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Molecular Epidemiology and Cluster Analysis of Human Listeriosis Cases in Three U.S. States

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ABSTRACT

To better understand the transmission and epidemiology of human listeriosis, 647 *Listeria monocytogenes* isolates obtained from human listeriosis cases in four U.S. locations (Michigan, Ohio, New York State, and New York City) over 61 months (1998 to 2003) were characterized by automated *Eco*RI ribotyping. A total of 65 ribotypes were differentiated among the characterized isolates; 393, 227, and 24 isolates were classified into lineages I, II, and III, respectively, and 3 isolates were not classified to lineage. The three most common ribotypes (responsible for 39% of all cases) represented *L. monocytogenes* epidemic clones, each of which had previously been linked to at least two human listeriosis outbreaks. Categorical analyses revealed that ribotypes and lineages were nonrandomly distributed among the four locations. Temporal cluster analysis of cases identified 13 statistically significant temporal subtype clusters, which represented 26% of all cases. Three of these clusters matched previously described human listeriosis outbreaks. Isolates involved in clusters belonged to nine ribotypes. Four, eight, and one cluster were caused by lineages I, II, and III, respectively. The two largest clusters were both caused by the epidemic clone representing ribotype DUP-1044A. Categorical analyses revealed no significant associations between lineage or ribotype and clinical manifestation (central nervous system infection, septicemia, fetal infection, or other infection) or disease outcome (fatal or not fatal). Although human listeriosis cases are caused by isolates belonging to a diversity of *Eco*RI ribotypes, specific lineage I epidemic clones cause a large number of human listeriosis cases. Many human listeriosis cases can be grouped into statistically significant temporal clusters, including widely distributed and region-specific clusters associated with isolates of various ribotypes. *L. monocytogenes* lineages and *Eco*RI ribotypes do not appear to differ in their likelihood of causing different clinical manifestations or mortality.

Listeria monocytogenes can cause severe invasive disease, including septicemia, spontaneous abortion, and central nervous system (CNS) infections *(32).* Human infections occur most often in particularly susceptible hosts, including pregnant women and elderly and immunocompromised individuals *(32).* Although *L. monocytogenes* has been isolated from a variety of raw and ready-to-eat foods, most human listeriosis infections appear to be caused by consumption of contaminated ready-to-eat foods *(34).* Human listeriosis is a somewhat rare disease with prevalence ranging from one to five cases per million people per year in most developed countries *(34).* Mead et al. *(23)* estimated that a total of 2,500 human listeriosis cases (500 of which result in death) occur annually in the United States. However, total numbers of cases seem to have declined over the last few years *(35).* Although a number of human listeriosis outbreaks have been described *(2, 22),* ranging in scope from relatively short duration and geographically defined *(21)* to multiyear and geographically dispersed, the majority of human listeriosis cases are thought to be sporadic *(32).*

L. monocytogenes strains have been grouped into two major lineages, I and II, based on various molecular subtyping strategies including ribotyping, multilocus enzyme electrophoresis, pulsed-field gel electrophoresis (PFGE), and virulence gene sequencing *(37).* Strains of serotypes 1/2b, 4b, 3b, and 3c group consistently into lineage I, whereas strains of serotypes 1/2a, 1/2c, and 3a group into lineage II *(25). L. monocytogenes* lineage III strains *(37)* include isolates with serotypes 4a, 4b, and 4c *(25, 36).* Some other researchers have suggested that *L. monocytogenes* subtypes and lineages appear to differ in their associations with specific host and nonhost environments *(13, 26, 27, 38).* The majority of sporadic human listeriosis cases appear to be caused by strains of serotypes 4b and 1/2b, whereas most human listeriosis outbreaks have been caused by serotype 4b strains *(37, 38).* Outbreaks have rarely been caused by non-4b serotypes; serotype 3a and 1/2a strains caused one outbreak each *(21, 28).* In a previous study *(17),* lineage I strains were more common among human cases and outbreaks than among animal cases, whereas lineage III strains were significantly more common among animal cases than among human cases.

Although molecular subtyping of 131 human *L. monocytogenes* isolates collected in a single region (New York

