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Molecular Characterization of *Listeria monocytogenes* from Natural and Urban Environments

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ABSTRACT

Characterization of 80 *Listeria monocytogenes* isolates from urban and natural environments differentiated 7 and 26 *Eco*RI ribotypes, respectively. Whereas the majority of isolates from the natural environment represented *L. monocytogenes* lineage II (12 of 13 isolates), urban isolates grouped evenly into lineages I and II (32 and 33 isolates, respectively) and included two lineage III isolates. Multilocus sequence typing of all natural isolates and a randomly selected subset of 30 urban isolates showed a higher overall diversity (Simpson index of discrimination [*D*] of 0.987 and 0.920, respectively) than did *Eco*RI ribotyping ($D = 0.872$ and 0.911, respectively). Combined analysis with ribotype and lineage data for 414 isolates from farm sources, 165 isolates from foods and food-processing environments, and 342 human clinical isolates revealed that lineage I was significantly more common among human ($P < 0.0001$) isolates, whereas lineage II was more common among isolates from the natural environment, farms, and foods ($P \leq 0.05$). Among a total of 92 ribotypes, 31 showed significant associations with specific isolate sources. One ribotype (DUP-1039C) was significantly associated with both natural environments and farms. A spatial analysis showed a marginal association between locations in the natural environment positive for *L. monocytogenes* and a proximity to farms. Our data indicate that (i) *L. monocytogenes* strains from different sources show a high level of diversity; (ii) *L. monocytogenes* subtypes differ significantly in their associations with different environments, even though populations overlap; and (iii) a higher proportion of isolates from environmental sources than from human clinical cases can be classified into *L. monocytogenes* lineage II, which supports the classification of this lineage as an environmentally adapted subgroup.

Listeria monocytogenes is a facultative intracellular human foodborne and animal pathogen. Human-invasive listeriosis is a rare, yet severe disease *(43)*, with a mortality rate of approximately 20% *(50)*. *L. monocytogenes* has not only been isolated from a variety of ready-to-eat food products *(9)*, but has also been found regularly in food processing plants *(1, 25, 26, 41, 46)* as well as in agricultural environments *(2, 16, 17, 20–22, 27, 34, 44, 45, 47)*. Only limited information is available on the ecology of *L. monocytogenes* in natural, nonagricultural, and non–food-processing environments. Although some early studies claimed that *L. monocytogenes* was widely distributed in the natural environment, including vegetation, soil, and surface water *(52, 53)*, these studies predated the definition of different *Listeria* species. Isolates identified as *L. monocytogenes* in these studies thus might have represented any of the currently recognized *Listeria* spp. (*L. innocua*, *L. ivanovii*, *L. seeligeri*, *L. welshimeri*, and *L. monocytogenes*). Although some more recently conducted studies have reported wide ranges of *L. monocytogenes* prevalence in soils, vegetation, surface water, and sewage *(15)*, including a study by our group (which showed a significantly higher *L. monocytogenes* prevalence in urban than in natural environments *(42)*), the relationship between these environmental *L. monocytogenes* isolates and foodborne transmission of *L. monocytogenes* is not well understood.

Although prevalence studies are useful for understanding the overall distribution of *L. monocytogenes* among different sources, the application of molecular subtyping methods is critical to an understanding of the ecology and transmission of *L. monocytogenes*. Molecular subtyping methods such ribotyping *(55, 57)*, pulsed-field gel electrophoresis *(28, 48)*, and, more recently, multilocus sequence typing (MLST) *(8, 39)* allow improved subtype discrimination over serotyping, which is the traditional method for the subtype characterization of *L. monocytogenes*. Traditional serotyping differentiates only 13 *L. monocytogenes* serotypes. A variety of molecular subtyping studies have shown considerable *L. monocytogenes* diversity among isolates from foods *(18)*, food processing plants *(12, 30, 31, 41)*, humans *(5, 18, 33, 40, 49)*, and animals *(2, 23, 34, 56, 57)*, with the species representing at least two if not three distinct genetic lineages. Although different nomenclatures have been used to designate these *L. monocytogenes* lineages *(24)*, we will refer to these lineages as I, II, and III following the designations previously used by our group *(54, 56)* and others *(13, 51)*. Lineage I predominantly includes serotype 1/2b, 3b, 3c, and 4b strains, whereas lineage II primarily includes serotypes 1/2a, 1/2c, and 3a *(32)*. Previous reports have shown that lineage I strains are sig-

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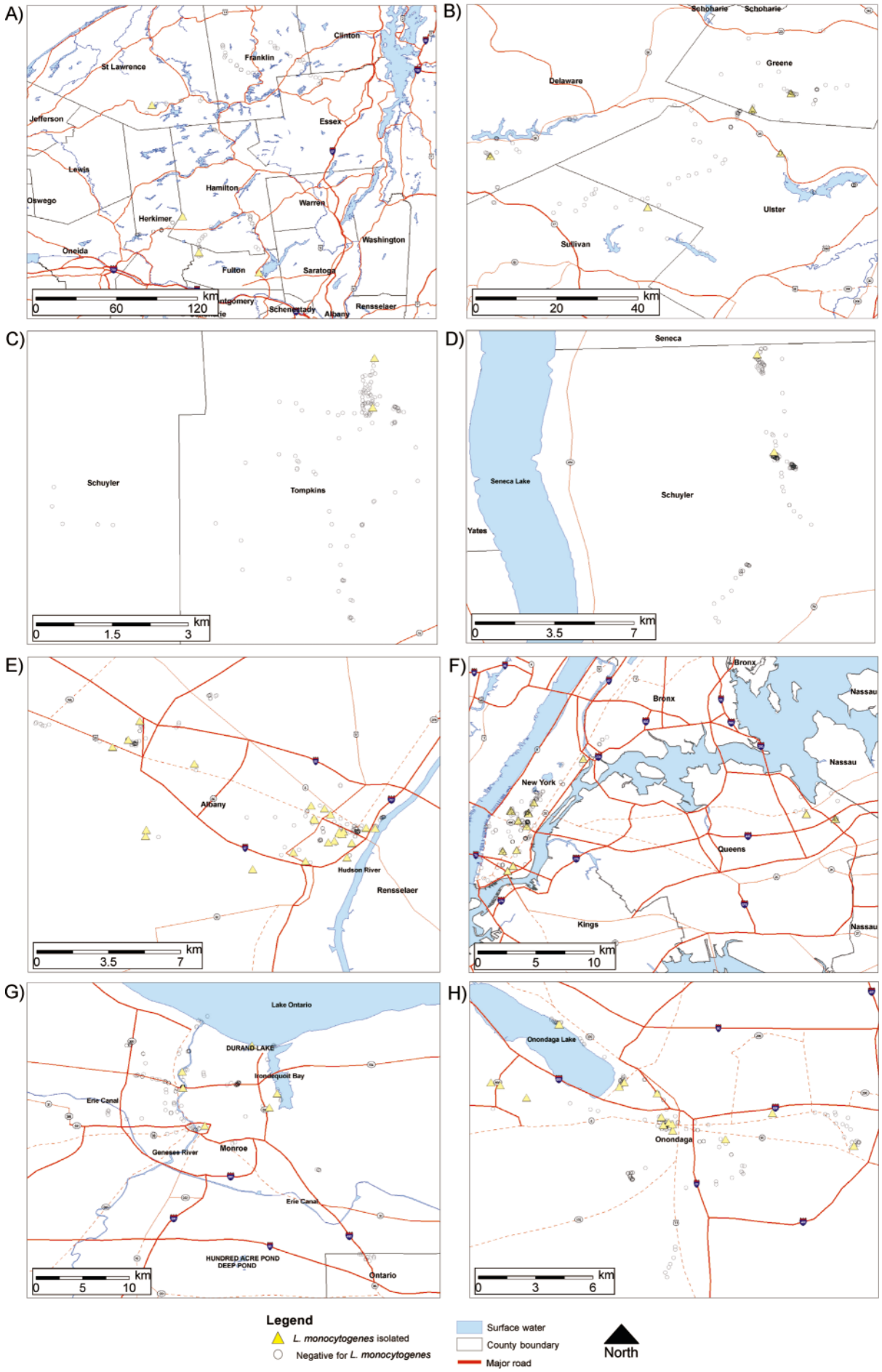

