APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Dec. 2002, p. 6273–6282 Vol. 68, No. 12
0099-2240/02/$04.00+0 DOI: 10.1128/AEM.68.12.6273–6282.2002

Direct Identification in Food Samples of *Listeria* spp. and *Listeria monocytogenes* by Molecular Methods

Luca Cocolin,[1]* Kalliopi Rantsiou,[1] Lucilla Iacumin,[1] Carlo Cantoni,[2] and Giuseppe Comi[1]

Dipartimento Scienze degli Alimenti, Università degli studi di Udine, 33100 Udine,[1] and Dipartimento di Scienze e Tecnologie Veterinarie per la Sicurezza degli Alimenti, Università degli studi di Milano, 20121 Milan,[2] Italy

Received 25 March 2002/Accepted 17 September 2002

A new molecular approach for the detection and identification of *Listeria* spp. and *Listeria monocytogenes* in food is presented here. The method is based on the PCR amplification of a fragment of the *iap* gene from the five species belonging to the genus and on the analysis of the PCR products obtained by denaturing gradient gel electrophoresis (DGGE). The protocol was first optimized by using strains from international collections. Based on the differences present in the sequences amplified, it was possible to obtain species-specific DGGE migration that allowed fast and easy identification of *L. monocytogenes*, *L. innocua*, *L. welshimeri*, *L. seeligeri*, and *L. ivanovii*. Moreover, for *L. monocytogenes* serotypes, partial differentiation was possible. The optimized protocol was used for identification of *Listeria* strains traditionally isolated from food and for direct detection and identification of *Listeria* members in food after an overnight enrichment. Identification of 48 food isolates and direct detection of *Listeria* spp. in 73 food samples show the potential of the method that can be used as a fast screening test to investigate the presence of *Listeria* spp. and *L. monocytogenes* in food.

The genus *Listeria* comprises a group of ubiquitous, gram-positive, nonsporulating bacteria. Previous results of DNA-DNA hybridization experiments unambiguously demonstrated that the species formerly called "*Listeria monocytogenes*" in fact contained five genomic groups deserving the species rank: *L. monocytogenes*, *L. ivanovii*, *L. innocua*, *L. welshimeri*, and *L. seeligeri* (48, 49). This subdivision has proven its usefulness for practical and epidemiological purposes, especially when problems caused by food contamination resulted in human listeriosis (29). Only the hemolytic species of *Listeria*, *L. monocytogenes*, *L. ivanovii*, and *L. seeligeri*, are associated with human pathogenicity. *L. monocytogenes* is the only species of the genus *Listeria* that has been involved in known food-borne outbreaks of listeriosis. *L. ivanovii* has been described to be involved in human pathology only rarely, and *L. seeligeri* has been reported only once to be the cause of meningitis in a nonimmunocompromised adult (35).

Several large outbreaks of listeriosis have been associated with contaminated commercial foodstuffs, such as vegetables, milk, and meat products, on which these bacteria can multiply even at low temperatures (52). Usually, the presence of any *Listeria* species in food is an indicator of poor hygiene (40).

Significant efforts have been dedicated to the development of enrichment media and protocols for *L. monocytogenes* and *Listeria* sp. isolation (1, 10, 11, 15, 24, 47). Ideal enrichment media would facilitate recovery of injured *Listeria* cells and enrichment of *Listeria* spp. and *L. monocytogenes* over competing microflora. In traditional culture-based assays, it becomes very difficult to detect *L. monocytogenes* at any level when it is greatly outnumbered by other *Listeria* spp., such as *L. innocua*, which appears to be present together with *L. monocytogenes* (10, 51). Consequently, detection of *Listeria* spp. is often used as an indication for the presence of *L. monocytogenes* (22).

Species-specific identification with biochemical standard methods, which include sugar fermentation or the CAMP test (53), is laborious and time-consuming and can require 1 to 2 weeks for species identification (28). Moreover, differentiation between species or strains within the same species is not always reached. For these reasons, little is known about the occurrence and distribution of *Listeria* species other than *L. monocytogenes*.

The first studies of isolation and detection of *L. monocytogenes* in food that exploited molecular methods date back to the 1990s (42, 50, 59). From then, protocols have been continuously proposed and only in the last few years a vast number of papers have been published (25, 27, 41, 44, 45). Only a few studies have considered the identification of the other members of the genus *Listeria*. Repetitive element sequence-based PCR (30), multiplex PCR (2, 6, 23, 32), rRNA gene restriction patterns (29), sequence analysis of the 16S-23S rRNA internal transcribed spacer loci (13, 18), temperature gradient gel electrophoresis (37), two-dimensional protein mapping (17), and colony-blot assay with anti-p60 antibodies (60) are some of the methods proposed to identify *Listeria* species. Often the protocols developed are able to detect only the genus *Listeria*, thus lacking the ability to identify different species of *Listeria* simultaneously. Moreover, the protocols described need a previous isolation of *Listeria* strains using traditional methods.

Currently, new methods are developed to directly characterize the microorganisms without the need for isolation (20). This approach eliminates the necessity for strain isolation, thereby negating the potential biases inherent to microbial enrichment. Studies that have employed such direct analysis have repeatedly demonstrated a tremendous variance between cultivated and naturally occurring species, thereby dramatically altering the perception of the true microbial diversity present in various habitats (26).

* Corresponding author. Mailing address: Food Science Department, Via Marangoni, 97, 33100 Udine, Italy. Phone: 39/0432/590-759. Fax: 39/0432/590-719. E-mail: luca.cocolin@dsa.uniud.it.

TABLE 1. Strains used in this study

Strain	Serotype	Strain no.	Source[a]
International collection strains			
L. monocytogenes	1/2a	NCTC 7979	NCTC
	1/2b	NCTC 10887	NCTC
	1/2c	NCTC 9862	NCTC
	3a	NCTC 5105	NCTC
	3b	CIP 78.35	CIP
	4b	NCTC 10527	NCTC
		ATCC 7644	ATCC
L. ivanovii		DSMZ 20750	DSMZ
L. innocua		DSMZ 20649	DSMZ
L. welshimeri		DSMZ 20650	DSMZ
L. seeligeri		DSMZ 20751	DSMZ
Human isolate strains			
L. monocytogenes	1/2a	26	Azienda Ospedaliera Policlinico di Modena
	1/2b	12	Azienda Ospedaliera Policlinico di Modena
	1/2b	15	Azienda Ospedaliera Policlinico di Modena
	1/2c	14	Azienda Ospedaliera Policlinico di Modena
	4b	1	Azienda Ospedaliera Policlinico di Modena
	4b	2	Azienda Ospedaliera Policlinico di Modena
	4b	3	Azienda Ospedaliera Policlinico di Modena
	4b	5	Azienda Ospedaliera Policlinico di Modena
	4b	6	Azienda Ospedaliera Policlinico di Modena
	4b	7	Azienda Ospedaliera Policlinico di Modena
	4b	8	Azienda Ospedaliera Policlinico di Modena
	4b	10	Azienda Ospedaliera Policlinico di Modena
	4b	11	Azienda Ospedaliera Policlinico di Modena
	4b	13	Azienda Ospedaliera Policlinico di Modena
	4b	17	Azienda Ospedaliera Policlinico di Modena
	4b	19	Azienda Ospedaliera Policlinico di Modena
	4b	20	Azienda Ospedaliera Policlinico di Modena
	4b	21	Azienda Ospedaliera Policlinico di Modena
	4b	23	Azienda Ospedaliera Policlinico di Modena
	4b	25	Azienda Ospedaliera Policlinico di Modena
Non-*Listeria* spp. strains			
Aeromonas hydrophila			Dipartimento di Scienze degli Alimenti
Campylobacter jejuni			Dipartimento di Scienze degli Alimenti
Escherichia coli			Dipartimento di Scienze degli Alimenti
Escherichia coli O157:H7			Dipartimento di Scienze degli Alimenti
Proteus vulgaris			Dipartimento di Scienze degli Alimenti
Proteus mirabilis			Dipartimento di Scienze degli Alimenti
Salmonella enterica serovar Typhimurium			Dipartimento di Scienze degli Alimenti
Salmonella enterica serovar Enteritidis			Dipartimento di Scienze degli Alimenti
Citrobacter freundii			Dipartimento di Scienze degli Alimenti
Shigella flexneri			Dipartimento di Scienze degli Alimenti
Yersinia enterocolitica			Dipartimento di Scienze degli Alimenti
Bacillus cereus			Dipartimento di Scienze degli Alimenti
Bacillus subtilis			Dipartimento di Scienze degli Alimenti
Enterococcus faecalis			Dipartimento di Scienze degli Alimenti
Lactobacillus plantarum			Dipartimento di Scienze degli Alimenti
Lactobacillus casei			Dipartimento di Scienze degli Alimenti
Staphylococcus aureus			Dipartimento di Scienze degli Alimenti
Staphylococcus carnosus			Dipartimento di Scienze degli Alimenti
Staphylococcus xylosus			Dipartimento di Scienze degli Alimenti

[a] NCTC, National Collection of Type Cultures; CIP, Collection Institut Pasteur; ATCC, American Type Culture Collection; DSMZ, Deutsche Sammlung von Mikroorganismen and Zellkulturen.

