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THE PRESENCE OF *LISTERIA* SPP. AND *LISTERIA MONOCYTOGENES* IN A CHOSEN FOOD PROCESSING ESTABLISHMENT IN SERBIA

BRANKICA LAKIĆEVIĆ[2], ALEKSANDRA STJEPANOVIĆ[2], M. MILIJAŠEVIĆ[2], AMARELA TERZIĆ-VIDOJEVIĆ[1], NATAŠA GOLIĆ[1*] and L. TOPISIROVIĆ[1]

[1]*Institute of Molecular Genetics and Genetic Engineering, University of Belgrade,* 11010 Belgrade, Serbia

[2]*Institute of Meat Hygiene and Technology,* 11000 Belgrade, Serbia

Abstract - The aim of the study was to establish a protocol to evaluate the presence of *Listeria* spp. in food processing environments. The presence of *Listeria* spp. was evaluated in a selected restaurant in Serbia on three occasions. Samples were collected from 47 sampling spots in the commercial kitchen equipment and environment. The presence of *Listeria* spp. and *Listeria monocytogenes* were detected by conventional culture methods and by the PCR method. The obtained results showed that 23 swab samples were positive for *Listeria* spp. Interestingly, the swabs from the bread-cutting board and meat defrosting sink were positive for *L. monocytogenes*.

Key words: Listeria monocytogenes, PCR, detection, environmental samples

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INTRODUCTION

Listeria spp. are ubiquitous in nature and can be isolated from soil, vegetables and natural waters as well as from healthy animals and man (Roberts and Wiedmann, 2003). *Listeria monocytogenes* is the most pathogenic species of this genus, although infections mainly occur in neonates, pregnant women, the elderly and immunocompromised individuals (McLauchlin, 1997). The primary mode of transmission of *L. monocytogenes* to humans is the consumption of contaminated, minimally processed food (Schlech, 2000; Kathariou, 2002; Shen et al., 2006). Accessibility to the public and relatively limited control interventions at food service establishments and the lack of a specific regulatory framework increase the likelihood of introduction of this pathogen into some foods in these establishments.

Various studies have indicated that certain strains of *L. monocytogenes* survive well within the food-processing environment (Kathariou, 2002; Tompkin, 2002; Pan et al., 2006) and the persistence of such strains is of concern as they have the potential to act as a continual source of contamination (Pan et al., 2006). Moreover, the detection of nonpathogenic *Listeria* spp. can be considered as a useful indicator of a deterioration in hygiene or process conditions during food production (PHLS, 2000), leading to an increased risk of contamination with pathogenic *Listeria* spp. Therefore, the detection of all *Listeria* spp. is necessary when testing food and environmental samples.

Advances in molecular technologies, particularly the PCR methodology, have allowed reliable microbial identification and surveillance. PCR techniques are sensitive, highly specific and allow rapid processing times, and they also enhance the likelihood of detecting *Listeria* spp. without the need for isolating pure cultures (Aznar & Alarcón, 2003; Cocolin et al., 2002). Moreover, PCR methods have been applied successfully to the detection and identification of pathogenic organisms in clinical and environmental samples (Simon, 1999; Soumet et al., 1994; Kaur et al., 2007).

Since studies on the prevalence of *Listeria* spp. in food processing establishments in Serbia are lacking, the aim of the present study was to establish a molecular protocol to evaluate the presence of *Listeria* spp. The samples were analyzed by either culture method or by PCR.

MATERIALS AND METHODS

Bacterial strains and growth conditions

The standard strains of *Listeria monocytogenes* 4b ATCC 19115 and *Listeria innocua* ATCC 33090 were obtained from the American Type Culture Collection (ATCC; Manassas, Va., USA). Standard strains were grown on brain-heart agar (BHA) (Merck, GmbH Darmstadt, Germany) and buffered peptone water (Merck) at 37°C.

Samples

For the analysis, 141 environmental samples from 47 sampling areas in a restaurant in Belgrade, Serbia, were selected. The samples were taken from different locations (goods reception area, raw material storage, food processing area) from drains, floors and food contact surfaces, including the equipment used to transport (carts), store (display case, coolers, freezers), or prepare food (slicers, knives, choppers, cutting boards, strainers). Samples (10 x 10 cm) were collected by using sterile commercial cotton swabs, on three independent visits. Whenever possible, swabs were collected from the same sites on each visit. All samples were kept refrigerated and analyzed within 2 h.