nificantly overrepresented among human clinical listeriosis cases when compared to their prevalence among animal listeriosis cases and contaminated foods *(18, 32, 36).* In contrast, lineage II strains show a significantly higher prevalence among food isolates and animal clinical cases than among human listeriosis cases *(18, 23).* In addition, lineage I isolates appear to have significantly greater pathogenic potential, as suggested by their ability to spread to the neighboring host cells in a cell culture plaque assay, when compared to lineage II isolates *(18, 36).* Lineage III includes serotypes 4a, 4b, and 4c *(32, 51).* Strains classified in this lineage are most commonly isolated from animals and are occasionally isolated from human listeriosis cases with clinical disease, but they are rarely isolated from foods *(18, 23, 56).*

Although we have previously *(42)* evaluated the overall distribution and molecular diversity of the genus *Listeria* in urban and natural environments by subtyping this organism by means of *sigB* sequencing, this report represents an expanded subtype analysis of the 80 *L. monocytogenes* strains isolated from urban and natural environments *(42)* by more discriminatory subtyping methods, including automated ribotyping and MLST. The goals of these studies were to (i) develop a better understanding of the ecology of *L. monocytogenes* outside the human food chain and (ii) compare the lineage and subtype diversity among *L. monocytogenes* isolates from outside the human food chain (i.e., those isolated from urban and natural environments) to the diversity observed in different compartments of the human food chain (i.e., human clinical cases, foods and food processing plants, farm animals, and farm environments). Specifically, this study allowed a comprehensive characterization of *L. monocytogenes* subtype diversity in these various compartments without potentially confounding temporal and spatial effects, because we have previously collected subtype data for isolates obtained from foods and food environments *(41),* farm animals, feeds and farm environments *(34),* and human clinical cases *(41)* from the same geographic area (New York State) and during the same time (from 1997 to 2003).

MATERIALS AND METHODS

Sample collection, culture, and identification of *L. monocytogenes* from natural and urban environments. Sample collection and isolation of the *L. monocytogenes* isolates characterized in this study have previously been described *(42).* Briefly, 1,805 samples were collected over a 2-year period (2001 and 2002) from various locations in four urban sites (Albany, New York City, Syracuse, and Rochester) and in four sites representing natural environments (Adirondack Park, Catskill Park, Connecticut Hill Wildlife Management Area, and the Fingerlakes National Forest) (Fig. 1). Geospatial location data for each sample were collected with a Garmin Emap (Garmin International Inc., Olathe, Kans.) handheld global positioning system and confirmed by the TopoUSA 4.0 software package (Delorme, Yarmouth, Maine). Sample types collected from natural sites included approximately equal numbers of soil, vegetation, and surface water. Samples collected from urban sites included soil and vegetation, surface water, and sponge swipes of floors, sidewalks, and human contact surfaces (e.g., door handles, hand railings, telephones). *L. monocytogenes* was cultured by selective enrichment in *Listeria* enrichment broth (Difco, Becton Dickinson, Sparks, Md.) and were then isolated on Oxford medium (Difco, Becton Dickinson) with the subsequent identification of *L. monocytogenes* by *L. monocytogenes* plating medium (Biosynth International, Inc., Naperville, Ill.) *(37)* and an *L. monocytogenes*–specific PCR assay targeting the *hly* gene sequence *(42).* All 80 *L. monocytogenes* isolates from our previous study *(42)* were used in this study.

***L. monocytogenes* isolates from human cases, foods, and farms.** Previously reported data sets *(34, 40, 41)* were used to compare the subtype diversity for the *L. monocytogenes* isolates from the natural and urban environments characterized in this study with the diversity associated with different compartments of the human food system. Specifically, data sets were available for (i) 342 human clinical isolates collected in New York State between 1998 and 2002 *(41)*; (ii) 125 food isolates and 40 isolates from food-processing environments collected in New York State between 1997 and 2002 *(41)*; and (iii) 414 isolates from animals, animal feedstuff, and farm environments, collected between 2001 and 2003 from 52 farms, which were located predominantly in New York State but also on a few farms in adjacent neighboring states (i.e., Vermont and Pennsylvania) *(34).*

Automated ribotyping and lineage classification. All isolates identified as *L. monocytogenes* were characterized by automated ribotyping by the restriction enzyme *Eco*RI and the RiboPrinter Microbial Characterization System (Qualicon Inc., Wilmington, Del.) *(6)* and classified to lineage as previously described *(18, 56). Eco*RI ribotype patterns for the 80 *L. monocytogenes* isolates from urban and natural environments in this study were not previously reported.

MLST analysis. All 13 *L. monocytogenes* isolates from natural environments, as well as a randomly selected subset of 30 isolates from urban environments, were characterized by a previously described MLST scheme based on sequencing partial open reading frames of the housekeeping genes *gap, prs, purM,* and *ribC,* as well as the stress-response gene *sigB,* and the virulence genes *actA* and *inlA* as previously described *(35).* Final sequence lengths used for analyses were as described by Nightingale et al. *(35),* with the exceptions of *gap, inlA,* and *sigB,* for which slightly shorter sequences were used (567, 755, and 660 nucleotides, respectively). Two gene sequences were classified as different allelic types if they differed by at least a single nucleotide. Sequence types (STs) were defined as unique combinations of allelic types for the seven genes sequenced. Based Upon Related Sequence Types (BURST) analysis *(14)* was used to assess the relationship between STs, including the grouping into clonal groups.

