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Comparison of Ribotyping and Multilocus Enzyme Electrophoresis for Subtyping of *Listeria monocytogenes* Isolates

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Ribotyping was compared with multilocus enzyme electrophoresis (MEE) for subtyping 305 *Listeria monocytogenes* isolates from clinical and nonclinical sources. For ribotyping, EcoRI-restricted genomic DNA fragments of *L. monocytogenes* strains were separated by agarose gel electrophoresis, and Southern blots were probed with a cloned *Escherichia coli rrnB* operon (plasmid pKK3535) labeled with digoxigenin. The *L. monocytogenes* isolates were divided into 28 distinct ribotypes, while MEE analysis divided the same isolates into 78 electrophoretic types (ETs). On the basis of their ribotype profiles, the strains were divided into two subgroups. The ribotype α (RTα) subgroup contained serotypes 1/2a, 1/2c, and 3a, and the ribotype β (RTβ) subgroup contained serotypes 1/2b, 3b, 4b, and 4ab. This division is in complete agreement with MEE analysis, which divides the species into two subgroups (ET groups A and B), with the same serotype distribution in each subgroup. Overall, MEE was more discriminating than ribotyping. However, in several instances ribotyping discriminated between isolates within the same ET. Ribotyping was more discriminating for serotypes 1/2a, 1/2c, and 3a (Simpson's Index for Diversity [DI] = 0.81) than for serotypes 1/2b and 4b (DI = 0.76). A substantial proportion (69%) of serotype 1/2b and 4b strains clustered in five ETs and five ribotypes. These data suggest that ribotyping and MEE do not provide adequate discrimination between strains of serotypes 1/2b and 4b. Methods such as pulsed-field gel electrophoresis and random amplified polymorphic DNA analysis should be explored for further discrimination of strains of these serotypes.

Listeria monocytogenes is recognized as a major foodborne pathogen. Although the incidence of sporadic listeriosis is much less common than other foodborne diseases such as salmonellosis, the high mortality rate (25 to 30%) associated with listeriosis makes it a serious public health problem (28). Furthermore, several major foodborne outbreaks of listeriosis occurred in North America and Europe between 1981 and 1992 (12, 21, 28). These outbreaks generated much publicity and caused greater awareness of foodborne listeriosis in the public health community.

A laboratory-based active surveillance study was undertaken by the Centers for Disease Control and Prevention in 1988 to assess the role of foods in sporadic listeriosis (24, 27). The study indicated that a large proportion of sporadic cases of listeriosis could be attributed to the transmission of *L. monocytogenes* through contaminated foods.

Subtyping of *L. monocytogenes* isolates is essential for studying the epidemiology of listeriosis and determining the role of foods in sporadic and epidemic cases of listeriosis. The subtyping methods used previously were inadequate in several respects. Serotyping lacked discriminating ability; bacteriophage typing is more discriminative and could be applied to large numbers of isolates, but many isolates, particularly from nonclinical sources, remain "not typeable." Also, bacteriophage typing can be done in only a few specialized reference laboratories. Molecular typing offers the advantages of high discriminating ability and 100% typeability. Also, molecular typing methods do not require specialized reagents such as typing sera and bacteriophages, which are available only in a few reference laboratories and are not easily accessible to other laboratories. For these reasons, molecular typing methods (multilocus enzyme electrophoresis [MEE], genomic restric-

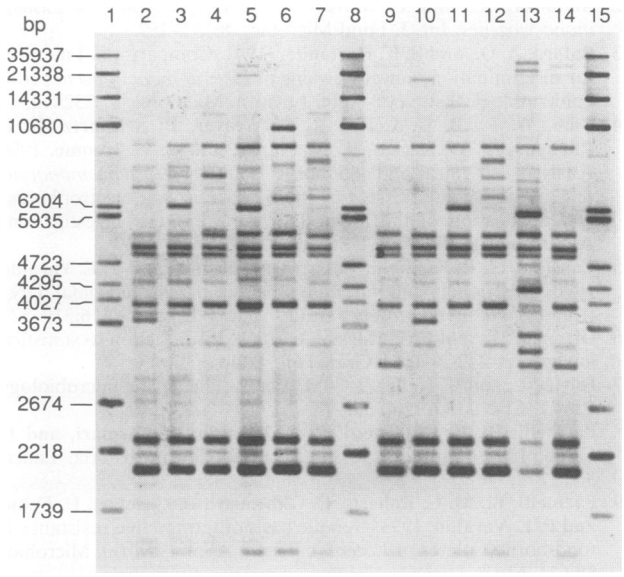

FIG. 1. Ribotype patterns of 12 *L. monocytogenes* strains (serotypes 1/2a, 1/2c, and 3a) obtained by digestion of the genomic DNA with *Eco*RI. Lanes 1, 8, and 15, adenovirus type 2 DNA marker; lanes 2 to 7, RTα01 to RTα06, (serotype 1/2a), respectively; lanes 9 to 12: RTα07 to RTα10 (serotype 1/2a), respectively; lane 13, RTα11 (serotype 3a); lane 14, RTα07 (serotype 1/2c).