In this paper a direct identification in food samples of *Listeria* spp. by a molecular method is described. PCR coupled with denaturing gradient gel electrophoresis (DGGE) allowed a direct identification of *Listeria* spp. in food, based on specific migration patterns. Moreover, *L. monocytogenes* serotypes could be partially distinguished on the basis of PCR product mobility in the denaturing gel. The method described could represent a fast and interesting tool to study the ecology of the members of the genus *Listeria*, monitoring their spread in food and environmental samples and for epidemiological purposes as well.

(Partial results of this work were presented at the 101st General Meeting of the American Society for Microbiology, Orlando, Fla., 21 to 25 May 2001.)

MATERIALS AND METHODS

Bacterial strains. The strains used in this study are listed in Table 1. *Listeria* spp. strains came from international collections or were isolated from patients with listeriosis. Non-*Listeria* strains came from the collection of the Dipartimento di Scienze degli Alimenti, Udine, Italy. Moreover, 48 strains of *Listeria* spp. previously isolated from food samples and identified by traditional methods were used. All the strains were cultured on brain heart infusion (BHI; Oxoid, Milan,

TABLE 2. Sequence and position of annealing of the primers and PCR product sizes for the different *Listeria* spp. tested in this study

Listeria sp.	Accession no. of reference sequence	5′-3′ Sequence (nucleotide position) of the forward primer annealing site[a]	5′-3′ Sequence (nucleotide position) of the reverse primer annealing site[a,b]	PCR product size (bp)[a]
L. ivanovii	M80350	ATGTCATGGAATAA (661–674)	AAAAACA**T**CTTGGAAAAGC (1250–1268)	610
L. seeligeri	M80353	ATGTCATGGAATAA (667–680)	AAAAACACCTTGG**T**AAAGC (1247–1265)	601
L. welshimeri	M80354	ATGTCATGGAATAA (661–674)	AAAAACA**T**CTTGGAAAAGC (1250–1268)	610
L. innocua	M80349	ATGTCATGGAATAA (670–683)	AAAAACA**T**CTTGGAAAAGC (1121–1139)	472
L. monocytogenes	M80351	ATGTCATGGAATAA (676–689)	AAAAACACCTTGGAAAAGC (1112–1130)	457

[a] Determined by using the molecular biology software Amplify (14).
[b] Nucleotides in bold represent positions with a mismatch with the primer used in the study.

Italy) broth and incubated at 30 or 37°C overnight before being subjected to analysis by molecular methods.

Traditional isolation and identification. Forty-eight *Listeria* strains were isolated from food according to the method of the U.S. Department of Agriculture, Food Safety and Inspection Service (9), and identified using morphological, cultural, and biochemical criteria. In particular, Gram stain, catalase test, mobility, β-hemolysis, CAMP test, and production of acids from rhamnose and xylose were used, as described in Bergey's manual (53), to identify the species belonging to the genus.

DNA extraction. One milliliter of an overnight culture was subjected to centrifugation at 14,000 × *g* at 4°C for 10 min, and the pellet was washed in 500 μl of lysozyme buffer solution (25% [wt/vol] sucrose, 0.1 g of lysozyme/ml). After a second centrifugation at 14,000 × *g* at 4°C for 10 min, the supernatant was discarded and the pellet was resuspended in 50 μl of lysozyme (50 mg/ml). A 30-min incubation at 37°C was performed, followed by a bead-beater treatment to break up the cells. In particular, the cell suspension was mixed with 300 μl of breaking buffer (2% Triton X-100, 1% sodium dodecyl sulfate, 100 mM NaCl, 10 mM Tris, 1 mM EDTA, pH 8) and transferred to a 1.5-ml screw-cap tube containing 0.3 g of glass beads. After the addition of 300 μl of phenol-chloroform-isoamyl alcohol (25:24:1) (pH 6.7) (Sigma, Milan, Italy), the tubes were subjected to three 30-s treatments, with a 15-s break in between them, at maximum speed in the Mini-Bead Beater 8 (Biospec Products, Inc., Bartlesville, Okla.). Three hundred microliters of 10 mM Tris–5 mM EDTA (pH 8) was added and a centrifugation at 12,000 × *g* at 4°C for 10 min was performed. The aqueous phase was collected, and the DNA was precipitated by the addition of 1 ml of ice-cold absolute ethanol and centrifugation at 14,000 × *g* at 4°C for 10 min. DNA pellets were dried under vacuum at room temperature, resuspended in 50 μl of RNase- and DNase-free sterile water, and treated with 1 μl of DNase-free RNase (500 μg/ml; Roche Diagnostics, Milan, Italy). After a 1-h incubation at 37°C, the tubes were stored at −20°C.

PCR amplification. PCR was performed in a final volume of 50 μl containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 3.5 mM $MgCl_2$, 0.2 mM (each) deoxynucleoside triphosphates, 1.25 IU of *Taq* polymerase (Applied Biosystems, Milan, Italy), and 0.2 μM (each) primers targeting the *iap* gene encoding the invasion-associated protein p60 in all *Listeria* spp. (7). Primers were named List-univ. 1 (5′-ATGTCATGGAATAA-3′) and List-univ. 2 (5′-GCTTTTCCA AGGTGTTTTT-3′) (L. Cocolin, M. Manzano, C. Cantoni, and G. Comi, Abstr. 101st Gen. Meet. Am. Soc. Microbiol. 2001, abstr. P-88, p. 575, 2001). A GC clamp was added to primer List-univ. 1 (5′-GCC AGC GGC CCG GCG CGG GCC CGG CGG CGG GGG CCG CGG C-3′) to improve its sensitivity in the detection of DNA sequence differences by DGGE analysis as previously described (55). After DNA addition (≈50 ng), the samples were subjected to amplification in a MiniCycler (Genenco, Florence, Italy) using the following program: 95°C for 5 min; 35 cycles of 95°C for 1 min, 36°C for 2 min, and 72°C for 3 min; and, finally, 72°C for 7 min. Five microliters of the product was analyzed by standard agarose gel electrophoresis before DGGE analysis.

DGGE analysis. The Dcode Universal Mutation Detection system (Bio-Rad, Hercules, Calif.) was used for the sequence-specific separation of the PCR products. Electrophoresis was performed in a 0.8-mm-thick polyacrylamide gel (8% [wt/vol] acrylamide-bisacrylamide [37.5:1]) containing a 20 to 40% urea-formamide denaturing gradient (100% corresponds to 7 M urea and 40% [wt/vol] formamide), increasing in the direction of the electrophoretic run. The gels were subjected to a constant voltage of 130 V for 4 h at 60°C. After electrophoresis, they were stained for 10 min in a SYBR Green solution (Molecular Probes, Eugene, Oreg.) and analyzed under UV illumination. Pictures of the gels were digitized by using a charge-coupled device camera (Polaroid, St. Louis, Mo.), and images were analyzed using the Gel Pro Analyzer 3.0 (Immagini e Computer, Milan, Italy) for the identification of the bands present in the gel.

