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A RE-PCR method to distinguish *Listeria monocytogenes* serovars

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Abstract

Strains (107) of *L. monocytogenes* were tested with a PCR-restriction enzyme analysis with two new original primers. A segment of 1395 bp containing the entire *iap* gene in *L. monocytogenes* was amplified by the PCR technique. The PCR product was cleaved with the restriction enzymes *Hin*dIII and *Rsa*I, and the fragments generated were separated by gel electrophoresis. Two groups of serovars were obtained: one group contained serovars 1/2a and 1/2c, the other group contained serovars 1/2b, 3b and 4b. The PCR-restriction enzyme analysis method described in this paper could be a useful tool for the unambiguous division of *L. monocytogenes* into two serovar groups, and it could be used to study the evolution of different serotypes and groups of serotypes in foods produced in the same processing plant and processed during the same month. The RE-PCR method used can give a rapid confirm at the subgroup level in the laboratory of an epidemiological association between human disease and suspected sources of contaminated food.

Keywords: *Listeria monocytogenes*; PCR; Restriction enzyme analysis

1. Introduction

Listeria monocytogenes is a Gram-positive, food-borne, human and animal pathogen responsible for serious infections in immunocompromised individuals and pregnant women [1,2]. It may cause invasive disease such as bacteraemia, meningitis and severe perinatal infection. Several large food-borne outbreaks have incriminated commercial food products as a primary source of infection for both epidemic and sporadic listerioses. The ubiquitous nature of *L. monocytogenes* in the environment and its potential presence in multiple food sources mean that highly discriminatory typing systems are necessary for epidemiological investigations [3]. The typing of isolates of *L. monocytogenes* presents a number of practical problems yet is of considerable importance. The existing typing methods, sero- or bacteriophage-typing, have either poor discrimination (serotyping) or good discrimination but leave many strains untyped. *L. monocytogenes* typing remains important in surveillance, epidemiology, or research with its applications in these areas related to the prevention of infection [4]. More recently genotypic techniques such as plasmid profiling, ribotyping (RT), analysis of chromosomal DNA either by restriction enzyme analysis or pulsed field gel electrophoresis (PFGE) and fingerprinting by arbitrarily primed PCR (AP-PCR) have been used for typing purposes [3]. Piffaretti et al. [5] and Bibb et al. [6] used multilocus enzyme electro-

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Table 1
Serotyped *L. monocytogenes* strains used in this study

	Serotype	Isolated from	Source or reference
L. monocytogenes			
035, 036	1/2a	Pork meat	Ist. Isp. Alim. Orig. Anim. Milano
056, 057	1/2a	Ground beef meat	Ist. Isp. Alim. Orig. Anim. Milano
061, 062, 063	1/2a	Sausages	Ist. Isp. Alim. Orig. Anim. Milano
026	1/2a	Human Listeriosis	Az. Osp., Policlinico di Modena
030, 031	1/2b	Pork meat	Ist. Isp. Alim. Orig. Anim. Milano
043	1/2b	Ground beef meat	Ist. Isp. Alim. Orig. Anim. Milano
058	1/2b	Sausages	Ist. Isp. Alim. Orig. Anim. Milano
012, 015, 016	1/2b	Human listeriosis	Az. Osp., Policlinico di Modena
027, 028, 029, 032, 033	1/2c	Pork meat	Ist. Isp. Alim. Orig. Anim. Milano
040, 041, 042	1/2c	Ground beef meat	Ist. Isp. Alim. Orig. Anim. Milano
064, 065,066, 067	1/2c	Sausages	Ist. Isp. Alim. Orig. Anim. Milano
014	1/2c	Human listeriosis	Az. Osp., Policlinico di Modena
CIP 78.35	3b	CBF*	Ist. Latt. Casear. Biotec. Agroalim. Thiene
009	3b	Human listeriosis	Az. Osp., Policlinico di Modena
NCTC 10527	4b	CBF*	Ist. Latt. Casear. Biotec. Agroalim. Thiene
047, 048, 049	4b	Pork meat	Ist. Isp. Alim. Orig. Anim. Milano
060, 061	4b	Ground beef meat	Ist. Isp. Alim. Orig. Anim. Milano
068, 069, 070	4b	Sausages	Ist. Isp. Alim. Orig. Anim. Milano
001, 002, 003, 004, 005	4b	Human listeriosis	Az. Osp., Policlinico di Modena
006, 007, 008, 009, 010	4b	Human listeriosis	Az. Osp., Policlinico di Modena
011, 013, 018, 019, 020	4b	Human listeriosis	Az. Osp., Policlinico di Modena
021, 022, 023, 024, 025	4b	Human listeriosis	Az. Osp., Policlinico di Modena
ATCC 7644	⊗	Blood	Ist. Latt. Caseario Lodi
OHIO	⊗	Blood	Ist. Latt. Caseario Lodi
SCOTT A	⊗	Blood	Ist. Latt. Caseario Lodi
V 7	⊗	Blood	Ist. Latt. Caseario Lodi
OSP 1	4b	Human listeriosis	Ist. Isp. Alim. Orig. Anim. Milano
OSP 2	4b	Human listeriosis	Ist. Isp. Alim. Orig. Anim. Milano
OSP 3	4b	Human listeriosis	Ist. Isp. Alim. Orig. Anim. Milano
OSP 4	4b	Human listeriosis	Ist. Isp. Alim. Orig. Anim. Milano
K 1	⊗	Human listeriosis	Ist. Superiore della Sanità Roma
K 2	⊗	Human listeriosis	Ist. Superiore della Sanità Roma