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FIGURE 1. *Geographic distribution of* L. monocytogenes*–positive sample locations. Individual maps show the spatial distribution of sample locations positive (yellow triangles) and negative (hollow circles) for* L. monocytogenes *in four natural (A through D; Adirondacks, Catskills, Connecticut Hill Wildlife Management Area, Fingerlakes National Forest) and four urban (E through H; Albany, New York City, Rochester, Syracuse) sites. County boundaries, major waterways, northerly direction, and scale (in kilometers) are as indicated.*

TABLE 1. *Sample source characteristics and molecular subtype data for 80* Listeria monocytogenes *isolates from natural and urban environments*

Environment	Site[a]	Season	Category[b]	Description	Date collected (day/mo/yr)	FSL-ID[c]	Ribotype	Lineage	Allelic profile[d]	ST[e]
Natural	ADK	Summer	S	Soil	07/08/01	S4-066	DUP-1045B	II	57, 4, 3, 4, 4, 4, 4	10
	ADK	Spring	V	Leaves/debris	04/21/02	S4-380	DUP-1039C	II	57, 3, 3, 6, 7, 7, 7	8
	ADK	Summer	V	Grass	09/08/02	S4-880	DUP-1039C	II	57, 9, 3, 7, 10, 6, 3	14
	ADK	Summer	W	River/stream	09/08/02	S4-887	DUP-1039E	II	57, 3, 1, 3, 3, 14, 3	5
	CAT	Summer	S	Soil	08/19/01	S4-189	DUP-1053C	II	59, 3, 3, 3, 5, 5, 5	19
	CAT	Autumn	S	Soil	11/24/01	S4-295	DUP-1045B	II	57, 5, 3, 5, 6, 4, 4	11
	CAT	Autumn	V	Leaves/debris	11/24/01	S4-304	DUP-1046A	II	57, 1, 3, 3, 4, 6, 6	2
	CAT	Summer	W	River/stream	08/18/02	S4-821	DUP-1039C	II	57, 6, 3, 7, 10, 6, 3	13
	CAT	Autumn	W	River/stream	11/10/02	S6-072	DUP-1045B	II	57, 6, 3, 5, 8, 8, 4	12
	CTHILL	Spring	S	Soil	06/06/01	S4-019	DUP-1045A	II	57, 1, 1, 1, 1, 1, 1	1
	CTHILL	Autumn	V	Leaves/debris	10/22/02	S4-941	DUP-1038B	I	58, 2, 2, 2, 2, 2, 2	17
	FLNF	Spring	S	Soil	05/15/02	S4-497	DUP-1039C	II	57, 6, 3, 7, 10, 6, 3	13
	FLNF	Summer	V	Leaves/debris	08/05/02	S4-766	DUP-1039E	II	57, 3, 1, 3, 3, 3, 3	6
Urban	ALB	Autumn	S	Soil	11/12/01	S4-257	DUP-1038B	I	58, 2, 2, 2, 2, 2, 2	17
	ALB	Autumn	S	Soil	11/12/01	S4-261	DUP-1038B	I	ND	ND
	ALB	Autumn	S	Soil	11/12/01	S4-268	DUP-1038B	I	ND	ND
	ALB	Autumn	V	Leaves/debris	11/12/01	S4-272	DUP-1038B	I	ND	ND
	ALB	Spring	S	Soil	06/09/02	S4-628	DUP-1038B	I	58, 2, 2, 2, 11, 2, 2	16
	ALB	Spring	V	Leaves/debris	06/09/02	S4-635	DUP-16635B	I	ND	ND
	ALB	Spring	V	Mulch	06/09/02	S4-640	DUP-1039C	II	57, 1, 3, 8, 12, 10, 4	4
	ALB	Spring	HC	Bench area	06/09/02	S4-643	DUP-1044B	I	ND	ND
	ALB	Spring	SW/F	Sidewalk	06/09/02	S4-645	DUP-1038B	I	ND	ND
	ALB	Spring	SW/F	Sidewalk	06/09/02	S4-649	DUP-1023A	II	ND	ND
	ALB	Spring	SW/F	Sidewalk	06/09/02	S4-652	DUP-1045B	II	57, 5, 3, 5, 6, 4, 4	11
	ALB	Spring	SW/F	Sidewalk	06/09/02	S4-655	DUP-1045B	II	57, 5, 3, 5, 6, 4, 4	11
	ALB	Spring	SW/F	Sidewalk	06/09/02	S4-658	DUP-1023B	II	ND	ND
	ALB	Spring	W	River/stream	06/09/02	S4-664	DUP-1038B	I	ND	ND
	ALB	Summer	SW/F	Sidewalk	07/14/02	S4-722	DUP-1053B	II	57, 1, 3, 8, 12, 10, 4	4
	ALB	Summer	SW/F	Sidewalk	07/14/02	S4-728	DUP-1044A	I	ND	ND
	ALB	Autumn	S	Soil	11/23/02	S6-093	DUP-1044B	I	ND	ND
	ALB	Autumn	V	Leaves/debris	11/23/02	S6-096	DUP-1038B	I	ND	ND
	ALB	Autumn	SW/F	Sidewalk	11/23/02	S6-106	DUP-1046A	II	ND	ND
	ALB	Autumn	SW/F	Sidewalk	11/23/02	S6-116	DUP-1038B	I	ND	ND
	ALB	Autumn	SW/F	Sidewalk	11/23/02	S6-124	DUP-1039E	II	ND	ND
	ALB	Autumn	W	River/stream	11/23/02	S6-131	DUP-1039A	II	57, 9, 3, 7, 3, 5, 13	15
	ALB	Autumn	W	River/stream	11/23/02	S6-134	DUP-1044A	I	ND	ND
	ALB	Autumn	W	Runoff	11/23/02	S6-136	DUP-1038B	I	ND	ND
	ALB	Autumn	W	River/stream	11/23/02	S6-142	DUP-1042A	I	ND	ND
	ALB	Autumn	W	Pond/lake	11/23/02	S6-147	DUP-1039C	II	ND	ND
	ALB	Autumn	W	Puddle	11/23/02	S6-151	DUP-1038A	I	58, 2, 2, 2, 2, 2, 2	17
	NYC	Summer	S	Soil	07/22/01	S4-154	116-931-S-4	II	57, 5, 3, 5, 6, 4, 4	11
	NYC	Spring	S	Soil	05/18/02	S4-548	DUP-1053B	II	57, 6, 3, 5, 8, 8, 4	12
	NYC	Spring	V	Mulch	05/18/02	S4-553	DUP-1052A	I	ND	ND
	NYC	Spring	S	Soil	05/18/02	S4-560	DUP-1045B	II	ND	ND