Direct analysis of food samples by PCR-DGGE. Seventy-three food samples were collected from local laboratories and represented beef, pork, and poultry

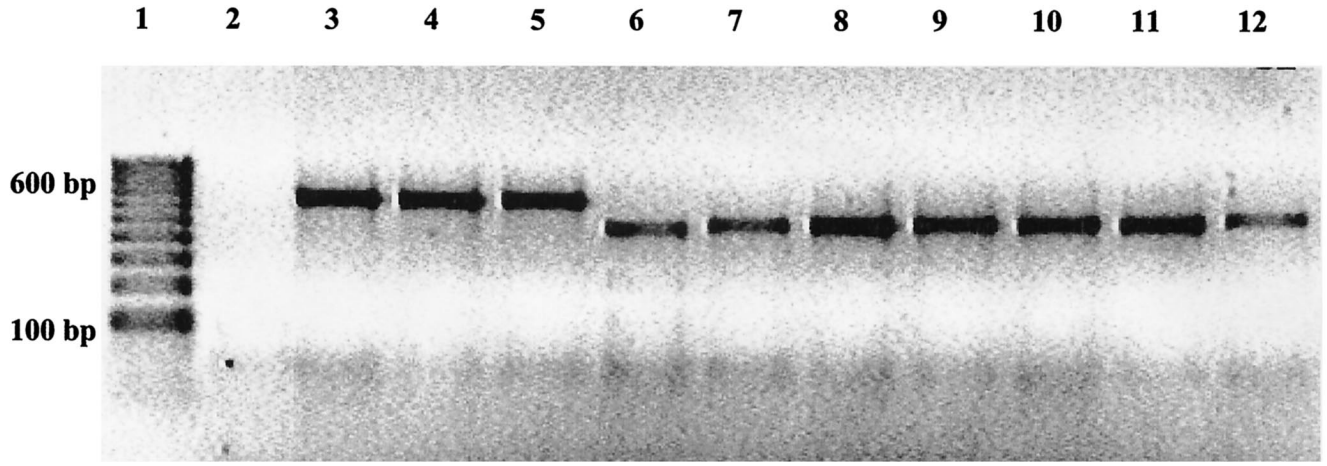

FIG. 1. Agarose gel electrophoresis of the products obtained after amplification of the *iap* gene from different *Listeria* species. Lane 1, 100-bp ladder (Sigma); lane 2, negative control; lane 3, *L. ivanovii* DSMZ 20750; lane 4, *L. seeligeri* DSMZ 20751; lane 5, *L. welshimeri* DSMZ 20650; lane 6, *L. innocua* DSMZ 20649; lane 7, *L. monocytogenes* 1/2a NCTC 7979; lane 8, *L. monocytogenes* 1/2b NCTC 10887; lane 9, *L. monocytogenes* 1/2c NCTC 9862; lane 10, *L. monocytogenes* 3a NCTC 5105; lane 11, *L. monocytogenes* 3b CIP 78.35; lane 12, *L. monocytogenes* 4b NCTC 10527. The numbers on the left indicate the molecular size of the DNA bands in base pairs.

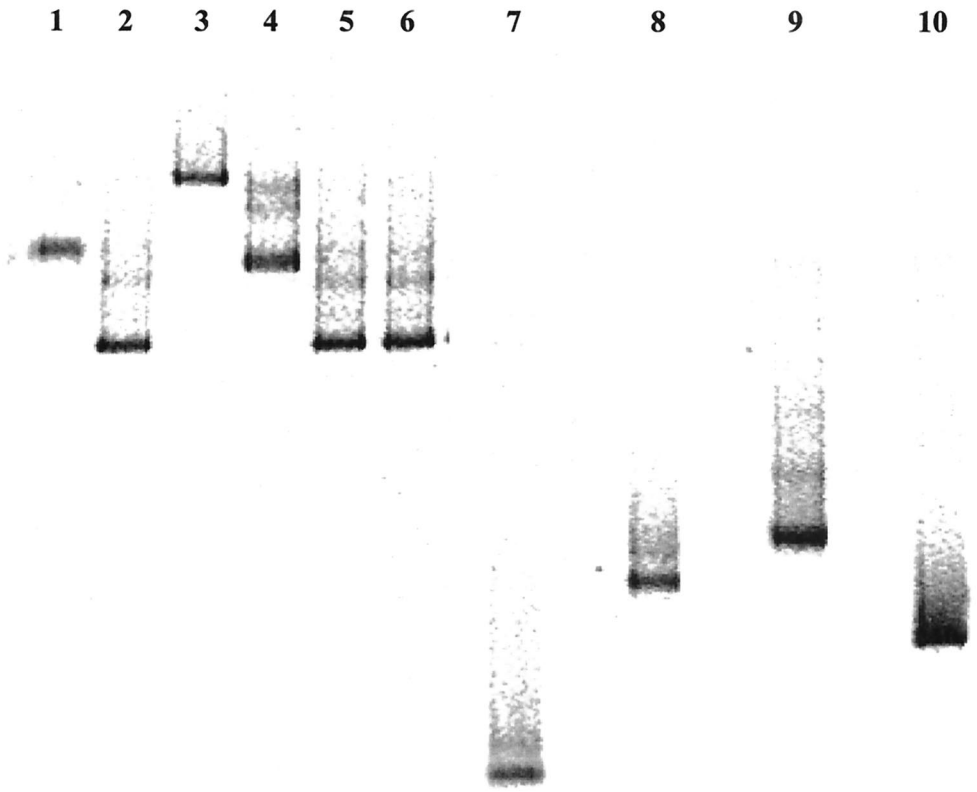

FIG. 2. DGGE profiles of *Listeria* species obtained from international collections. Lane 1, *L. monocytogenes* 1/2a NCTC 7979; lane 2, *L. monocytogenes* 1/2b NCTC 10887; lane 3, *L. monocytogenes* 1/2c NCTC 9862; lane 4, *L. monocytogenes* 3a NCTC 5105; lane 5, *L. monocytogenes* 3b CIP 78.35; lane 6, *L. monocytogenes* 4b NCTC 10527; lane 7, *L. innocua* DSMZ 20649; lane 8, *L. welshimeri* DSMZ 20650; lane 9, *L. seeligeri* DSMZ 20751; lane 10, *L. ivanovii* DSMZ 20750.

meats. Twenty-five grams of sample was added to 225 ml of BHI broth in a sterile bag and homogenized in a stomacher machine (P.B.I., Milan, Italy) for 1.5 min. After an overnight enrichment step at 37°C, 1 ml was collected and centrifuged at 14,000 × *g* at 4°C for 10 min. The pellet obtained was then subjected to DNA extraction, PCR amplification, and DGGE analysis as described above. One aliquot of the overnight enrichment was also streaked onto Oxford agar (Oxoid), and plates were incubated at 37°C for 48 h. After incubation, suspected *Listeria* sp. colonies were purified on BHI agar and, after propagation in BHI broth, were subjected to molecular identification by PCR-DGGE. The direct identification of *Listeria* spp. was also confirmed by direct sequencing of the bands migrating at the same level with appropriate controls loaded on the gel. In particular, bands of interest were punched out of the gel by use of sterile tips, resuspended in sterile water, and after an overnight incubation at 4°C, reamplified using the GC-clamped List-univ. 1 primer. A DGGE run was performed to check for the presence of a single band migrating at the same position with respect to the band isolated from the food sample, and after amplification using the List-univ. 1 primer lacking the GC tail, the PCR product was purified using a QiaQuick PCR purification kit (Qiagen, Milan, Italy) and sequenced by a commercial facility (MWG Biotech, Edelsberg, Germany). Moreover, the presence of *L. monocytogenes* was confirmed by specific amplification using the primers Mar 1 and Mar 2 (38) and restriction enzyme analysis (REA) as previously described (36).