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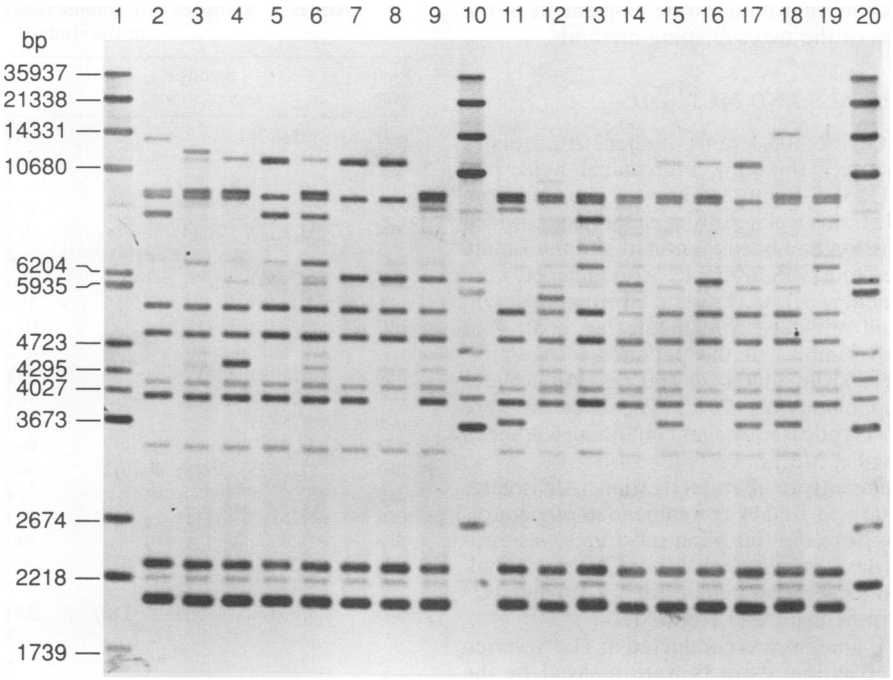

FIG. 2. Ribotype patterns of 17 *L. monocytogenes* strains of serotypes 1/2b, 3b, 4b, and 4ab obtained by digestion of genomic DNA with *Eco*RI. Lane 1, 10, and 20, *Ad2* adenovirus type 2 DNA marker; lanes 2 to 6, RTβ01 to RTβ05 (serotype 1/2b), respectively; lanes 7 to 9, RTβ06 to RTβ08 (serotype 4b), respectively; lane 11, RTβ09 (serotype 4b); lane 12, RTβ10 (serotype 1/2b); lane 13, RTβ11 (serotype 4b); lanes 14 to 17, RTβ12 to RTβ15 (serotype 1/2b), respectively; lane 18, RTβ16 (serotype 4b); lane 19, RTβ17 (serotype 1/2b).

tion endonuclease analysis by conventional gel electrophoresis [DNA fingerprinting] and pulsed-field gel electrophoresis [PFGE], and ribosomal DNA fingerprinting [ribotyping]) are increasingly used to subtype *L. monocytogenes* isolates. Of these methods, only MEE has been extensively evaluated to demonstrate its utility as a subtyping method (4, 6). Although substantial numbers of *L. monocytogenes* isolates have been ribotyped, most were isolated from limited epidemiologic settings. Thus, its usefulness for subtyping *L. monocytogenes* is unclear.

In the study described here, we directly compared ribotyping with MEE for subtyping more than 300 *L. monocytogenes* isolates (primarily from the United States) from clinical and nonclinical sources. Isolates that caused epidemic and sporadic cases of listeriosis were included in the study. Because the descriptive epidemiology of most of the isolates was known,

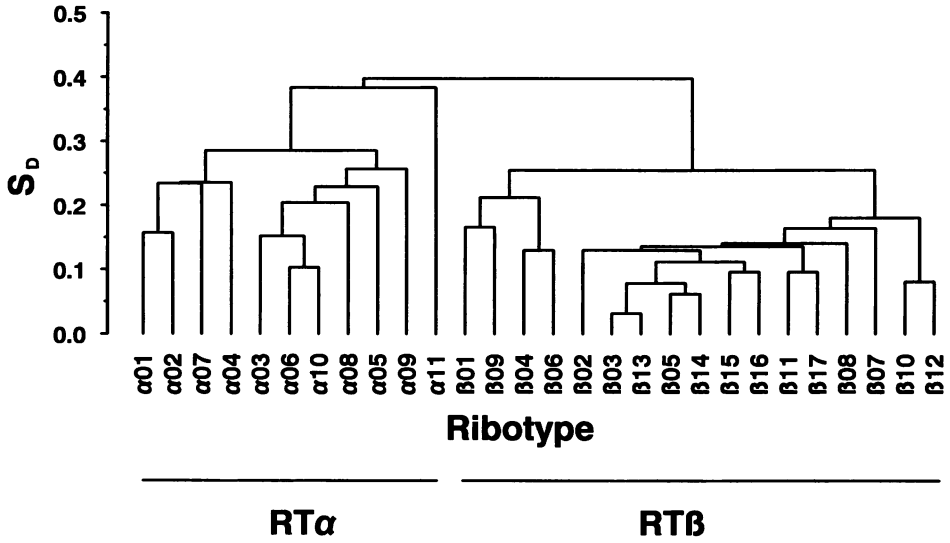

FIG. 3. Dendrogram showing the relationship among ribotypes of *L. monocytogenes*. S_D, Dice similarity coefficient.

the study was expected to offer an accurate assessment of the discriminating abilities of the two subtyping methods.

