APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Sept. 2011, p. 6290–6294 Vol. 77, No. 17
0099-2240/11/$12.00 doi:10.1128/AEM.00429-11

Novel Multiplex Single Nucleotide Polymorphism-Based Method for Identifying Epidemic Clones of *Listeria monocytogenes*▽

Sara Lomonaco,[1] Stephen J. Knabel,[2] Alessandra Dalmasso,[1] Tiziana Civera,[1] and Maria Teresa Bottero[1]*

Department of Animal Pathology, Faculty of Veterinary Medicine, University of Turin, Grugliasco, Italy,[1] and Department of Food Science, The Pennsylvania State University, University Park, Pennsylvania 16802[2]

Received 25 February 2011/Accepted 27 June 2011

A novel primer extension-based, multiplex minisequencing assay targeting six highly informative single nucleotide polymorphisms (SNPs) in four virulence genes correctly identified and differentiated all four epidemic clones (ECs) of *Listeria monocytogenes* and 9 other strains initially misclassified as non-ECs. This assay allows rapid, accurate, and high-throughput screening for all known ECs of *L. monocytogenes*.

Different serotypes of *Listeria monocytogenes* have been isolated from foods, but only a few (e.g., 1/2a, 1/2b, and 4b) account for the vast majority of clinical cases, and most outbreaks of listeriosis have involved a small number of closely related clones (1). An epidemic clone (EC) of *L. monocytogenes* has been defined as groups of genetically related isolates implicated in different, geographically and temporally unrelated epidemics and presumably of a common ancestor (5, 12). In particular, four ECs are currently recognized: ECI, ECII, and ECIV of serotype 4b and ECIII of serotype 1/2a (5, 12).

Pulsed-field gel electrophoresis (PFGE) is currently the gold standard technique for subtyping food-borne pathogens, as it has high discriminatory power and good repeatability. However, it is time-consuming, is laborious, has relatively low throughput, requires extensive standardization, and may confound epidemiological investigations of ECs (4, 20). Consequently, alternative methods for subtyping EC strains have been examined. Multilocus sequence typing, which utilizes sequences of multiple housekeeping genes (15) to determine genetic relatedness, considers specific nucleotide base changes rather than DNA fragment size. Multi-virulence-locus sequence typing (MVLST), which utilizes only virulence genes, was subsequently shown to correctly detect all four ECs with excellent discriminatory power and epidemiological concordance (4, 5). Instead of sequencing multiple genes, various methods can detect individual polymorphisms at defined SNP (single nucleotide polymorphism) locations (8). The purpose of this study was to develop a multiplex minisequencing assay detecting multiple SNPs that can correctly identify and differentiate all four ECs of *L. monocytogenes*.

All 84 selected *L. monocytogenes* strains (Tables 1, 2, and 3) were grown overnight at 37°C in tryptic soy broth (TSB; Acumedia, MI). Genomic DNA was extracted using an UltraClean microbial DNA extraction kit (MoBio Laboratories, Solana Beach, CA), quantified by spectrophotometry (Biophotometer 6131; Eppendorf AG, Hamburg, Germany), and stored at −20°C.

Four virulence genes, internalin A, B, and J and sortase A genes (*inlA*, *inlB*, *inlJ*, and *srtA*), were selected based on their more rapid rate of evolution compared to that of housekeeping genes (2, 15) and their ability to provide profiles specific for each EC and to differentiate the four ECs from other non-EC strains. Sequences of internal fragments in these genes (14) were aligned using Mega 2.0 software (22), and then diagnostic SNPs were selected and preliminary PCR and minisequencing primers (SP) were designed (Table 4). All sequences available in GenBank for each EC were examined to confirm the absence of intraclone variations of the selected SNPs.