RESULTS

Use of the *iap* gene as a target for specific PCR amplification of *Listeria* spp. The set of primers developed was designed based on the partial alignments of the *iap* genes from the *Listeria* spp. considered in the study. The accession numbers of the reference sequences, primer sequences, positions of primer annealing sites, and expected PCR product sizes are reported in Table 2. The primers chosen are able to prime the amplification of all *Listeria* spp. tested, due to their high or complete homology to the sequence of the *iap* genes considered. More specifically, the forward primer was completely homologous to the reference sequence of all five species considered. In contrast, the reverse primer was identical to the *L. monocytogenes* sequence only, while a mismatch in one nucleotide position for each of the other four species was observed. The primers are able to amplify a region that contains significant differences in the sequence between the species of *Listeria* studied. *L. monocytogenes* and *L. innocua* are characterized by the presence of different deletions in the sequence compared to *L. ivanovii*, *L. seeligeri*, and *L. welshimeri*. Because of these deletions in the sequence for *L. monocytogenes* and *L. innocua*, PCR amplification gives amplicons with different molecular sizes as follows: *L. ivanovii*, *L. seeligeri*, and *L. welshimeri* are characterized by an amplicon of between 600 and 610 bp, whereas *L. monocytogenes* and *L. innocua* give PCR products of 457 and 472 bp, respectively. The expected sizes of the products, determined by using molecular biology software (14), were confirmed by agarose gel electrophoresis of the PCR amplicons. As shown in Fig. 1, *L. ivanovii*, *L. seeligeri*, and *L. welshimeri* (Fig. 1, lanes 3, 4, and 5) have a higher molecular weight band than *L. monocytogenes* and *L. innocua* (Fig. 1, lanes 6 to 12). No differences in the size of the PCR products were noticed when different serotypes of *L. monocytogenes* were amplified (Fig. 1, lanes 7 to 12). The primers designed for this study were proven to be highly specific for *Listeria* spp. When DNA from non-*Listeria*

TABLE 3. Comparison of the results obtained from the biochemical and PCR-DGGE identification on the 48 *Listeria* strains isolated from food samples using traditional methods (9)[a]

Strain no.	Biochemical identification	PCR-DGGE identification
1	*L. innocua*	*L. innocua*
4	*L. monocytogenes*	*L. monocytogenes* serotype 1/2c
5	*L. innocua*	*L. innocua*
6	*L. monocytogenes*	*L. monocytogenes* serogroup a
7	*L. monocytogenes*	*L. monocytogenes* serogroup b
9	*L. monocytogenes*	*L. monocytogenes* serotype 1/2c
11	*L. innocua*	*L. innocua*
13	*L. monocytogenes*	*L. monocytogenes* serogroup a
15	*L. innocua*	*L. innocua*
16	*L. innocua*	*L. innocua*
21	*L. innocua*	*L. innocua*
24	*L. monocytogenes*	*L. monocytogenes* serogroup a
26	*L. monocytogenes*	*L. monocytogenes* serogroup a
30	*L. innocua*	*L. innocua*
35	*L. innocua*	*L. innocua*
37	*L. monocytogenes*	*L. monocytogenes* serotype 1/2c
38	*L. monocytogenes*	*L. monocytogenes* serotype 1/2c
43	***L. monocytogenes***	***L. innocua***
45	*L. innocua*	*L. innocua*
46	*L. innocua*	*L. innocua*
47	***L. monocytogenes***	***L. innocua***
48	*L. monocytogenes*	*L. monocytogenes* serotype 1/2c
49	***L. monocytogenes***	***L. innocua***
50	*L. innocua*	*L. innocua*
51	*L. innocua*	*L. innocua*
52	*L. innocua*	*L. innocua*
53	*L. innocua*	*L. innocua*
54	***L. monocytogenes***	***L. innocua***
57	***L. monocytogenes***	***L. innocua***
60	*L. innocua*	*L. innocua*
64	***L. monocytogenes***	***L. innocua***
66	***L. monocytogenes***	***L. innocua***
68	***L. monocytogenes***	***L. innocua***
69	*L. innocua*	*L. innocua*
70	***L. monocytogenes***	***L. innocua***
71	*L. innocua*	*L. innocua*
79	*L. innocua*	*L. innocua*
81	*L. innocua*	*L. innocua*
83	*L. monocytogenes*	*L. monocytogenes* serogroup b
86	*L. monocytogenes*	*L. monocytogenes* serotype 1/2c
87	*L. monocytogenes*	*L. monocytogenes* serotype 1/2c
89	*L. monocytogenes*	*L. monocytogenes* serotype 1/2c
91	*L. monocytogenes*	*L. monocytogenes* serotype 1/2c
92	*L. monocytogenes*	*L. monocytogenes* serogroup a
94	*L. monocytogenes*	*L. monocytogenes* serotype 1/2c
96	*L. monocytogenes*	*L. monocytogenes* serotype 1/2c
98	*L. monocytogenes*	*L. monocytogenes* serogroup b
99	*L. monocytogenes*	*L. monocytogenes* serotype 1/2c

[a] Strains reported in boldface were identified differently by biochemical and molecular methods.

strains, as reported in Table 1, was used in the PCR, no PCR amplicon, of any size, was obtained, underlining the feasibility of the use of the primers directly on DNA extracted from microbial mixtures containing *Listeria* spp. for their specific detection by PCR amplification.

DGGE differentiation of *iap*-derived PCR products within *Listeria* spp. The DGGE profiles of the *iap*-derived PCR products for the different *Listeria* spp. considered in this study are shown in Fig. 2. Species-specific migrations were obtained for all the species tested. *L. monocytogenes*, *L. innocua*, *L. ivanovii*, *L. seeligeri*, and *L. welshimeri* all showed a different profile, making the identification simple and immediate. Moreover, for *L. monocytogenes*, differentiation between serotypes was possible. The PCR products obtained from serotypes 1/2a and 3a (Fig. 2, lanes 1 and 4) showed the same mobility in the gel, but it was different from that of serotype 1/2c (Fig. 2, lane 3) as well as those of 1/2b, 3b, and 4b (Fig. 2, lanes 2, 5, and 6). In addition, PCR-DGGE-obtained differentiation of *L. monocytogenes* from humans with listeriosis (Table 1) agreed with the previous serotyping (data not shown).

Identification of *Listeria* strains isolated from food by traditional methods. The results obtained from the biochemical and molecular identification of 48 *Listeria* strains isolated from food by traditional methods are reported in Table 3, and an example of DGGE strain identification is shown in Fig. 3. Only strains belonging to the species *L. monocytogenes* and *L. innocua* were identified, whereas no *L. ivanovii*, *L. seeligeri*, and *L. welshimeri* were found. Almost all of the strain identifications that were obtained by use of traditional methods were in agreement with the DGGE patterns. Among the 48 strains isolated, 9 were identified as *L. monocytogenes* by traditional methods but had the DGGE profile of *L. innocua*. Moreover, thanks to the capability of the DGGE protocol to differentiate *L. monocytogenes* serotypes, 20 strains of *L. monocytogenes* were grouped as follows: 12 serotype 1/2c strains, 5 serogroup a strains, and 3 serogroup b strains.

Identification of *Listeria* spp. in food samples. Seventy-three food samples of different origins were subjected to PCR-DGGE detection and isolation of *Listeria* spp. Of the 73 samples, 24 were positive for *Listeria*, whereas 49 showed no *Listeria* spp. The PCR products obtained by amplification of the DNA extracted directly from food, using the primers List-univ. 1 and List-univ. 2, were subjected to DGGE analysis, and suspected colonies grown on Oxford agar were isolated and identified by PCR-DGGE. Moreover, the samples containing *L. monocytogenes* were also subjected to PCR with specific primers for *L. monocytogenes*, Mar 1 and Mar 2, to validate the results obtained from the DGGE gels. The PCR product obtained was cut with the *Hin*dIII restriction endonuclease to confirm the differentiation of serotypes obtained by DGGE. Table 4 reports the profiling of *Listeria* sp. populations present in the positive food samples. Sixteen foods contained single populations of *Listeria*, or a single serogroup of *L. monocytogenes*, whereas eight samples showed mixed populations. Figure 4 shows the PCR products obtained directly from food samples by use of List-univ. 1 and List-univ. 2 primers, their relative DGGE profiles, and the results of the specific Mar 1 and Mar 2 *L. monocytogenes* amplification for nine food samples. The presence of different species in food samples was already determined by gel electrophoresis of the PCR products obtained from the DNA extracted from food samples, using primers List-univ. 1 and List-univ. 2. As shown in Fig. 4A, samples 4, 9, 55, 58, 68, and 72 presented a doublet, indicating populations belonging to the *L. monocytogenes-L. innocua* group and to the *L. ivanovii-L. seeligeri-L. welshimeri* group. Samples 3, 4, and 5 gave a single band that could correspond to mixed *L. monocytogenes* and *L. innocua*, to different *L. monocytogenes* serotypes, or to single populations of *L. monocytogenes* or *L. innocua.* In Fig. 4B, the profiles obtained by DGGE analysis of the amplicons shown in Fig. 4A are reported. Only samples 32 and 38 presented a single population of *L. mono-*

