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Typing of *Listeria monocytogenes* Strains by Repetitive Element Sequence-Based PCR

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Listeria monocytogenes **strains possess short repetitive extragenic palindromic (REP) elements and enterobacterial repetitive intergenic consensus (ERIC) sequences. We used repetitive element sequence-based PCR (rep-PCR) to evaluate the potential of REP and ERIC elements for typing *L. monocytogenes* strains isolated from humans, animals, and foods. On the basis of rep-PCR fingerprints, *L. monocytogenes* strains were divided into four major clusters matching origin of isolation. rep-PCR fingerprints of human and animal isolates were different from those of food isolates. Computer evaluation of rep-PCR fingerprints allowed discrimination among the tested serotypes 1/2a, 1/2b, 1/2c, 3b, and 4b within each major cluster. The index of discrimination calculated for 52 epidemiologically unrelated isolates of *L. monocytogenes* was 0.98 for REP- and ERIC-PCR. Our results suggest that rep-PCR can provide an alternative method for *L. monocytogenes* typing.**

Listeria monocytogenes is an important food-borne pathogen, and various kinds of food have been implicated as sources of animal and human listeriosis (12). Since there are great differences in pathogenic potential among strains of *L. monocytogenes* (18), useful information can be obtained from typing.

Serotyping and phage typing have traditionally been used for the differentiation and characterization of *L. monocytogenes* isolates (2, 31). Since the vast majority of clinical isolates belong to serotype 1/2a, 1/2b, or 4b, serotyping has limited value as an epidemiological tool (12, 32). Phage typing has been useful in studies of some listeriosis outbreaks but also has limited value, as not all strains are typeable (12, 29). Improved discrimination between *L. monocytogenes* isolates has been attained by the development of molecular typing methods such as restriction enzyme analysis (3, 9, 36), pulsed-field gel electrophoresis (7, 8, 27), multilocus enzyme electrophoresis (MEE) (6, 17), esterase typing (14, 15), ribotyping (3), and random amplification of polymorphic DNA (RAPD) (4, 5, 11, 24).

Repetitive element sequence-based PCR (rep-PCR) is a recently described method which generates DNA fingerprints that allow discrimination between bacterial strains (34). The term rep-PCR refers to the general methodology involving the use of oligonucleotide primers based on short repetitive sequence elements that are dispersed throughout the prokaryotic kingdom. The palindromic units, or repetitive extragenic palindromes (REP), constitute the best-characterized family of repetitive bacterial sequences (16).

REP sequences are 35 to 40 bp long and include an inverted repeat. A second family of repetitive elements, called intergenic repeat units or enterobacterial repetitive intergenic consensus (ERIC) sequences, are larger elements of 124 to 127 bp and contain a highly conserved central inverted repeat (19). REP and ERIC sequences were used as primer binding sites to amplify the genomes of a variety of bacteria by PCR (10, 22, 23, 28, 30, 33, 37). Our previous study showed that REP and ERIC sequences are also present in the genus *Listeria* and that they are useful for species and strain discrimination (21).

In this work, REP- and ERIC-PCR were used to generate DNA fingerprints of *L. monocytogenes* strains isolated from humans, animals, and foods. The objective of this investigation was to evaluate the potential of rep-PCR for typing *L. monocytogenes* isolates of various origins.

MATERIALS AND METHODS

Isolates. Sixty-four *L. monocytogenes* isolates were examined by rep-PCR. Among them, 52 strains were of unrelated origin (Table 1). Geographical origin, time of isolation, and maintenance of *L. monocytogenes* isolates prior to the beginning of this research are shown in Table 2. Bacteria were maintained on brain heart infusion agar (Oxoid Ltd., London, United Kingdom) at 4°C after the beginning of this research. All isolates were biochemically confirmed to be *L. monocytogenes* and serotyped at the Belgian National Reference Center for Listeriosis (Institute of Hygiene and Epidemiology, Brussels) (32).

Preparation of genomic DNA. DNA was extracted from bacterial cells grown overnight at 37°C in brain heart infusion broth (Oxoid) by the method of Flamm et al. (13) as follows. Bacterial cells from 2 ml of culture were pelleted by centrifugation at 13,000 × *g* for 2 min, washed in 1 ml of 1× SSC (150 mM NaCl plus 15 mM sodium citrate [pH 7.0]), suspended in 100 μl of lysozyme solution (10 mM sodium phosphate [pH 7.0], 20% sucrose, 4 mg of lysozyme [Boehringer, Mannheim, Germany] per ml), and incubated for 45 min at 37°C. To these suspensions, 200 μl of TE buffer (20 mM Na_2-EDTA, 50 mM Tris-HCl [pH 8.0]), 100 μl of Sarkosyl solution (5% Sarkosyl [Boehringer] in TE buffer), and 100 μl of proteinase K solution (25 mg/ml [Boehringer] in TE buffer) were added and incubated at 37°C for 1 h. Cell lysates were extracted once with phenol and twice with chloroform. Precipitation of nucleic acids was done with sodium acetate (final concentration, 0.3 M) and 2 volumes of ethanol (100%). The DNA was dissolved in sterile water, and the concentration of the DNA was determined spectrophotometrically at 260 nm.