Multiplex PCRs were performed in 50 μl containing 75 mM Tris-HCl (pH 8.8); 1 unit of recombinant *Taq* DNA polymerase (Invitrogen, Carlsbad, CA); a 0.2 mM concentration of each deoxynucleoside triphosphate (dNTP) (Pharmacia, Uppsala, Sweden); 25 pmol for *inlA*, *inlB*, and *inlJ* primers and 40 pmol for *srtA* primers; 1.5 mM $MgCl_2$; and 250 ng of DNA template. PCR conditions were 5 min at 95°C followed by 35 cycles at 94°C for 30 s, 55°C for 1 min, and 72°C for 40 s and a final extension of 72°C for 7 min. All thermocycling reactions were performed using a GeneAmp PCR System 2720 (Applied Biosystems, Foster City, CA). Amplicons were resolved by electrophoresis on a 2.0% agarose gel (Invitrogen, Carlsbad, CA). Amplification of specific fragments of 495 bp, 304 bp, 387/402 bp, and 193 bp for *inlA*, *inlB*, *inlJ*, and *srtA*, respectively, was observed in all but four strains, which lacked *inlB* and *inlJ* (Fig. 1; Table 2). These genes were missing in 57% of lineage III strains of *L. monocytogenes*. In particular, strain J1-023 (Table 2), although belonging to the International Life Sciences Institute diversity subset and originally classified as a lineage III *L. monocytogenes*, was later reclassified as a hemolytic strain of *Listeria innocua* (10, 11). Lineage III strains show high genetic variability and do not possess a number of genes specific for *L. monocytogenes*, such as internalin genes and other virulence genes (7, 13, 18). Moreover, reclassification of these strains has been proposed (23), thus corroborating their pronounced genetic divergence from other *L. monocytogenes* strains.

Multiplex PCR products were used as templates for subse-

* Corresponding author. Mailing address: Department of Animal Pathology, Faculty of Veterinary Medicine, University of Turin, Via Leonardo da Vinci 44, 10095 Grugliasco (TO), Italy. Phone: 390116709216. Fax: 390116709224. E-mail: mariateresa.bottero@unito.it.

▽ Published ahead of print on 8 July 2011.

TABLE 1. Description of the 37 strains representative of the four ECs of *Listeria monocytogenes* analyzed in this study

Strain[a]	Source	Year isolated, location, and association of outbreak	Lineage and serotype	EC	Minisequencing profile
13.83	Food	1985, California—Mexican-style cheese	I, 4b	ECI	AGGACA
13.123	Human	1985, California—Mexican-style cheese			
J1-119	Human	1985, California—Mexican-style cheese			
J1-002	Human	1985, California—Mexican-style cheese			
J1-123	Human	1983-1987, Switzerland—soft cheese	I, 4b	ECI	AGGACA
103322	Human	1983-1987, Switzerland—soft cheese			
13.43	Human	1983-1987, Switzerland—soft cheese			
13.41	Food	1983-1987, Switzerland—soft cheese			
13.54	Human	1983-1987, Switzerland—soft cheese			
J1-003	Human	1981, Canada—coleslaw	I, 4b	ECI	AGGACA
N3-008	Food	1981, Canada—coleslaw			
J1-108	Human	1981, Canada—coleslaw			
13.84	Food	1981, Canada—coleslaw			
N1-227	Food	1998, United States—hot dog	I, 4b	ECII	AGGGTA
N1-225	Human	1998, United States—hot dog			
H7355	Human	1998, United States—hot dog			
H7557	Human	1998, United States—hot dog			
H7596	Food	1998, United States—hot dog			
H7858	Food	1998, United States—hot dog			
R2-764	Food	2002, United States—turkey deli	I, 4b	ECII	AGGGTA
J1736	Food	2002, United States—turkey deli			
J1776	Food	2002, United States—turkey deli			
J1816	Environmental	2002, United States—turkey deli			
J1817	Environmental	2002, United States—turkey deli			
J1926	Human	2002, United States—turkey deli			
J1928	Human	2002, United States—turkey deli			
J1-101	Human	1989, United States—hot dog-associated infection	II, 1/2a	ECIII	TAAACA
N3-031	Food	1989, United States—hot dog-associated infection			
R2-603	Food	2000, United States—turkey deli	II, 1/2a	ECIII	TAAACA
R2-499	Food	2000, United States—turkey deli			
F6-154	Food	2000, United States—turkey deli			
J1-220	Human	1979, Boston, MA—vegetable	I, 4b	ECIV	AGAATG
J1-116	Human	1989, United Kingdom—pâté	I, 4b	ECIV	AGAATG
J1-129	Human	1989, United Kingdom—pâté			
N3-010	Food	1989, United Kingdom—pâté			
N3-013	Food	1989, United Kingdom—pâté			
12426	Human	1989, United Kingdom—pâté			
12480	Human	1989, United Kingdom—pâté			