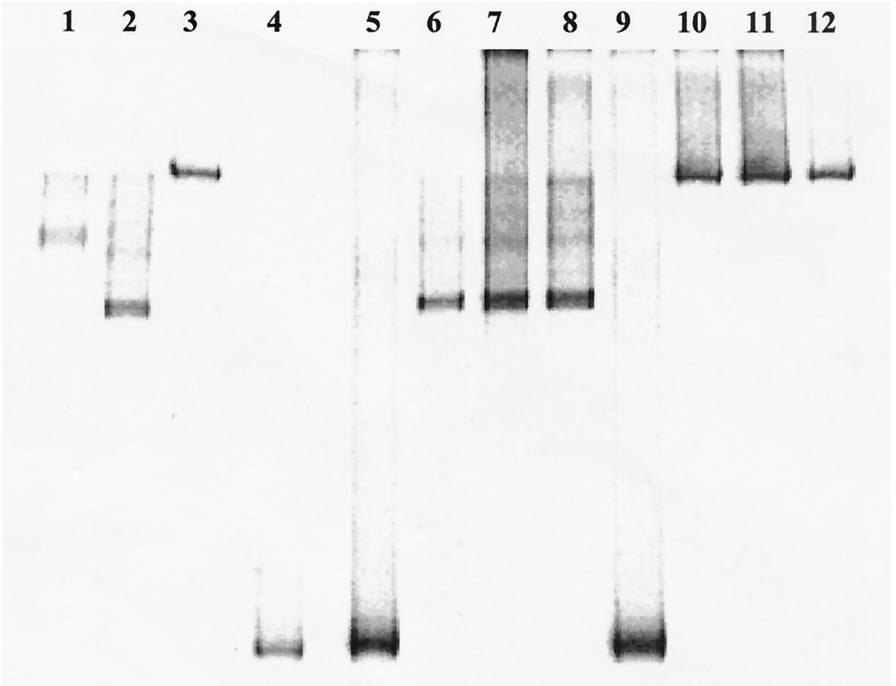

FIG. 3. DGGE patterns of *Listeria* strains isolated from food. Lanes 1 to 3, *L. monocytogenes* serotypes 1/2a NCTC 7979, 1/2b NCTC 10887, and 1/2c NCTC 9862, respectively; lane 4, *L. innocua* DSMZ 20649; lanes 5 to 12, strains isolated from food.

cytogenes or mixed *L. monocytogenes* strains belonging to the same serogroup, whereas the other samples contained two to three different species of *Listeria*. Sample 4, in particular, showed the simultaneous presence of *L. monocytogenes* serogroup b, *L. welshimeri*, and *L. innocua* (faint DGGE band). To confirm the identification obtained by comparing electrophoretic mobilities, bands indicated in Fig. 4B were excised and sequenced. All of them showed high similarities (>99%) to the species previously identified by analyzing the electrophoretic pattern.

The DNA extracted directly from food samples was also subjected to PCR using primers Mar 1 and Mar 2, and the results obtained for the samples shown in Fig. 4A are reported in Fig. 4C. As shown, only samples 4, 12, 32, 38, and 68, which contained *L. monocytogenes*, gave the specific amplicon, confirming the DGGE differentiation and identification.

The identification of *Listeria* spp. by molecular methods directly in food samples was last compared with the characterization of the strains isolated from Oxford agar. Up to five suspected *Listeria* sp. colonies per sample were isolated and subjected to DNA extraction, PCR amplification, and DGGE analysis. The results obtained are reported in Table 4. For almost all of the samples, the results obtained were in agreement with the identification of the isolates and, in fact, the species isolated and identified were represented in the DGGE gels. Only for sample 4, containing three different populations, *L. welshimeri* was present in the DGGE gel, but no isolates belonged to this species.

DISCUSSION

The detection and identification of *Listeria* spp. have attracted the attention of many authors. This specific interest is related to the presence of *L. monocytogenes*, one of the most important food-borne pathogens, in the genus. It is often found in various uncooked foods, such as meat, cheese, and vegetables. It is widely diffused in the environment and this fact can cause the contamination of food during production and distribution. However, *L. monocytogenes* has been the main representative of the genus to be studied.

The advances in biotechnology over the past decades have resulted in the development of many methods for the detection of pathogenic microorganisms such as *L. monocytogenes* in food (56). Molecular methods have been applied with certain success to the classification and typing of *Listeria*. Multilocus enzyme electrophoresis (4, 46), REA (5, 43, 49), and ribotyping (19, 57) have been developed. The use of faster procedures like PCR and immunological or bacteriophage lysis techniques which might allow a more rapid monitoring of all *Listeria* species is limited because they detect only the genus *Listeria* or only *L. monocytogenes* (8, 12, 16, 31, 34), thus lacking the ability to simultaneously characterize all the species of *Listeria*. Moreover, the methods developed so far require pure cultures isolated by traditional methods.

In this study, the development of a PCR-DGGE method to directly identify the members of the genus *Listeria* in food samples is described. This approach exploited the potential of PCR to amplify, with specific primers, variable regions within the *iap* gene as well as the discriminatory power of DGGE to differentiate DNA molecules on the basis of differences in their sequence (33).

The *iap* gene has been demonstrated to be a reliable PCR target for differentiation of *Listeria* spp. (6, 21, 39, 58). Bubert et al. (7) characterized the *iap* gene from different *Listeria* spp. and demonstrated the presence of conserved

TABLE 4. Results of the detection of *Listeria* spp. by the PCR-DGGE method and specific amplification and serogrouping of *L. monocytogenes* by the PCR-REA protocol directly in food samples and comparison with the identification by PCR-DGGE of the strains isolated on Oxford agar after overnight enrichment[a]

Sample no.	Species identified by direct PCR-DGGE	*L. monocytogenes* direct specific amplification	*L. monocytogenes* direct REA serogrouping	Species isolated from Oxford agar and identified by PCR-DGGE
2	*L. monocytogenes* group b	+	b	*L. monocytogenes* group b
4	*L. monocytogenes* group b, *L. welshimeri*, and *L. innocua*	+	b	*L. monocytogenes* group b and *L. innocua*
5	*L. monocytogenes* group a	+	a or 1/2c	*L. monocytogenes* group a
8	*L. monocytogenes* group a and *L. monocytogenes* group b	+	a or 1/2c plus b	*L. monocytogenes* group a and *L. monocytogenes* group b
9	*L. innocua* and *L. welshimeri*	−	NA[b]	*L. innocua* and *L. welshimeri*
11	*L. monocytogenes* group a and *L. monocytogenes* group 1/2c	+	a or 1/2c	*L. monocytogenes* group a and *L. monocytogenes* group 1/2c
12	*L. monocytogenes* group b	+	b	*L. monocytogenes* group b
22	*L. innocua*	−	NA	*L. innocua*
28	*L. innocua*	−	NA	*L. innocua*
32	*L. monocytogenes* group b	+	b	*L. monocytogenes* group b
38	*L. monocytogenes* group a	+	a or 1/2c	*L. monocytogenes* group a
39	*L. monocytogenes* group 1/2c	+	a or 1/2c	*L. monocytogenes* group 1/2c
46	*L. innocua*	−	NA	*L. innocua*
51	*L. innocua*	−	NA	*L. innocua*
52	*L. welshimeri*	−	NA	*L. welshimeri*
55	*L. innocua* and *L. welshimeri*	−	NA	*L. innocua* and *L. welshimeri*
58	*L. innocua* and *L. seeligeri*	−	NA	*L. innocua*
61	*L. monocytogenes* group 1/2c	+	a or 1/2c	*L. monocytogenes* group 1/2c
64	*L. monocytogenes* group a	+	a or 1/2c	*L. monocytogenes* group a
65	*L. monocytogenes* group b	+	b	*L. monocytogenes* group b
66	*L. innocua*	−	NA	*L. innocua*
67	*L. innocua*	−	NA	*L. innocua*
68	*L. monocytogenes* group b and *L. ivanovii*	+	b	*L. monocytogenes* group b and *L. ivanovii*
72	*L. monocytogenes* group b and *L. ivanovii*	+	b	*L. monocytogenes* group b and *L. ivanovii*

[a] Samples in bold are reported in Fig. 5.
[b] NA, not applicable.

regions at the 5′ and 3′ ends and a species-specific internal region.

