Development of a Biomolecular Assay for the Identification of *Listeria* at Species Level

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Abstract

In this article, we developed a biomolecular assay specifically designed for the identification of *Listeria* at species level. The proposed test was based on (i) a duplex PCR targeting a specific fragment for *L. monocytogenes* (*plcA* gene) and a common fragment for all the *Listeria* (16S rRNA gene) and (ii) a minisequencing of the common fragment to detect diagnostic sites for the differentiation of the other five species: *L. innocua*, *L. welshimeri*, *L. seeligeri*, *L. ivanovii*, and *L. grayi*. The specificity of the assay was first tested on a total of 25 certified strains representing 6 *Listeria* species and 14 non-*Listeria* strains as negative control and validated on 124 wild isolates obtained from food samples. The proposed assay provides an appropriate tool for rapid identification of *Listeria* at species level and it should be of great benefit to the food industry as well as to regulatory or public health laboratories engaged in establishing the safety of food products and the management of listeriosis.

Introduction

THE GENUS *LISTERIA* comprises ubiquitous, Gram-positive bacteria, nonsporulating even though rather resistant to adverse environmental conditions. Up to now the following species have been identified: *Listeria monocytogenes*, *Listeria innocua*, *Listeria ivanovii*, *Listeria seeligeri*, *Listeria welshimeri*, *Listeria grayi*, and *Listeria marthii* (Gasanov *et al.*, 2005; Graves *et al.*, 2009).

L. monocytogenes has been particularly studied as it has been recognized as responsible for causing severe disease in humans and animals (Paillard *et al.*, 2003).

The epidemiology of listeriosis in Europe is developing: the incidence is increasing and the distribution of cases is shifting, primarily affecting elderly persons and those with predisposing medical condition (Goulet *et al.*, 2008).

Approximately 2500 human listeriosis cases occur annually in the United States, resulting in 500 deaths (Barbuddhe *et al.*, 2008).

L. ivanovii is considered to be specific to ruminants (Snampir *et al.*, 2006); however, reports indicate that this species, just as *L. seeligeri*, can also cause infection in humans (Gasanov *et al.*, 2005). No pathogenic effect has been described for *L. innocua*, *L. welshimeri*, *L. grayi*, and *L. marthii* (Paillard *et al.*, 2003; Graves *et al.*, 2009).

The unambiguous differentiation of *Listeria* genus in general, and of pathogenic species in particular, is extremely important in the control of food safety and public health.

The traditional cultural and serological methods are scarcely discriminating. In the last few years, a considerable number of biomolecular assays has been developed; so, at present, different laboratories can choose among numerous tests depending on the equipment at their disposal.

The first assays were based on the amplification of a unique fragment common to the whole genus (Bubert *et al.*, 1992; Gray and Kroll, 1995). Later on, the amplification of specific fragments for *L. innocua* (Liu *et al.*, 2003), *L. monocytogenes* (Liu *et al.*, 2004a), *L. ivanovii* (Liu *et al.*, 2004b), *L. welshimeri* (Liu *et al.*, 2004c), *L. seeligeri* (Liu *et al.*, 2004d), and *L. grayi* (Liu *et al.*, 2005) were applied.

To shorten the time of analysis, some multiplex PCR have been proposed; in this way, the simultaneous detection of more than one pathogen in the same sample, such as *Listeria* and *Salmonella* (Li *et al.*, 2000; Hsieh and Tsen, 2001) or *L. monocytogenes* and other *Listeria* species (Wesley *et al.*, 2002; Rodríguez-Lázaro *et al.*, 2004), has been achieved.