Enrichment and isolation of Listeria spp. from environmental swabs

The conventional culture method was carried out by two-step enrichment (Anonymous, 1996). For primary enrichment, commercial cotton swabs were homogenized in 20 ml of half-concentrated Fraser broth and incubated at 30°C for 24 h, followed by a secondary enrichment. After that the primary enrichment broth (100 µl) was transferred into 10 ml of Fraser broth and incubated for a further 48 h at 37°C. After 48 h, a loopful of the enrichment broth culture was plated onto polymyxin-acriflavine-lithium chloride-ceftazidime-aesculin-mannitol (PALCAM) (Merck) and OXFORD (Oxoid, UK) agar plates, and incubated for 48 h at 37°C. For each sample *Listeria* presumptive colonies were tested for catalase and oxidase reactions, Gram staining and motility.

Biochemical identification

Hemolysis was assessed by streaking isolated colonies on 5% sheep blood agar (Base: meat peptone 15 g l^{-1}, liver digest 2.5 g l^{-1}, yeast extract 5 g l^{-1}, sodium chloride 5 g l^{-1}, agar 9 g l^{-1}. To 100 ml base, 5 ml of defibrinated sheep blood was added). After 24 h of incubation at 37°C the plates were examined for the presence of a zone of hemolysis. Isolates that were small, Gram-positive rods, catalase positive, oxidase negative, with motility, were further identified by using the API-*Listeria* identification system (BioMerieux, France), and confirmed by PCR analysis.

DNA extraction

DNA was extracted by using the DNeasy Tissue Kit (Qiagen GmbH, Germany) according to the manufacturer's protocol for Gram-positive bacteria.

PCR amplification conditions

PCR was performed in a final volume of 50 µl containing 1xPCR buffer (10xPCR buffer: 500 mM KCl, 100 mM Tris-HCl, 0.8% Nonidet P40), 2.5 mM $MgCl_2$, 200 µM of each dNTP, 2.5 µM of each primer, 1 U of *Taq* polymerase (Fermentas UAB, Lithuania) and 0.1-1 µg of DNA template. The samples were amplified in a DNA thermal cycler (Flexigene, Techne, UK) with primers complementary to the *hlyA* gene (LM1: 5′-CCTAAGACGCCAATCGAA -3′ and LM2: 5′-AAGCGCTTGCAACTGCTC -3′) and 16S rRNA gene (LI1: 5′- CTCCATAAAGGTGACCCT -3′

Table 1. Natural swabs, positive for the presence of *Listeria* spp., collected during three visits

Sample code	Location	Culture method	Pre-enrichment (24 h)		Confirmation	
			hlyA	iap	hlyA	iap
S1		Goods reception area				
S1-02-2/I	*Dairy products case* last level	–	+	+	nd	nd
S2		Row material storage				
S2-01-2/II	*Cooling chamber* walk-in entry 2	*L. m.*	–	–	nd	–
S2-02-1/III	*Cooling chamber* walk-in 4 floor tiles 1	–	–	+	nd	nd
S2-02-2/I	Walk-in 4 floor tiles 2	*L. w. 96.8%*	–	+	–	+
S2-02-2/III	Walk-in 4 floor tiles 2	–	–	+	nd	nd
S2-04-1/I	*Freezing chamber* walk-in 4 floor tiles 1	–	–	+	nd	nd
S2-05-1/III	Floor	*L. w. 65.7%*	–	+	–	+
S2-06-2/III	*Hall floor* a corner of the wall	*L. i. 99.6%*	–	+	–	+
S2-08-1/II	Drain	*L. m.*	–	+	–	+
S2-08-1/III	Drain	*L. w. 65.7%*	+	+	–	+
S3		Food processing area - kitchen				
S3-01-1/III	*Meat defrosting sink* wall	–	–	+	nd	nd
S3-01-2/I	Drain	*L. w. 99.9%*	–	+	–	+
S3-01-2/III	Drain	*L. m.*	+	+	+	+
S3-02-1/III	*Hand wash sink* wall	–	–	+	nd	nd
S3-03-2/I	Bread cutting board	*L. m. 98.6%*	–	+	+	+
S3-05-1/II	Floor drain 1	*L. i.*	–	–	nd	–
S3-05-1/III	Floor drain 1	–	–	+	nd	nd
S3-05-2/I	Floor drain 2	*L. i. 99.6%*	–	–	–	+
S3-05-2/II	Floor drain 2	*L. m.*	–	–	nd	–
S3-05-2/III	Floor drain 2	*L. w. 65.7%*	+	+	–	+
S3-08-2/II	*Cooling chamber* walk-in 4 floor tiles 2	*L. m.*	–	–	nd	–
S3-11-1/III	Kitchen sink	–	–	+	nd	nd
S3-11-2/II	Guard back of the meat slicer	*L. m.*	–	–	nd	–
T o t a l			4	17	2	10