MATERIALS AND METHODS

Bacterial strains. Of the 305 strains studied, 202 isolates were from clinical sources (blood, cerebrospinal fluid, placenta, ear, cervix, and stool). Eighty strains were isolated from foods; these included strains isolated from outbreaks in which the vehicle of transmission had been identified and the isolate from the contaminated food was available. Also included were six isolates from Sweden, which were collected from a patient, the cerebrospinal fluid and feces of an infected goat, goat cheese, and two swab samples of the patient's refrigerator. Seventeen strains were from unknown sources. All isolates were stored in sheep blood at −70°C. The bacteria were cultured by plating on Trypticase soy agar containing 5% sheep blood (BBL, Cockeysville, Md.) at 35°C for 24 h.

Biochemical and phenotypic characterization. All isolates were biochemically characterized by recommended procedures (33). Acid production from the following substrates was evaluated: D-glucose, D-xylose, D-mannitol, lactose, sucrose, maltose, L-rhamnose, and α-methyl-D-mannoside. Serotyping was done by the method of Seeliger and Hohne (30).

MEE analysis. MEE analysis was conducted in 11.5% starch gels at pH 8.0 (26). Bacterial extracts were assayed by the method of Selander et al. (31) for enzyme mobilities as described previously (3). The electrophoretic mobilities of 16 enzymes were determined as described by Bibb et al. (4). Electrophoretic mobility variants of each enzyme were assigned different allele numbers. Each unique combination of alleles was designated an electrophoretic type (ET). Genetic relationships among ETs were demonstrated by a dendrogram generated by the average-linkage method of clustering from a matrix of pairwise coefficients of weighted distance (31, 32) by using an SAS macro subroutine developed by Jacobs (16).

DNA isolation. DNA isolation and purification were done by the method of Graves and Swaminathan (13).

Digestion with restriction endonuclease, gel electrophoresis, and Southern blotting. *L. monocytogenes* genomic DNA (1 μg) was restricted with *Eco*RI (New England Biolabs, Beverly, Mass.) in a total volume of 50 μl according to the enzyme manufacturer's instructions. Restricted DNA was electrophoresed on 1.0% agarose (Molecular biology grade; BRL, Gaithersburg, Md.) in Tris-acetate buffer at 4.5 V/cm in a horizontal electrophoresis chamber (Horizon 20 · 25 Gel Electrophoresis System; BRL) for 16 to 18 h. A molecular size marker (an *Eco*RI-*Bam*HI mixed digest of adenovirus type 2 DNA; International Biotechnologies, New Haven, Conn.) was included in the two end lanes and in the middle of the gel. DNA fragments in the gel were stained with ethidium bromide and were then placed on a UV transilluminator and photographed. DNA restriction fragments were transferred from the gel to a nylon membrane (Magnagraph; MSI Micron Separations, Westboro, Mass.) by Southern blotting (19). The DNA was cross-linked to the nylon membrane at 120,000 μJ/cm^2 with a Stratalinker 2400 instrument (Stratagene, La Jolla, Calif.). The membranes with the immobilized DNA were stored at 4 to 6°C until use.

Isolation and labeling of plasmid DNA. Plasmid pKK3535, a pBR322-derived plasmid containing the *rrnB* rRNA operon of *Escherichia coli* (8), was used as the probe for rDNA fingerprinting. Plasmid pKK3535 was isolated from *E. coli* ED8654 by the method of Ish-Horowicz and Burke (15). The plasmid was digested with *Eco*RI. Restricted DNA was purified by phenol-chloroform extraction, concentrated by ethanol precipitation in the presence of 0.3 M sodium acetate, washed once with 70% ethanol, and dried. The restricted plasmid was resuspended in deionized water, denatured by heating at 95°C for 10 min, and quick chilled on ice. Denatured DNA (2 μg) was labeled with digoxigenin-11-dUTP by random priming by the protocol furnished by Boehringer Mannheim (Indianapolis, Ind.). Each batch of newly synthesized labeled DNA was tested for uptake of the digoxigenin label by the protocol recommended by the manufacturer. The digoxigenin-labeled probe was either used immediately or stored at −20°C for up to 1 year.