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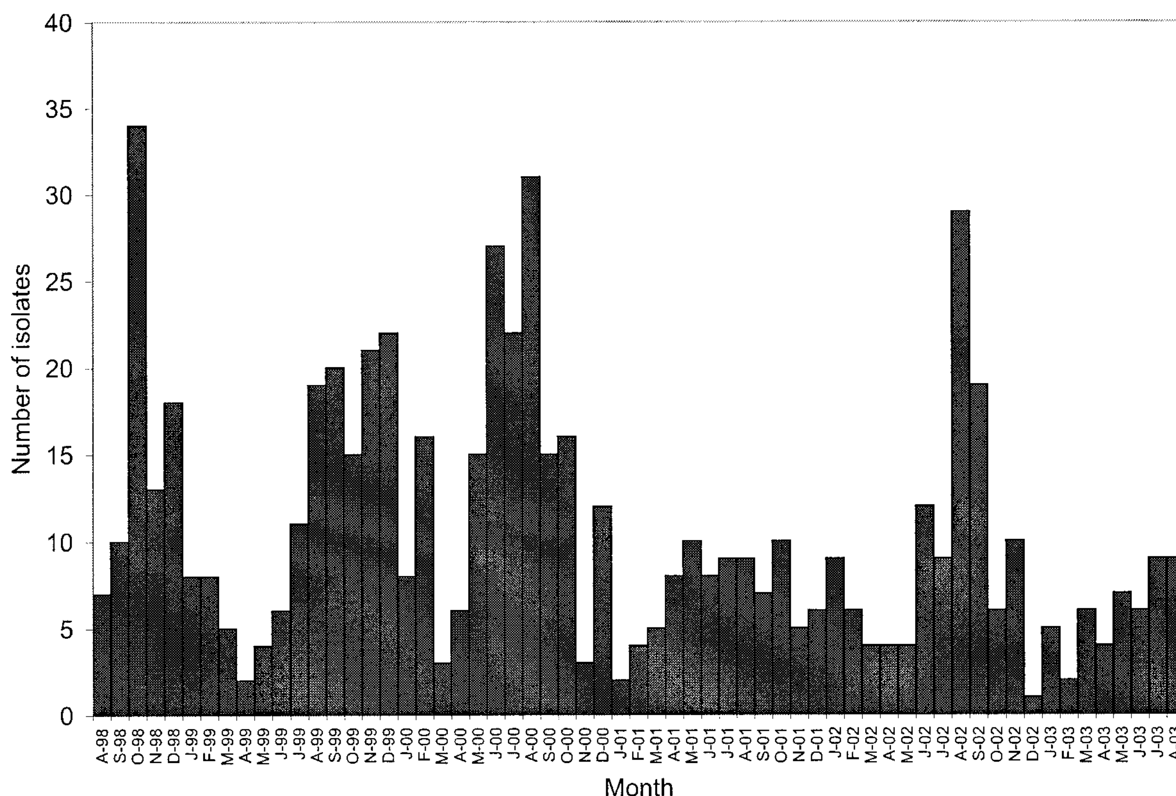

FIGURE 1. *Distribution of human listeriosis cases by month (August 1998 through August 2003). Numbers represent all* L. monocytogenes *isolates from human listeriosis cases reported to health departments in Michigan, New York City, New York State, and Ohio in a given month.*

State excluding New York City) revealed that a larger number of human listeriosis cases than previously assumed may be clustered *(30),* studies of larger data sets of human *L. monocytogenes* isolates from different geographic regions are needed to further understand the transmission and epidemiology of human listeriosis, including the prevalence of listeriosis clusters. Thus, we characterized a large set of human *L. monocytogenes* isolates from different regions (i) to determine the prevalence of listeriosis cases representing clusters and (ii) to determine whether *L. monocytogenes* genetic subtypes and lineages differ in their likelihood of causing specific clinical manifestations or fatalities. Although PFGE is considered the "gold standard" for subtype discrimination of *L. monocytogenes* isolates and often discriminates multiple PFGE types within a given ribotype *(30),* we chose *Eco*RI ribotyping for our study because of the reproducibility that can be achieved with a fully automated ribotyping system *(8),* an important consideration when a large set of >600 isolates were to be characterized. Although only limited *L. monocytogenes* PFGE data sets are currently available for comparisons, use of *Eco*RI ribotyping also allowed subtype comparisons with results of previous studies, including a recent study in which more than 500 *L. monocytogenes* food isolates collected in the United States were characterized by *Eco*RI ribotyping *(14).*

MATERIALS AND METHODS

Isolates and case reporting. *L. monocytogenes* isolates were collected from human listeriosis cases reported to health departments in three U.S. states (Michigan [MI], New York State [NYS], and Ohio [OH]) and one large U.S. city (New York City [NYC]). These regions were chosen based on the willingness of the health departments to participate in this collaborative study. There are two health registration districts in New York. Communicable diseases occurring in residents of the five counties of New York City (Bronx, Kings, New York, Queens, and Richmond) are reported to the New York City Department of Health and Mental Hygiene, and those occurring in residents of the remaining 57 NYS counties are reported to the New York State Department of Health. For the purposes of this analysis, NYC and NYS were considered separate regions because of differences in the communicable disease surveillance systems of the two areas. The isolates included in this analysis represent all isolates submitted to the four health departments between 1 August 1998 and 31 August 2003 (5 years and 1 month). For NYS, this isolate set included some but not all of the isolates previously reported in an evaluation of subtyping and statistical methods used to detect human listeriosis clusters *(30)*; of the isolates from this previous study, only those that were collected between 1 August 1998 and 30 June 2000 were also included in the analyses reported here. Some isolates in the NYS data set also were used in a comparison of *L. monocytogenes* subtypes from human, food, and food environment sources *(31).* Overall, a total of 647 human isolates of *L. monocytogenes* were obtained from residents of MI (87), NYC (163), NYS (222), and OH (175) for a period of 61 months. The number of isolates obtained per month ranged from 1 (December 2002) to 34 (October 1998) (Fig. 1). The annualized average listeriosis rate ranged from a low of 0.17 cases per 100,000 people per year in MI to a high of 0.40 cases per 100,000 people per year in NYC.

In addition to routine identification of isolates as *L. monocytogenes* by each public health department laboratory (by conventional phenotypic and/or genetic methodology), all isolates

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also were confirmed as *L. monocytogenes* by *Eco*RI automated ribotyping as described here. One isolate per case was analyzed; therefore, each isolate in this study represents a single unique human listeriosis case. In cases where isolates were obtained from multiple specimen sites, we preferentially analyzed isolates from CNS sites over other sterile sites and isolates from other sterile sites over nonsterile sites. If multiple isolates were obtained from both mother and fetus, only the fetal isolate was included in the analysis.