rep primers and rep-PCR conditions. For REP-PCR, the (18-mer) primers REP 1R-I (5′-IIIICGICGICATCIGGC-3′) and REP 2-I (5′-ICGICTTATCIG GCCTAC-3′) and for ERIC-PCR, the (22-mer) primers ERIC 1R (5′-ATGTA AGCTCCTGGGGATTCAC-3′) and ERIC 2 (5′-AAGTAAGTGACTGGGGT GAGCG-3′) (34) were used. The REP 1R-I and REP 2-I primers contain the nucleotide inosine (I) at ambiguous positions in the REP consensus (34). Inosine can form Watson-Crick base pairs with A, T, G, or C. PCRs were carried out as described by Versalovic et al. (34) with 25 ng of template DNA per reaction for

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TABLE 1. Characteristics of *L. monocytogenes* strains isolated from humans, animals, and foods and REP and ERIC types

Designation		Origin[b]	Sero-type	REP type		ERIC type	
This study	Source[a]			VC[c]	CA[d]	VC	CA
1	MF1	Child 1, CSF	1/2b	1	A4	1	A1
2	MF2	Child 1, navel	1/2b	1	A4	1	A1
3	MF3	Child 1, auditory passage	1/2b	1	A4	1	A1
4	MF4	Child 1, eyes	1/2b	1	A4	1	A1
5	MF5	Child 1, skin	1/2b	1	A3	1	A1
6	MF6	Child 1, blood	1/2b	1	A3	1	A1
7	MF7	Mother 1, cervix	1/2b	1	A3	1	A1
8	MF8	Mother 1, lochia	1/2b	1	A3	1	A1
9	MF9	Human 2, CSF	4b	2	A1	2	A2
10	MF10	Human 3, CSF	4b	2	A2	2	A2
11	MF11	Human 4, auditory passage	4b	2	A1	2	A2
12	MF12	Human 4, blood	4b	2	A1	2	A2
13	MF13	Human 5, /	1/2c	3	A5	3	A4
14	MF14	Human 5, /	1/2c	3	A5	3	A4
15	MF15	Child 6, auditory passage	4b	2	A1	2	A2
16	MF16	Mother 6, cervix	4b	2	A1	2	A2
17	MF17	Child 6, blood	4b	2	A1	2	A2
18	MF18	Human 7, /	4b	2	A1	2	A2
19	MF19	Human 8, CSF	4b	2	A1	2	A2
20	MF20	Human 9, blood	1/2b	4	B3	4	B5
21	MF21	Human 10, CSF	1/2b	5	B2	5	B2
22	MF22	Human 11, /	1/2b	5	B2	6	B6
23	MF24	Human 12, /	4b	6	B4	5	B4
24	MF25	Human 13, /	4b	6	B4	5	B4
25	MF26	Human 14, /	1/2a	7	B6	7	B8
26	MF27	Human 15, blood	4b	8	B1	8	B1
27	MF28	Human 15, auditory passage	4b	8	B1	8	B1
28	B574	Sheep 1, brain	4b	6	B4	5	B3
29	B282	Sheep 2, brain	4b	6	B4	5	B3
30	B139	Sheep 3, brain	4b	6	B4	5	B3
31	B429	Sheep 4, brain	1/2a	9	B5	7	B7
32	B254	Sheep 5, brain	1/2a	9	B5	7	B7
33	VF321/17	Sheep 6, brain	4b	6	B4	5	B3
34	IPH61	A, Dairy Queen salad	4b	10	C10	9	C14
35	IPH68	A, chicken salad	1/2c	11	C1	10	C7
36	IPH77	B, vienna salad	1/2a	12	C8	10	C3
37	IPH129	C, turkey salad	1/2b	13	C2	11	C9
38	IPH132	D, French salad	1/2a	14	C9	10	C1
39	IPH139	E, frozen kajmak	1/2a	11	C3	10	C6
40	IPH143	F, cheese with vegetables	1/2b	15	C15	12	C15
41	IPH144	F, Smoked cheese	1/2b	15	C13	12	C15
42	IPH145	B, Vienna salad	1/2b	15	C13	9	C11
43	IPH151	D, French salad	1/2a	16	C4	10	C5
44	IPH152	D, squid salad	1/2a	16	C4	10	C5
45	IPH153	G, paste	4b	15	C11	9	C12
46	IPH156	H, Russian salad	3b	15	C12	9	C11
47	IPH160	H, Russian salad	1/2a	17	C6	9	C11
48	IPH161	H, liptaver	1/2b	18	C14	13	C4
49	IPH165	B, Alps salad	1/2c	16	C5	10	C8
50	IPH173	B, egg grease	3b	18	C14	9	C13
51	IPH134	I, rough seitan	1/2a	12	C7	10	C2
52	IPH123	G, Tatarian beefsteak	1/2b	18	C14	12	C10
53	IPH141	J, Tatarian beefsteak	1/2b	19	D4	9	D3
54	IPH146	K, Tatarian beefsteak	4b	19	D6	9	D3
55	IPH147	L, Tatarian beefsteak	1/2b	19	D4	14	D1
56	IPH149	M, Tatarian beefsteak	1/2b	19	D5	14	D2
57	IPH150	D, Tatarian beefsteak	1/2a	20	D2	15	D5
58	IPH166	N, Tatarian beefsteak	1/2a	21	D3	15	D5
59	VF2648	O, steamed chicken	1/2a	20	D1	15	D4
60	VF2746/11	O, steamed chicken	1/2a	20	D1	15	D4
61	VF2856/15	O, steamed chicken	1/2a	20	D1	15	D4
62	VF2855/17	O, steamed chicken	1/2a	20	D1	15	D4
63	VF2856/18	O, steamed chicken	1/2a	20	D1	15	D4
64	VFM10	O, steamed chicken	1/2b	22	D4	16	A3