TABLE 1. Subtypes of *L. monocytogenes* isolates included in the study

Ribotype	Serotype (no. of strains)	Enzyme type (no. of strains)
α01	1/2a (26)	08 (2), 11 (1), 12 (1), 13 (22)
α02	1/2a (7)	02 (1), 03 (2), 04 (1), 15 (2), 32 (1)
α03	1/2a (1)	05 (1)
α04	1/2a (7), 1/2c (4)	01 (1), 16 (1), 20 (3), 28 (1), 29 (1), 30 (3), 31 (1)
α05	1/2a (1)	10 (1)
α06	1/2a (6)	19 (6)
α07	1/2a (6), 3a (1)	07 (1), 17 (1), 22 (2), 23 (1), 24 (1), 26 (1)
α08	1/2a (2), 3a (1)	09 (1), 19 (1), 27 (1)
α09	1/2a (3), 3a (1)	18 (1), 21 (1), 25 (2)
α10	1/2a (2)	14 (2)
α11	3a (1)	06 (1)
β01	1/2b (25), 3b (3), 4b (6)	36 (5), 39 (21), 41 (3), 43 (1), 49 (1), 74 (1), 75 (2)
β02	1/2b (3), 4b (1)	50 (1), 51 (1), 52 (1), 63 (1)
β03	1/2b (5)	54 (5)
β04	1/2b (9), 3b (2)	33 (1), 50 (1), 69 (1), 71 (1), 72 (7)
β05	1/2b (32), 4ab (1), 4b (58)	35 (5), 36 (42), 37 (1), 38 (1), 39 (9), 40 (1), 42 (2), 44 (1), 45 (3), 46 (1), 47 (1), 48 (1), 50 (9), 54 (1), 56 (1), 57 (1), 60 (1), 63 (1), 72 (9)
β06	1/2b (6), 4b (54)	36 (1), 39 (4), 63 (41), 64 (1), 65 (2), 67 (1), 68 (3), 70 (1), 72 (2), 73 (1), 74 (1), 76 (2)
β07	4b (2)	63 (2)
β08	4b (9)	34 (1), 53 (2), 55 (2), 56 (4)
β09	4b (3)	39 (1), 53 (1), 59 (1)
β10	1/2b (1), 4b (1)	58 (1), 63 (1)
β11	1/2b (3), 4b (1)	48 (1), 57 (1), 77 (1), 78 (1)
β12	1/2b (1)	39 (1)
β13	1/2b (3)	50 (1), 62 (1), 66 (1)
β14	1/2b (2), 4b (1)	61 (2), 63 (1)
β15	1/2b (1)	72 (1)
β16	4b (2)	36 (1), 38 (1)
β17	1/2b (1)	39 (1)

Hybridization and color development. Prehybridization, hybridization with digoxigenin-labeled probe, posthybridization washing, and immunologic detection were performed by the protocol in the "Genius" nonisotopic labeling and detection kit (Boehringer Mannheim), except that posthybridization washing and blocking of nonspecific reactions were done at 60°C instead of the recommended 68°C.

Data acquisition. The data were acquired by use of a BioImage System (BioImage/Millipore, Ann Arbor, Mich.) with Whole-Band Analysis software and by visual examination. The molecular sizes of the different fragments were determined by interpolation from the sizes of the fragments generated from a molecular size standard (adenovirus type 2 DNA). Ribotype

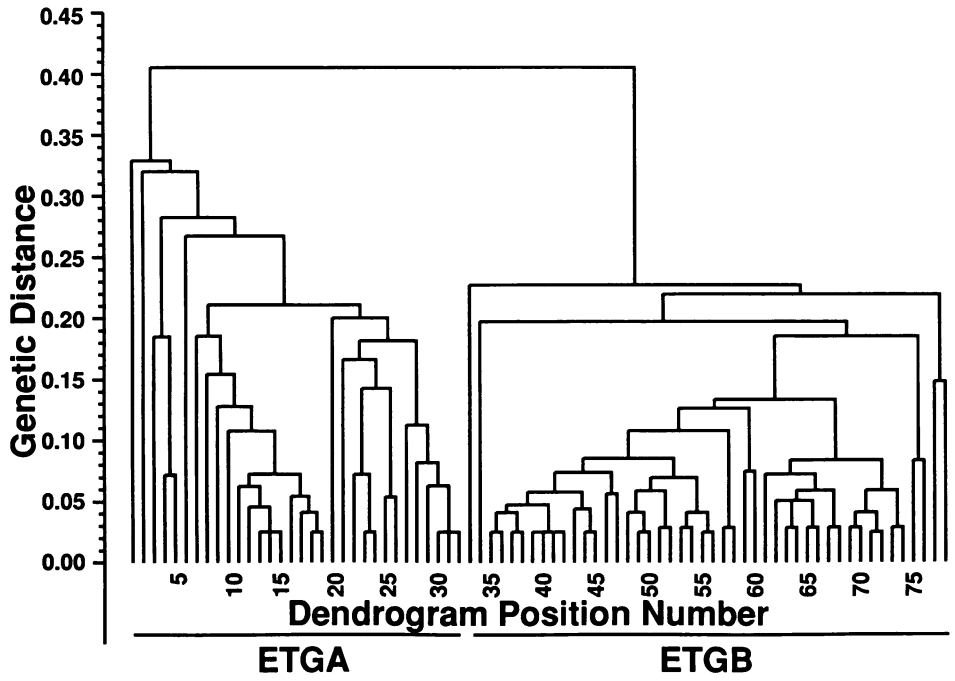

FIG. 4. Dendrogram showing the relationship among electrophoretic types of *L. monocytogenes*.

patterns not analyzed by Whole-Band Analysis software were visually compared with the ribotype patterns of representative strains which had been analyzed by Whole-Band Analysis software.

