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Multiple-Locus Variable-Number Tandem-Repeat Analysis as a Tool for Subtyping *Listeria monocytogenes* Strains▽

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***Listeria monocytogenes*, like many other food-borne bacteria, has certain strains that are commonly linked to outbreaks. Due to the relatively low numbers of affected individuals, outbreaks of *L. monocytogenes* can be difficult to detect. The current technique of molecular subtyping in PulseNet laboratories to identify genetically similar strains is pulsed-field gel electrophoresis (PFGE). While PFGE is state-of-the-art, interlaboratory comparisons are difficult because the results are highly susceptible to discrepancies due to even minor variations in experimental conditions and the subjectivity of band marking. This research was aimed at the development of a multiple-locus variable-number tandem-repeat analysis (MLVA) that can be implemented in PulseNet laboratories to replace or complement existing protocols. MLVA has proven to be a rapid and highly discriminatory tool for subtyping many bacteria. In this study, a novel MLVA method for *L. monocytogenes* strains was developed utilizing eight loci multiplexed into two PCRs. The PCR products were separated by capillary gel electrophoresis for high throughput and accurate sizing, and the fragment sizes were analyzed and clustered based on the number of repeats. When tested against a panel of 193 epidemiologically linked and nonlinked isolates, this MLVA for *L. monocytogenes* strains demonstrates strong epidemiological concordance. Since MLVA is a high-throughput screening method that is fairly inexpensive, easy to perform, rapid, and reliable, it is well suited to interlaboratory comparisons during epidemiological investigations of food-borne illness.**

Listeria monocytogenes, the causative agent of listeriosis, is a dangerous food-borne pathogen. Listeriosis causes meningitis, encephalitis, and septicemia, primarily in the elderly or in immunocompromised patients. It is most severe, however, in pregnant women and neonates due to its ability to cross the placenta and infect the fetus, causing congenital defects, stillbirth, and miscarriage. *L. monocytogenes* is most commonly acquired through consumption of contaminated foods such as unpasteurized or incompletely pasteurized cheeses and ready-to-eat foods, especially deli-type meats, due to its ability to grow at 4°C and to contaminate the food-processing environment. Given the severity of *L. monocytogenes* infections and potentially tragic outcomes, improving the detection of outbreaks and the discriminatory power of molecular subtyping methods is clearly a priority for food safety initiatives (7, 48). Public health laboratory scientists and epidemiologists play a critical role in this initiative by subtyping food-borne bacteria and performing outbreak investigations.

Bacterial subtyping is used to determine the relatedness among different isolates as part of an epidemiologic investigation. PulseNet, the international molecular subtyping network for food-borne bacteria developed and managed by the Centers for Disease Control and Prevention (CDC), utilizes pulsed-field gel electrophoresis (PFGE) as one key method for early detection of strains linked to potential outbreaks. This subtyping method compares DNA fragment patterns generated by macrorestriction digests of total genomic DNA that are separated by electrophoresis. The resulting banding patterns are compared to determine similarity. When clusters of isolates with similar PFGE profiles are detected, public health laboratories share these data with the epidemiologists, who then perform food history investigations to track the source of the organism. PulseNet PFGE protocols have been standardized and disseminated to public health laboratories by the CDC (32). These protocols must be strictly followed to ensure that the results are comparable from laboratory to laboratory.

The CDC developed an international database so that PulseNet laboratories can submit normalized PFGE patterns, thus enabling interlaboratory comparisons. PulseNet laboratories use the patterns submitted to the database to detect clusters of cases, to identify increases in the occurrence of a specific subtype, and to identify outliers to an outbreak. Subtyping by PulseNet laboratories in concert with epidemiological investigations has in many cases identified and/or confirmed the source of an outbreak (50). At the end of 2006, the *L. monocytogenes* database consisted of a total of 7,753 gel images (tiff files) including 920 unique AscI patterns and 1,262 unique ApaI patterns. Since the inception of the *L. monocytogenes* database, 77 clusters have been identified including 17 outbreaks linked to likely sources (Steven G. Stroika, PulseNet National Database Team, CDC, Atlanta, GA, personal communication).

L. monocytogenes has many serotypes, although serotypes 1/2a, 1/2b, and 4b are implicated in most cases of human disease (20). Serotype 4b is responsible for the majority of outbreaks and has been shown to be highly clonal (11, 17).

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TABLE 1. *L. monocytogenes* isolate information