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[b] Human subjects were labelled 1 to 15; sheep were labelled 1 to 6; and food manufacturers were labelled A to O. For human subjects, those in whom isolates of related origin were found are given the same number (e.g., mother 1 and child 1 produced related isolates). Kajmak is salted cream fermented for a short time; Alps salad contains mayonnaise, sliced sausages, and mixed vegetables; liptaver is fresh cheese with red paprika, onion, and salt; seitan is a vegetarian substitute for meat. A slash mark indicates that no data about exact origin were available. CSF, cerebrospinal fluid.

[c] VC, determined by visual comparison of banding patterns.

[d] CA, determined by computer analysis on the basis of the Jaccard coefficient ($S_J \geq 80\%$).

TABLE 2. Geographical origin, time of isolation, and maintenance of *L. monocytogenes* isolates

Geographical region of origin of isolate[a]	Isolate designation	Date of isolation	Maintenance of isolates before the present research was begun
Ljubljana	1–27	1980–1985	Bacterial strain storage medium (Sanofi Diagnostics, Pasteur, Marnes-la-Coquette, France) at 4°C without subculturing
Smoljan (Rodopi mountain)	28 30 31, 32	1972 1983 1984	Lyophilized
Sofia (Kostenez)	29	1972	Lyophilized
Ljubljana	33	1985	Nutrient agar at 4°C subcultured every 4 mo
Ljubljana	34–58	1992–1993	Microbank system (Pro-Lab Diagnostics, Richmond Hill, Ontario, Canada) subcultured every 6 mo
Ptuj	59–64	1993	Nutrient agar at 4°C subcultured every 4 mo

[a] The regions of Ljublana and Ptuj are in central and northeastern Slovenia, respectively.

REP-PCR and 35 ng of template DNA for ERIC-PCR. Amplification reactions were performed in 25 μl of a solution containing 25 pmol of each of the two opposing primers (Isogen, Amsterdam, The Netherlands), 200 μM each deoxynucleoside triphosphate (Pharmacia, Uppsala, Sweden), 2.5 mM $MgCl_2$ (Perkin-Elmer, Norwalk, Conn.), 50 mM KCl–10 mM Tris-HCl (pH 8.3), and 0.35 U of Goldstar DNA polymerase (Eurogentec S. A., Seraing, Belgium). Amplifications were performed with a DNA thermocycler (Cetus model 9600 [Perkin-Elmer]) with the following temperature profiles: for REP-PCR, 1 cycle at 95°C for 3 min; 30 cycles at 90°C for 30 s, at 40°C for 1 min, at 72°C for 1 min; and 1 cycle at 72°C for 8 min; for ERIC-PCR, 1 cycle at 95°C for 5 min; 30 cycles at 90°C for 30 s, at 50°C for 30 s, at 52°C for 1 min, at 72°C for 1 min; and 1 cycle at 72°C for 8 min.

Analysis of rep-PCR products. rep-PCR products (12 μl) were separated by electrophoresis on a 1.5% agarose gel (SeaKem LE agarose; FMC Bioproducts, Rockland, Maine) in 1× TBE buffer (2 mM EDTA, 0.1 M Tris-HCl, 0.1 M boric acid [pH 8]) at a constant voltage of 4 V/cm. After being stained with ethidium bromide, the gel was photographed under UV transillumination with Polaroid 665 film. The DNA molecular weight marker X from Boehringer was used as a size standard.

First, DNA fingerprints of the isolates were compared for similarity by visual inspection of the band patterns. Two fingerprints were considered different if the presence or absence of at least two bands differed in one of the patterns. Variations in band intensity were not considered to be differences. Bands that were too faint to be interpreted when reproduced were not considered.