Analysis of data. The similarity between any two restriction patterns was evaluated by the method of Plikaytis et al. (25) by using a window size equal to 2% of the fragment's molecular size. A matrix of similarity coefficients was created, and a dendrogram was generated by the unweighted pair group method with arithmetic averages (32) and the SAS programming language (SAS Institute, Inc., Cary, N.C.).

Discrimination index. Simpson's Index for Diversity (DI) was determined by the method of Hunter and Gaston (14).

RESULTS

Serotypes of *Listeria* species. The serotype distribution of the 305 *L. monocytogenes* isolates included in the study was as

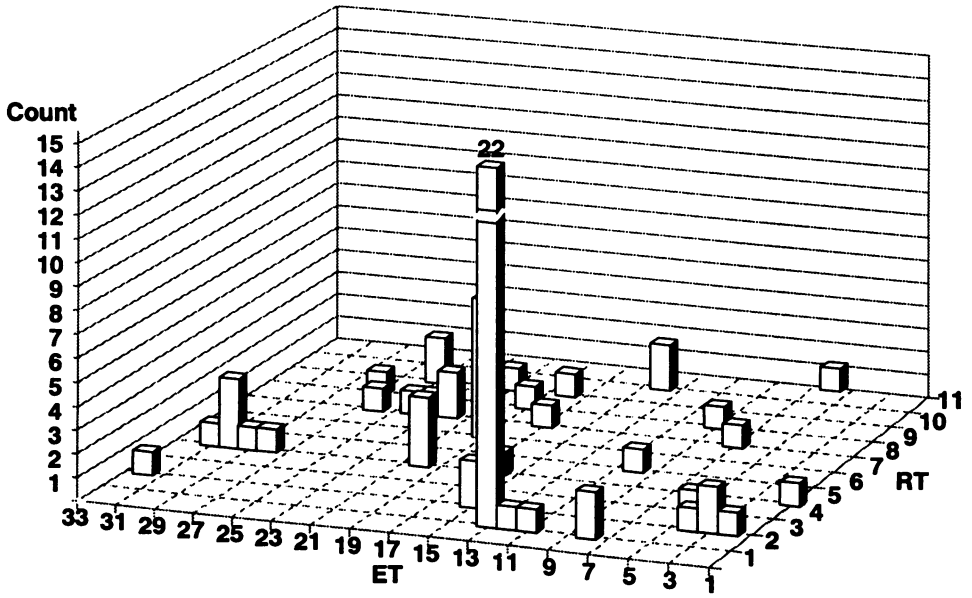

FIG. 5. Three-dimensional frequency histogram showing the separation of *L. monocytogenes* serotypes 1/2a, 1/2c, and 3a (group A) by a combination of MEE and ribotyping. ET indicates the ET determined by MEE. RT, ribotype.

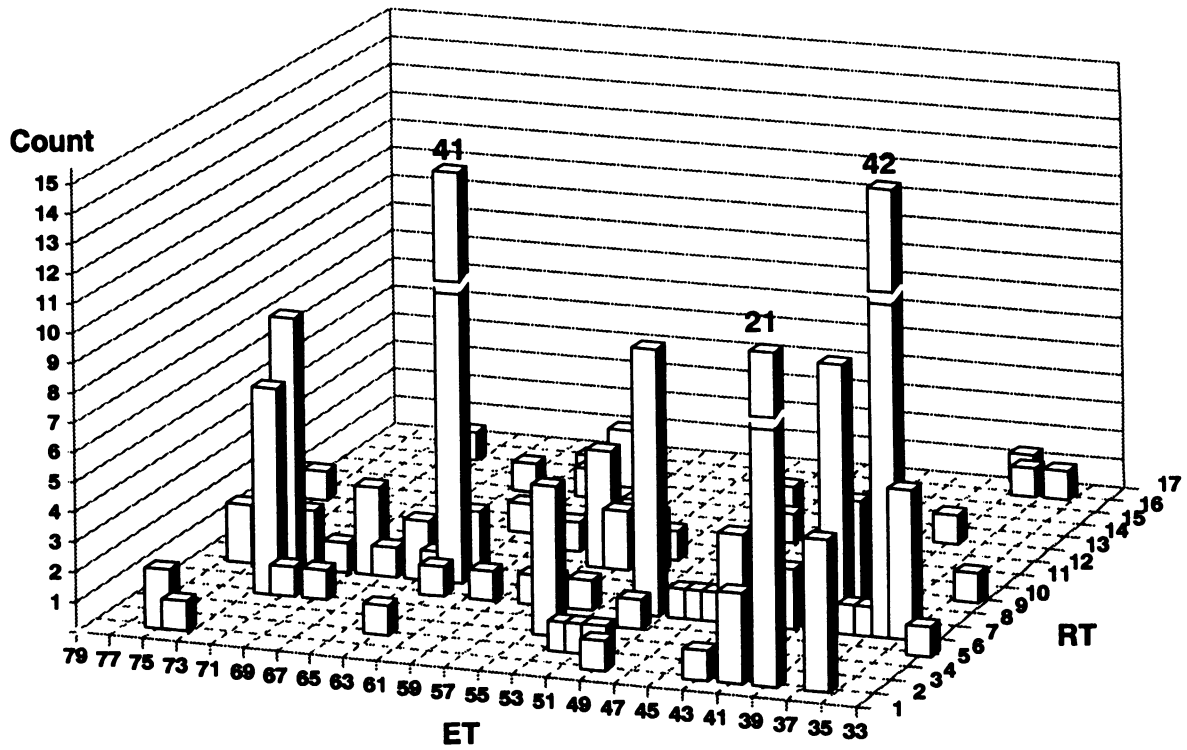

FIG. 6. Three-dimensional frequency histogram showing the separation of *L. monocytogenes* serotypes 1/2b, 3b, 4b, and 4ab (group B) by a combination of MEE and ribotyping.