*CBF, cerebrospinal fluid; ⊗, not serotyped.

phoretic (MEE) analysis to divide *L. monocytogenes* into two subgroups. Rasmussen et al. [7] found that strains belonging to different serovars of the species *L. monocytogenes* could be divided into two major groups with part of the sequence of the *hly* gene. Vines et al. [8] presented two groups of strains within the species by a PCR method with restriction enzyme analysis (REA) on four virulence-associated genes.

The aim of this study was to develop a method to divide *L. monocytogenes* into two serogroups by in vitro amplification of the *iap* gene fragments, with two new, original primers and a restriction endonuclease enzyme assay.

2. Materials and methods

2.1. Bacterial strains and culture collections

The serotyped *L. monocytogenes* strains (Table 1) used in this study came from human and environmental sources and were obtained from the Istituto di Ispezione degli Alimenti di Origine Animale, Milan, Italy [9]; the Azienda Sanitaria, Policlinico di Modena, Italy; the Istituto Lattiero Caseario e di Biotecnologie Agroalimentari, Thiene, Italy; the Istituto Lattiero Caseario, Lodi, Italy, and the Istituto Superiore della Sanità, Rome, Italy.

Other *L. monocytogenes* strains (44) were isolated

from naturally contaminated meat, sausages and cheese by traditional methods (Table 2).

The isolation and identification of *L. monocytogenes* from the food sources was performed according to USDA-FSIS protocols [10]. Presumptive strains grown on brain heart infusion agar (Oxoid) were identified by morphological, cultural and biochemical criteria according to the USDA-FSIS protocol. Api-Listeria (BioMerieux, France) was also used for biochemical tests.

In order to confirm the biochemical identifications, *L. monocytogenes* strains were also tested with a PCR method with two specific primers for *L. monocytogenes* [11].

All *L. monocytogenes* strains were routinely grown for 24 h at 37°C on brain heart infusion agar and used to evaluate the RE-PCR method for serotyping.

2.2. Oligonucleotides

Universal primers, synthesised with *t*-methoxyphosphoramidite chemistry using an Applied Biosystem (USA) 392-A DNA synthesizer, derived from a conserved sequence of the *iap* gene, were used for the PCR methods. The base composition of primers denoted CLM 1 (sense) and CLM 2 (antisense) was: 5′-ACAGCTGGGATTGCGGT-3′ and 5′-CCCAGCCAGAGCCGTGGA-3′ respectively. Primers CLM 1 and CLM 2 were located within the *iap* gene of *L. monocytogenes* at position 31 to 47 bp and 1406 to 1423 bp respectively, resulting in the amplification of a 1395 bp fragment, determined with a molecular biology software (AMPLIFY, for analyzing PCR experiments, Madison, WI). The *iap* gene from *Listeria* spp. can be used for the development of a more versatile procedure by PCR, as suggested by other authors [12,13].