[a] Strains were obtained from the *Listeria* strain collection at the Cornell University Food Safety Laboratory (Ithaca, NY), the Health Protection Agency Culture Collections (Salisbury, United Kingdom), and the Centre de Resources Biologiques de l'Institut Pasteur (Paris, France).

TABLE 2. Description of the 25 strains of *Listeria monocytogenes* in the International Life Science Institute diversity subset (10) analyzed in this study

Strain	Source	Year isolated, location, and association of outbreak	Lineage and serotype	Minisequencing profile (EC)	Confirmation method and result[a]
J1-225	Human	1983, Boston, MA—dairy	I, 4b	AGAATG (ECIV)	MVLST, ECIV
N1-225	Human	1998, United States—hot dog	I, 4b	AGGGTA (ECII)	–
J2-020	Animal	1986—cow	II, 1/2a	AAAACA	–
J1-110	Food	1985, California—soft cheese	I, 4b	AGGACA (ECI)	–
C1-122	Human	1998—sporadic case	I, 4b	AGGACA (ECI)	PCR, ECII
J2-064	Animal	1989—cow	I, 1/2b	AGAACA	–
J1-177	Human	1997—sporadic case	I, 1/2b	AGAACA	–
J2-035	Animal	1993—goat	I, 1/2b	AGAATA	–
J1-169	Human	1996—sporadic case	I, 3b	AGAACA	–
J1-049	Human	NA[b]—sporadic case	I, 3c	AGAACA	–
C1-056	Human	1998—sporadic case	II, 1/2a	TAAATA	–
J2-054	Animal	1993—sheep	II, 1/2a	AAAACA	–
M1-004	Human	1997—sporadic case	II, NA	AGAACA	–
J2-031	Animal	1996—cow	II, 1/2a	AGAACA	–
J2-066	Animal	1994—sheep	II, 1/2a	AGAACA	–
J2-063	Animal	1993—sheep	II, 1/2a	AAAACA	–
J1-094	Human	NA—sporadic case	II, 1/2c	TGAACA	–
C1-115	Human	1998—sporadic case	II, 3a	AAAACA	–
J1-031	Human	NA—sporadic case	III, 4a	AGAATA	–
J1-168	Human	1996—sporadic case	III, 4a	AAAACA	–
W1-111	NA	NA	III, 4c	GAAA	–
W1-112	NA	NA	III, 4a	GAAA	–
W1-110	NA	NA	III, 4c	AGAACA	–
J1-158	Animal	1997—goat	III, 4b	GAAA	–
J1-023	NA	NA	III, 3a	GAAA	–

[a] –, confirmation was not carried out for these samples, as they were already known to be EC strains (N1-225 and J1-110) or did not show an EC profile.
[b] NA, not available.