Isolate and case report data included demographic, epidemiologic, and clinical characteristics such as state and county of residence, age, sex, clinical manifestation, outcome (fatal or nonfatal), and perinatal transmission. Clinical manifestation was categorized as fetal infection, CNS infection, septicemia, or other, based on the site of specimen isolation and/or case report. Fetal infections included those in babies <30 days old and/or those in which *L. monocytogenes* was isolated from amniotic fluid, placenta, or aborted fetuses. Case reports for pregnant women indicating fetal involvement also were classified as fetal infection. CNS infections were defined as those in which *L. monocytogenes* was isolated from cerebrospinal fluid or CNS tissue regardless of clinical symptoms or was isolated from blood with case reports of meningitis and/or encephalitis. Septicemia cases were defined as those in which *L. monocytogenes* was isolated from blood or an otherwise sterile site, excluding cerebrospinal fluid, CNS tissue, amniotic fluid, or placenta. Case reports involving isolations from nonsterile sites were categorized as "other infections." Overall, 52.6% of listeriosis cases occurred in females (Table 1). Septicemia was the most common clinical manifestation (74.2%), followed by fetal infection (9.4%), CNS infection (9.1%), and other infection (1.9%). There was insufficient case history data available to categorize the clinical manifestation for 5.4% of cases (Table 1). The majority of cases occurred in patients older than 60 years (60.1%), although 7.9% of isolates were obtained from patients of age 30 days or younger.

Automated ribotyping and genetic lineage classification. All isolates identified as *L. monocytogenes* were characterized by automated ribotyping using the restriction enzyme *Eco*RI and the RiboPrinter Microbial Characterization System (Qualicon Inc., Wilmington, Del.) *(8)*. Ribotype designations (e.g., DUP-1039) assigned by the RiboPrinter were confirmed by visual inspection of the bands. When visual inspection indicated that a given DuPont ID included more than one distinct ribotype pattern, each pattern was designated by an alphabetically assigned letter suffix (e.g., DUP-1044A and DUP-1044B represent two distinct ribotype patterns within DuPont ID DUP-1044). Distinct ribotype patterns within a given DuPont ID generally differed only by position of a single weak band. When a ribotype pattern did not match a DuPont ID pattern with a similarity greater than 0.85, a type designation was assigned manually based on the ribogroup assigned by the instrument (e.g., ribogroup 116-363-S-2). Isolates were assigned to one of the three *L. monocytogenes* lineages (I, II, or III) based on *Eco*RI ribotypes *(38)*.

Categorical analysis. Associations between geographic origin (i.e., MI, NYC, NYS, and OH), molecular subtypes (ribotype and lineage), clinical manifestation (fetal infection, CNS infection, septicemia, or other infection), and disease outcome (fatal or nonfatal) were determined based on categorical analysis with a chi-square test or Fisher's exact test when expected values were less than 5. All categorical analyses were performed with SAS 9.1 (SAS Institute Inc., Cary, N.C). Results with P values of $\leq$0.05 were considered significant and were not adjusted for multiple comparisons. Because of the large number of associations that were tested, the probability of a type 1 error may have been inflated, necessitating the adjustment of the significance threshold, e.g., through a Bonferroni correction. We choose to provide observed P values to avoid missing possible associations (as could occur with a very conservative P value); thus, the reader is not limited to an arbitrary interpretation of the threshold for significance and can evaluate significance according to any preferred criterion *(14, 29)*.

Temporal cluster detection. A scan statistic was used for the detection of temporal clusters of cases in each region separately and in all regions combined. The scan statistic maintains the assumption that an underlying Poisson distribution and a stable at-risk population over time describes the occurrence of rare events and tests the null hypothesis that the incidence of events within a given time window is equal to the incidence of events outside the time window. In a previous study *(30)*, we evaluated the use of both 1- and 3-month window sizes and found that 3-month windows were more suitable for detecting listeriosis clusters. In the present study, we calculated the scan statistic with the Clusterseer software package (version 2.2.4, http://www.terraseer.com) using a 3-month scanning window with Monte Carlo simulation (999 replicates) estimation of P values. Results were considered significant at $P \leq 0.05$.

Isolate and data curation. *L. monocytogenes* isolates were frozen at −80°C in brain heart infusion broth containing 15% glycerol. Isolate information and subtyping data from this study are archived and freely available through the Pathogen Tracker 2.0 database (http://www.pathogentracker.net).

RESULTS

***Eco*RI ribotype and lineage analysis.** Sixty-five different ribotypes were identified among the 647 isolates. Lineages I, II, and III represented 393, 227, and 24 isolates, respectively. Three isolates represented two ribotypes (116-967-S-4 and DUP-14003) that had not previously been identified and thus were not classified to lineage. The three most common ribotypes (DUP-1044A, DUP-1042B, and DUP-1038B) were all classified as lineage I and represented 16.8, 13.0, and 9.4% of isolates, respectively. The two most common lineage II ribotypes (DUP-1039B and DUP-1053A) represented 7.0 and 6.2% of the isolates, respectively.

Distribution of ribotypes and lineages by region. Overall categorical analyses were performed to determine whether genetic lineages and/or *Eco*RI ribotypes were significantly associated with any of the four regions. Categorical analysis for lineages (chi-square for four lineages [I, II, III, and UNK] by four regions) revealed that lineages were not randomly distributed among regions ($P = 0.0097$). Subsequent 2×2 chi-square analyses revealed that lineage I and II isolates were significantly more common in NYC and OH, respectively (Table 2).