Here, the information available on the *iap* gene was used to design primers that specifically amplify *Listeria* spp. and that could be used for specific identification using the DGGE method. Two primers were identified for the region of the sequences that were conserved among different *Listeria* spp. The regions amplified contained a high degree of heterogeneity among the sequences considered, allowing their differentiation by DGGE. In addition, just by PCR amplification, it was possible to differentiate two groups of species on the basis of the molecular weight of the amplicon obtained (Fig. 1). The primers developed showed high specificity toward *Listeria* spp., since no positive amplification was obtained by using DNA extracted from different non-*Listeria* organisms.

The PCR products produced from control strains obtained from international collections were used to optimize the experimental conditions (denaturant gradients, temperature, voltage, and length of the electrophoretic run) of the DGGE. Denaturants from 20 to 40% showed the best differentiation power, allowing the identification, on the basis of specific migration, of the five species considered in the study (Fig. 2). Moreover, *L. monocytogenes* strains were characterized by different electrophoretic patterns depending on the serotype. Profiles of serotypes 1/2a and 3a were different from those of serotype 1/2c and serotypes 1/2b, 3b, and 4b. The results obtained by analyzing different serotypes of *L. monocytogenes* isolated from humans with listeriosis by PCR-DGGE confirmed the discriminatory power of the method.

The PCR-DGGE protocol described was then first used to identify 48 strains that were previously isolated from food samples and identified by traditional methods. All the strains tested showed DGGE patterns matching with those of control strains, allowing a rapid identification at the species level. Moreover, for *L. monocytogenes* it was also possible to differentiate serogroups. Nine strains identified as *L. monocytogenes* by traditional methods showed DGGE profiles of *L. innocua*. The explanation of the different identification must be related to the subjective analysis of the biochemical tests used. Hemolysis is the only marker that can be used to differentiate *L. monocytogenes* from *L. innocua* (3) and its misinterpretation could lead to mistakes in the identification of these two species as described in this paper.

Due to the specificity of the primers developed, the protocol was then applied to the direct detection of *Listeria* spp. in food samples. After an overnight enrichment step to increase the number of target cells and to avoid the amplification of dead cells, DNA was extracted from the enriched broth and subjected to PCR and DGGE. Enrichments were also streaked onto Oxford agar, and after incubation, suspected colonies were isolated and identified to confirm the results obtained by direct analysis. The identification of the isolated strains agreed

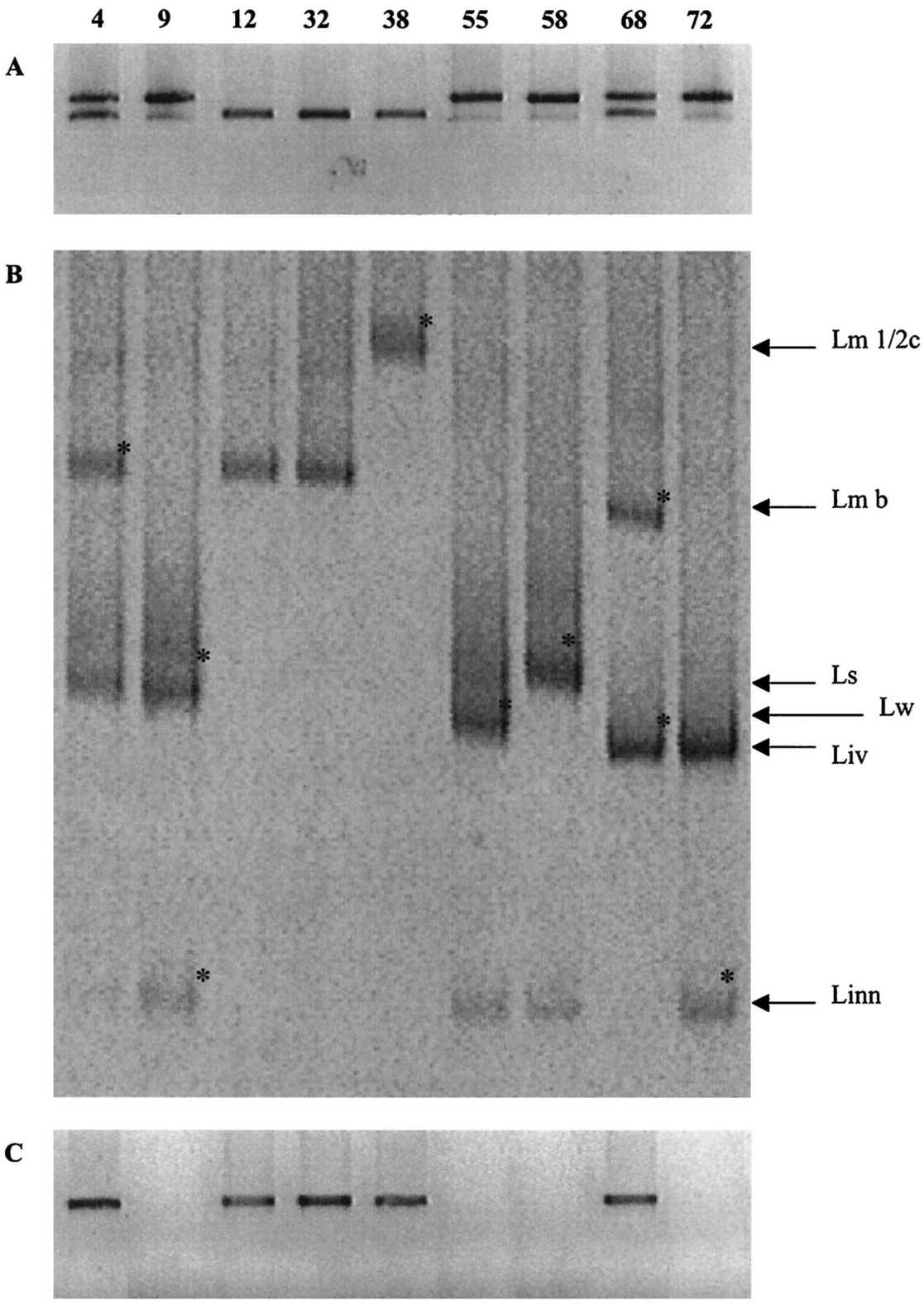

FIG. 4. Results obtained from the direct amplification and characterization of *Listeria* spp. in food. Lane labels indicate the number of the food sample shown in the gel and also reported in Table 4. (A) List-univ. 1 and List-univ. 2 PCR amplification. (B) DGGE profiles of food samples. (C) Specific *L. monocytogenes* PCR using primers Mar 1 and Mar 2 (38). The DGGE bands indicated by an asterisk were excised, reamplified, sequenced, and identified by sequence analysis. Abbreviations: Lm 1/2c, *L. monocytogenes* serotype 1/2c; Lm b, *L. monocytogenes* serogroup b; Linn, *L. innocua*; Liv, *L. ivanovii*; Ls, *L. seeligeri*; Lw, *L. welshimeri*.

with the detection of *Listeria* spp. directly in the food samples. Only for sample 4, *L. welshimeri* was detected in the DGGE gels, but no colony belonging to this species was isolated, thus underscoring the biases of traditional methods. Of the 73 food samples tested, 24 gave PCR products indicating the presence of *Listeria* species, while 49 did not. After DGGE analysis it was possible to identify single populations of *Listeria* spp. or single *L. monocytogenes* serogroups in 16 samples and mixed *Listeria* spp. in 8 samples. The results of the direct identification from food samples, on the basis of comigration with control strains, were also confirmed by direct sequencing of different bands (indicated in Fig. 4A).

DNA extracted directly from food samples was also amplified by use of specific primers for *L. monocytogenes*, Mar 1 and Mar 2 (38), and the positive samples were subjected to restriction analysis as described previously (36) to confirm the results of the identification of *L. monocytogenes* by direct PCR-DGGE protocol. As described in Table 4, no differences in the results were obtained, underscoring the reliability and accuracy of the PCR-DGGE method.