Moreover, the identification of *Listeria* at the species level may be obtained by means of other biomolecular techniques such as a PCR coupled with denaturing gradient gel electrophoresis based on specific migration patterns (Coccolin *et al.*, 2002), or a PCR coupled with restriction fragment length polymorphism (RFLP) (Paillard *et al.*, 2003). More recently, other alternative methods have been proposed, for example, Fourier transform infrared spectroscopy applied to differentiate five *Listeria* species (Janbu *et al.*, 2008; Rebuffo-Scheer *et al.*, 2008) and matrix-assisted laser desorption ionization-time of flight

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mass spectrometry for a rapid identification and typing of *Listeria* species (Barbuddhe *et al.*, 2008).

Another promising molecular technique is minisequencing, which consists on the analysis of the diagnosis sites. In particular, this method is based on the dideoxynucleotide triphosphate (ddNTP) single base extension of an unlabeled oligonucleotide (sequencing primer) to the 3' end. Each sequencing primer is designed immediately adjacent to the diagnosis sites and their length is modified by addition of nonhomologous tails (poly-T) at the 5' end. Each ddNTP is labeled with different fluorescent dyes and a fifth color is used to label the internal size marker. The extended sequencing primers used to interrogate different diagnosis sites differ in color and size. This approach has been recently successfully applied to differentiate closely related species of *Vibrio* in seafood samples (Dalmasso *et al.*, 2009).

In this article, we propose a biomolecular assay based on (i) a duplex PCR with a specific fragment for *L. monocytogenes* and another fragment common to the *Listeria* genus and (ii) a minisequencing of the common fragment to interrogate diagnosis sites for five species: *L. innocua*, *L. welshimeri*, *L. seeligeri*, *L. ivanovii*, and *L. grayi*.

Materials and Methods

Strains

All reference species of *Listeria* (*L. monocytogenes*, *L. innocua*, *L. welshimeri*, *L. seeligeri*, *L. ivanovii*, and *L. grayi*) used to develop the assay are listed in Table 1. Bacterial strains belonging to phylogenetically related genera were also tested to verify possible cross-reactions (Table 1). To evaluate the applicability of the proposed assay, 124 *Listeria* wild isolates (WIs) were used. These WIs were isolated from dairy and meat products and identified by means of sequencing (MicroSeq[®] 500 16S rDNA Bacterial Identification Kits; Applied Biosystems, Foster City, CA). They comprised 29 *L. monocytogenes*, 65 *L. innocua*, 14 *L. seeligeri*, 7 *L. welshimeri*, 6 *L. ivanovii*, and 3 *L. grayi*.

DNA extraction

All the isolates were grown overnight at 37°C in tryptone soya broth (Acumedia, Lansing, MI). DNA was extracted using the following protocol: 1 mL of broth culture (10⁸ CFU/mL) was centrifuged at 12,000 g for 5 min, and then the pellet was resuspended in 1 mL of phosphate-buffered saline, boiled for 5 min, and centrifuged again (Bottero *et al.*, 2004). The supernatant was stored at -20°C until use. DNA was quantified by means of a spectrophotometer at 260 nm (Bio-Photometer 6131; Eppendorf AG, Hamburg, Germany) and diluted at a concentration of 50 ng/μL.

Primers design

For the development of the duplex PCR, the primers were designed on the phosphatidylinositol-specific phospholipase C (*plcA*) gene and the 16S rRNA gene.

In particular, sequences of *plcA* gene for several *L. monocytogenes* belonging to all serotypes (accession no. AL591974, AF532205–AF532216) were obtained from the GenBank database (National Center for Biotechnology Information, www.ncbi.nlm.nih.gov) and aligned with the ClustalV program (Higgins *et al.*, 1992) to design species-specific primers