TABLE 1. *Continued*

Environment	Site[a]	Season	Category[b]	Description	Date collected (day/mo/yr)	FSL-ID[c]	Ribotype	Lineage	Allelic profile[d]	ST[e]
Urban (continued)	NYC	Spring	V	Leaves/debris	05/18/02	S4-567	DUP-1045B	II	57, 5, 3, 5, 6, 4, 4	11
	NYC	Summer	SW/F	Sidewalk	08/25/02	S4-835	DUP-1039C	II	ND	ND
	NYC	Summer	SW/F	Sidewalk	08/25/02	S4-839	DUP-1061A	III	66, 7, 5, 10, 14, 12, 9	23
	NYC	Summer	SW/F	Sidewalk	08/25/02	S4-843	DUP-1044C	I	ND	ND
	NYC	Summer	SW/F	Sidewalk	08/25/02	S4-848	DUP-1042B	I	58, 8, 4, 2, 15, 2, 10	18
	NYC	Summer	W	Puddle	08/25/02	S4-859	DUP-1044C	I	64, 8, 4, 11, 16, 13, 11	22
	NYC	Autumn	SW/F	Storm drain	11/02/02	S6-016	116-931-S-4	II	ND	ND
	NYC	Autumn	W	Pond/lake	11/02/02	S6-025	DUP-1042B	I	67, 8, 4, 12, 17, 16, 11	24
	NYC	Autumn	SW/F	Sidewalk	11/03/02	S6-023	DUP-1042C	I	ND	ND
	NYC	Autumn	W	Puddle	11/03/02	S6-029	DUP-1042C	I	61, 8, 2, 2, 18, 17, 12	21
	NYC	Autumn	W	Puddle	11/03/02	S6-033	DUP-1039C	II	ND	ND
	ROCH	Summer	S	Soil	08/12/01	S4-169	DUP-1038B	I	58, 2, 2, 2, 2, 2, 2	17
	ROCH	Summer	V	Mulch	08/12/01	S4-172	DUP-1038B	I	58, 2, 2, 2, 2, 2, 2	17
	ROCH	Summer	V	Mulch	07/15/02	S4-742	DUP-1039C	II	57, 3, 3, 9, 13, 11, 1	9
	ROCH	Autumn	S	Soil	10/12/02	S4-908	DUP-1045B	II	57, 4, 3, 4, 4, 4, 4	10
	ROCH	Autumn	S	Soil	10/12/02	S4-914	DUP-1039C	II	57, 3, 3, 3, 3, 15, 3	7
	RCCH	Autumn	W	River/stream	10/12/02	S4-926	DUP-1038B	I	58, 2, 2, 2, 2, 2, 2	17
	ROCH	Autumn	W	Pond/lake	10/12/02	S4-929	DUP-1045B	II	57, 5, 3, 5, 6, 4, 4	11
	SYR	Summer	SW/F	Sidewalk	06/24/01	S4-024	DUP-1045B	II	ND	ND
	SYR	Summer	S	Soil	06/24/01	S4-032	DUP-1058C	II	ND	ND
	SYR	Summer	W	Pond/lake	06/24/01	S4-049	DUP-1038B	I	58, 2, 2, 2, 2, 2, 2	17
	SYR	Summer	SW/F	Sidewalk	06/24/01	S4-061	DUP-1039E	II	57, 3, 1, 3, 3, 3, 3	6
	SYR	Summer	SW/F	Sidewalk	06/24/01	S4-064	DUP-1039E	II	ND	ND
	SYR	Autumn	SW/F	Sidewalk	11/06/01	S4-236	DUP-1039E	II	ND	ND
	SYR	Spring	S	Soil	04/28/02	S4-407	DUP-1045B	II	ND	ND
	SYR	Spring	V	Leaves/debris	04/28/02	S4-410	DUP-1045B	II	57, 6, 3, 5, 8, 8, 4	12
	SYR	Spring	HC	Trash can	04/28/02	S4-470	DUP-1057B	II	ND	ND
	SYR	Spring	SW/F	Sidewalk	04/28/02	S4-474	DUP-1045B	II	57, 6, 3, 5, 8, 8, 4	12
	SYR	Spring	SW/F	Sidewalk	04/28/02	S4-436	DUP-1025A	I	ND	ND
	SYR	Spring	SW/F	Sidewalk	04/28/02	S4-440	DUP-1042B	I	61, 2, 4, 2, 9, 2, 8	20
	SYR	Spring	W	River/stream	04/28/02	S4-452	DUP-1054A	II	57, 1, 3, 6, 4, 9, 5	3
	SYR	Spring	W	River/stream	04/28/02	S4-461	DUP-1030B	II	ND	ND
	SYR	Spring	W	Puddle	04/28/02	S4-465	DUP-1061A	III	ND	ND
	SYR	Summer	SW/F	Sidewalk	08/11/02	S4-774	DUP-1038B	I	58, 2, 2, 2, 2, 2, 2	17
	SYR	Summer	W	Drain	08/11/02	S4-780	DUP-1038B	I	ND	ND
	SYR	Autumn	SW/F	Sidewalk	10/06/02	S4-899	DUP-1053C	II	ND	ND

[a] ADK, Adirondack Park; CAT, Catskill Park; CTHILL, Connecticut Hill Wildlife Management Area; FLNF, Finger Lakes National Forest; ALB, Albany; NYC, New York City; ROCH, Rochester; SYR, Syracuse.

[b] S, soil; V, vegetation; W, water; HC, human contact; SW/F, sidewalk/floor.

[c] Abbreviation of FSL-ID number; full isolate identification numbers include the prefix ''FSL'' (e.g., ''FSL S4-066''). Full isolate name is required for searching the Pathogen Tracker on-line database (http://www.pathogentracker.net).

[d] Allelic profile represents a concatenation of the allelic types based on the partial gene sequences for *sigB, gap, prs, ribC, purM, inlA,* and *actA*; ND, not done.

[e] ST, sequence type.

Simpson index of discrimination. The Simpson index of diversity (*D*) with 95% confidence intervals was calculated as previously described *(19).*

Categorical analysis. Associations between environments (natural and urban) or other compartments (e.g., human clinical) and molecular subtypes (ribotype and lineage) were determined by categorical analyses with a chi-square test or the Fisher exact test if expected values were less than 5. All categorical analyses were performed by SAS 9.1 (SAS Institute Inc., Cary, N.C.). *P* values ≤ 0.05 were considered statistically 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 was likely to be inflated, and it could be contended that the significance threshold should be adjusted. Therefore, we provide observed *P* values to avoid missing possible associations of genetic characteristics (caused by a very conservative *P* value); readers are thus not limited to an arbitrary interpretation of the threshold for significance and can evaluate significance levels according to their preferred criteria *(18, 38).*

Spatial analysis. Global positioning system data for all samples collected in our initial study *(42)* and for all sample locations that yielded *L. monocytogenes* isolates had previously been imported into ArcGIS 9 (ESRI, Redland, Calif.) and were thus available for spatial analysis. Spatial analyses were conducted to determine whether the occurrence of *L. monocytogenes* in natural environments was related to a proximity to farms. Locations for the *L. monocytogenes*–positive samples ($n = 13$) were plotted, and the nearest straight-line distance to known bovine farms was measured with the measure tool in ArcMap 9.0 (a component of the ArcGIS package). For comparison, straight-line distances to known farms were also measured for 65 (representing five times the number of *L. monocytogenes*–positive locations) randomly selected sampling locations, which were selected proportionately to the number of *L. monocytogenes*–positive locations (e.g., 5 positive locations and 25 randomly selected locations for the site ''Catskills'' were used). The mean distance of the *L. monocytogenes*–positive sample locations to the nearest farm was then compared to the mean distance of the randomly selected sample locations to the nearest farm by a two-sample *t* test. Significance was considered at $P \leq 0.05$.