follows: 1/2a, 61 (20%); 1/2c, 4 (1.3%); 3a, 4 (1.3%); 4b, 138 (45.3%), 1/2b, 92 (30.2%); 3b, 5 (1.6%); and 4ab, 1 (0.3%).

Ribotypes. Each unique ribosomal DNA restriction profile was designated a ribotype. Lanes 9 and 14 in Fig. 1 were initially thought to be different ribotype patterns but were later found to be the same ribotype. Twenty-eight ribotypes were found among the 305 strains (Fig. 1 and 2). The 28 ribotypes clustered into two groups. A dendrogram generated from the ribotyping data with a 2% window (Fig. 3) shows the separation of the strains into the two distinct clusters at a Dice similarity coefficient of 0.41. The two clusters were designated ribotype α (RTα) and ribotype β (RTβ). The 11 ribotypes in RTα contained serotypes 1/2a, 1/2c, and 3a, and the 17 ribotypes in RTβ contained serotypes 1/2b, 3b, 4b, and 4ab. The overall DI for ribotyping for all strains was 0.85 ($DI_{RT\alpha}$ = 0.81; $DI_{RT\beta}$ = 0.76).

A total of 211 (69%) of the strains were distributed among four ribotypes. The distribution of strains in each of these four ribotypes was as follows: RTα01, 26 (9%); RTβ01, 34 (11%); RTβ05, 91 (30%); and RTβ06, 60 (20%). Ninety-one (30%) strains were distributed over the 24 remaining ribotypes, with 1 to 11 strains (mean = 3.83 strains) per ribotype (Table 1).

MEE analysis. A total of 78 distinct ETs were found among the 305 strains tested. Fifteen of 16 enzymes tested were polymorphic; aconitase was monomorphic. Indophenol oxidase and phosphoglucose isomerase varied only in three strains, two patient isolates of serotype 1/2b and one patient isolate of serotype 1/2a. One hundred eighty-six strains clustered in six major ETs: ET 13 (7% of all strains), ET 36 (16%), ET 39 (12%), ET 50 (4%), ET 63 (15%), and ET 72 (6%). The remaining 119 (39%) strains were separated into 72 ETs.

Analysis of the dendrogram generated from MEE data revealed that two large groups (groups A and B) existed among the strains tested and are separated by a genetic distance of 0.410 (Fig. 4). Group A contained 69 (23%) strains consisting of serotypes 1/2a, 1/2c, and 3a. In this group the strains were more heterogeneous (32 ET types). ET 13 contained 22 (32%) strains, which were all serotype 1/2a. Forty-seven (68%) strains were separated into 31 ETs. Group B contained serotypes 1/2b, 4b, 3b, and 4ab. In this group 164 (69%) strains clustered into five ETs (ETs 36, 39, 50, 63, and 72). Seventy-two (31%) strains were separated into 41 ETs. The overall DI for all electrophoretic types was = 0.92 (DI_{ETGA} = 0.89 and DI_{ETGB} = 0.88, where ETGA and ETGB are ET groups A and B, respectively).

MEE and ribotyping. The three-dimensional frequency histograms in Fig. 5 and 6 show the separation of *L. monocytogenes* strains into different subtypes when the results of MEE and ribotyping were combined and plotted. Figure 5 shows one major cluster (defined as the grouping of 10 or more strains) of 22 strains which were found in RTα01 and ET13.

Figure 6 shows three major clusters of serotypes 1/2b, 3b, 4b, and 4ab, in which the strains grouped in the same ET and ribotype. Twenty-one strains clustered in ET 39 and RTβ01, 42 strains clustered in ET 36 and RTβ05, and 41 strains clustered in ET 63 and RTβ06.

The DI for MEE and ribotyping for all strains was 0.95 ($DI_{MEE + RT}$[ETGA/RTα] = 0.89; $DI_{MEE + RT}$[ETGB/RTβ] = 0.92), where RT is ribotyping).