TABLE 1. LIST OF TARGET AND NON-TARGET BACTERIAL SPECIES USED FOR SPECIFICITY TESTS

Species	Source	Strain
<i>Listeria grayi</i>	NCTC	20601
<i>L. grayi</i>	CCM	4029
<i>L. grayi</i>	CCM	5990
<i>Listeria innocua</i>	ATCC	33090
<i>L. innocua</i>	NCTC	20649
<i>L. innocua</i>	CIP	8011
<i>L. innocua</i>	CCM	4030
<i>Listeria ivanovii</i>	DMSZ	20750
<i>L. ivanovii</i>	CCM	5884
<i>Listeria monocytogenes</i>	NCTC	7979
<i>L. monocytogenes</i>	NCTC	9862
<i>L. monocytogenes</i>	NCTC	5105
<i>L. monocytogenes</i>	NCTC	10527
<i>L. monocytogenes</i>	CIP	55143
<i>L. monocytogenes</i>	CIP	78.39
<i>L. monocytogenes</i>	CIP	105459
<i>L. monocytogenes</i>	CIP	78.36
<i>L. monocytogenes</i>	CIP	105458
<i>L. monocytogenes</i>	CIP	78.43
<i>L. monocytogenes</i>	CIP	105457
<i>L. monocytogenes</i>	CRBP	13.34
<i>Listeria seeligeri</i>	DMSZ	20751
<i>L. seeligeri</i>	CCM	3971
<i>L. welshimeri</i>	NCTC	20650
<i>Listeria welshimeri</i>	CCM	3971
<i>Aeromonas caviae</i>	CIP	102629
<i>Alteromonas macleodii</i>	CCUG	16128
<i>Campylobacter jejuni</i>	Our collection ^a	—
<i>Bacillus cereus</i>	DMSZ	350
<i>Brochotrix thermosphacta</i>	Our collection	—
<i>Enterococcus faecalis</i>	Our collection	—
<i>Enterococcus faecium</i>	Our collection	—
<i>Escherichia coli</i>	ATCC	25922
<i>Lactobacillus plantarum</i>	Our collection	—
<i>Lactococcus garviae</i>	Our collection	—
<i>Lactococcus lactis</i>	Our collection	—
<i>Plesimonas shigelloides</i>	CCUG	10616
<i>Pseudomonas aeruginosa</i>	CCUG	38935
<i>Salmonella enterica</i> serovar Typhimurium	Our collection	—
<i>Staphylococcus xylosus</i>	DMSZ	6179
<i>Streptococcus thermophilus</i>	DMSZ	20617
<i>Yersinia enterocolitica</i>	CCUG	12369

NCTC, National Collection of Type Cultures (London, UK); CCM, Czech Collection of Microorganism (Brno, Czech Republic); ATCC, American Type Culture Collection, (Teddington, Middlesex, UK); CIP, Collection de l'Institut Pasteur (Paris, France); DMSZ, Deutsche Sammlung von Mikroorganismen und Zellkulturen (Braunschweig, Germany); CCUG, Culture Collection University of Göteborg (Göteborg, Sweden).

^aStrains were previously identified by 16S rDNA sequencing.

for *L. monocytogenes* (*plcA* primers) (Table 2). In the same way, 16S rRNA gene sequences for *L. monocytogenes* (accession no. S55472), *L. innocua* (accession no. X56152), *L. welshimeri* (accession no. X56149), *L. seeligeri* (accession no. DQ065845), *L. ivanovii* (accession no. X56151), *L. grayi* (accession no. X56150), and *L. marthii* (accession no. EU545983) were retrieved from GenBank and aligned with ClustalV software to design primers specific for *Listeria* genus (16S rDNA primers). The design of the *Listeria* genus-specific primers was per-

TABLE 2. PRIMERS FOR PRELIMINARY POLYMERASE CHAIN REACTION AND MINISEQUENCING

	Primers	Position	Oligonucleotides
Duplex PCR	16S rDNA forward	45	5'-CCTAATACATGCAAGTCGAA-3'
	16S rDNA reverse	480	5'-ACAAGCAGTTACTCTTATCCTT-3'
	<i>plcA</i> forward	1	5'-CCAGGTACACATGATACGAT-3'
	<i>plcA</i> reverse	632	5'-GCTGCAGCATACTGACG-3'
Minisequencing	L1 reverse	108	5'-CCGTCCGCCACTAAC-3'
	L2 forward	161	5'-27(T)GGGGCTAATACCGAAT-3'
	L3 reverse	240	5'-16(T)GGGCCCATCTGTAAG-3'
	L4 reverse	284	5'-22(T)TTGCCTTGGTAGGCC-3'