Subsequently, gel images were scanned (ScanJet4p; Hewlett-Packard Co., Palo Alto, Calif.), digitized, and stored as TIFF files. These files were converted (track resolution, 450 pixels [px]), normalized (normalization settings: resolution, 350 px; smoothing, 3; background subtraction: rolling disk, intensity 6), and analyzed (band settings: band search filters: minimal profiling, 0.27%; minimal area, 0.25%; band comparison settings: position tolerance, 0.85%; increase, 0%; clustering bands, unweighted pair group method using arithmetic averages (UPGMA), and Jaccard coefficients were compared with GelCompar software (version 3.1; Applied Maths, Kortrijk, Belgium) (1). DNA bands detected by computer were carefully verified by visual examination to correct unsatisfactory detection. The normalization program allowed alignment of gels by associating internal reference bands. The similarities between DNA fingerprints were calculated with the band-matching Jaccard coefficient (S_J) (28). The proportion of bands common to two strains, A and B, is defined as $S_J = n_{AB}/(n_A + n_B - n_{AB})$, where n_{AB} is the number of bands common to A and B, and n_A and n_B are the total numbers of bands for A and B, respectively. This Jaccard coefficient ranges from 0 to 1.0, where 1.0 represents 100% identity (presence and position) for all bands in the two fingerprints being compared. A band-matching tolerance of 0.8% was chosen. DNA fingerprints from 298 to 6,100 bp were compared. Cluster analysis of similarity matrices was performed by UPGMA.

RESULTS

rep-PCR of genomic DNA from *L. monocytogenes* isolates generated multiple DNA fragments in sizes ranging between 298 and 6,100 bp (Fig. 1 and 2). One common band of about

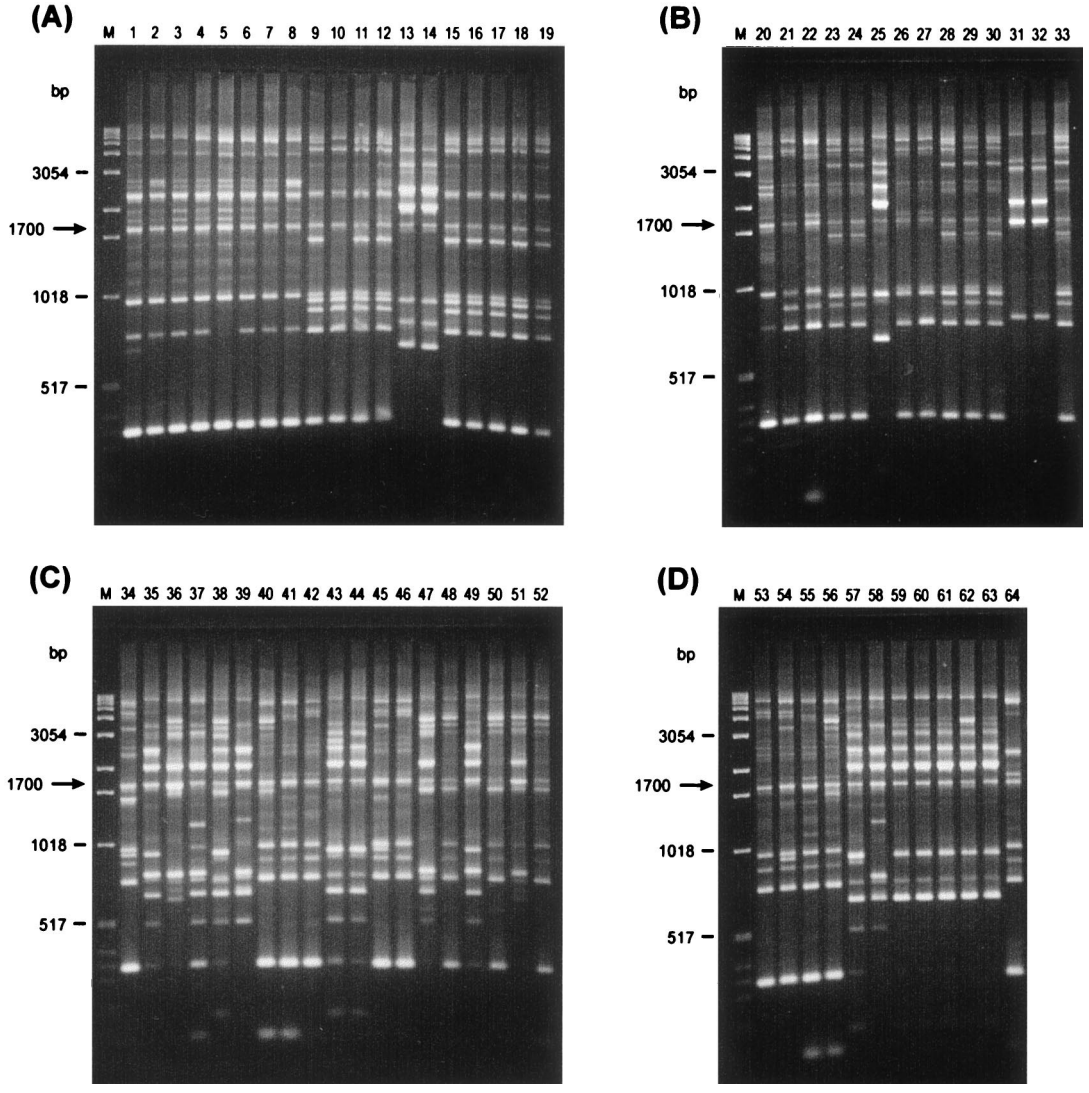

FIG. 1. REP-PCR fingerprints of human (lanes A1 to A19 and B20 to B27), animal (lanes B28 to B33), and food (lanes C34 to C52 and D53 to D64) isolates of *L. monocytogenes*. Lanes M contain molecular size markers.