Categorical analysis of *Eco*RI ribotypes (chi-square analysis of 20 individual ribotypes with five or more occurrences and ribotypes that occurred less than five times classified as a single group by four regions) revealed that ribotypes were not randomly distributed among regions ($P < 0.0001$). Subsequent chi-square analyses revealed that five lineage I and seven lineage II ribotypes were signifi-

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TABLE 1. *Descriptive epidemiology (location, sex, age, clinical manifestation, and number of fatalities) for 647 human listeriosis cases from four regions*

Region[a]	Sex	No. of cases evaluated	Median age of patient (range)[b]	No. of cases where patient age was unknown	No. of cases with infection reported as:					No. (%) of fatal cases		
					Fetal	CNS	Septicemia	Other	Unknown	Fatal	Nonfatal	Unknown
MI	Male	43	66 yr (13 days–85 yr)	2	3	4	33	3	0	0	0	43 (100)
	Female	44	64 yr (0 day–91 yr)	5	5	4	35	0	0	0	0	44 (100)
	Unknown	0	NA	0	0	0	0	0	0	0	0	0
NYC	Male	81	57 yr (1 day–92 yr)	0	4	4	68	0	5	8 (9.9)	22 (27.2)	51 (62.9)
	Female	81	63 yr (<1 day–92 yr)	1	9	3	61	0	8	9 (11.1)	15 (18.5)	57 (70.4)
	Unknown	1	NA	1	0	0	1	0	0	0	0	1 (100)
NYS	Male	100	70 yr (<1 day–98 yr)	0	11	13	74	2	0	18 (18)	54 (54)	28 (28)
	Female	121	71 yr (1 day–98 yr)	0	12	14	94	1	0	17 (14)	64 (52.9)	40 (33.1)
	Unknown	1	NA (1 day)	0	1	0	0	0	0	0	1 (100)	0
OH	Male	78	70 yr (<1 day–92 yr)	1	8	9	54	3	4	10 (12.8)	2 (2.6)	66 (84.6)
	Female	94	70 yr (8 days–94 yr)	1	8	8	60	3	15	7 (7.4)	0	87 (92.6)
	Unknown	3	NA	3	0	0	0	0	3	0	0	3 (100)
Total	Male	302	67 yr (<1 day–98 yr)	3	26	30	229	8	9	36 (11.9)	78 (25.8)	188 (62.3)
	Female	340	68 yr (<1 day–98 yr)	7	34	29	250	4	20	33 (9.7)	79 (23.2)	228 (67.1)
	Unknown	5	34.5 yr (<1 day–69 yr)	3	1	0	1	0	3	0	1 (20)	4 (80)

[a] MI, Michigan; NYC, New York City; NYS, New York State; OH, Ohio.
[b] 0 day indicates unborn fetuses.

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TABLE 2. *Distribution of* L. monocytogenes *ribotypes and genetic lineages by region (Michigan, New York City, New York State, and Ohio)*

Lineage	Ribotype[a]	No. of isolates with specific characteristics[b]				Total
		Michigan	NY City	NY State	Ohio	
I	<5 total occurrences	3	9	6	11	29
	116-363-S-2	1	0	4 (+)[c]	0	5
	DUP-1042C (18595)[d]	0	0	0	5 (+)[e]	5
	DUP-1043A	1	5	5	5	16
	DUP-1042A	4	10 (+)[c]	3	3	20
	DUP-1044B	4	4	11	7	26
	DUP-1052A	3	8	19 (+)[c]	8	38
	DUP-1038B	12	25 (+)[f]	17	7 (−)[e]	61
	DUP-1042B	9	24	32	19	84
	DUP-1044A (18611)[d]	11	34	38	26	109
	All lineage I	48	119 (+)[f]	135	91 (−)[c]	393
II	<5 total occurrences	6	8	17	4	35
	DUP-1030B	5 (+)[f]	0	2	0	7
	DUP-1062A	0	2	2	3	7
	DUP-1039E	0	0	3	5 (+)[c]	8
	DUP-1045B	2	4	5	1	12
	DUP-1062C	9 (+)[f]	1	2	0 (−)[c]	12
	DUP-1030A	3	1	6	3	13
	DUP-1039C	2	1	10 (+)[c]	3	16
	DUP-1039A	0 (−)[c]	3 (−)[c]	10	19 (+)[f]	32
	DUP-1053A	4	17 (+)[c]	16	3 (−)[e]	40
	DUP-1039B (16619)[d]	3	3 (−)[e]	4 (−)[f]	35 (+)[f]	45
	All lineage II	34	40 (−)[e]	77	76 (+)[e]	227
III	<5 total occurrences	2	2	4	7	15
	DUP-1061A	2	2	4	1	9
	All lineage III	4	4	8	8	24
Unknown	<5 total occurrences	1	0	2	0	3
	All unknown	1	0	2	0	3
	TOTAL	87	163	222	175	647

[a] Individual ribotypes with fewer than five occurrences among all four regions were grouped together for analysis.
[b] Within a specific region, *L. monocytogenes* genetic lineage or *Eco*RI ribotype prevalence was significantly higher (+) or lower (−) as determined by categorical analyses. A complete listing of all isolate subtype data is available online (http://www.pathogentracker.net).
[c] Genetic lineage or ribotype with a significantly higher or lower prevalence for a specific region at $P \leq 0.05$.
[d] Ribotypes DUP-1042C, DUP1044A, and DUP-1039B were recently added to the Riboprinter database and are now identified as DUP-18595, DUP-18611, and DUP-16619, respectively. Both previous and current designations are listed.
[e] Genetic lineage or ribotype with a significantly higher or lower prevalence for a specific region at $P \leq 0.005$.
[f] Genetic lineage or ribotype with a significantly higher or lower prevalence for a specific region at $P \leq 0.0005$.

cantly more common in specific regions (Table 2). For example, ribotypes DUP-1038B and DUP-1042A were significantly more common than expected in NYC (Table 2).