The positions are referred to sequence number X56152 for 16S rDNA primers, sequencing primers, and AF532205 for *plcA* primers.

formed to include the amplified fragment diagnosis sites that are able to identify all *Listeria* species with the exception of *L. monocytogenes* (Tables 2 and 3). All the sequences available for each species in GenBank were then examined to confirm the absence of intraspecific variations of the diagnosis positions selected.

For the development of minisequencing, sequencing primers (L1 reverse, L2 forward, L3 reverse, L4 reverse) were designed upstream from the diagnosis sites and had varying

lengths of poly(dT) nonhomologous tails attached to the 5' end (Tables 2 and 3). Primers were all synthesized by Sigma (St. Louis, MO).

Duplex PCR

In a preliminary phase of the investigation, *plcA* and 16S rDNA primers' specificity was assessed on DNA extracted from all reference strains by simplex PCRs.

TABLE 3. PRIMER BINDING SITES FOR SEQUENCING PRIMERS AND DIAGNOSIS SITES

Species	GenBank accession no.	Aligned sequences 5–3 (the position numbers are referred to sequence number X56152)	
		93	108
<i>L. marthii</i>	EU545983	A - A G T T A G T G G C G G A C G G G T G A G T A A C A C G T G G G C A A C C T G C C T G T A A	
<i>L. monocytogenes</i>	S55472		
<i>L. seeligeri</i>	DQ065845		
<i>L. welshimeri</i>	X56149		
<i>L. innocua</i>	X56152		
<i>L. ivanovii</i>	X56151		
<i>L. grayi</i>	X98526	G T C	
		162	178
<i>L. marthii</i>	EU545983	G T T G G G G A T A A C T C C G G G A A A C C G G G G C T A A T A C C G A A T A A T A A G A T G	
<i>L. monocytogenes</i>	S55472		G A G .
<i>L. seeligeri</i>	DQ065845		G G A .
<i>L. welshimeri</i>	X56149		. G T G .
<i>L. innocua</i>	X56152		G G A G .
<i>L. ivanovii</i>	X56151		G T A .
<i>L. grayi</i>	X98526	. A . T	. A T C A .
		226	270
<i>L. marthii</i>	EU545983	T G G C G C A T G C C A C G C C T T T G A A A G A T G G T T T C G G C T A T C A C T T A C A G A	
<i>L. monocytogenes</i>	S55472		G
<i>L. seeligeri</i>	DQ065845	. . A T T G	G
<i>L. welshimeri</i>	X56149		G
<i>L. innocua</i>	X56152		G
<i>L. ivanovii</i>	X56151	. . A T T C A	G
<i>L. grayi</i>	X98526	C T C G A G G G G C G	. A
		241	270
<i>L. marthii</i>	EU545983	T G G G C C C C G C G G T G C A T T A G C T A G T T G G T A G G G T A A T G G C C T A C C A A G G	
<i>L. monocytogenes</i>	S55472		. A
<i>L. seeligeri</i>	DQ065845		. A
<i>L. welshimeri</i>	X56149		. A
<i>L. innocua</i>	X56152		. A
<i>L. ivanovii</i>	X56151		. A
<i>L. grayi</i>	X98526		. A
		285	
<i>L. marthii</i>	EU545983	C A A C G A T G C A T A G C C G A C C T G A G A G G G T G A T C G G C C A C A C T G G G A C T G	
<i>L. monocytogenes</i>	S55472		
<i>L. seeligeri</i>	DQ065845		
<i>L. welshimeri</i>	X56149		
<i>L. innocua</i>	X56152		
<i>L. ivanovii</i>	X56151		
<i>L. grayi</i>	X98526	. G	

Underlined position numbers refer to the 5' position of each sequencing primers. Primer binding sites of sequencing primers L1 reverse, L2 forward, L3 reverse, and L4 reverse correspond to positions 108–94, 162–177, 241–227, and 285–271, respectively, and are highlighted in gray. Bold position numbers mark the diagnosis sites and correspond to positions 93, 178, 226, and 270 (as referred to GenBank accession n. X56152).