TABLE 3. Description of the 22 strains of *Listeria monocytogenes* isolated from other outbreaks, clinical sporadic cases, and other sources, not classified as ECs

Strain	Source	Year isolated, location, and association of outbreak	Lineage and serotype	Minisequencing profile (EC)	Confirmation method and result[a]
R2-500	Food	2000, North Carolina—soft cheese	I, 4b	AGAACA	–
R2-501	Human	2000, North Carolina—soft cheese	I, 4b	AGAACA	–
R2-502	Food	1994, Illinois—chocolate milk	I, 1/2b	AGAACA	–
R2-503	Human	1994, Illinois—chocolate milk	I, 1/2b	AGAACA	–
R2-578	Human	1983, Boston, MA—dairy	I, 4b	AGAATG (ECIV)	MVLST, ECIV
R2-583	Human	1983, Boston, MA—dairy	I, 4b	AGAATG (ECIV)	MVLST, ECIV
J1-012	Human	1987, Pennsylvania—ice cream	I, 4b	AGAATG (ECIV)	MVLST, ECIV
J1-105	Human	1981, United Kingdom, Carlisle—NA[b]	II, 1/2a	TAAATA	–
13.42	Human	1981, United Kingdom, Carlisle—NA	II, 1/2a	TAAATA	–
13.34	Human	1998, Switzerland—sporadic case	I, 4b	AGGACA (ECI)	PCR, ECI
F2-601	Human	2001, New York—sporadic case	I, 4b	AGAACC	–
F2-525	Human	2000, New York—sporadic case	III, 4b	AAAACA	–
U1	Human	2005, Italy—sporadic case	I, 4b	AGGACA (ECI)	PCR, ECI
U2	Human	2004, Italy—sporadic case	I, 4b	AGAACA	–
U3	Human	2004, Italy—sporadic case	I, 4d	AGAATG (ECIV)	MVLST, ECIV
U4	Human	2002, Italy—sporadic case	II, 1/2a	TAAATA	–
U5	Human	2006, Italy—sporadic case	I, 4b	AGGGTA (ECII)	PCR, ECII
N1-014	Food	NA	II, 1/2a	AGAACA	–
F2-032	Food	1999, New York—smoked fish	II, 1/2a	AAAACA	–
F2-373	Food	NA	II, 1/2a	AGAACA	–
C1-387	Food	1999, New York—turkey breast	II, 1/2a	AGAACA	–
J2-044	Animal	1989, New York—primate	I, 4b	AGAACA	–

[a] –, confirmation was not performed for these samples, as they did not show an EC profile.
[b] NA, not available.

quent minisequencing reactions after enzymatic cleanup with Exo-Sap (USB Europe GmbH, Staufen, Germany), according to the manufacturer's instructions. Primer extension multiplex minisequencing reactions were performed according to the SNaPshot multiplex kit protocol (Applied Biosystems, Foster City, CA) in a total volume of 10 μl with minor modifications: 3 μl of purified PCR products, 3 μl of SNaPshot multiplex ready reaction mix, and 1 μl of sequencing primer mix (2 pmol of SP2 and SP3, 3 pmol of SP5, 7 pmol of SP1 and SP6, 15 pmol of SP4). After extension, the final volume was treated with 1 unit of calf intestinal alkaline phosphatase (CIAP) (Fermentas, Burlington, CA) according to the manufacturer's instructions. Finally, 1 μl of the postextension product was mixed with 24.6 μl of Hi-Di formamide and 0.4 μl of GeneScan 120 LIZ size standard (Applied Biosystems, Foster City, CA) and analyzed with an ABI 310 genetic analyzer (Applied Biosystems, Foster

TABLE 4. PCR and minisequencing (SP) primers, fragment sizes, and allelic locations for the four *Listeria monocytogenes* virulence genes and 6 diagnostic SNPs interrogated in this study[a]