and U1: 5′- CAGCMGCCGCGGTAATWC -3) for 5 min at 94°C, 35 cycles of 30 s at 94°C, 45 s at 50°C, 45 s at 72°C and a final extension of 5 min at 72°C (Aznar and Alarcón, 2003) and to the *iap* gene (List. univ. 1: 5′-GCCAGCGGCCCGGCGCGGGCCCGGCGGGGGCCGCGGCATGTCATGGAATAA -3′ and List. univ. 2: 5′-GCTTTTCCAAGGTGTTTTT -3′) for 5 min at 95°C; 35 cycles of 1 min at 95°C, 2 min at 36°C and 3 min at 72°C; and, finally, 7 min at 72°C (Cocolin et al., 2002). All PCR products were analyzed by agarose gel electrophoresis on 1% and 2% (wt/vol) agarose gels in a 1xTBE buffer (10xTBE: 89 mM Tris, 89 mM boric acid, 2 mM EDTA) (Fermentas), at a constant voltage of 80 V for 1 h, and visualized by CCD camera Bio Doc Analyze Darkhood (Biometra, Gottingen, Germany). All PCR products were run next to the DNA molecular standards "MassRuler™ DNA Ladder" (Fermentas) and „GeneRuler™ DNA Ladder Mix" (Fermentas).

Sequencing of 16S rDNA amplicons

The 16S rDNA amplicons were purified by QIAquick PCR Purification KIT/250 (QIAGEN GmbH, Hilden, Germany), and sequenced by CRIBI-BMR Genomics Service sequencing of DNA (University of Padua, Italy). The BLAST algorithm was used to determine the close-matching sequence relatives in the NCBI nucleotide sequence database (http://www.ncbi.nlm.nih.gov/BLAST).

RESULTS

In this study, 141 environmental samples were taken from 47 sampling spots in a restaurant in Belgrade on three independent visits. The samples were analyzed by either culture method or by PCR. Results obtained by culture method showed that during the first visit 4 out of 47 swabs were positive for the presence of *Listeria* spp. (Table 1).

Among them, *L. welshimeri* was detected on the swabs S2-02-2/I and S3-01-2/I isolated from the floor and drain, respectively; *L. innocua* was detected on the swab S3-05-2/I isolated from the floor drain and, interestingly, *L. monocytogenes* was identified on the swab S3-03-2/I, isolated from bread-cutting board. The isolates were further checked by PCR using primers LM1/LM2, and List-univ. 1/List-univ. 2. The results confirmed that the isolate S3-03-2/I belonged to *L. monocytogenes* (Fig. 1, Table 1). Interestingly, the isolate S3-05-2/I was identified by PCR as *L. monocytogenes* (Fig. 2). The results of the ambiguous identification were further checked by sequencing of the 16S rRNA, and nucleotide sequence analysis showed homology with *L. innocua* (92%). The results obtained by PCR using List-univ. 1/List-univ. 2 primers showed that the other discordant isolate (S2-02-2/I), misidentified by culture method as *L. welshimeri*, belonged to *L. innocua* (Fig. 2).

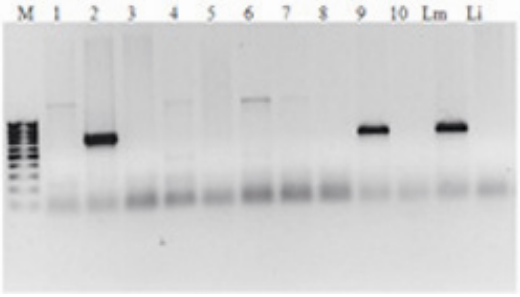

Fig. 1. Agarose gel electrophoresis of the PCR obtained by using LM1/LM2 primers from samples obtained during the first, second and third sampling: M - marker (MassRuler™ DNA Ladder), 1. sample from walk-in 4 floor tiles (S2-02-2/I), 2. sample from bread-cutting board (S3-03-2/I), 3. sample from drain (S3-01-2/I), 4. sample from floor drain (S3-05-2/I), 5. sample from drain (S2-08-1/II), 6. sample from floor (S2-05-1/III), 7. sample from corner of the wall (S2-06-2/III), 8. sample from drain (U2-08-1/III), 9. sample from meat defrosting sink (S3-01-2/III), 10. sample from floor drain (U3-05-2), *Lm - L. monocytogenes* 4b ATCC 19115, *Li - L. innocua* ATCC 33090.