2.3. PCR assay

PCR was done in a volume of 50 μl containing 10 mM Tris HCl pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.125 nM of the deoxynucleoside triphosphate dATP, dGTP, dCTP and dTTP solution, 0.1 pM of each primer, 1.25 U of *Taq*-DNA polymerase (Pharmacia Biotech, Italy) and 5.0 μl of template DNA, extracted as described above [14]. The reaction mixture was overlaid with 50 μl sterile mineral oil and subjected to a Thermalcycler process (MJ, Genenco, USA). Template DNA was initially denatured at 95°C for 5 min, followed by 35 cycles of 90 s denaturation at 95°C, 60 s primer annealing at 54°C, 3 min extension at 72°C and 72°C for 7 min as the final extension.

A total of 10 μl of amplified PCR product was analysed by electrophoresis in 1.5% w/v agarose gel containing 0.5 μg ml^{-1} ethidium bromide with a 100 base-pair ladder (Pharmacia Biotech, Italy) as DNA molecular weight marker.

PCR assay results were compared with negative and positive control results: the positive control was an 'in house' standard containing approximately 10^5 cells ml^{-1} *L. monocytogenes* serovar 4b. In the negative control, the bacterial DNA was replaced with 5.0 μl sterile distilled water.

2.4. Restriction endonuclease enzyme assay

A total of 5.0 μl of amplified PCR product was digested with the restriction enzymes (RE) *Alu*I, *Apa*I, *Dpn*I, *Hin*fI, *Hin*dIII, *Not*I, *Rsa*I, *Sma*I (Boeringer Mannheim, Germany). Enzymes were used as recommended by the manufacturers.

After digestion, the fragments were analysed by electrophoresis in 2.5% w/v agarose gel and stained in a solution containing 0.5 μg ml^{-1} ethidium bromide. Uncut DNA was included as a control to determine cleaved and uncleaved fragment size.

The DNA bands visualized under ultra-violet light, using a transilluminator apparatus, were photographed with DS-34 camera system (Polaroid, USA).

Table 2
L. monocytogenes strains isolated from food used in this study

L. monocytogenes	Isolated from
090, 091, 092, 093, 094	Pork meat
095, 096, 097, 098, 099	Pork meat
100, 101, 102, 103, 104	Ground beef meat
105, 106, 107, 108	Ground beef meat
109, 110, 111, 112, 113	Sausages
114, 115, 116, 117, 118	Sausages
119, 120, 121	Sausages
122, 123, 124, 125, 126	Cheese
127, 128, 129, 130, 131	Cheese
132, 133	Cheese

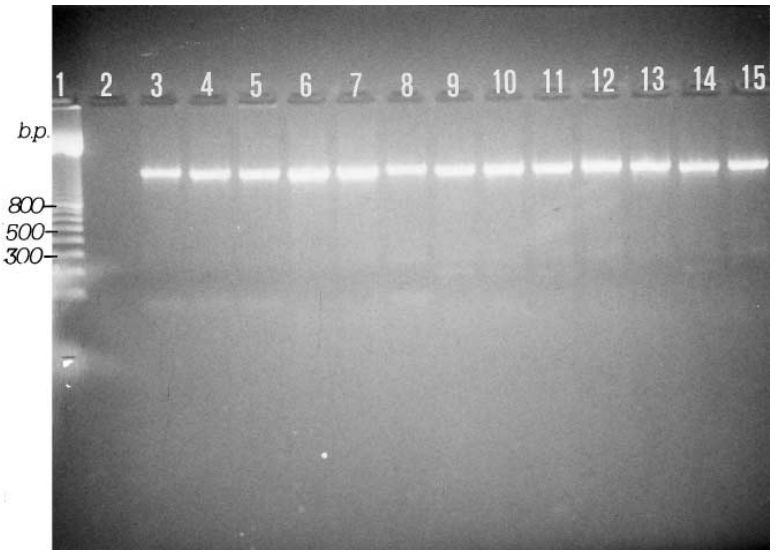

Fig. 1. Agarose gel of CLM 1-CLM 2 primers PCR products obtained by the amplification of 12 *L. monocytogenes* strains: lane 1: 100 bp ladder molecular weight (Pharmacia Biotech, Italy), the numbers to the left indicate fragment size (bp); lane 2: negative control; lanes 3–5: *L. monocytogenes* serovar 1/2a; lanes 6–8: *L. monocytogenes* serovar 1/2c; lanes 9–10: *L. monocytogenes* serovar 1/2b; lane 11: *L. monocytogenes* serovar 3b; lanes 12–14: *L. monocytogenes* serovar 4b; lane 15: positive control *L. monocytoge*nes NCTC 10527 serovar 4b.