Isolate and data curation. Isolates were stored frozen in brain heart infusion broth containing 15% glycerol at −80°C. 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

Spatial and temporal distributions of *L. monocytogenes* in natural and urban environments. Although we previously reported that a total of 67 and 13 *L. monocytogenes* isolates (Table 1) were obtained from 898 and 907 samples collected from urban and natural environments (yielding prevalences of 7.5 and 1.4%, respectively) *(42)*, no detailed analysis of the spatial and temporal distributions of these isolates has been reported, because our previous study *(42)* focused on the ecology of all *Listeria* spp. with only very limited subtype analysis (i.e., sequencing of part of the *sigB* open reading frame). *L. monocytogenes* prevalence was similar for the four natural sites, ranging from 0.77% (Finger Lakes National Forest) to 2.1% (Adirondacks). Within these four sites, *L. monocytogenes* was isolated from various sampling locations (Fig. 1A through 1D), with no evidence of clustering of positive sites *(42)*. *L. monocytogenes* prevalence among the four urban sites was more variable and ranged from 2.9 (Syracuse) to 12.6% (Albany). Although *L. monocytogenes* was also isolated from a wide range of locations in the four urban sites (Fig. 1E through 1H), we have previously shown a spatial clustering of locations positive for *L. monocytogenes sigB* allelic type 58 in Albany *(42)*. Seasonal *L. monocytogenes* prevalences were 1.3 and 11.9% for spring, 1.8 and 4.8% for summer, and 1.2 and 7.9% for fall for samples from natural and urban environments, respectively. Whereas the *L. monocytogenes* prevalence among various sample types (e.g., soil, vegetation) (Fig. 2) was very similar for natural environments (Fig. 2), *L. monocytogenes* prevalences varied considerably among samples for urban environments—from 1.6% for human contact surfaces to 10.7% for soil samples (Fig. 2).

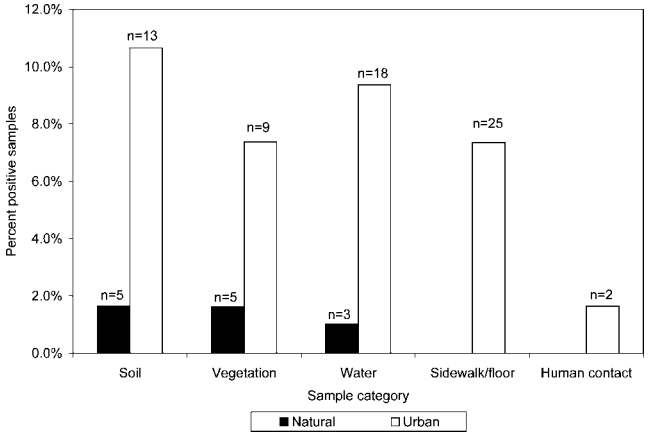

FIGURE 2. *Prevalence of* L. monocytogenes *among various sample types from natural and urban environments. Whereas soil, vegetation, and surface water were collected from both natural and urban environments, sidewalks, floors, and human contact surfaces were sampled only in urban environments.*

***Eco*RI ribotypes and lineage diversity for *L. monocytogenes* isolated from natural and urban environments.** A total of 27 *Eco*RI ribotypes were differentiated among the 80 isolates characterized, with 26 and 7 ribotypes found among isolates from urban environments ($n = 67$) and natural environments ($n = 13$), respectively (Table 1). Only four ribotypes (DUP-1039E, DUP-1039C, DUP-1045B, and DUP-1038B) occurred more than five times. Whereas 20 of the ribotypes isolated from urban locations were not found among isolates from natural environments, only one of the ribotypes isolated from natural environments was not found among the isolates from urban locations. Although ribotype diversity, as determined by the Simpson index of discrimination (*D*), was higher among urban than environmental isolates (Table 2), this difference was not significant, as evidenced by overlapping 95% confidence intervals.

Classification, based on *Eco*RI ribotype data, of isolates into genetic lineage showed that nearly all of the isolates from natural environments were grouped into lineage

TABLE 2. *Simpson index of diversity for lineages and ribotypes based on* Listeria monocytogenes *isolates (*n *= 1,001) from different sources*

Subtype	Simpson index by isolate source (no. of individual subtypes)					
	Natural	Urban	Farm[a]	Food[b]	Human[c]	All sources
Lineage	0.154 (2)	0.536 (3)	0.461 (3)	0.483 (2)	0.471 (3)	0.516 (3)
Ribotype	0.872 (7)	0.911 (26)	0.936 (59)	0.929 (32)	0.918 (48)	0.950 (92)

[a] Data used to calculate values in the column Farm represented 414 *L. monocytogenes* isolates from animals, animal feedstuff, and farm environments collected between 2001 and 2003 from 52 farms located predominantly in New York State *(34)*.

[b] Data used to calculate values in the column Food represented 125 *L. monocytogenes* food isolates and 40 *L. monocytogenes* isolates from food-processing environments collected in New York State between 1997 and 2002 *(41)*.

[c] Data used to calculate values in the column Human represented 342 *L. monocytogenes* isolates from human clinical cases collected in New York State between 1998 and 2002 *(41)*.

II (i.e., 12 of 13 isolates). Isolates from urban environments represented approximately equal numbers of lineage I and II isolates (32 and 33 isolates, respectively) and also included two lineage III isolates. Consistent with these data, urban *L. monocytogenes* isolates showed a significantly higher lineage diversity than did isolates from natural environments (Table 2).

MLST analysis of isolates from natural and urban environments. MLST analysis of all 13 isolates from natural environments and a randomly selected subset of 30 urban *L. monocytogenes* isolates identified between 5 (*prs*) and 18 (*purM*) allelic types for a total of 24 STs among all 43 isolates (Table 3). A total of 17 STs were isolated only once, including 6 and 11 STs found among isolates from natural and urban environments, respectively (Table 1). Among the seven STs found more than once, one ST was found only among isolates from natural environments, one ST was found only among isolates from urban environments, and five STs were found among isolates from both natural and urban environments (Table 1).

TABLE 3. *Simpson index of diversity for multilocus sequence typing (MLST) data from all natural (*n *= 13) and a subset of randomly selected urban (*n *=* 30) Listeria monocytogenes *isolates*

Subtype	Simpson index by isolate source (no. of unique STs or ATs)[a]		
	Natural	Urban	All sources
Sequence type	0.987 (12)	0.920 (17)[b]	0.941 (24)
sigB	0.295 (3)	0.639 (6)	0.560 (7)[c]
gap	0.872 (7)	0.860 (9)	0.874 (9)
prs	0.500 (3)	0.662 (5)	0.631 (5)
ribC	0.872 (7)	0.805 (11)	0.850 (12)
purM	0.936 (9)	0.910 (14)	0.924 (18)
inlA	0.910 (9)	0.853 (13)	0.891 (17)
actA	0.833 (7)	0.805 (11)	0.833 (13)

[a] The number of unique sequence types (STs) is given, while for each individual gene, the number of unique allelic types (ATs) is given.