Temporal cluster analysis. Initial temporal cluster analysis using all isolates without stratification by *Eco*RI ribotype and by region revealed four temporal clusters of cases (August 1998 to March 1999, May 1999 to December 2000, March 2001 to November 2002, and June 2003 to August 2003). This clustering is largely consistent with the temporal case distribution (Fig. 1), which also indicated a seasonal pattern of lower case numbers in winter and early spring.

Temporal cluster analysis with isolates stratified by *Eco*RI ribotypes revealed 11 significant temporal ribotype clusters with data pooled for all regions. Temporal cluster analysis by region revealed a total of 16 ribotype clusters. All 11 clusters detected by overall analysis were also detected as regional clusters in at least one region, including three clusters that were detected in more than one region. Two clusters (clusters L and M, Table 3) were detected only in the regional cluster analyses; both occurred in NYS. Overall, a total of 13 distinct clusters were detected by our analyses (Table 3 and Fig. 2). The number of cases included in these clusters ranged from 3 to 46; overall, 168 (26%) of all human cases were part of significantly different temporal clusters.

The temporal clusters detected were associated with nine different ribotypes; four ribotypes (DUP-1039A, DUP-1053A, DUP-1039B, and DUP-1044A) were associated with more than one cluster (Fig. 2). Although the two clusters associated with ribotype DUP-1044A represented two distinct time periods (August 1998 to February 1999 and

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TABLE 3. *Temporal clustering of* L. monocytogenes *ribotypes among human listeriosis cases*

Lineage	Cluster	Ribotype	Total no. of cases in cluster	Date	No. of cases that clustered in[a]:				
					MI	NYC	NYS	OH	All regions
I	A	DUP-1044A	46	Aug. 98	0	1	0	0	1[c]
				Sep. 98	0	2	1[c]	0	3[c]
				Oct. 98	1	0	7[c]	8[c]	16[c]
				Nov. 98	1	0	2[c]	7[c]	10[c]
				Dec. 98	0	1	4[c]	6[c]	11[c]
				Jan. 99	0	1	0	2[c]	3[c]
				Feb. 99	0	1	0	1[c]	2[c]
	B	DUP-1044A	28	Jul. 02	0	1[c]	1[c]	0	2[c]
				Aug. 02	4	8[c]	7[c]	0	19[c]
				Sep. 02	0	3[c]	3[c]	0	6[c]
				Oct. 02	0	1[c]	0	0	1[c]
	C	116-363-S-2	4	Aug. 99	1	0	1[b]	0	2[c]
				Sep. 99	0	0	2[b]	0	2[c]
	D	DUP-1042C	3	Dec. 01	0	0	0	1[c]	1[b]
				Jan. 02	0	0	0	1[b]	1[b]
				Feb. 02	0	0	0	1[b]	1[b]
II	E	DUP-1039B	17	Oct. 99	1	0	0	1[c]	2[c]
				Nov. 99	0	0	0	5[c]	5[c]
				Dec. 99	0	0	0	9[c]	9[c]
				Jan. 00	0	0	0	0	0
				Feb. 00	0	0	0	1[c]	1[c]
	F	DUP-1039B	12	Jun. 00	0	0	0	1[c]	1[c]
				Jul. 00	0	0	1	2[c]	3[c]
				Aug. 00	0	0	1	4[c]	5[c]
				Sep. 00	0	0	0	2[c]	2[c]
				Oct. 00	0	0	0	1[c]	1[c]
	G	DUP-1053A	8	Sep. 99	0	2	1[b]	0	3[c]
				Oct. 99	0	1	0[b]	0	1[c]
				Nov. 99	0	1	2[b]	0	3[c]
				Dec. 99	0	0	1[b]	0	1
	H	DUP-1053A	18	May. 00	0	0	1[b]	0	1[c]
				Jun. 00	0	0	2[b]	0	2[c]
				Jul. 00	0	4[c]	1[b]	0	5[c]
				Aug. 00	2	3[c]	2[b]	0	7[c]
				Sep. 00	0	1[c]	0[b]	0	1[c]
				Oct. 00	0	1[c]	1[b]	0	2[c]
	I	DUP-1030B	6	Sep. 99	3[c]	0	1	0	4[c]
				Oct. 99	2[c]	0	0	0	2[c]
	J	DUP-1039A	10	Nov. 99	0	0	1	4[c]	5[c]
				Dec. 99	0	0	0	2[c]	2[c]
				Jan. 00	0	0	0	0	0
				Feb. 00	0	0	1	2[c]	3[c]
	K	DUP-1039A	10	Jun. 00	0	0	2	4[c]	6[c]
				Jul. 00	0	0	0	1[c]	1[c]
				Aug. 00	0	0	1	2[c]	3[c]
	L	DUP-1045B	3	Apr. 00	0	0	1[b]	0	1
				May. 00	0	0	1[b]	0	1
				Jun. 00	0	0	1[b]	0	1
III	M	DUP-1061A	3	Aug. 00	0	0	3[b]	0	3

[a] Isolates that were part of significant temporal clusters of *L. monocytogenes* ribotypes, as determined with a scan statistic using 3-month sliding windows, are indicated.
[b] Statistically significant temporal cluster with $0.05 \geq P > 0.01$.
[c] Statistically significant temporal cluster with $P \leq 0.001$.