DISCUSSION

Ribotyping divided the strains into two distinct groups, RTα and RTβ. The strains that clustered in each of these groups still shared some common fragments. For example, RTα03 and RTβ08 each had 15 fragments, but only 8 fragments were

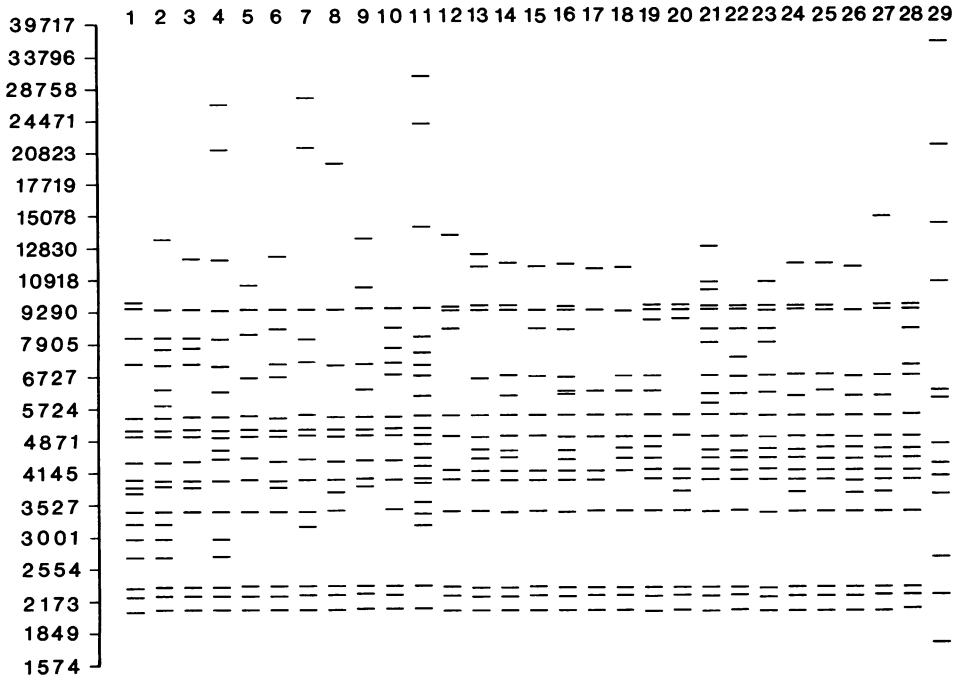

FIG. 7. Composite representation of 28 *L. monocytogenes* ribotypes after computer-based image acquisition, normalization, and analysis by the BioImage system. Lanes 1 to 11, RTα01 to RTα11 (serotype 1/2a), respectively; lanes 12 to 16, RTβ01 to RTβ05 (serotype 1/2b), respectively; lanes 17 to 20, RTβ06 to RTβ09 (serotype 4b), respectively; lane 21, RTβ10 (serotype 1/2b); lane 22, RTβ11; (serotype 4b); lanes 23 to 26, RTβ12 to RTβ15 (serotype 1/2b), respectively; lane 27, RTβ16 (serotype 4b); lane 28, RTβ17 (serotype 1/2b); lane 29, adenovirus type 2 DNA marker (13 DNA fragments with the following lengths [in base pairs]: 35,937, 21,338, 14,331, 10,680, 6,204, 5,935, 4,723, 4,295, 4,027, 3,673, 2,674, 2,218, and 1,739). (Some faint bands seen in Fig. 1 and 2 do not appear in this normalized composite pictogram either because they were not recognized by the "Find Band" function by using the band-intensity and band-width parameters chosen for BioImage Whole-Band Analysis software or because they were deleted because of inconsistent results in replicate runs.)

common to both ribotypes (Fig. 7). Five fragments (9.40, 5.40, 4.95, 2.30, and 2.10 kbp) were common to all isolates of RTα and RTβ. Interestingly, RTα has a unique fragment of 5.10 kbp; this fragment was absent from all isolates of RTβ.

Ribotyping discriminated between RTα strains (mean of 6.3 strains per ribotype) than it did between RTβ strains (mean of 13.9 strains per ribotype). However, two RTα ribotypes contained more than 10 strains (RTα01, 26 strains; RTα04, 11 strains). Of the 26 strains in RTα01, 18 (69%) were epidemiologically associated with a single sporadic cases of listeriosis (9, 24). They included 1 patient isolate, 13 isolates from foods obtained from the patient's refrigerator, and 4 isolates from the factory where the contaminated turkey franks were made. The same strains also clustered with four other strains in a single ET (ET 13). Because they were associated with a single sporadic case of listeriosis, they probably represent a clone.

Four large clusters were found in the RTβ group (RTβ01, 34 strains; RTβ04, 11; RTβ05, 91; RTβ06, 60). Twenty-one of the 34 strains in RTβ clustered in ET 39. All were serotype 1/2b. Thirteen isolates were from San Francisco (nine patient isolates and four food isolates). All four food isolates were from raw chicken obtained from a patient's refrigerator and were probably a single clone. No obvious epidemiologic associations were evident for the other isolates that clustered in RTβ01 and ET 39.

RTβ04 contained 4.7% of the RTβ isolates. Of 11 isolates in RTβ04, nine were serotype 1/2b and 2 were serotype 3b. Seven of the isolates (Five of serotype 1/2b and two of serotype 3b) were ET 72 and four were divided into four separate ETs. The strains in this group were not associated with epidemics.