1,700 bp, for REP-PCR (Fig. 1), and two common bands, of about 1,696 and 3,500 bp, for ERIC-PCR (Fig. 2), were present in all *L. monocytogenes* strains tested. Visual comparison of banding patterns revealed 22 distinct REP profiles and 16 distinct ERIC profiles (Fig. 1 and 2 and Table 1) for the 52 unrelated strains tested. Related strains (isolated from a mother and a newborn or different isolates from the same patient) produced identical REP and ERIC profiles. REP and ERIC profiles of *L. monocytogenes* strains isolated from foods were clearly distinct from REP and ERIC profiles of human and animal *L. monocytogenes* isolates (Fig. 1 and 2). In contrast, four of six *L. monocytogenes* animal isolates (no. 28 to 30 and 33) had the same DNA banding pattern as that of two human isolates (no. 23 and 24). All those isolates (no. 23, 24, 28 to 30, and 33), which are REP type 6 and ERIC type 5, belong to serotype 4b. It is, however, worth noting that not all ERIC type 5 strains are serotype 4b strains, as the human isolate no. 21 is ERIC type 5, serotype 1/2b (Table 1). Subsequently, DNA fingerprints were analyzed by using a computer program for comparative analysis of DNA electrophoresis patterns. After normalization and alignment of the different DNA profiles, the relative genetic similarity among *L. monocytogenes* isolates was calculated and visualized by cluster analysis. The estimated relationships among isolates on the basis of REP and ERIC fingerprints are indicated on the dendrograms in Fig. 3 and 4,, respectively. The dendrograms clearly show that the strains examined are divided into four distinct groups designated A, B, C, and D. Strains were assigned to group A, B, C, or D regardless of which type of PCR (REP or ERIC) was used. The low degree of relative genetic similarity between these four groups is less than 20%. Group A consists of human isolates, group B consists of human and animal isolates, and groups C and D consist of food isolates. Within each of the four groups A, B, C, and D, a further differentiation of rep profiles was established at 80% relative genetic similarity. This second level of clustering enabled the same or greater differentiation among strains than did serotyping, with the exception of REP type C14 and ERIC types C11 and D3 (no. 53 and 54) (Table 1). This computer evaluation (at $S_J = 80\%$) suggested the existence of 33 different REP patterns and 35 different ERIC patterns (Fig. 3 and 4). Computers work with a certain resolution to discriminate bands from each other, and this can