The protocol developed in this paper allows for fast and easy identification of all the species belonging to the genus *Listeria*. DGGE analysis of *Listeria* sp. PCR products results in species-specific migration patterns. For identification, reference strains must be included in the PCR-DGGE analysis. Comigration in the gel of a PCR product obtained from an unknown sample with a control amplicon gives the identification. Further confirmation can be obtained by direct sequencing of the DGGE band under consideration. The method can be used for rapid identification of traditionally isolated strains or it can be applied directly in food samples to detect *Listeria* spp., avoiding time-consuming classical isolation and identification. The results reported in this study prove the tremendous impact of the protocol. Even if a pre-enrichment step is necessary, in 18 h it is possible to determine the presence of any *Listeria* spp. in the sample. The method described is also able to identify a single cell of *Listeria* present before enrichment (data not shown), becoming an important tool especially for the detection of *L. monocytogenes* in food samples, since no *L. monocytogenes* cell can be present in ready-to-eat or cooked foods (54). Moreover, the possibility for quickly screening food samples to assess the presence of *L. monocytogenes* represents a big benefit for both plant surveillance and safety and spoilage matters.

The protocol described here makes possible the study of *Listeria* sp. ecology in food samples. Its application allows for reliable monitoring of all *Listeria* species and it can be exploited for a better understanding of the occurrence and distribution of *Listeria* in the environment. Lastly, since the method allows for distinguishing *L. monocytogenes* serotypes directly in food, it may potentially be exploited for epidemiological purposes, too.

REFERENCES

1. **Asperger, H., H. Heistinger, M. Wagner, A. Lehner, and E. Brandl.** 1999. A contribution of *Listeria* enrichment methodology-growth of *Listeria monocytogenes* under varying conditions concerning enrichment broth composition, cheese matrices and competing microbial flora. Food Microbiol. **16:**419–431.
2. **Bansal, N. S., F. H. Y. McDonell, A. Smith, G. Arnold, and G. F. Ibrahim.** 1996. Multiplex PCR assay for the routine detection of *Listeria* in food. Int. J. Food Microbiol. **33:**293–300.
3. **Beumer, R. R., M. C. T. Giffel, M. T. C. Kok, and F. M. Rombouts.** 1996. Confirmation and identification of *Listeria* spp. Lett. Appl. Microbiol. **22:** 448–452.
4. **Bibb, W. F., B. G. Schwartz, B. G. Gellin, B. D. Plikaytis, and R. E. Weaver.** 1989. Analysis of *Listeria monocytogenes* by multilocus enzyme electrophoresis and application of the method to epidemiologic investigations. Int. J. Food Microbiol. **8:**233–239.
5. **Brosch, R., C. Buchrieser, and J. Rocourt.** 1991. Subtyping of *Listeria monocytogenes* serovar 4b by use of low-frequency-cleavage restriction endonucleases and pulsed-field gel electrophoresis. Res. Microbiol. **142:**667–675.
6. **Bubert, A., I. Hein, M. Rauch, A. Lehner, B. Yoon, W. Goebel, and M. Wagner.** 1999. Detection and differentiation of *Listeria* spp. by a single reaction based on multiplex PCR. Appl. Environ. Microbiol. **65:**4688–4692.
7. **Bubert, A., S. Kohler, and W. Goebel.** 1992. The homologous and heterologous regions within the *iap* gene allow genus-specific and species-specific identification of *Listeria* spp. by polymerase chain reaction. Appl. Environ. Microbiol. **58:**2625–2632.
8. **Bubert, A., P. Schubert, S. Kohler, R. Frank, and W. Goebel.** 1994. Synthetic peptides derived from the *Listeria monocytogenes* p60 protein as antigens for the generation of polyclonal antibodies specific for secreted cell-free *L. monocytogenes* p60 proteins. Appl. Environ. Microbiol. **60:**3120–3127.
9. **Carnevale, R. A., and P. Johnston.** 1989. Method for the isolation and identification of *Listeria monocytogenes* from meat and poultry products. Laboratory communication no. 57. U.S. Department of Agriculture, Washington, D.C.
10. **Curiale, M. S., and C. Lewus.** 1994. Detection of *Listeria monocytogenes* in samples containing *Listeria innocua*. J. Food Prot. **57:**1048–1051.
11. **Curtis, G. D. W., and W. H. Lee.** 1995. Culture media and methods for the isolation of *Listeria monocytogenes*. Int. J. Food Microbiol. **26:**1–13.
12. **Deneer, H., and I. Boychuk.** 1991. Species-specific identification of *Listeria monocytogenes* by DNA amplification. Appl. Environ. Microbiol. **57:**606–609.
13. **Drebot, M., S. Neal, W. Schlech, and K. Rozee.** 1996. Differentiation of *Listeria* isolates by PCR amplicon profiling and sequence analysis of 16S-23S rRNA internal transcribed spacer loci. J. Appl. Bacteriol. **80:**174–178.
14. **Engels, B.** 1992. Amplify, for analyzing PCR experiments. University of Wisconsin, Madison.
15. **Flanders, K. J., and C. W. Donnelly.** 1994. Injury, resuscitation and detection of *Listeria* spp. from frozen environments. Food Microbiol. **11:**473–480.
16. **Furrer, B., U. Candrian, C. Hoefelein, and J. Luethy.** 1991. Detection and identification of *Listeria monocytogenes* in cooked sausage products and in milk by in vitro amplification of haemolysin gene fragments. J. Appl. Bacteriol. **70:**372–379.
17. **Gormon, T., and L. Phanthanh.** 1995. Identification and classification of *Listeria* by two-dimensional protein mapping. Res. Microbiol. **146:**143–154.
18. **Graham, T. A., E. J. Golsteyn Thomas, J. E. Thomas, and V. P. J. Gannon.** 1997. Inter- and intraspecies comparison of the 16S-23S rRNA operon intergenic spacer regions of six *Listeria* spp. Int. J. Syst. Bacteriol. **47:**863–869.
19. **Graves, L. L., B. Swaminathan, M. W. Reeves, and J. Wenger.** 1991. Ribosomal fingerprinting of *L. monocytogenes* using a digoxigenin-labeled DNA probe. Eur. J. Epidemiol. **7:**77–82.
20. **Head, I. M., J. R. Saunders, and R. W. Pickup.** 1998. Microbial evolution, diversity, and ecology: a decade of ribosomal RNA analysis of uncultivated microorganisms. Microb. Ecol. **35:**1–21.
21. **Hein, I., D. Klein, A. Lehner, A. Bubert, E. Brandl, and M. Wagner.** 2001. Detection and quantification of the *iap* gene of *Listeria monocytogenes* and *Listeria innocua* by a new real-time quantitative PCR assay. Res. Microbiol. **152:**37–46.
22. **Heisick, J. E., L. I. Rosas-Marty, and S. R. Tatini.** 1995. Enumeration of viable *Listeria* species and *Listeria monocytogenes* in foods. J. Food Prot. **58:**733–736.
23. **Herman, L. M. F., H. F. M. Deridder, and G. M. M. Vlaemynck.** 1995. A multiplex PCR method for the identification of *Listeria* spp. and *Listeria monocytogenes* in dairy samples. J. Food Prot. **58:**867–872.
24. **Hitchins, A. D., and R. E. Duvall.** 2000. Feasibility of a defined microflora challenge method for evaluating the efficacy of foodborne *Listeria monocytogenes* selective enrichments. J. Food Prot. **63:**1064–1070.
25. **Hudson, J. A., R. J. Lake, M. G. Savill, P. Scholes, and R. E. McCormick.** 2001. Rapid detection of *Listeria monocytogenes* in ham samples using immunomagnetic separation followed by polymerase chain reaction. J. Appl. Microbiol. **90:**614–621.
26. **Hugenholtz, P., B. M. Goebel, and N. R. Pace.** 1998. Impact of culture-independent studies on the emerging phylogenetic view of bacterial diversity. J. Bacteriol. **180:**4765–4774.
27. **Ingianni, A., M. Floris, P. Palomba, A. Madeddu, M. Quartuccio, and R. Pompei.** 2001. Rapid detection of *Listeria monocytogenes* in foods, by a combination of PCR and DNA probe. Mol. Cell. Probes **15:**275–280.
28. **International Dairy Federation.** 1995. Milk and milk products—detection of *Listeria monocytogenes.* IDF standard 143A:1995. International Dairy Federation, Brussels, Belgium.
29. **Jacquet, C., S. Aubert, N. Elsolh, and J. Rocourt.** 1992. Use of rRNA gene restriction patterns for the identification of *Listeria* species. Syst. Appl. Microbiol. **15:**42–46.
30. **Jersek, B., E. Tcherneva, N. Rijpens, and L. Herman.** 1996. Repetitive element sequence-based PCR for species and strain discrimination in the genus *Listeria*. Lett. Appl. Microbiol. **23:**55–60.