3. Results and discussion

All 63 serotyped *L. monocytogenes* and all 44 *L. monocytogenes* strains isolated from food yielded amplimers of the expected size when amplified with CLM 1 and CLM 2, which anneal on the *iap* gene region (Fig. 1).

CLM 1 and CLM 2 original primers located at position 31 to 47 bp and 1406 to 1423 bp within the *iap* gene of *L. monocytogenes* have a stability of match of 79% and 86% respectively and a primability of match of 100%. Their high specificity means that they yield the only 1395 bp amplicon. The absence of aspecific PCR products enabled us to obtain unambiguous results by RE analysis.

Table 3 lists the restriction enzymes which produced restriction fragment length polymorphism (RFLP) for the *iap* gene. Of the 8 restriction endonuclease evaluated *Alu*I, *Hin*fI, *Hin*dIII and *Rsa*I restricted the amplimers and only *Hin*dIII and *Rsa*I gave polymorphic bands.

The RE profiles of the tested strains, 63 *L. monocytogenes* serotyped and 44 *L. monocytogenes* not serotyped, are shown in Fig. 2 (A and B). All the strains used for the RE analysis were subdivided into two groups which corresponded to serogroups 1/2a, 1/2c, and 1/2b, 3b, 4b respectively. One restriction profile was obtained with *Hin*dIII and *Rsa*I for all strains of serovars 1/2a, 1/2c, and another restriction profile was observed for all strains of serovars 1/2b, 3b and 4b.

The RE fragments of the two *L. monocytogenes*

Table 3
Restriction fragment sizes obtained by digestion with different restriction enzymes of the amplimer obtained with CLM 1–CLM 2 primers pair

L. monocytogenes serovars	Restriction enzymes	Restriction fragment size (bp)	
		Observed	Expected*
1/2a–1/2c	*Hin*dIII (A/AGCTT)	693-425-277	693-425-277
	*Rsa*I (GT/AC)	1043-204-148	1043-204-148
1/2b–3b–4b	*Hin*dIII (A/AGCTT)	1118-277	1118-277
	*Rsa*I (GT/AC)	792-251-204-148	792-251-204-148

*Determined with DNA Strider 1.0 Sotfware for Macintosh.