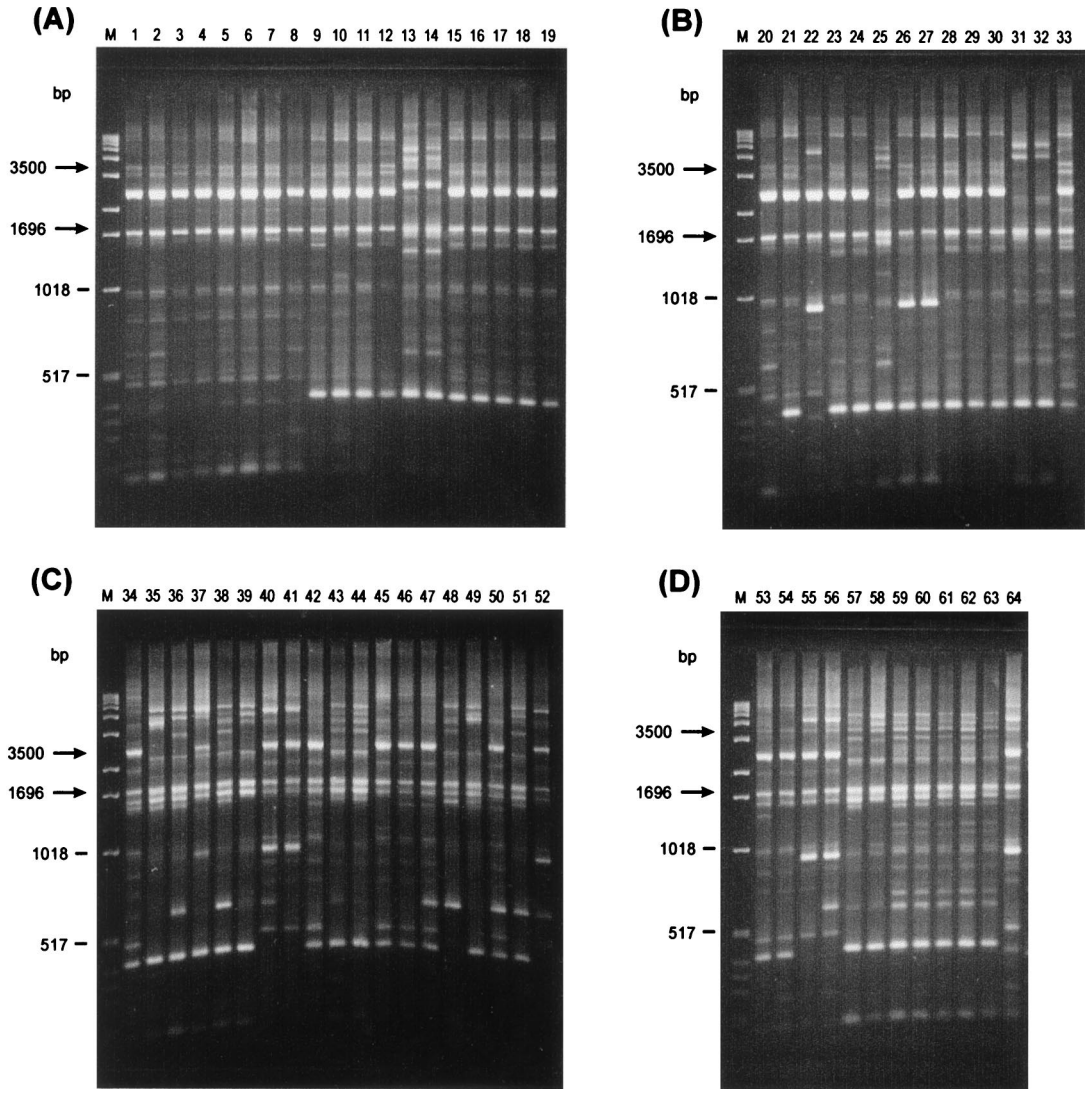

FIG. 2. ERIC-PCR fingerprints of human (lanes A1 to A19 and B20 to B27), animal (lanes B28 to B33), and food (lanes C34 to C52 and D53 to D64) isolates of *L. monocytogenes*. Lanes M contain molecular size markers.

differ from visual interpretation by eye. The index of discrimination (DI) of Hunter and Gaston (20) calculated for 52 epidemiologically unrelated isolates was 0.98 for REP- and ERIC-PCR. As expected, we found a lower DI (0.72) for serotyping. By visual analysis, the DIs calculated for 52 epidemiologically unrelated isolates were 0.96 for REP-PCR and 0.94 for ERIC-PCR. The lower DI obtained by visual analysis may be compensated for by better REProducibility, and this is a very important but often neglected criterion for the assessment of a typing method.

REP- and ERIC-PCR provided a similar degree of discrimination within the tested serotypes (Table 3). At a relative genetic similarity of 80%, there was complete discrimination between serotypes 1/2a, 1/2b, and 4b. Strains 50 and 46, both serotype 3b, could be discriminated from the other serotypes at a relative genetic similarity of 90%. In general, fewer *L. monocytogenes* rep types were found among human and animal isolates than among food isolates. As for visual comparison of banding patterns, rep types found among human and animal isolates are different from those found among food isolates, regardless of the serotype. Serotype 4b is present in human, animal, and food isolates, but human and animal serotype 4b isolates clearly belong to other rep types than food isolates. The different rep types present among food isolates show a relative genetic similarity lower than 20% to rep types found among human and animal isolates, with the exception of one strain (no. 64) isolated from chicken, which is more closely related to human *L. monocytogenes* isolates than to the other food isolates (Fig. 3 and 4). REP- and ERIC-PCR for strain 64 were repeated with freshly prepared DNA in order to confirm the results.

DISCUSSION

In this study, rep-PCR was used as a tool to characterize *L. monocytogenes* strains isolated from humans, animals, and foods. Both methods, REP- and ERIC-PCR, showed great possibilities for the typing of *L. monocytogenes* isolates. *L. monocytogenes* isolates which are closely linked epidemiologically as well as some unrelated strains (animal isolates 28 to 30 and 33 and human isolates 23 and 24) showed very similar REP- and ERIC-PCR fingerprints by visual inspection. Com-

