOA medium, due to the presence of a mixture of *Listeria* spp. Even after the repeated isolation by streaking on ALOA medium, no typical *L. monocytogenes* colonies could be observed. Similar problems with underestimation of *L. monocytogenes* by traditional microbiological analysis were reported by ALESSANDRIA et al. [31] and DALMASSO et al. [32] investigating the occurrence of *L. monocytogenes* in dairy processing factories.

Among 20 positive samples, 18 (3.6% from 499 analysed) were associated with production environment including four product-contact and 14 non-product-contact surfaces and equipment. Only two samples of food products (1.4% from 140 analysed) were positive, namely, one sample of bryndza (final product) and one sample of ewes' lump cheese (semi-product).

According to the presented results, sporadic *L. monocytogenes* contamination was observed in all three factories. The levels of contamination ranged from 2.8% to 3.4%, with 90% of positivity associated with environmental samples. The prevalence of *L. monocytogenes* in all three factories was very low, particularly in comparison with the results published by other researchers, 7.1% [16] or 19.5% [33]. Higher prevalence



Strain	Plant	Sampling	Sampling site, material, location	Molecular serogroup	PFGE AscI-profile
P/63/25	A	02-2013	Rag for shoes cleaning	IIa	7
P/77/22	A	04-2014	Rag for udders cleaning	IIa	7
T/65/15	B	03-2013	Container for raw cheese	IIa	7
T/51/42	B	05-2012	Ewes' lump cheese	IVb	5
K/83/11	C	08-2014	Rag from washbasin near dispatch	IVb	16
K/66/3	C	05-2013	Floor in milk house	IIa	3st[a]
K/66/16	C	05-2013	Rag for floor cleaning	IIa	3st[a]
P/63/20	A	02-2013	Cellulose filter for raw milk	IIa	3
P/67/4	A	05-2013	Rag for udders cleaning	IIa	3st[b]
P/67/14	A	05-2013	Rag for shoes cleaning	IIa	10
P/67/3	A	05-2013	Part of milk machine	IIa	10
T/72/17	B	09-2013	Waste drain water, admission room	IIa	14
T/72/7	B	09-2013	Wall in admission room	IIa	14
T/84/12	B	10-2014	Floor in production room	IIa	14st
T/38/11	B	06-2011	Bryndza cheese	IIc	1
T/38/4	B	06-2011	Packing machine	IIc	1
P/59/26	A	07-2012	Fly-paper	IIa	6
P/63/27	A	02-2013	Fermented feed	IIa	8
T/70/11	B	07-2013	Washbasin in production room	IIa	12
T/70/12	B	07-2013	Floor in production room	IIa	13

Fig. 1. Dendrogram of PFGE AscI patterns of 20 *L. monocytogenes* isolates with sample identification data and results of molecular serogrouping.

Strains P/63/20, K/66/3, K/66/16 and P/67/4 grouped in one cluster with a similarity of approx. 95% for the first three of them, and approx. 90% for the fourth one. Therefore, these four strains were assigned to one PFGE Asc-profile designated 3, 3sta and 3stb. In a similar case for strains T/72/7, T/72/17 and T/84/12 with a similarity of 90%, designation 14 and 14st was used.

was observed by WAGNER et al. [34], who isolated *L. monocytogenes* from 50 small factories in Austria at a prevalence of 27.6%, and by SPANU et al. [35], who isolated *L. monocytogenes* from the environment of 12 ewes' cheese-producing plants in Italy at a prevalence ranging between 3.0% and 22.6%.

Molecular analysis of serogroups, linages and epidemic clones

By molecular serotyping, *L. monocytogenes* isolates were classified into three serogroups, with the majority of strains falling in serogroup IIa (80%), followed by serogroups IIc (10%) and IVb (10%). Regarding the classification of lineages, 90% of strains represented lineage II (serogroups IIa and IIc) and 10% lineage I (serogroup IVb) (Tab. 1). Markers specific for three epidemic clones (ECI–ECIII) of *L. monocytogenes* were investigated with negative results for all analysed strains.

The results obtained in our survey are in agreement with observations in other European countries, where *L. monocytogenes* strains of lineage II and serotype 1/2a were the most frequently isolated from dairy products and from factory environments [9, 35, 36]. On the other hand, 85 *L. monocytogenes* isolates from cheese manufacturing plants in São Paulo, Brazil, were classified to serotypes 1/2b, 1/2c and 4b, with predominance of serotype 4b [13].

PFGE typing

Analysis of PFGE AscI patterns of 20 *L. monocytogenes* isolates resulted in strain discrimination into 14 clusters at a similarity level of 100%, and into 11 clusters at a similarity level of 90% (Fig. 1). At the similarity level of 90%, two clusters of similar types (ST) of profiles 3 (four isolates) and 14 (three isolates) were identified. These ST PFGE patterns presented less than three bands difference per profile, and could be therefore considered as closely related according to the "3-band rule" [37]. At the level of 50% similarity, most of strains were grouped into one main cluster, only two strains of serogroup IVb and three identical IIa strains (profile 7) were outliers. Dendrogram of PFGE profiles, complemented with relevant data on isolates identity, factory, sampling date, sampling site, location, material or product, and results of serogrouping and AscI profile indication, are given in Fig. 1.

Diversity and tracing of *L. monocytogenes* isolates

PFGE profiling was used to determine the genetic variability of isolates, and to trace the contamination in the factories. In fact, the strains with the same PFGE profiles repeatedly isolated from the same place at subsequent samplings over a longer period could be considered potentially persistent. However, no evidence of persistent strains was observed in our survey. According to the results obtained in all three cheese-processing factories, accidental incidence of *L. monocytogenes* of a relatively high strain diversity suggested that there were random external sources of contamination.

The dendrogram of PFGE profiling (Fig. 1), clusters of strains of AscI profile 3, 7 or 14 isolated from different samples and from different locations, indicate the spread of certain biotypes in the environment of two factories. This suggests a wide distribution of the pathogen in the natural environment. Our results suggest that some PFGE types may be associated with a single source, whereas other PFGE types may be disseminated wider or even globally [38].

CONCLUSION

Listeria monocytogenes is a food-borne pathogen with significant public health and economic impact. Since it can persist in the production environment and cross-contaminate food products, the pathogen is of great concern for food producing companies. For the purpose of tracing *L. monocytogenes* in the food production environment, to reveal the character, sources and routes of contamination, appropriate analytical methods with sufficient discrimination power need to be used, such as molecular typing methods. In our survey, low and sporadic contamination of three small and medium-sized milk processing factories was detected. By application of PCR serogrouping and PFGE typing to *L. monocytogenes* isolates, high strain diversity was determined, which suggested that the contamination originated from the external environment.

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