[b] The 30 randomly selected *L. monocytogenes* isolates from urban environments characterized by MLST represented a total of 13 ribotypes.

[c] While 11 *sigB* allelic types were reported previously *(42)* among all 67 *L. monocytogenes* isolates from urban environments, the 30 isolates characterized by MLST represented only 7 *sigB* allelic types.

Overall ST diversity ($D = 0.941$) was higher than overall ribotype diversity ($D = 0.887$) when *D*-values were calculated for the 43 isolates for which both ST and ribotype data were determined. For isolates from both natural and urban environments, ST diversity ($D = 0.987$ and 0.920, respectively) (Table 3) was considerably higher than ribotype diversity ($D = 0.872$ and 0.887, respectively).

Correlation between STs and ribotypes. Combined analysis of ribotype and ST data for the 43 isolates for which both subtype data were available showed that both subtyping methods together provided higher discrimination than MLST or ribotyping alone for the urban isolates ($D = 0.949$), whereas no improved discrimination was observed for isolates from natural environments ($D = 0.987$). Among all 43 isolates, six ribotypes were represented more than once, and six could be differentiated into two or more (up to six) STs, whereas among the seven STs represented more than once, four could each be differentiated into two ribotypes, and three were represented by only one ribotype.

A dendogram generated by the unweighted pairs group matching algorithm (UPGMA) analysis of allelic profiles showed that STs formed two main clusters, which represent lineages I and II (Fig. 3); this clustering of isolates into lineages was consistent with a lineage designation based on the *Eco*RI ribotype. BURST cluster analysis showed three clonal complexes (A, B, and C) (Fig. 3) representing 16 isolates as well as 17 singleton STs representing 27 isolates.

Ribotype and lineage distributions and diversity among *L. monocytogenes* isolates from natural and urban environments, farm environments and farm animals, foods and food-processing environments, and human clinical cases. Subtype data for a total of 1,001 *L. monocytogenes* isolates collected over a period of 5 years in New York State from various sources were analyzed to assess the distribution and diversity of specific lineages and ribotypes from different sources. In addition to the 80 isolates described in this study, this data set included (i) 342 human clinical isolates *(40)*; (ii) 125 food isolates and 40 isolates from food-processing environments *(41)*; and (iii) 414 isolates from animals, animal feedstuff, and farm en-

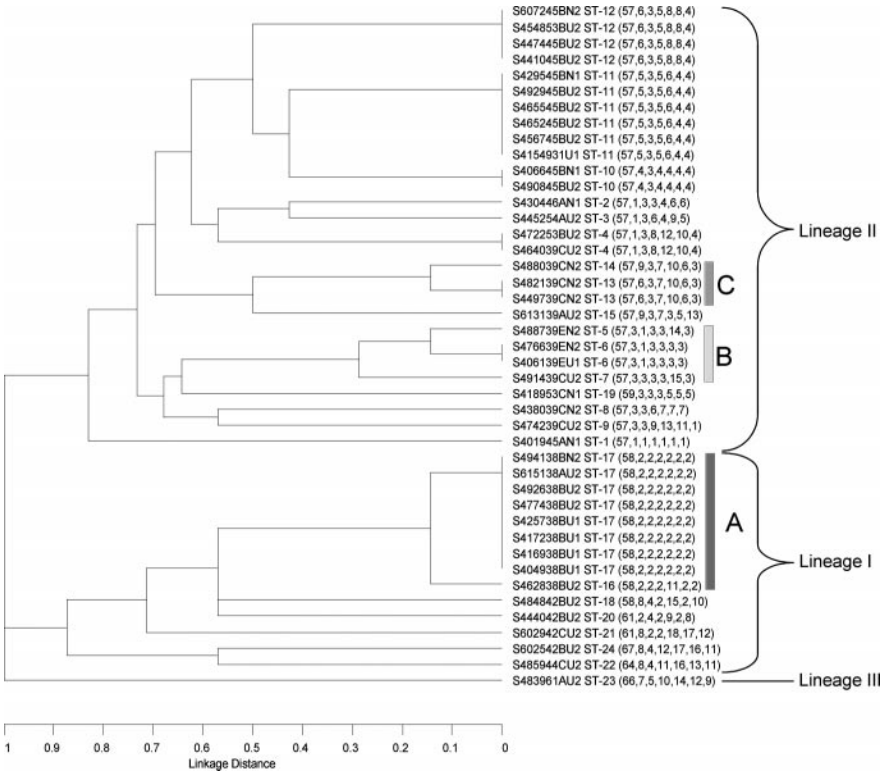

FIGURE 3. *UPGMA dendogram showing the relationship between ribotypes and sequence types (STs) for* L. monocytogenes *isolated from natural and urban environments. The dendogram was constructed on the basis of MLST using allelic profiles derived from the sequencing of seven genes (*sigB, gap, prs, ribC, purM, inlA, *and* actA*). Each isolate is labeled with a concatenated code consisting of a five-character FSL-ID (i.e., the label that includes S6072 as the first five letters represents isolate FSL S6-072), a three-character ribotype (e.g., DUP-1045B = 45B), a one-character code for natural or urban environment (e.g., natural = N), and a one-digit year of isolation (e.g., 2001 = 1). Each isolate is also labeled with an allelic profile and corresponding ST. Clonal complexes identified by Based Upon Related Sequence Types (BURST) cluster analysis are indicated as A, B, and C.*

vironments *(34)*. Isolates thus represented five source categories, including (i) natural environments; (ii) urban environments; (iii) farm animals, farm animal feedstuff, and farm environments; (iv) foods and food-processing environments; and (v) human clinical cases. A total of 92 different ribotypes were identified among these isolates; 91% of the isolates grouped into 35 ribotypes that occurred more than five times, whereas 36 ribotypes occurred only once.

Categorical analysis of lineage distribution using an overall 3×5 table (3 lineages $\times$ 5 source categories) showed that lineages were not independently distributed among source categories ($P < 0.0001$). Individual 2×2 tables of specific sources versus lineages showed that lineage I was significantly more common among human isolates ($P < 0.0001$) and significantly less common among isolates from natural environments ($P = 0.0049$) and farms ($P < 0.0001$), whereas lineage II was significantly more common among isolates from natural environments ($P = 0.0034$), farms ($P < 0.0001$), and foods ($P = 0.0235$) and significantly less common among isolates from human clinical cases ($P < 0.0001$) (Table 4). Lineage III was significantly ($P = 0.0074$) associated with human clinical case isolates. Lineage diversity was highest among urban isolates ($D = 0.536$) (Table 2).