PCR or minisequencing[b]	Primer	Primer sequence (5′ to 3′)	Fragment size (bp)	Allelic location on strain EGD-e (GenBank no. NC_003210)
Preliminary PCR	inlA_F	5′-CAACSTTTGAKAATGACGGTGT-3′	496	96242–96263
	inlA_R	5′-GGTATATTTGCGGAAGGTGG-3′		96737–96718
	inlB_F	5′-ATGGATAATTATTGGAAACGG-3′	305	97089–97109
	inlB_R	5′-GCCATCCTAAATTTTTCAAG-3′		97393–97374
	inlJ_F	5′-ACTGAGCCAAAAACTATCGA-3′	388–403	190193–190212
	inlJ_R	5′-TCACTTCGGTTGTCTTTAAAT-3′		190595–190575
	srtA_F	5′-AACCATGCGTTCTGATCA-3′	195	76571–76588
	srtA_R	5′-CACTTACTTCTGTTTCATCAAT-3′		76764–76743
Minisequencing	SP1 (inlA234)	5′-TTTGATGTTGATGGAAAA-3′	20	96457–96474
	SP2 (inlA71)	5′-12(T)GCTTGCTACAAGAACCTAC-3′	31	96293–96311
	SP3 (srtA31)	5′-12(T)GTTCTGATCAAGTMATGGGTAA-3′	34	76579–76600
	SP4 (inlA303)[c]	5′-20(T)TCATACCAACCTTTGAAAG-3′	39	96563–96545
	SP5 (inlB53)[c]	5′-24(T)ATTTGCTTGATTGGCGTTG-3′	43	97160–97142
	SP6 (inlJ350)	5′-30(T)CAACCTTTACCAGMTAAAA-3′	49	190523–190541

[a] All primers were synthesized by Sigma Genosys (St. Louis, MO).
[b] Primers for the preliminary multiplex PCR were designed upstream and downstream from the diagnostic SNPs. Sequencing primers (SPs) were designed immediately flanking the diagnostic sites and with varying lengths of nonhomologous poly(dT) tails attached to their 5′ ends.
[c] Reverse primer was designed for the interrogation of this SNP.

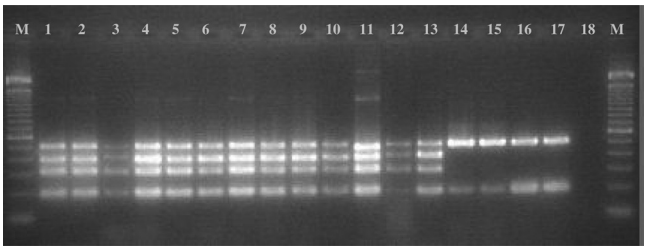

FIG. 1. Preliminary multiplex amplification of four *Listeria monocytogenes* virulence genes: *inlA*, *inlB*, *inlJ*, and *srtA*. M, 100-bp marker (Invitrogen). Lanes 1 to 3, ECI; lanes 4 and 5, ECII; lanes 6 and 7, ECIII; lanes 8 and 9, ECIV; lane 10, R2-500; lane 11, R2-578; lane 12, U1; lane 13, C1-056; lane 14, W1-111; lane 15, W1-112; lane 16, 1-158; lane 17, J1-023; lane 18, reagent control.

City, CA), using parameters described previously (6). Electropherograms were evaluated with GeneMapper 4.0 software (Applied Biosystems, Foster City, CA).

The multiplex minisequencing assay not only was able to correctly identify all known ECs with specific profiles (Fig. 2) but also identified nine non-EC strains as ECI, ECII, or ECIV (Tables 2 and 3). These findings were confirmed by a previously described multiplex PCR (3) for ECI, ECII, and ECIII and by MVLST (14) for ECIV.

In particular, classification of strain 13.34 (Table 3) as ECI suggested a possible epidemiological link between this strain and the 1987 Switzerland ECI outbreak. ECI strains may have persisted in the environment after 1987 and thus may have been the source of this sporadic clinical strain. Moreover, the perfect sequence identity observed between strain 13.34 and a 4b strain isolated in Switzerland in 1988 from dairy products (7) further strengthens the epidemiological link between strain 13.34 and ECI.