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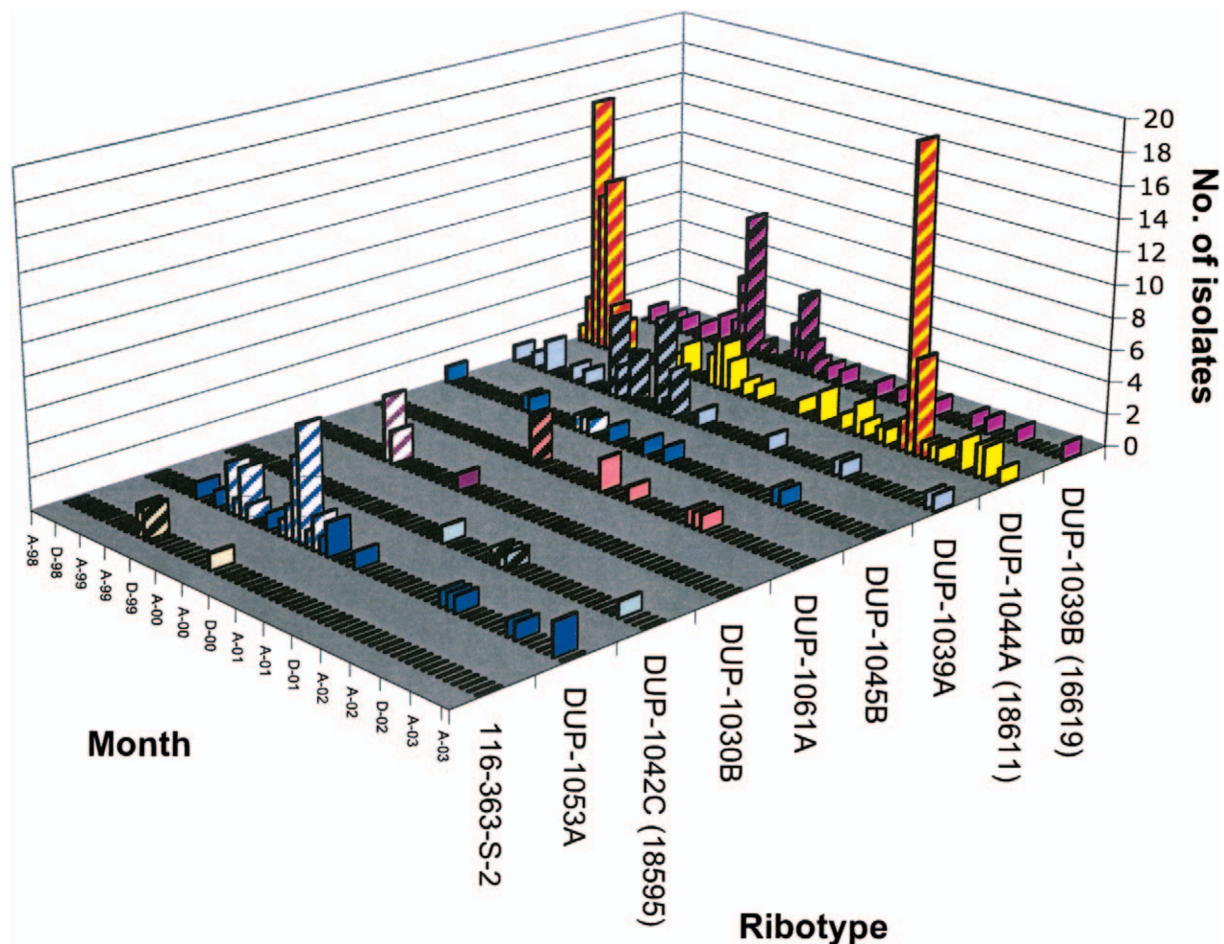

FIGURE 2. *Temporal distribution of ribotype clusters as detected with a scan statistic. The monthly numbers for the nine* L. monocytogenes *isolate ribotypes that caused at least one significantly distinct temporal cluster (*P $\leq$ *0.05) (Table 3) are shown. Isolate numbers represent all four regions (Michigan, New York City, New York State, and Ohio). Isolates that are part of a significant temporal cluster (*P $\leq$ *0.05) are indicated by diagonal lines.*

July 2002 to October 2002), the clusters for each of the other three ribotypes were only separated by 3 to 7 months (Table 3). Of the nine different ribotypes associated with clusters, three were lineage I, five were lineage II, and one was lineage III. The numbers of cases in clusters associated with lineage I, II, and III ribotypes were 81, 84, and 3, respectively.

Association between subtype and lineage and disease type and outcome. Categorical analyses were performed to test for associations between clinical manifestation (fetal infection, septicemia, CNS infection, other infection, and unknown) and lineage or *Eco*RI ribotype. Chi-square analysis revealed no significant association between lineage and clinical manifestation ($P = 0.4941$). Similarly, there was no significant association between *Eco*RI ribotype and clinical manifestation ($P = 0.4297$).