31. **Johnson, W. M., S. D. Tyler, E. P. Ewan, F. E. Ashton, G. Wang, and K. R. Rozee.** 1992. Detection of genes coding for listeriolysin and *Listeria monocytogenes* antigen A (*lmaA*) in *Listeria* spp. by the polymerase chain reaction. Microb. Pathog. **12:**79–86.
32. **Lawrence, L. M., and A. Gilmour.** 1994. Incidence of *Listeria* spp. and *Listeria monocytogenes* in a poultry processing environment and in poultry products and their rapid confirmation by multiplex PCR. Appl. Environ. Microbiol. **60:**4600–4604.
33. **Lerman, L. S., S. G. Fischer, I. Hurley, K. Silverstein, and N. Lumelsky.** 1984. Sequence-determined DNA separation. Annu. Rev. Biophys. Bioeng. **13:**399–423.
34. **Loessner, M. J., C. E. D. Rees, G. S. A. B. Stewart, and S. Scherer.** 1996. Construction of luciferase reporter bacteriophage A511::*luxAB* for rapid and sensitive detection of viable *Listeria* cells. Appl. Environ. Microbiol. **62:**1133–1140.
35. **Lovett, J., and R. Twedt.** 1988. *Listeria*. Outstanding symposia in food science and technology. Food Technol. **8:**188–191.
36. **Manzano, M., L. Cocolin, C. Cantoni, and G. Comi.** 1998. A rapid method for the identification and partial serotyping of *Listeria monocytogenes* in food by PCR and restriction enzyme analysis. Int. J. Food Microbiol. **42:**207–212.
37. **Manzano, M., L. Cocolin, C. Cantoni, and G. Comi.** 2000. Temperature gradient gel electrophoresis of the amplified product of a small 16S rRNA gene fragment for the identification of *Listeria* species isolated from food. J. Food Prot. **63:**659–661.
38. **Manzano, M., L. Cocolin, P. Ferroni, C. Cantoni, and G. Comi.** 1997. A simple and fast PCR protocol to detect *Listeria monocytogenes* from meat. J. Sci. Food Agric. **74:**25–30.
39. **Manzano, M., L. Cocolin, P. Ferroni, V. Gasparini, D. Narduzzi, C. Cantoni, and G. Comi.** 1996. Identification of *Listeria* species by a semi-nested polymerase chain reaction. Res. Microbiol. **147:**637–640.
40. **McLauchlin, J.** 1997. The identification of *Listeria* spp. Int. J. Food Microbiol. **38:**77–81.
41. **Medeiros, D., and J. M. Farber.** 2001. A single space polymerase chain reaction for combined gene detection and epidemiological typing of *Listeria monocytogenes*. Food Microbiol. **18:**375–386.
42. **Niederhauser, C., U. Candrian, C. Hofelein, M. Jermini, H. P. Buhler, and J. Luthy.** 1992. Use of polymerase chain reaction for detection of *Listeria monocytogenes* in food. Appl. Environ. Microbiol. **58:**1564–1568.
43. **Nocera, D., E. Bannerman, J. Rocort, J. Jaton-Ogay, and J. Bille.** 1990. Characterization by DNA restriction endonuclease analysis of *Listeria monocytogenes* strains related to the Swiss epidemic of listeriosis. J. Clin. Microbiol. **28:**2259–2263.
44. **Nogva, H. K., K. Rudi, K. Naterstad, A. Holck, and D. Lillehaug.** 2000. Application of 5′-nuclease PCR for quantitative detection of *Listeria monocytogenes* in pure cultures, water, skim milk, and unpasteurized whole milk. Appl. Environ. Microbiol. **66:**4266–4271.
45. **Norton, D. M., M. A. McCamey, K. L. Gall, J. M. Scarlett, K. J. Boor, and M. Wiedmann.** 2001. Molecular studies on the ecology of *Listeria monocytogenes* in the smoked fish processing industry. Appl. Environ. Microbiol. **67:**198–205.
46. **Piffaretti, J. C., H. Kressbuch, M. Aeschbacher, J. Bille, E. Bannerman, J. M. Musser, R. K. Salander, and J. Rocourt.** 1989. Genetic characterization of clones of the bacterium *Listeria monocytogenes* causing epidemic diseases. Proc. Natl. Acad. Sci. USA **86:**3818–3822.
47. **Pritchard, T. J., and C. W. Donnelly.** 1998. Combined secondary enrichment of primary enrichment broths increases *Listeria* detection. J. Food Prot. **62:**532–535.
48. **Rocourt, J., J. M. Alonso, and H. P. R. Seeliger.** 1983. Virulence comparée des cinq groupes génomiques de *Listeria monocytogenes* (sensu lato). Ann. Microbiol. (Inst. Pasteur) **134A:**359–364.
49. **Rocourt, J., F. Grimont, P. A. D. Grimont, and H. P. R. Seeliger.** 1982. DNA relatedness among serovars of *Listeria monocytogenes* sensu lato. Curr. Microbiol. **7:**383–388.
50. **Rossen, L., K. Holmstrom, J. E. Olsen, and O. F. Rasmussen.** 1991. A rapid polymerase chain reaction (PCR)-based assay for the identification of *Listeria monocytogenes* in food samples. Int. J. Food Microbiol. **14:**145–151.
51. **Ryser, E. T., S. M. Arimi, M. Bunduki, and C. W. Connelly.** 1996. Recovery of different *Listeria* ribotypes from naturally contaminated, raw refrigerated meat and poultry products with two primary enrichment media. Appl. Environ. Microbiol. **62:**1781–1787.
52. **Schuchat, A., B. Swaminathan, and C. V. Broome.** 1991. Epidemiology of human listeriosis. Clin. Microbiol. Rev. **4:**169–183.
53. **Seeliger, H. P. R., and D. Jones.** 1986. Genus *Listeria* Pirie 1940, 383^{AL}, p. 1235–1245. *In* P. H. A. Sneath, N. S. Mair, M. E. Sharpe, and J. G. Holt (ed.), Bergey's manual of systematic bacteriology, vol. 2. Williams & Wilkins, Baltimore, Md.
54. **Shank, F. R., E. L. Elliot, I. K. Wachsmuth, and M. E. Losikoff.** 1996. U.S. position on *Listeria monocytogenes* in foods. Food Control **7:**229–234.
55. **Sheffield, V. C., D. R. Cox, L. S. Lerman, and R. M. Myers.** 1989. Attachment of a 40-base pair G+C-rich sequence (GC-clamp) to genomic DNA fragments by the polymerase chain reaction results in improved detection of single-base changes. Proc. Natl. Acad. Sci. USA **86:**232–236.
56. **Swaminathan, B., and P. Feng.** 1994. Rapid detection of foodborne pathogenic bacteria. Annu. Rev. Microbiol. **48:**401–425.
57. **Vogt, R. L., C. Donelly, B. Gellin, W. Bibb, and B. Swaminathan.** 1990. Linking environmental and human strains of *Listeria monocytogenes* with isoenzyme and ribosomal RNA typing. Eur. J. Epidemiol. **6:**229–230.
58. **Wagner, M., A. Lehner, D. Klein, and A. Bubert.** 2000. Single-strand conformation polymorphisms in the *hly* gene and polymerase chain reaction analysis of a repeat region in the *iap* gene to identify and type *Listeria monocytogenes*. J. Food Prot. **63:**332–336.
59. **Wang, R. F., W. W. Cao, and M. G. Johnson.** 1992. 16S rRNA-based probes and polymerase chain reaction method to detect *Listeria monocytogenes* cells added to foods. Appl. Environ. Microbiol. **58:**2827–2831.
60. **Wieckowska-Szakiel, M., A. Bubert, M. Rozalski, U. Krajewska, W. Rudnicka, and B. Rozalska.** 2002. Colony-blot assay with anti-p60 antibodies as a method for quick identification of *Listeria* in food. Int. J. Food Microbiol. **72:**63–71.