An ECIV profile was found in three strains: J1-225 (Table 2), R2-578, and R2-583 (Table 3), belonging to the 1983 Boston dairy outbreak. Originally the 1983 Boston, 1979 Boston, and 1981 United Kingdom outbreaks were all classified as ECIa (12). However, ECIa was recently reclassified as ECIV, while excluding the 1983 Boston dairy outbreak, based on results from an MVLST scheme evidencing an SNP difference

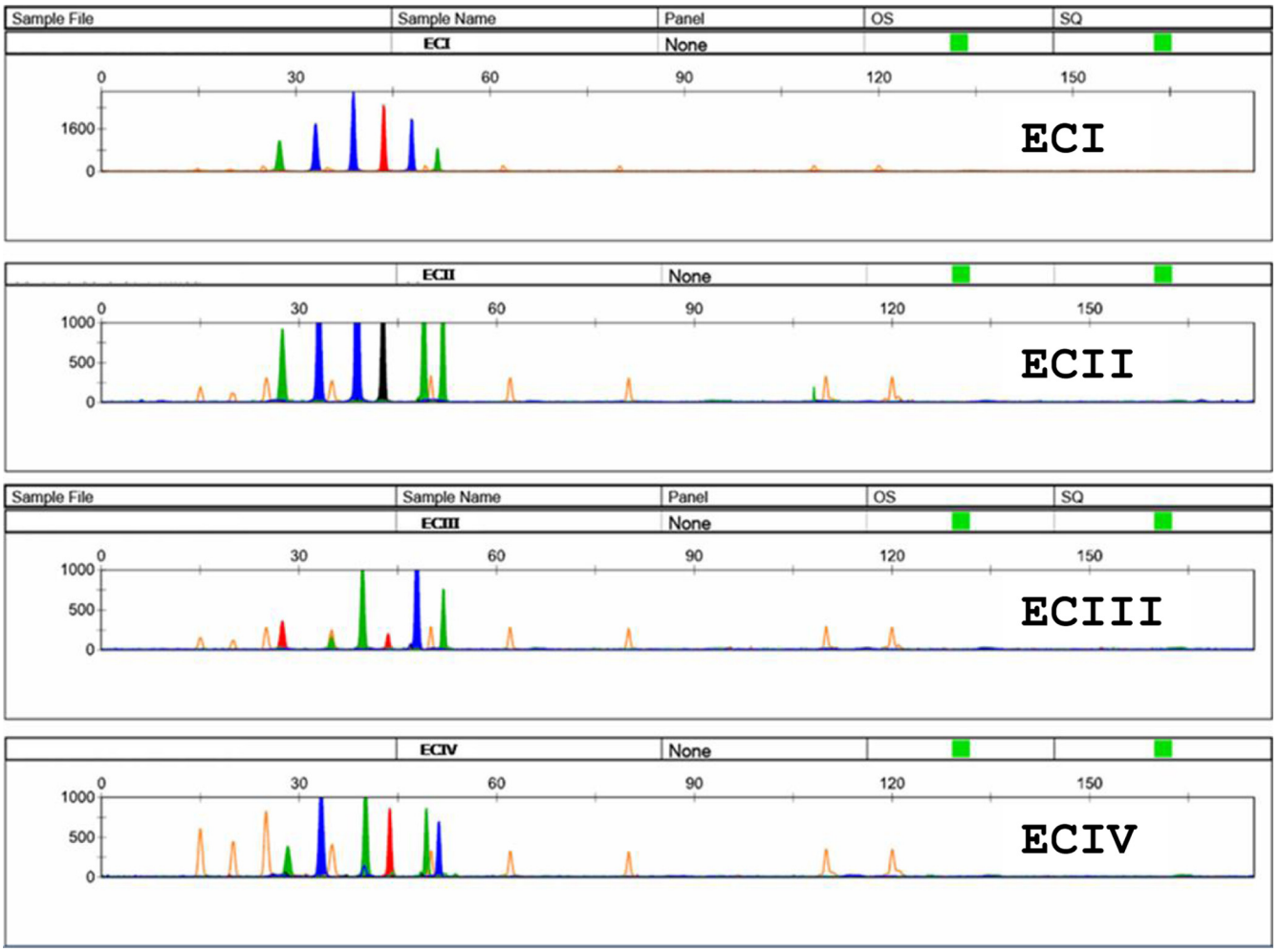

FIG. 2. Representative EC-specific profiles obtained by multiplex minisequencing reaction. Colors are specific to the fluorescently labeled dideoxynucleoside triphosphates (ddNTPs) incorporated: green (A), black (C), blue (G), and red (T). The figure was created using GeneMapper 4.0 software (Applied Biosystems, Foster City, CA).