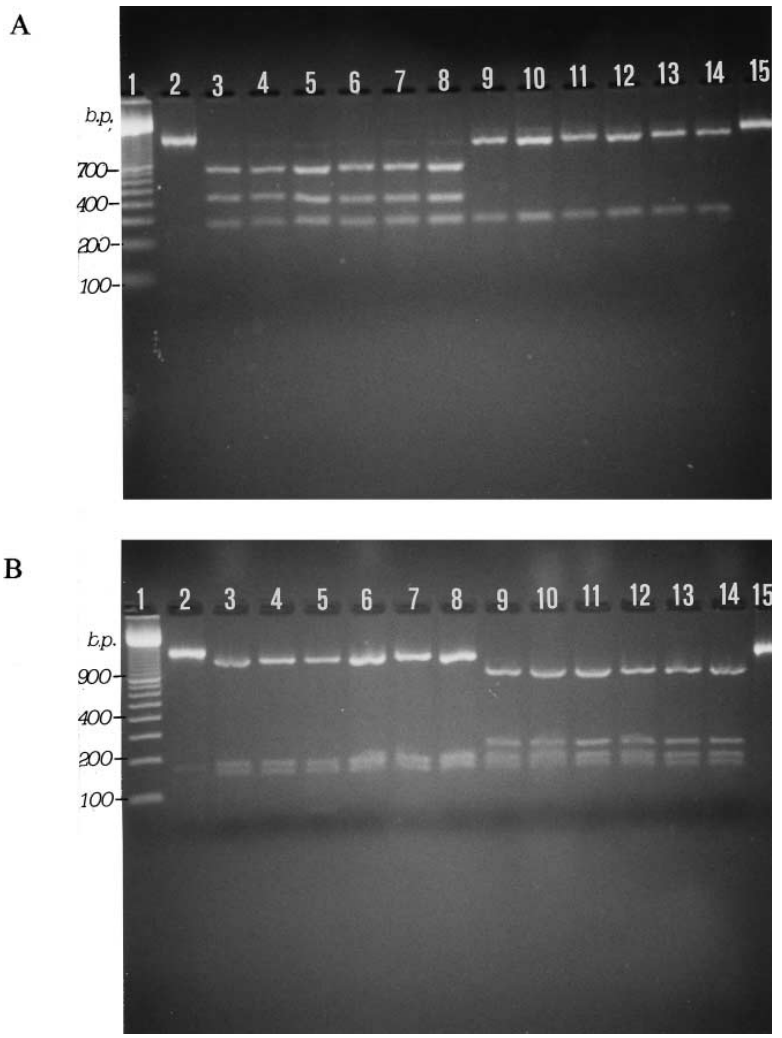

Fig. 2. Restriction fragments of PCR products generated by CLM 1-CLM 2 primers pair. (**A**) Digested with *Hin*dIII; (**B**) Digested with *Rsa*I: lane 1: 100 bp ladder molecular weight (Pharmacia Biotech, Italy), the numbers to the left indicate fragment size (bp); lane 2: *L. monocytogenes* serovar 1/2a, undigested control; lanes 3–5: *L. monocytogenes* serovar 1/2a; lanes 6–8: *L. monocytogenes* serovar 1/2c; lanes 9–10: *L. monocytogenes* serovar 1/2b; lane 11: *L. monocytogenes* serovar 3b; lanes 12–14: *L. monocytogenes* serovar 4b; lane 15: *L. monocytogenes* serovar 4b, undigested control.

serogroups had different molecular weights and so could be distinguished. *Hin*dIII had two restriction sites on the *L. monocytogenes* of 1/2a and 1/2c serogroups, yielding specific bands of 277, 425 and 693 bp, whereas for serogroups 1/2b, 3b and 4b *Hin*dIII had one restriction site which produced two specific bands of 277 and 1118 bp. *Rsa*I had two restriction sites on the *L. monocytogenes* of 1/2a and 1/2c serogroups yielding three specific bands of 1043, 204 and 148 bp, while serogroups 1/2b, 3b and 4b had three restriction sites yielding four specific bands of 148, 204, 251 and 792 bp.

In this study a combined PCR and RE technique was used to distinguish and divide into two serogroups *L. monocytogenes* taken from culture collections (NCTC and ATCC), or isolated from foods.

While Vines et al. [8] and Gutekunst et al. [15] presented two groups of strains within the species, based on PCR and RE on the *iap* gene using only

*Hin*dIII, in our study the amplimers of the *iap* gene from all serovars were cut by *Hin*dIII and *Rsa*I.

The data indicated that the uncut *iap* gene amplimers from all 5 serovars of *L. monocytogenes* tested were the same size, so it is possible that the differences resulted from nucleotide substitutions or inversion of sequences within the *iap* gene.

Different *L. monocytogenes* strains, isolated from patients with listeriosis, gave a similar restriction profile using *Hin*dIII and *Rsa*I. These were all referable to 1/2b, 3b and 4b serogroups. The fact that these strains belong to the serogroups 1/2b, 3b and 4b enable us to state that the serogroups 1/2b and 4b are the major ones involved in outbreaks, as other authors have reported [8,15,16]

The RE-PCR method should be useful for a rapid subgroup characterisation of *L. monocytogenes* in the investigation of listeriosis outbreaks, indeed DNA extraction, PCR amplification, restriction analysis and electrophoresis of restricted amplimers can be completed in 12 h. The RE-PCR method used, should allow the study of the distribution of different serovars or populations of *Listeria monocytogenes* in food and food processing plants and should be useful for a first rapid subgroup characterization in the investigation of listeriosis outbreaks.

Considering that the identification of distinct serovars or groups of serovars could lead to a better understanding of virulence factors and the epidemiology of human listeriosis, the RE-PCR method used can give a rapid confirm at the subgroup level in the laboratory of an epidemiological association between human disease and suspected sources of contaminated food. Furthermore, the materials used for this method are easily obtainable, while specific antisera required for the traditional method are not easily found in commerce.

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