The simplex PCRs were carried out in a final volume of 50 μ L consisting of 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 1 unit of Recombinant Taq DNA Polymerase (Invitrogen, Carlsbad, CA), 0.2 mM each of dATP, dCTP, dGTP, and dTTP (Invitrogen), 2 mM MgCl₂, 25 pmol of each primer, and 50 ng of DNA template.

The specificity of duplex PCR was also tested on DNA extracted from all reference strains to evaluate potentially undesirable pairings of primers. The duplex PCR reactions were performed in a 50 μ L volume consisting of 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 2 units of Recombinant Taq DNA Polymerase (Invitrogen), 0.2 mM each of dATP, dCTP, dGTP, and dTTP (Invitrogen), 2 mM di MgCl₂, 20 pmol of 16S rDNA primers, 50 pmol of *plcA* primers, and 50 ng of DNA template.

Thermocycling conditions, both for simplex and duplex PCR, were the following: initial denaturation at 94°C for 3 min followed by 35 cycles of 1 min denaturation at 94°C, 1 min annealing at 55°C, and 1 min extension at 72°C. The final extension was carried out at 72°C for 5 min. Amplification was performed in a GeneAmp PCR System 2720 thermal cycler (Applied Biosystems). Amplicons were resolved by electrophoresis on a 2.5% agarose gel (Invitrogen), run in Tris acetate ethylenediaminetetraacetic acid buffer (pH 8.3) for 70 min at 110 V and stained with ethidium bromide (0.4 ng/mL) for 20 min.

Minisequencing

The 16S rRNA gene fragment was used as template for minisequencing reaction after the enzymatic clean-up. To remove primers and unincorporated nucleotides, 2 μ L of Exo-Sap (USB Europe GmbH, Stauf, Germany) were added to 5 μ L of PCR products. Tubes were incubated at 37°C for 1 h and then at 80°C for 15 min.

Tailed sequencing primers were tested individually to assess performance and validate migration size before the four primers were tested in multiplex reaction. Singleplex minisequencing reactions were performed following the SNaPshot Multiplex Kit protocol (Applied Biosystems) with minor modifications: 2.5 μ L of SNaPshot Multiplex Ready Reaction Mix; 3 μ L of purified preliminary PCR products diluted to obtain a range of 0.4–0.01 pmol; and 0.2 μ M of sequencing primer in a total volume 10 μ L.

Subsequently, a multiplex minisequencing was performed using the following concentrations of sequencing primers: 0.28 μ M of L1 reverse and 0.56 μ M each of L2, L3 reverse, and L4 reverse. Primer concentrations varied depending on the efficiency of each primer and were selected after the preliminary assays. All reactions underwent 25 single base extension cycles of denaturation at 96°C for 10 sec, annealing at 50°C for 5 sec, and extension at 60°C for 30 sec and were carried out in a GeneAmp PCR System 2720 thermal cycler. A postextension treatment to remove the 5'-phosphoryl group of the F-ddNTPs helped to prevent unincorporated F-ddNTPs comigrating with the extended primers, thereby producing a high background signal. For this purpose the final volume (10 μ L) was treated with 1 unit of calf intestine alkaline phosphatase (Fermentas, Burlington, CA). The tubes were incubated at 37°C for 1 h and at 75°C for 15 min. Finally, samples were prepared by adding 1 μ L of the postminisequencing product to 24.6 μ L of formamide (Applied Biosystems) and 0.4 μ L of

GeneScan 120 LIZ size standard (Applied Biosystems). Each sample was loaded on the ABI 310 Genetic Analyzer (Applied Biosystems) and analyzed using Pop4 polymer (Applied Biosystems), a 47-cm capillary column (Applied Biosystems), and the ABI GeneScan E5 Run Module. Parameters for this analysis were as follows: injection time 12 sec and run time 15 min. Electropherograms were analyzed using the GeneScan 3.1.2 software (Applied Biosystems).