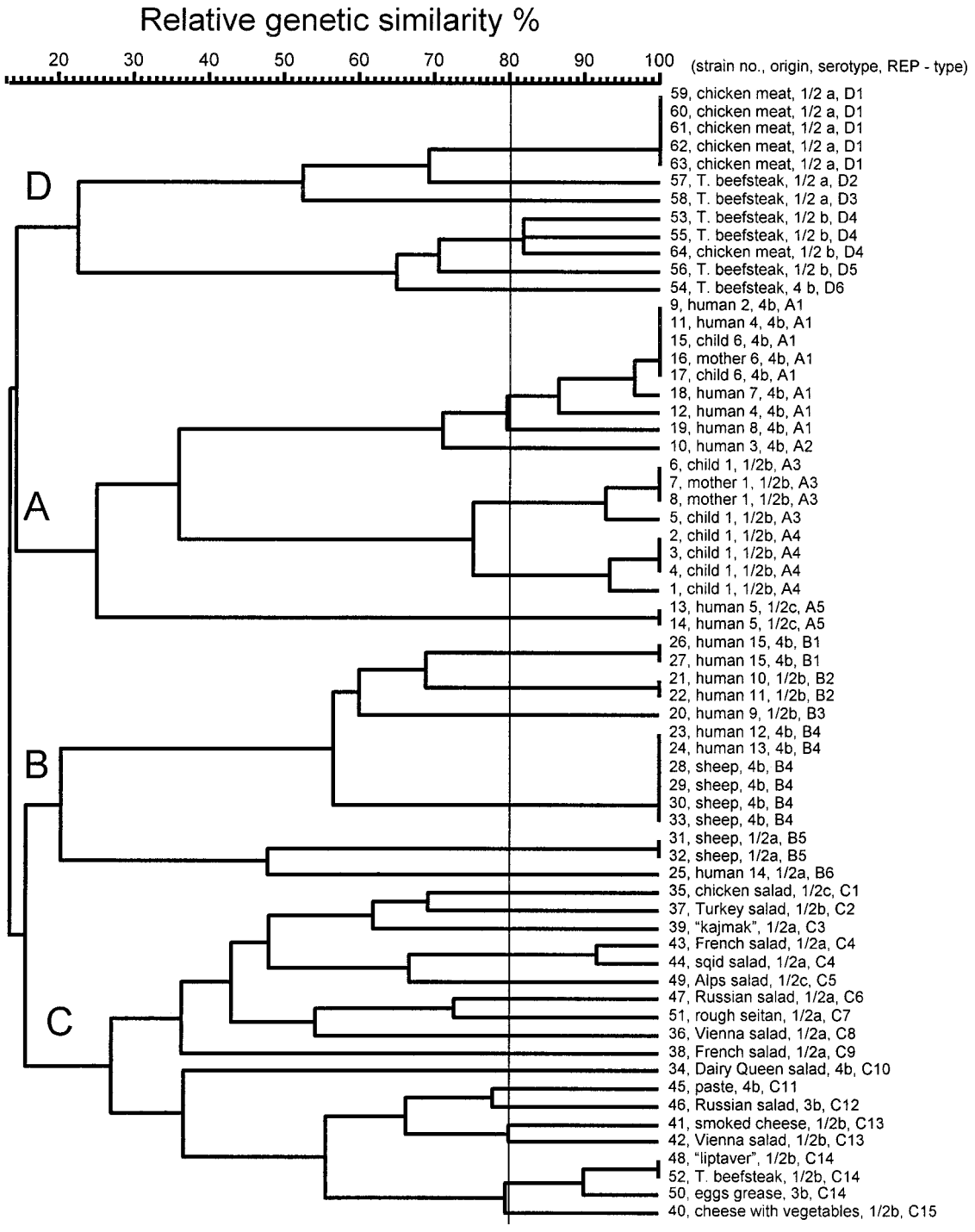

FIG. 3. Dendrogram representing genetic relationships between *L. monocytogenes* isolates based on REP-PCR fingerprints.

puter-aided comparison of electrophoresis patterns confirmed this visual impression (Table 1). REP and ERIC types correlated well with serotypes and generally provided greater differentiation within and between human and animal isolates.

Among food isolates of *L. monocytogenes*, a great diversity of fingerprints was observed. REP and ERIC profiles for food isolates were clearly different from the profiles obtained for human and animal isolates of *L. monocytogenes*.

The 64 *L. monocytogenes* isolates were divided into four groups, A, B, C, and D, separated at a relative genetic similarity of less than 20%. This first level of clustering was based on the origin of the *L. monocytogenes* strains—human, animal, or food. The second level of clustering (at $S_J = 80\%$) allowed at least the same level of differentiation between strains as serotyping. The results of our clustering of *L. monocytogenes* strains are different from the division of *L. monocytogenes* strains by restriction fragment length polymorphism (33) or MEE (17). Vines et al. (35) examined 29 strains of *L. monocytogenes* and divided them into one group containing serovars 1/2a, 3a, and 1/2c and another group comprising serovars 1/2b,