In parallel, PCR detection of *Listeria* spp. in the collected samples was carried out by using primers LM1/LM2, and List-univ. 1/List-univ. 2, after 24 h of pre-enrichment. In total, 5 out of 47 samples were found to be positive for the presence of *Listeria* spp. in direct identification from the swab samples, during the first visit (S1-02-2/I, S2-02-2/I, S2-04-1/I, S3-01-2/I and S3-03-2/I) (Table 1).

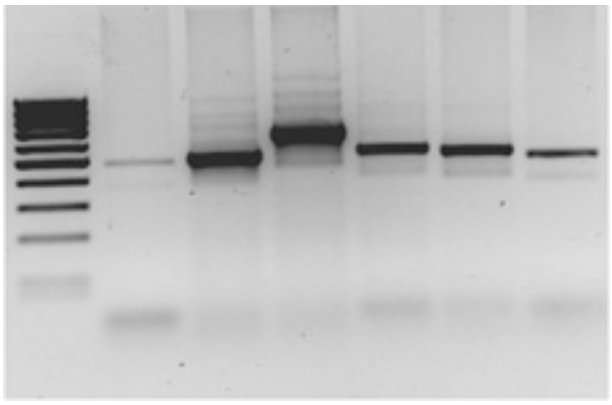

Fig. 2. Agarose gel electrophoresis of the PCR products obtained by using List-univ.1/List-univ.2 primers from samples obtained during the first visit: M - MassRuler^{TM} DNA Ladder, 1. sample from walk-in 4 floor tiles (S2-02-2/I), 2. sample from bread-cutting board (S3-03-2/I), 3. sample from meat defrosting sink (S3-01-2/I), 4. sample from floor drain (S3-05-2/I), *Lm - L. monocytogenes* 4b ATCC 19115, *Li - L. innocua* ATCC 33090.

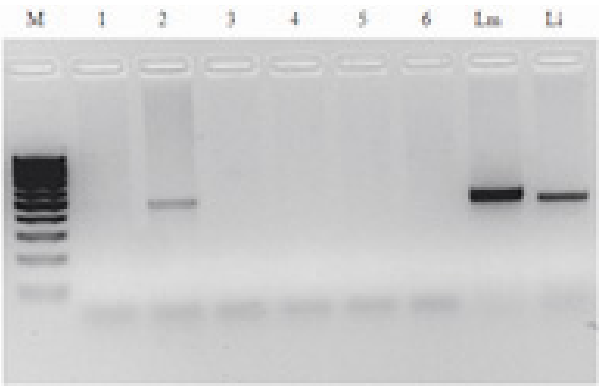

Fig. 3. Agarose gel electrophoresis of the PCR products obtained by using List-univ.1/List-univ.2 primers, from samples obtained during the second visit: M marker (MassRuler^{TM} DNA Ladder), 1. sample from cooling chamber (S2-01-2/II), 2. sample from drain (S2-08-1/II), 3. sample from drain (S3-05-1/II), 4. sample from drain (S3-05-2/II), 5. sample from cooling chamber (S3-08-2/II), 6. sample from meat slicer (S3-11-2/II), *Lm - L. monocytogenes* 4b ATCC 19115, *Li - L. innocua* ATCC 33090.

Results obtained by culture method showed that during the second visit six out of 47 swabs were positive for the presence of *Listeria* spp. (Table 1). *L. monocytogenes* was identified in five samples S2-01-2/II, S2-08-1/II, S3-05-2/II, S3-08-2/II and S3-11-2/II, and *L. innocua* was detected on the swab S3-05-1/II. The isolates were further checked by PCR using primers LM1/LM2, and List-univ. 1/List-univ. 2. The results obtained by PCR analyses showed that only isolate S2-08-1/II, isolated from drain, (misidentified by culture method as *L. monocytogenes*) belonged to *Listeria* spp. and it was identified as *L. innocua* (Table 1, Fig. 3).

The results showed that the same sample S2-08-1/II was positive for the presence of *Listeria* spp. in direct identification from the swab samples, isolated during the second visit (Table 1). According to the length of the PCR products obtained with List-univ. 1/List-univ. 2 primers, it could be concluded that the sample contained two different *Listeria* spp., identified as *L. innocua* and *L. welshimeri* (Fig. 4). The results of the PCR analyses by using LM1/LM2 primers confirmed that there were no *L. monocytogenes* on the swab samples taken during the second visit (Table 1).