Categorical analysis of ribotype distribution using an overall 5×36 table (ribotypes with fewer than five occurrences were combined into a single category) (see Table 4) showed that ribotypes were not independently distributed across isolate sources ($P < 0.00001$). Individual 2×2 tables used to analyze the distribution of individual ribotypes among individual sources (''ribotype X versus not-ribotype X'' $\times$ ''source Y versus not-source Y'') (Table 4) showed that 2, 2, 7, 8, and 12 ribotypes were significantly more common than expected under a random distribution among isolates from natural environments, urban environments, foods, human clinical cases, and farms, respectively (Table 4). All but one of the lineage I ribotypes associated with human clinical cases were significantly underrepresented among farm or food isolates, and most lineage II isolates that were significantly underrepresented among human clinical isolates were overrepresented among farm or food isolates. Whereas farm isolates showed the highest overall ribotype diversity, isolates from natural environments showed the lowest overall ribotype diversity (Table 2).

Spatial analysis for proximity of *L. monocytogenes*–positive locations in natural environments to known farms. The observation that the *L. monocytogenes* ribotype (DUP-1039C) most commonly found among isolates from natural environments was also significantly overrepresented among farms (and foods) led us to test the hypothesis that the presence of *L. monocytogenes* in natural environments is related to its proximity to farms. Although the mean distance of *L. monocytogenes*–positive sample locations to the closest cattle farm was 4.41 km (SD = 3.75 km), the mean distance between the randomly selected sample locations in natural environments and the closest farm was 6.74 km (SD = 6.13 km). The difference between these distances was marginally significant ($P = 0.081$; two-sample t test).

DISCUSSION

L. monocytogenes isolated from urban and natural environments were characterized by molecular subtyping in an attempt to establish a broader understanding of the *L. monocytogenes* genetic diversity, distribution, and ecology

TABLE 4. *Distribution of* Listeria monocytogenes *lineages and ribotypes among isolates from natural, urban, and farm environments, foods and food environments, and human clinical cases*

		No. of *L. monocytogenes* isolates from[a,b]:					
Lineage	Ribotype	Natural	Urban	Farm[c]	Food[d]	Human[e]	Total
I	<5 occurrences[f]	0	3	18	7	14	42
	DUP-1027B	0	0	0	1	4 (+)*	5
	DUP-1025A	0	1	3	4 (+)*	1	9
	DUP-16635B	0	1	8 (+)**	0	0 (−)*	9
	DUP-1043A	0	0	2 (−)*	4	9 (+)*	15
	DUP-1042A	0	1	3 (−)*	3	12 (+)**	19
	DUP-1044B	0	2	9	0 (−)*	13 (+)*	24
	DUP-1042C	0	2	8	15 (+)***	0 (−)***	25
	DUP-1052A	0	1	7 (−)***	8	26 (+)***	42
	DUP-1038B	0	16 (+)***	13 (−)***	6 (−)*	38 (+)**	74
	DUP-1044A	0	2	9 (−)***	9	60 (+)***	80
	DUP-1042B	0	3	60 (+)*	9 (−)**	48	120
	All lineage I	1 (−)**	32	140 (−)***	66	225 (+)***	464
II	<5 occurrences	1	7	15	10	10	43
	DUP-1048B	0	0	0	5 (+)***	0	5
	DUP-1062C	0	0	2	0	3	5
	DUP-1023B	0	1	5	0	0	6
	DUP-1062F	0	0	6 (+)**	0	0	6
	DUP-18041	0	0	6 (+)**	0	0	6
	DUP-1046A	1	1	1	0	4	7
	DUP-1054A	0	1	5	0	1	7
	DUP-1039D	0	0	3	6 (+)**	3	12
	DUP-1062D	0	0	8	3	1	12
	DUP-1062E	0	0	10 (+)**	0	2	12
	DUP-1023A	0	1	12 (+)***	0	0 (−)**	13
	DUP-1030B	0	1	11 (+)**	0	1 (−)*	13
	DUP-1030A	0	0	3	5	7	15
	DUP-1039B	0	0	1 (−)**	9 (+)***	5	15
	DUP-1045D	0	0	14 (+)***	0	1 (−)*	15
	DUP-1045E	0	0	15 (+)***	0	0 (−)**	15
	DUP-1045A	1	0	24 (+)***	0 (−)*	2 (−)**	27
	DUP-1062A	0	0	0 (−)***	26 (+)***	2 (−)**	28
	DUP-1039A	0	1	16	1 (−)*	12	30
	DUP-1045B	3 (+)**	10 (+)***	11	3	8	35
	DUP-1053A	0	0	2 (−)***	6	31 (+)***	39
	DUP-1039E	2	4	39 (+)***	0 (−)**	3 (−)***	48
	DUP-1039C	4 (+)*	6	61 (+)***	25 (+)*	10 (−)***	106
	All lineage II	12 (+)**	33	270 (+)***	99 (+)*	106 (−)***	520
III	<5 occurrences	0	0	3	0	5	8
	DUP-1061A	0	2	1	0	6	9
	All lineage III	0	2	4	0	11 (+)*	17
	Total	13	67	414	165	342	1,001

[a] *L. monocytogenes Eco*RI ribotype or genetic lineage prevalences that were significantly higher (+) or lower (−) from a specific source as determined by categorical analyses were labeled as * (indicating $P \leq 0.05$), ** (indicating $P \leq 0.005$), or *** (indicating $P \leq 0.0005$).

[b] A complete listing of all isolate subtype data is available on-line (http://www.pathogentracker.net).

[c] Data in the column Farm represented 414 *L. monocytogenes* isolates from animals, animal feedstuff, and farm environments collected between 2001 and 2003 from 52 farms located predominantly in New York State (*34*).

[d] Data in the column Food represented 125 *L. monocytogenes* food isolates and 40 isolates of *L. monocytogenes* from food-processing environments collected in New York State between 1997 and 2002 (*41*).

[e] Data in the column Human represented 342 *L. monocytogenes* human clinical isolates collected in New York State between 1998 and 2002 (*41*).

[f] Individual ribotypes with fewer than five occurrences among all sources were grouped together for analysis.

in the natural environment, foods, food-processing environments, farms, and human disease. Our data indicate that (i) *L. monocytogenes* isolates from different sources show a high level of diversity; (ii) *L. monocytogenes* subtypes differ significantly in their associations with different environments, even though populations overlap; and (iii) a higher proportion of isolates from environmental sources than from human clinical cases can be classified in *L. monocytogenes* lineage II, which supports the classification of this lineage as an environmentally adapted subgroup.