RTβ05 contained 38.6% of the RTβ isolates. Of the 91 isolates in RTβ05, 42 clustered in ET 36 and the remaining isolates were distributed among 18 ETs. The isolates that clustered in RTβ05 and ET 36 included eight isolates associated with a sporadic case of listeriosis in which Boo Dan (Boudin Link), a traditional Cajun pudding of cooked rice and pork in a 5-in. (12.7-cm) raw sausage casing, was implicated as the probable vehicle of transmission (1). This group also included nine isolates from the outbreak of listeriosis in Massachusetts in which 2% pasteurized milk was epidemiologically implicated (10).

Of 60 isolates in RTβ06, 41 clustered in ET 63 and the remaining strains were distributed among 11 ETs. The 41 isolates in RTβ06 and ET 63 included 12 isolates associated with the 1985 listeriosis outbreak attributed to contaminated Mexican-style cheese, 5 isolates from the 1981 outbreak in Nova Scotia attributed to contaminated cole slaw, 2 isolates from the 1987 to 1989 outbreak in Switzerland associated with the consumption of contaminated soft cheese, and 7 isolates from an outbreak in Pennsylvania (29).

Several studies describing the application of ribotyping to the subtyping of *L. monocytogenes* were cited by Nocera et al. (22). In some of these studies, investigators subtyped small numbers of isolates. Although their observations demonstrated the potential of the method, additional conclusions could not be drawn from these studies because of the small number of isolates examined.

Norrung and Gerner-Smidt (23) subtyped 88 *L. monocytogenes* isolates (30 of serotype 1 and 58 of serotype 4). The discriminating ability of ribotyping was better for serotype 1/2

(DI = 0.87) than for serotype 4 (DI = 0.53). Jacquet et al. (17) ribotyped 94 isolates of *L. monocytogenes* obtained from diverse sources and separated them into 14 ribotypes by using *Eco*RI for restriction. Unlike our study and other studies, Jacquet et al. (17) used a probe containing *Bacillus subtilis* 16S rDNA as the probe; therefore, their results are not directly comparable to ours. Nevertheless, 58% of the isolates ribotyped by Jacquet et al. (17) were clustered in a single ribotype; 74% of the isolates in the cluster belonged to serotype 4b.

Clustering of serotype 4b isolates in a few ribotypes was also observed by Nocera et al. (22). Of 96 serotype 4b strains examined, 61 clustered in a single ribotype. Of these, 34 were associated with epidemic listeriosis in Switzerland (5) and could be divided into two distinct phage types. Even if all 34 Swiss outbreak-associated strains are considered to be a single clone, 28 of 63 serotype 4b strains still clustered in a single ribotype.

MEE was generally more discriminating than ribotyping, although there were several instances in which ribotyping differentiated between strains within a single enzyme type. The combination of MEE and ribotyping increased the discrimination index for serotypes 1/2b and 4b from 0.88 (MEE) and 0.76 (ribotyping) to 0.92 (MEE plus ribotyping), but 55% of the strains still clustered in three ET groups by ribotyping. When epidemiologically linked isolates were considered to be a single strain and DIs were recalculated, the DIs were as follows: $DI_{RT\ overall\ +\ MEE\ overall} = 0.97$, $DI_{RT\alpha\ +\ MEE/ETGA} = 0.97$, $DI_{RT\beta\ +\ MEE/ETGB} = 0.95$, $DI_{RT\ overall} = 0.86$, $DI_{RT\alpha} = 0.88$, $DI_{RT\beta} = 0.78$, $DI_{MEE\ overall} = 0.95$, $DI_{MEE/ETGA} = 0.97$, and $DI_{MEE/ETGB} = 0.92$.

Because MEE and ribotyping measure changes in different loci on the chromosome, it is tempting to conclude that serotypes 1/2b and 4b are of a recent lineage and have not diverged to the extent that serotypes 1/2a and 1/2c have. However, recent data from the subtyping of *L. monocytogenes* by PFGE argue against such a conclusion. Brosch et al. (7) demonstrated that strains of *L. monocytogenes* serotype 4b that clustered in a single ribotype could be divided into several PFGE types. The greater discriminating ability of PFGE for other gram-positive bacteria (*Enterococcus faecalis*) compared with that of ribotyping has also been reported (11).

Recently, random amplified polymorphic DNA (RAPD) analysis has been shown to be useful for subtyping *L. monocytogenes* strains that cluster in the same ET and same ribotype. With respect to its discriminating ability, RAPD analysis may be equal to (20) or better than (18) bacteriophage typing. Because RAPD analysis is a PCR-based technique, it can be completed in a short time.

In conclusion, ribotyping is an acceptable method for subtyping *L. monocytogenes* serotypes 1/2a, 1/2c, and 3a but does not appear to possess adequate discriminating ability for subtyping *L. monocytogenes* serotypes 1/2b and 4b. Increasing the number of restriction enzymes will increase the work but may not produce a concomitant increase in the discriminating ability (2, 23). Of the other established *L. monocytogenes* subtyping methods, phage typing suffers from the lack of reproducibility and many strains are not typeable, MEE also causes clustering of serotypes 1/2b and 4b, and restriction endonuclease analysis is not suitable for large-scale analysis and comparison of profiles. Therefore, there is a need to explore new and promising subtyping methods such as PFGE and RAPD for subtyping *L. monocytogenes* serotypes 1/2b and 4b.

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