Results obtained by culture method showed that during the third visit 5 out of 47 swabs were positive for the presence of *Listeria* spp. (Table 1). *L. welshimeri* was detected in three samples isolated from the floor (S2-05-1/III) and drain (S2-08-1/III and S3-05-2/III), and *L. innocua* was identified in the sample isolated from the corner of the wall (S2-06-2/III). The results of the PCR analyses showed the presence of *L. innocua* in all samples (Table 1). Moreover, *L. monocytogenes* was identified in the sample isolated from the meat defrosting sink (S3-01-2/III), by either culture method or PCR analyses (Fig. 1).

The results of the direct PCR detection by using List-univ. 1/ List-univ. 2 primers from the swab samples taken during the third visit showed the presence of *Listeria* spp. in 11 samples. Moreover, the results of the PCR analyses by using LM1/LM2 primers showed the presence of *L. monocytogenes* in samples isolated from the drain (S2-08-1/III), meat defrosting sink (S3-01-2/III) and floor drain (S3-05-2/III) (Table 1).

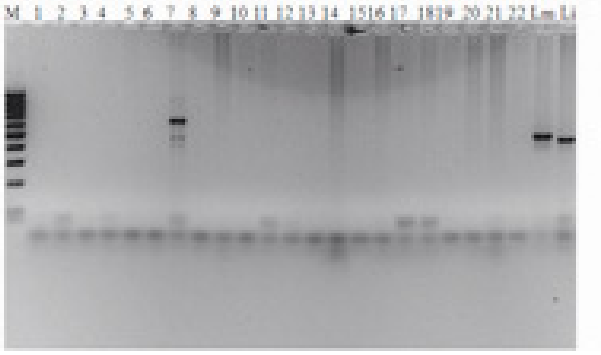

Fig. 4. Agarose gel electrophoresis of the PCR products obtained by using List-univ.1/List-univ.2 primers, from sample swabs obtained during the second visit: 1. M marker (MassRuler™ DNA Ladder), 2. S1-01-1/II, 3. S1-01-2/II, 4. S1-03-1/II, 5. S1-03-2/II, 6. S1-03-3/II, 7. S2-01-1/II, 8. S2-01-2/II, 9. S2-02-1/II, 10. S2-04-2/II, 11. S2-05-1/II, 12. S2-06-1/II, 13. S2-06-2/II, 14. S2-07-1/II, 15. S2-08-1/II, 16. S3-01-1/II, 17. S3-01-2/II, 18. S3-02-1/II, 19. S3-02-2/II, 20. S3-02-3/II, 21. S3-05-1/II, 22. S3-05-2/II, 23. S3-11-1/II, *Lm - L. monocytogenes* 4b ATCC 19115, *Li - L. innocua* ATCC 33090.

DISCUSSION

The detection and identification of *Listeria* spp. have attracted the attention of many authors. For the purposes of public health protection, it is important to recover nonpathogenic *Listeria* spp., as these act as markers for the likelihood of the presence of *L. monocytogenes* and allow preventive action to be taken.

In total, 23 samples were identified as positive for the presence of *Listeria* spp. (Table 1). Out of them, 17 samples were found to be positive for the presence of *Listeria* spp. in direct identification from the swab samples. Among them, *Listeria* spp. was most commonly isolated from the drains and floors, as was shown previously. The results of Gudmundsdóttir et al. (2005) suggested that the cleaning procedures were not sufficient to eliminate cross-contamination from drains and floors and that the in-house flora represents a potential health risk. Several other studies have concluded that the plant equipment and the processing environment (in-house flora) rather than the raw material is the source of *L. monocytogenes* (Rørvik et al., 1995; Autio et al., 1999; Giovannacci et al., 1999; Miettinen and Wirtanen, 2006). However, this does not exclude the possibility that the raw material may be an important, initial source for contaminating the processing equipment and environment (Vogel et al., 2001). This is in concordance with our findings that the swabs from the bread-cutting board and meat defrosting sink were positive for the presence of *L. monocytogenes*.

Furthermore, definitive identification, revealed by both culture and PCR methods, was obtained for 10 samples, and all but two samples were correctly identified. One discordant sample (S3-05-2/I) was misidentified as *L. monocytogenes* by PCR assay by using primers complementary to the *iap* gene (Fig. 2). On the other hand, the results of the culture method showed that it could be *L. innocua*, thus underscoring the biases of traditional methods. The results obtained by PCR using List-univ. 1/List-univ. 2 primers showed that the other discordant sample (S2-02-2/I), misidentified by culture method as *L. welshimeri*, belonged to *L. innocua* (Fig. 2). These results underscore the reliability and accuracy of the PCR method. Finally, ambiguous identification was resolved by direct sequencing of the 16S rRNA and nucleotide sequence analysis revealed homology with *L. innocua* (92%). The obtained results suggest the need to use combined methods in order to obtain accurate identification of *Listeria* spp.

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