***L. monocytogenes* from different sources shows a high level of diversity.** Combined analysis of more than 1,000 *L. monocytogenes* isolates obtained from various sources in New York State over a time span of approximately 5 years identified a total of 92 *Eco*RI ribotypes and an overall ribotype diversity of 0.950, as determined by Simpson's index of discrimination. Overall ribotype diversity was highest among farm isolates and lowest among isolates from natural environments. By comparison, only 63 *Eco*RI ribotypes were found among 994 human and food isolates collected over 2 years in two U.S. states *(18).* In conjunction with our observation that the ribotype diversity for all isolates analyzed was higher than the diversity observed among isolates from any single source, these data support the observation that overall *L. monocytogenes* diversity increases when isolates from various environments and niches are characterized. Our findings thus further support the observation that *L. monocytogenes* is a widely distributed, highly diverse species, even though *L. monocytogenes* prevalence seems to vary widely, e.g., from 1.4% among natural environments *(42)* to 20.1% among bovine and small ruminant herds, their feedstuff, and their farm environments *(34).*

Our finding that *Eco*RI ribotype diversity was higher among isolates from urban environments than from natural environments is consistent with our initial characterization of the 80 *L. monocytogenes* isolates used in this study by the sequencing of the stress-response gene *sigB (42),* which showed that *sigB* allelic diversity was higher among urban isolates ($D = 0.639$) than among isolates from natural environments ($D = 0.295$) *(42).* In contrast to both ribotype and *sigB* sequencing data, MLST analysis showed a higher diversity among isolates from natural environments than from urban environments. Because diversity detected by MLST may often be generated by horizontal gene transfer events, our observations may reflect that most isolates from natural environments represent lineage II organisms, which are more commonly involved in horizontal gene transfer than lineage I organisms *(29, 35),* and that horizontal gene transfer of housekeeping gene fragments is not likely to affect ribotype patterns. Further characterization of the *L. monocytogenes* isolate collection from the various sources analyzed in this study will provide an opportunity to further probe the importance and frequency of different mechanisms of diversity generation (e.g., point mutations, horizontal gene transfer) in isolates obtained from different environments and will thus ultimately provide a better understanding of the mechanisms behind the observed genetic diversity in *L. monocytogenes.*

Even though populations overlap, *L. monocytogenes* subtypes differ significantly in their associations with different environments. Categorical analysis of subtype data for 1,001 *L. monocytogenes* isolates from different sources showed that *L. monocytogenes* ribotypes and lineages differed significantly in their associations with different compartments, including (i) natural environments; (ii) urban environments; (iii) foods and food processing plants; (iv) farm animals, feeds, and farm environments; and (v) human clinical cases. Although many subtypes were found in all or nearly all compartments, other subtypes were almost exclusively found in single or comparatively few compartments. These findings extend previous reports, which indicate that *L. monocytogenes* isolates from animal clinical cases, human clinical cases, and foods form distinct yet overlapping populations *(3, 4, 18, 24, 41).*

L. monocytogenes lineages have previously been reported to show strong associations with specific sources, including an association of lineage I with human clinical illness and lineage II with foods and food-processing environments *(18, 23, 24, 36, 41, 51).* We found that, although lineages I and II were isolated from all sources, lineage I was strongly associated with human isolates but significantly underrepresented among isolates from natural and farm environments, which further supports the classification of this lineage as a human host–adapted lineage. Lineage II, which has been strongly associated with foods and food-processing environments in previous studies *(18, 23, 24, 36, 41, 51),* was strongly associated with natural environments, farms, and foods and was significantly underrepresented among human isolates, further confirming that this lineage is likely to represent environmental adaptation. Although these findings are also consistent with tissue culture plaque assay data, which have consistently shown that lineage I isolates show higher pathogenic potential (as determined by plaque size) than lineage II isolates *(18, 36, 55, 56),* it cannot be ruled out that culture bias that may favor the recovery of lineage II over lineage I isolates may contribute to these findings *(7).* However, this is unlikely to explain the high prevalence of lineage II isolates in natural environments, because the low prevalence of *L. monocytogenes* in these environments makes the presence of multiple *L. monocytogenes* lineages in a single sample highly unlikely.

Ribotype data also showed significant differences in the distribution of specific *L. monocytogenes Eco*RI ribotypes between different compartments. It is meaningful that three of the four ribotypes with the highest overall prevalence represented lineage I (DUP-1042B, DUP-1044A, and DUP-1038B). These three high-prevalence lineage I ribotypes represented the only three ribotypes that have been associated with multiple human listeriosis outbreaks; ribotype DUP-1038B has been linked to outbreaks in Anjou, France (1976), Nova Scotia, Canada (1981), Los Angeles (1985), and Switzerland *(23);* ribotype DUP-1042B has been linked to outbreaks in Boston (1979) and Massachu-

setts (1983) *(23)*; and ribotype DUP-1044A has been linked to two outbreaks in the United States in 1998 to 1999 and in 2002 caused by the consumption of contaminated hot dogs *(10)* and sliced turkey *(11)*, respectively. Consistent with their association with human listeriosis outbreaks, two of these ribotypes (DUP-1044A and DUP-1038B) were also significantly associated with human clinical cases. In addition, a previous study *(18)* reported that all three of these ribotypes were significantly less likely to occur among food isolates, which supports the observation that these subtypes show increased human virulence *(18)*. Ribotype DUP-1038B was found among all isolate sources (and was overrepresented among isolates from urban environments), which may suggest that its widespread distribution also contributes to its high prevalence among human listeriosis cases. Overall, that many human disease–associated ribotypes are also found among urban and pristine environments suggests that these environments play a role as potential primary sources for the introduction of human pathogenic *L. monocytogenes* subtypes into the human food chain and food processing plants.

***L. monocytogenes* lineage II represents an environmentally adapted subgroup.** A higher proportion of isolates from environmental sources than from human clinical cases can be classified in *L. monocytogenes* lineage II, which supports the classification of this lineage as an environmentally adapted subgroup. Specifically, we found that *L. monocytogenes* lineage II isolates were significantly associated with natural environments, farms, and foods and food processing plants yet underrepresented among human clinical isolates, a finding consistent with other reports, which found that lineage II isolates or serotypes grouped within lineage II were generally more common among foods than human isolates *(18, 23, 24, 32, 36, 41, 51)*. *L. monocytogenes* isolated from natural environments included ribotypes commonly found in farm environments (e.g., ribotype DUP-1039C), and spatial analyses showed a potential and marginally significant relationship between *L. monocytogenes*–positive locations in natural environments and a proximity to cattle farms. This could possibly indicate that, although *L. monocytogenes* isolates in farms and natural environments represent largely separate populations, some spillover occurs into natural environments from high-density *L. monocytogenes* populations on farms *(34)*.

Our data not only support the observation that *L. monocytogenes* is widely distributed and found in various environments, but they also show that *L. monocytogenes* is a genetically diverse species that includes genetic subtypes that differ significantly in their associations with different environments and compartments. Whereas *L. monocytogenes* lineages I and II appear to represent human foodborne disease transmission and environmental survival–adapted lineages, respectively, both natural and urban environments may represent sources of human disease–associated *L. monocytogenes*. However, the extent to which these sources contribute to the introduction of *L. monocytogenes* into the human food system and their consequent role in the transmission of disease remain to be ascertained.

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