Categorical analyses also were performed to test for associations between clinical outcome (fatal, nonfatal, and unknown) and lineage or *Eco*RI ribotype. Chi-square analysis revealed no significant association between lineage or ribotype and disease outcome ($P = 0.4553$ and 0.0539, respectively). However, disease outcome was unknown for a large number of cases (420 cases, 64.9%), so these results should be considered preliminary.

DISCUSSION

Retrospective molecular subtyping and temporal cluster analyses of 647 human *L. monocytogenes* isolates obtained in four regions during 61 months revealed that although human listeriosis cases are caused by a diverse group of *Eco*RI ribotypes, specific lineage I epidemic clones cause a large number of human listeriosis cases. A large number of human listeriosis cases also can be grouped into statistically significant temporal clusters, including widely distributed and region-specific clusters. *L. monocytogenes* lineages and *Eco*RI ribotypes do not appear to differ in either their likelihood of causing specific clinical manifestations or their likelihood of causing fatal infections in humans.

Although human listeriosis cases are caused by a diversity of *Eco*RI ribotypes, specific lineage I epidemic clones cause a large number of human listeriosis cases. The overall distribution of human isolates among *L. monocytogenes* lineages is consistent with previous evidence that also indicated that lineage I strains are much more common

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among human listeriosis cases than are lineage II strains *(14, 17, 37)*. The three *Eco*RI ribotypes most prevalent among the human isolates characterized here (DUP-1038B, DUP-1042B, and DUP-1044A) all have previously been classified as *L. monocytogenes* epidemic clones, and each have caused multiple human listeriosis outbreaks *(17)*, including two recent outbreaks in the United States caused by ribotype DUP-1044A and linked to consumption of contaminated hot dogs *(2, 22)* and sliced turkey *(5)*. Because of their historical association with human listeriosis clusters, the strains represented by ribotypes DUP-1038B and DUP-1042B have been referred to as epidemic clone I, whereas the strains represented by ribotype DUP-1044A have been referred to as epidemic clone II *(18)*.

Although both outbreaks caused by ribotype DUP-1044A occurred during the time frame covered by this study (and consequently appear to represent clusters A and B detected here), likely contributing to the high prevalence of this ribotype, neither of the other two epidemic ribotypes were responsible for any of the temporal clusters detected here. All three of these epidemic ribotypes have previously been shown to be overrepresented among human isolates as compared with food isolates and had increased cytopathogenicity in a tissue culture plaque assay *(14)*, further supporting the classification of these subtypes as highly virulent epidemic clones. Epidemic clone III *(18)*, representing ribotype DUP-1053A, was only the fifth most prevalent human disease–associated ribotype identified, even though the outbreak caused by this ribotype was included as a cluster with 18 human cases in the present study. Although this ribotype was also previously reported *(14)* to be significantly more common among human isolates than among food isolates, differences in its prevalence among human and food isolates were less significant ($P < 0.05$) than those reported for ribotypes DUP-1038B, DUP-1042B, and DUP-1044A ($P < 0.0001$). Ribotype DUP-1053A isolates were not more cytopathogenic in a tissue culture plaque assay *(14)*. Thus, multiple lines of evidence support the conclusion that lineage I *Eco*RI ribotypes DUP-1038B, DUP-1042B, and DUP-1044A (representing epidemic clones I and II *(18)*) are widely distributed and have a high likelihood of causing human disease. However, although the lineage II ribotype DUP-1053A (representing epidemic clone III *(18)*) is clearly a human health hazard, it seems less prevalent among human cases and does not seem to have the same increased cytopathogenicity as do isolates classified into the other two epidemic clones *(14)*.

Many human listeriosis cases can be grouped into statistically significant temporal clusters, both widely distributed and region specific. Although most human listeriosis infections are generally considered to represent sporadic cases *(34)*, the apparent increase in reported human listeriosis outbreaks in the United States over the last few years *(1–5, 22, 28)*, which probably is due to increased molecular subtype surveillance (e.g., PulseNet surveillance *(12)* in the United States), provides anecdotal evidence that outbreaks and common source clusters of *L. monocytogenes* infections may be more common than previously assumed. The data reported here provide clear evidence that many human listeriosis cases in the United States can be grouped into molecular subtype clusters. We used only ribotyping to identify molecular subtypes in the current study, but application of PFGE, with its typically higher discriminatory ability, may have revealed that some of the ribotype clusters we identified were made up of multiple PFGE types. For example, in our earlier study *(30)* we found that one temporal cluster of five cases associated with ribotype DUP-1042B that occurred from December 1998 to March 1999 was actually made up of cases with isolates having five different PFGE patterns. In our current study, which was conducted during a different but overlapping time period, we found no evidence for temporal clustering of cases associated with ribotype DUP-1042B; the overall occurrence of DUP-1042B was distributed relatively evenly over the study period. Thus, although ribotyping provides an automated, highly discriminatory, and quick (<8 h of run time) means of identifying putative single-source disease clusters, for public health investigations PFGE typically provides higher sensitivity for identifying differences in molecular subtypes. Overall, three of the subtype clusters reported here appear to match previously reported human listeriosis outbreaks, supporting the conclusion that the subtype clusters reported here are biologically meaningful and probably are single-source clusters. Specifically, cluster A appears to represent a multistate listeriosis outbreak linked to consumption of sliced deli meats and hot dogs *(2, 22)*. Cluster H appears to represent a previously reported outbreak in the northeastern United States, which occurred between May and December 2000 *(28)*. Cluster B appears to represent a previously reported outbreak in eight U.S. states that occurred between July 2002 and September 2002 *(5)*. Although no specific epidemiologic investigations were conducted to confirm the clusters described in this report or to determine outbreak sources, our findings indicate that aggressive foodborne disease surveillance efforts will lead to a reduction in the overall incidence of human listeriosis through early outbreak detection, which can reduce the number of cases in individual outbreaks.