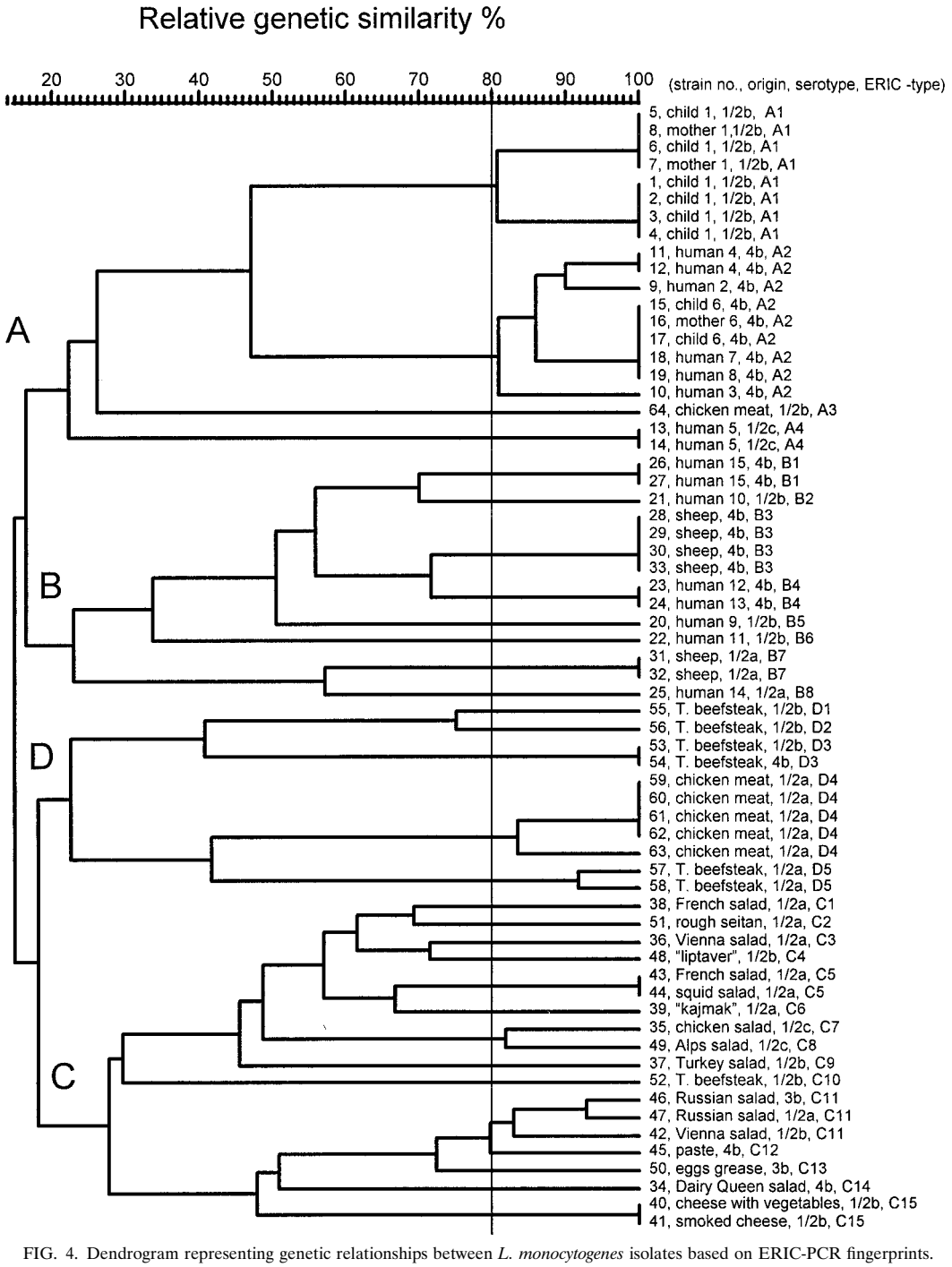

FIG. 4. Dendrogram representing genetic relationships between *L. monocytogenes* isolates based on ERIC-PCR fingerprints.

3b, and 4b. These groups did not correlate with human or environmental origin. Harvey and Gilmour (17) divided 141 *L. monocytogenes* strains into ET group I, containing serovars 1/2b, 4a, 4b, 4c, 4d, and 4e, and ET group II, containing serovars 1/2a, 1/2c, and 3a. The DI of Hunter and Gaston (20), being 0.98 for REP-PCR or for ERIC-PCR, is in the same range as the one determined by RAPD with the combination of results obtained with two or three primers (5).

The division of the 64 strains of *L. monocytogenes* by rep-PCR into two groups (A and B) containing human and animal isolates and another two groups (C and D) containing food

TABLE 3. Ability of rep-PCR to discriminate between *L. monocytogenes* isolates

Serotype	No. of unrelated isolates	No. of REP types[a]	No. of ERIC types[a]
1/2a	17	11	10
1/2b	14	10	13
1/2c	3	3	3
3b	2	2	2
4b	16	7	7

[a] REP and ERIC types were based on determination by computer analysis with the Jaccard coefficient ($S_J \geq 80\%$).

isolates showed that no similarity between strains isolated from humans or animals and strains isolated from foods is present (Fig. 3 and 4). The exception, strain 64, isolated from chicken, showed by ERIC-PCR higher similarity with human isolates than with other food isolates (S_J = 26%). This observation could lead to the hypothesis that only a minor portion of the *L. monocytogenes* strains present in food products are infectious for humans and animals. McLauchlin (25) and Notermans et al. (26) reached a similar conclusion that not all *L. monocytogenes* bacteria present in food cause disease because of the interstrain differences in their virulence properties. Because only 64 strains were included in this study, these observations should be validated by the examination of a larger set of strains.

The potential of rep-PCR as an efficient and sensitive molecular typing tool for *L. monocytogenes* should be further evaluated by the examination of *L. monocytogenes* isolates associated with food-borne epidemics. rep-PCR may serve as a rapid screening method to classify a large number of isolates into clusters. Results of this study add further evidence to the idea that rep-PCR may be broadly applicable for fingerprinting bacteria which possess repetitive elements such as REP or ERIC sequences.

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