DNA sequencing

Confirmatory sequencing of the amplified *plcA* and 16S rDNA fragments was carried out for all reference strains. Amplified products were purified with Exo-Sap according to the manufacturer's recommendations (USB Europe GmbH).

Sequencing was carried out directly on purified fragments with ABI 310 Genetic Analyzer using ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kit, version 1.1 (Applied Biosystems). Sequenced fragments were purified by DyeEX (Qiagen, Hilden, Germany) and resolved by capillary electrophoresis using an ABI 310 Genetic Analyzer. The

FIG. 1. Specificity of duplex PCR of DNA from *Listeria* certified strains. (A) M: 100-bp ladder; lane 1: *L. monocytogenes* NCTC 7979; lane 2: *L. monocytogenes* NCTC 9862; lane 3: *L. monocytogenes* NCTC 5105; lane 4: *L. monocytogenes* CIP 55143; lane 5: *L. monocytogenes* CIP 105459. (B) M: 100-bp ladder; lane 1: *L. innocua* NCTC 20649; lane 2: *L. ivanovii* CCM 5884; lane 3: *L. seeligeri* CCM 3971; lane 4: *L. welshimeri* NCTC 20650; lane 5: *L. grayi* NCTC 20601.

nucleotide sequences were analyzed using the BLASTN sequence similarity search at the National Center for Biotechnology Information database (Altschul *et al.*, 1990).

Results and Discussion

Although only *L. monocytogenes* is recognized as a major cause of human listeriosis outbreaks, a correct identification of *Listeria* species in environmental and food microbiology and in human and animal veterinary cases can contribute to a thorough monitoring of the diffusion of these microorganisms and to the implementation of effective strategies of prevention.

Further, the differentiation of nonpathogenic species such as *L. innocua* could represent a useful marker of potential contamination with *L. monocytogenes* (Gasnov *et al.*, 2005). Given the strict similarity of *L. monocytogenes* with other *Lis-*

teria species, specific and sensitive diagnostic methods to be used on different matrices are needed.

The aim of our study was to develop an assay for the specific identification of the six *Listeria* species (*L. monocytogenes*, *L. innocua*, *L. welshimeri*, *L. seeligeri*, *L. ivanovii*, and *L. grayi*) by targeting the 16S rRNA and *plcA* genes. These genes have been reported to be useful targets for the differentiation among *Listeria* species (Sallen *et al.*, 1996; Volokhov *et al.*, 2002; Schmid *et al.*, 2003).

In our work, we assembled a duplex PCR characterized by a specific fragment for *L. monocytogenes* (632 bp long) and by a common fragment for all *Listeria* sp. (437 bp long). When both bands were detected in the agarose gels, *L. monocytogenes* was immediately identified (Fig. 1A). When only the 437-bp band was present, it was possible to identify the *Listeria* genus but not to discriminate between the other *Listeria* species, except for *L. monocytogenes*, which was readily excluded as a possible

FIG. 2. Species-specific patterns observed in *Listeria* reference strains. (Color figure is available at www.liebertonline.com.)

identification because of the absence of the species-specific fragment (Fig. 1B). However, minisequencing of this fragment allowed identification of the five other species. This aspect of the method developed is of particular interest because, even though it is crucially important to identify *L. monocytogenes* because of its pathological potential, sometimes the epidemiological surveys require the differentiation of the other species as well.