from the newly classified ECIV (5). However, when other virulence genes were analyzed, strain J1-225 was shown to be highly homologous to ECIV, sharing the same sequence in every SNP location, including the ECIV-specific SNP (*inlJ350* in this study). The only difference was observed in *actA7*, which was in the primer binding site (14). These findings suggest that strain J1-225 might not be considered belonging to ECIV; however, further studies are needed in order to correctly evaluate its classification.

The ECIV minisequencing profile obtained for strain J1-012 (Table 3) could indicate that this strain may need to be reclassified. Some authors did not classify this strain as an EC (5, 12), while others stated that the 1987 Pennsylvania outbreak was characterized by the presence of both ECI and ECIa/ECIV strains, and strain J1-012 was to be considered an ECI strain (19, 21). However, based on *inlA* sequencing results, J1-012 was recently shown to be more closely related to an ECIV strain (19), which is consistent with our minisequencing results.

In this study, ECI, ECII, and ECIV minisequencing profiles were also found in four sporadic strains; however, no epidemiological correlation with specific outbreak clones/ECs could be established. Notwithstanding, ECs (particularly ECI and ECII) continue to be represented among apparent sporadic cases of listeriosis (9, 16, 17). In Italy, 27% to 38% of sporadic clinical isolates isolated between 1994 and 2007 showed PCR amplicons specific for EC strains (9). Recently in Portugal, two strains isolated from sporadic cases showed PFGE profiles ascribable to ECI and ECIV (17).

Findings from this study may suggest that EC strains have specific environmental reservoirs during the intervals between outbreaks and sporadic cases. Given the repeated and widespread incidences of listeriosis due to ECs in the past, it is reasonable to assume that ECs will again be involved in listeriosis cases in the future. The novel multiplex minisequencing method described represents a simple, accurate, rapid, high-throughput SNP-based subtyping method for use in surveillance, detection, risk assessment, and epidemiological investigation of *L. monocytogenes*. Detection and differentiation of strains within *L. monocytogenes*, in particular detection of EC strains, will be crucial to effectively create prevention plans, thus contributing to the improvement of food safety.

This work was supported by funds from MIUR—Ministero dell'Istruzione, dell'Università e della Ricerca and Regione Piemonte.