The 13 clusters detected in this study include both single-region and multiregional clusters, further supporting the hypothesis that human listeriosis clusters can be either geographically well defined or widely distributed *(30)*. This hypothesis also is supported by the epidemiology of reported listeriosis outbreaks, which can be grouped into spatially confined clusters *(11, 20)* or multistate and multiregional clusters *(2, 5, 22, 28)*. In the present study, region-specific clusters appear to contribute to the significant differences observed in the distribution of *Eco*RI subtypes among the four regions studied. For example, the significant association of ribotype DUP-1053A with NYC was largely caused by the occurrence of two significant DUP-1053A clusters in NYC. Other ribotypes associated with specific regions do not appear to reflect regional temporal clusters but may reflect temporally drawn out clusters (such as the 1983 through 1987 listeriosis outbreak in Switzerland *(7)*) or may represent other regional factors (e.g., consumption of regional or ethnic foods, which may be more likely

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to be contaminated with specific *L. monocytogenes* subtypes).

Because three of the four ribotypes that were associated with more than one cluster each were associated with two temporal clusters that were only separated by a few months, one hypothesis is that in all or at least some of these cases, both clusters had the same source. This hypothesis is supported by the fact that in each case of two temporally close clusters associated with the same ribotype, both the first and second cluster had similar or identical spatial distributions. These observations indicate that some clusters may be temporally drawn out (e.g., due to long-term production of contaminated food products in a facility persistently contaminated with a given subtype *(18, 31)*). These data further support the idea that sensitive outbreak detection coupled with aggressive follow-up source tracking to identify food sources may provide an opportunity to curtail outbreaks before a large number of cases occur.

Our data indicate that a larger number of *L. monocytogenes* subtypes than previously reported may be associated with clusters of cases. Overall, nine different *Eco*RI ribotypes were responsible for the clusters detected here, including ribotypes representing lineages I, II, and III. This finding is particularly important because previous reports have indicated that lineage II strains generally are less likely to cause human disease *(16, 26, 27)* and that lineage III strains are associated with animal hosts *(17)*. Based on our findings, we propose the following hypotheses on the association of different *L. monocytogenes* lineages and subtypes with human listeriosis cases and clusters. First, many lineage I strains (such as the lineage I epidemic clones) have enhanced transmissibility and/or virulence and thus cause a disproportionately high number of human listeriosis cases relative to their prevalence in foods. Second, some *L. monocytogenes* subtypes, predominantly those of lineage II, have premature *inlA* stop codons and thus have a very reduced ability to cause human disease *(14, 15)*, contributing to a disproportional underrepresentation of lineage II isolates among human disease cases relative to their prevalence among food isolates. Third, a variety of *L. monocytogenes* strains can become persistent in food processing plants *(19)* and, if sufficiently virulent, can cause human listeriosis outbreaks; however, the scope of the associated outbreaks may differ based on the relative virulence and transmissibility of the specific subtypes. Fourth, isolates of *L. monocytogenes* lineage III, which have high pathogenic potential (as indicated by the large plaque size when tested in tissue culture plaque assays, *(38)*), are predominantly associated with animal hosts *(17)* and have a reduced ability to grow and multiply in foods *(9, 14)*, only rarely cause human listeriosis because the likelihood of human exposure is very limited. Consistent with these hypotheses, the only lineage III cluster we identified contained just three cases.

L. monocytogenes lineages and *Eco*RI ribotypes do not appear to differ in their association with specific clinical manifestations or fatal outcomes. In our analyses, there was no evidence for differences among *L. monocytogenes* subtypes and lineages in the likelihood of causing distinct clinical manifestations or fatal outcomes. Although there is growing evidence among other foodborne pathogens that specific subtypes within a given species may differ in the ability to cause different clinical manifestations and/or fatal outcomes *(10, 24)*, only very limited data on mortality rates and clinical manifestations associated with different *L. monocytogenes* subtypes have been reported. Aouaj et al. *(6)* reported that in The Netherlands specific *L. monocytogenes* lineage II serotypes were significantly associated with nonneonatal cases with underlying disease, whereas our data suggest that subtype association with disease manifestation may be limited and may reflect epidemiological factors other than strain-specific characteristics.

The data reported here provide evidence supporting the hypothesis that a larger number of human listeriosis cases than previously assumed occur as clusters, highlighting the importance of aggressive outbreak detection efforts. These data also indicate that although the previously defined *L. monocytogenes* epidemic clones (in particular epidemic clones I and II) are associated with a high proportion of human listeriosis cases, a variety of subtypes can be associated with temporal listeriosis clusters. Although further epidemiological studies are needed, our observations are consistent with the hypothesis that the ability of *L. monocytogenes* subtypes to colonize and persist in processing plants may be an important factor contributing to listeriosis clusters *(18, 31, 33)*. Although many *L. monocytogenes* subtypes, even if they have become established in a given processing plant, may cause only a few cases of listeriosis, persistence of epidemic clones and other subtypes with enhanced transmissibility and/or virulence may cause particularly large clusters and/or outbreaks. Further studies on the biology of these epidemic clones are thus particularly crucial.

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