Concerning the experimental design, the alignment of the sequences of *L. monocytogenes* *plcA* gene allowed the design of the specific primers for this species. In the same way, analyzing the 16S rRNA gene for the other *Listeria* sp., it was possible to observe base differences at positions 93, 178, 226, and 270 (numbering referred to GenBank accession no. X56152) so that 16S rRNA gene could differentiate *L. innocua*, *L. welshimeri*, *L. seeligeri*, *L. ivanovii*, and *L. grayi* (Table 3). These chosen diagnosis sites could also allow, at least from a theoretical point of view, the differentiation of *L. marthii*, recently described and currently available only as a sequence (Graves *et al.*, 2009). The absence of intraspecific variation of the chosen sites was verified by the alignments of all the sequences available in GenBank for each species object of this study (data not shown).

Simplex *plcA* PCR allowed the amplification of a 632-bp fragment in all *L. monocytogenes* reference strains and no cross-reaction was detected when the primers were tested for other *Listeria* species or for other genera phylogenetically related. Similarly, simplex 16S rRNA gene PCR allowed the amplification of a 437-bp fragment in all reference *Listeria* strains and no cross-reaction was detected when the primers were tested with non-*Listeria* strains. Similar results were obtained when duplex PCR was optimized.

When DNA was challenged in singleplex minisequencing, it gave rise to a peak of the expected color that was specific to the fluorescent-labeled dideoxynucleotides (F-ddNTPs) incorporated: green (adenine), black (cytosine), blue (guanine), and red (thymine).

To optimize the resolution of the profile in capillary electrophoresis, the length of the sequencing primers had to be increased of at least six nucleotides (dT). The sequencing primers ranged in size from 15 to 43 bp, although migration did not exactly correspond to primers' size, in agreement with that observed in previous works (Bottero *et al.*, 2007; La Neve *et al.*, 2008; Dalmasso *et al.*, 2009). The multiplex minisequencing was optimized and the DNA extracted from five *Listeria* species gave species-specific patterns (Fig. 2). As *L. marthii* strains are not publicly available yet, it was not possible to demonstrate its differentiation.

Minisequencing has been recently used for rapid identification of various microorganisms such as *Brucella* isolates at the species level (Scott *et al.*, 2007) and pathogenic species of *Vibrio* (Dalmasso *et al.*, 2009). These investigations have demonstrated that the analysis of diagnosis sites represents a valuable strategy for the differentiation of closely related species. In this respect, Paillard *et al.* (2003) have described another genotypic method based on the analysis of the mutations employing the RFLP for the identification of the six species of *Listeria*. This procedure, however, seems rather complicated because it requires the combination of several enzymes, given the high number of species to be discriminated (Ram *et al.*, 1996; Quinteiro *et al.*, 1998; Pardo and Perez-Villareal, 2004). This also precludes the possibil-

ity of an automation of the method. Further, because of possible secondary conformation, enzyme access to target site may be difficult, leading to the presence of some undigested PCR products (Quinteiro *et al.*, 1998).

Minisequencing, together with the simultaneous interrogation of different diagnosis sites, allows high automation with the same precision achievable with the sequencing. For these reasons the proposed assay may be considered a valid alternative to PCR-RFLP, especially when several species must be identified.

Finally, concerning the validation of the test, all 29 WIs of *L. monocytogenes* gave rise to two amplicers (632 and 437 bp) in duplex PCR, whereas the other *Listeria* strains gave rise to the expected species-specific patterns in minisequencing technique. Regarding the 95 non-*L. monocytogenes* WIs, it was possible to observe a perfect match between the minisequencing results and the identification of these isolates as obtained by MicroSeq 500 16S rDNA Bacterial Identification Kit. Further, the test was validated by submitting to sequencing of both 16S rDNA and *plcA* fragments.

In conclusion, in this study, an assay based on duplex PCR and minisequencing for rapid detection and identification of *Listeria* genus or species was established. This technique has the potential to become a rapid method of great benefit to the food industry as well as to regulatory or public health laboratories engaged in establishing the safety of food products and the management of listeriosis.

Disclosure Statement

No competing financial interests exist.

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