REFERENCES

1. **Bibb, W. F., et al.** 1990. Analysis of clinical and food-borne isolates of *Listeria monocytogenes* in the United States by multilocus enzyme electrophoresis and application of the method to epidemiologic investigations. Appl. Environ. Microbiol. **56:**2133–2141.
2. **Cai, S., et al.** 2002. Rational design of DNA sequence-based strategies for subtyping *Listeria monocytogenes*. J. Clin. Microbiol. **40:**3319–3325.
3. **Chen, Y., and S. J. Knabel.** 2007. Multiplex PCR for simultaneous detection of bacteria of the genus *Listeria*, *Listeria monocytogenes*, and major serotypes and epidemic clones of *L. monocytogenes*. Appl. Environ. Microbiol. **73:** 6299–6304.
4. **Chen, Y., W. Zhang, and S. J. Knabel.** 2005. Multi-virulence-locus sequence typing clarifies epidemiology of recent listeriosis outbreaks in the United States. J. Clin. Microbiol. **43:**5291–5294.
5. **Chen, Y., W. Zhang, and S. J. Knabel.** 2007. Multi-virulence-locus sequence typing identifies single nucleotide polymorphisms which differentiate epidemic clones and outbreak strains of *Listeria monocytogenes*. J. Clin. Microbiol. **45:**835–846.
6. **Dalmasso, A., T. Civera, and M. T. Bottero.** 2009. Multiplex primer-extension assay for identification of six pathogenic vibrios. Int. J. Food Microbiol. **129:**21.
7. **Doumith, M., et al.** 2004. New aspects regarding evolution and virulence of *Listeria monocytogenes* revealed by comparative genomics and DNA arrays. Infect. Immun. **72:**1072–1083.
8. **Foley, S., A. Lynne, and R. Nayak.** 2009. Molecular typing methodologies for microbial source tracking and epidemiological investigations of Gram-negative bacterial foodborne pathogens. Infect. Genet. Evol. **9:**430–440.
9. **Franciosa, G., et al.** 2007. Distribution of epidemic clonal genetic markers among *Listeria monocytogenes* 4b isolates. J. Food Prot. **70:**574–581.
10. **Fugett, E., E. Fortes, C. Nnoka, and M. Wiedmann.** 2006. International Life Sciences Institute North America *Listeria monocytogenes* strain collection: development of standard *Listeria monocytogenes* strain sets for research and validation studies. J. Food Prot. **69:**2929–2938.
11. **Johnson, J., et al.** 2004. Natural atypical *Listeria innocua* strains with *Listeria monocytogenes* pathogenicity island 1 genes. Appl. Environ. Microbiol. **70:** 4256–4266.
12. **Kathariou, S.** 2002. *Listeria monocytogenes* virulence and pathogenicity, a food safety perspective. J. Food Prot. **65:**1811–1829.
13. **Liu, D., et al.** 2006. *Listeria monocytogenes* subgroups IIIA, IIIB, and IIIC delineate genetically distinct populations with varied pathogenic potential. J. Clin. Microbiol. **44:**4229–4233.
14. **Lomonaco, S., Y. Chen, and S. J. Knabel.** 2008. Analysis of additional virulence genes and virulence gene regions in *Listeria monocytogenes* confirms the epidemiologic relevance of multi-virulence-locus sequence typing. J. Food Prot. **71:**2559–2566.
15. **Maiden, M., et al.** 1998. Multilocus sequence typing: a portable approach to the identification of clones within populations of pathogenic microorganisms. Proc. Natl. Acad. Sci. U. S. A. **95:**3140–3145.
16. **Mammina, C., et al.** 2009. Characterization of *Listeria monocytogenes* isolates from human listeriosis cases in Italy. J. Clin. Microbiol. **47:**2925–2930.
17. **Neves, E., A. Lourenço, A. C. Silva, R. Coutinho, and L. Brito.** 2008. Pulsed-field gel electrophoresis (PFGE) analysis of *Listeria monocytogenes* isolates from different sources and geographical origins and representative of the twelve serovars. Syst. Appl. Microbiol. **31:**387–392.
18. **Roberts, A., et al.** 2006. Genetic and phenotypic characterization of *Listeria monocytogenes* lineage III. Microbiology **152:**685–693.
19. **Roberts, A. J., S. K. Williams, M. Wiedmann, and K. K. Nightingale.** 2009. Some *Listeria monocytogenes* outbreak strains demonstrate significantly reduced invasion, *inlA* transcript levels, and swarming motility in vitro. Appl. Environ. Microbiol. **75:**5647–5658.
20. **Sauders, B., et al.** 2003. Molecular subtyping to detect human listeriosis clusters. Emerg. Infect. Dis. **9:**672–680.
21. **Schwartz, B., et al.** 1989. Investigation of an outbreak of listeriosis: new hypotheses for the etiology of epidemic *Listeria monocytogenes* infections. J. Infect. Dis. **159:**680–685.
22. **Tamura, K., J. Dudley, M. Nei, and S. Kumar.** 2007. MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. Mol. Biol. Evol. **24:**1596–1599.
23. **Ward, T., T. Ducey, T. Usgaard, K. Dunn, and J. Bielawski.** 2008. Multilocus genotyping assays for single nucleotide polymorphism-based subtyping of *Listeria monocytogenes* isolates. Appl. Environ. Microbiol. **74:** 7629–7642.