Serotype and isolate name	Alternate name	Strain information			Source and/or reference	MLVA locus copy number							
		EG[b]	Date/location	Origin (type)[c]		Lm-2	Lm-8	Lm-10	Lm-11	Lm-3	Lm-15	Lm-23	Lm-32
Serotype 1/2a													
G3965[a]	TS-4/F6854	1	1988	Turkey franks (S)	CDC; 3, 6	16	3	5	1	1	2	32	14
G3975[a]	TS-14/F6900	1	1988	Clinical; turkey franks (S)	CDC; 3, 6	16	3	5	1	1	2	32	14
G4013[a]	TS-52/F7125	1	1988	Turkey franks (S)	CDC; 3, 6	16	3	5	1	1	2	32	14
G4000	TS-39/F7390	3	1988–1990	Food (S)	CDC; 6, 43	16	3	5	1	1	2	32	14
G4017	TS-56/F6801	3	1988–1990	Clinical (S)	CDC; 6, 43	16	3	5	1	1	2	32	14
G4028	TS-67/F6953	3	1988–1990	Clinical (S)	CDC; 6, 43	16	3	5	1	1	2	32	14
G4038	TS-77	3	1988–1990	Clinical (S)	CDC; 6, 43	16	3	5	1	1	2	32	14
G3972	TS-11/F7249	10	1988–1990	Processing plant (S)	CDC; 6, 53	16	3	5	1	1	2	32	14
G3976	TS-15/F7272	10	1988–1990	Processing plant (S)	CDC; 6, 53	16	3	5	1	1	2	32	14
G3979[a]	TS-18/F7222	10	1988–1990	Processing plant (S)	CDC; 6, 53	16	3	5	1	1	2	32	14
G3981	TS-20/F7283	10	1988–1990	Processing plant (S)	CDC; 6, 53	16	3	5	1	1	2	32	14
G3997[a]	TS-36/F7228	10	1988–1990	Processing plant (S)	CDC; 6, 53	16	3	5	1	1	2	32	14
G4010	TS-49/F7273	10	1988–1990	Processing plant (S)	CDC; 6, 53	16	3	5	1	1	2	32	14
G4023	TS-62/F7294	10	1988–1990	Processing plant (S)	CDC; 6, 53	16	3	5	1	1	2	32	14
G4029	TS-68/F7295	10	1988–1990	Processing plant (S)	CDC; 6, 53	16	3	5	1	1	2	32	14
H8395[a]	H7764			Food	CDC	16	3	5	1	1	2	32	14
J0161[a]			2000	Clinical; turkey (E)	CDC; 42	16	3	5	1	1	2	32	14
NC2001-021[a]	J0335		2000/NC	Clinical	NCSLPH	16	3	5	1	1	2	32	14
G3984[a]	TS-23/L745	14	1981/England	Clinical (E)	CDC; 6, 38	15	3	5	−2	1	2	20	13
G3994[a]	TS-33/L735	14	1981/England	Clinical (E)	CDC; 6, 38	15	3	5	−2	1	2	20	13
G4014	TS-53/L6483	23	Scotland	Animal strain	CDC; 6	16	3	5	4	1	2	27	13
G4020	TS-59			Animal strain	CDC	17	3	5	−2	5	2	39	13
G4039	TS-78	23	Scotland	Animal strain	CDC; 6	17	3	5	−2	5	2	39	13
G3991	TS-30/L5151	24	United Kingdom	Clinical (S)	CDC; 6	17	3	5	−2	6	2	39	13
G4005	TS-44/L5669	24	United Kingdom	Clinical (S)	CDC; 6	17	3	5	−2	6	2	39	13
EGDe[a]	BAA-679/ AL591824				ATCC; 29	18	3	5	4	4	2	19	13
H2446[a]			1996	Clinical (S)	CDC	15	3	5	−2	1	2	20	13
H3281[c]				Clinical	CDC	15	3	5	5	1	2	20	13
NC2001-025[a]	J0303		2001/NC	Clinical	NCSLPH	16	3	5	−2	6	1	24	13
NC2001-082	J0834		2001/NC	Clinical	NCSLPH	16	3	5	4	1	2	27	13
NC2001-236[a]	J1090		2001/NC	Clinical	NCSLPH	19	3	5	4	7	2	32	13
NC2002-001[a]	J1240		2002/NC	Clinical	NCSLPH	16	3	5	4	1	2	27	13
NC2002-002[a]	J2147		2002/NC	Clinical	NCSLPH	14	3	5	4	5	3	22	13
NC2002-119[a]	J1369		2002/NC	Clinical	NCSLPH	16	3	5	4	1	2	27	13
NC2002-327[a]	J2148		2002/NC	Clinical	NCSLPH	16	3	5	1	1	2	32	14
NCAg2002-890[a]			2002/NC	Deli meat	NCSLPH	16	3	5	4	1	2	27	13
NC2003-184[a]	J2416		2003/NC	Clinical	NCSLPH	16	3	5	−2	6	2	35	13
NC2004-363[a]	J3009		2004/NC	Clinical	NCSLPH	17	3	5	−2	5	2	39	13
NC2004-454	J3111		2004/NC	Clinical	NCSLPH	16	3	5	1	1	2	32	14
NC2005-397	J3496		2005/NC	Clinical	NCSLPH	17	3	5	4	4	2	30	13
NC2005-462	J3591		2005/NC	Clinical	NCSLPH	16	3	5	−2	3	1	18	13
NC2005-513	J3620		2005/NC	Clinical	NCSLPH	16	3	5	−2	2	1	18	13
NC2005-585	J3692		2005/NC	Clinical	NCSLPH	20	3	5	−2	3	2	20	13
NC2006-644	J4244		2006/NC	Clinical	NCSLPH	16	3	5	−2	9	1	26	13
Serotype 1/2b													
G3967	TS-6/F7473	4	1988–1990	Clinical (S)	CDC; 6, 43	15	3	5	4	4	4	33	14

G4019	TS-58/F7493	4	1988–1990	Food (S)	CDC; 6, 43	15	3	5	4	4	4	33	14
G3978	TS-17/F7271	5	1988–1990	Clinical (S)	CDC; 6, 43	16	3	5	5	6	5	29	17
G4022	TS-61/F7378	5	1988–1990	Food (S)	CDC; 6, 43	16	3	5	5	6	5	29	17
G4009[a]	TS-48/F7432	7		Clinical[d] (S)	CDC; 6	16	3	5	5	3	4	35	13
G4027[a]	TS-66/F2433	7		Clinical[d] (S)	CDC; 6	16	3	5	5	3	4	35	13
G3985[a]	TS-24/F7598	8		Clinical[e] (S)	CDC; 6	16	3	5	5	3	4	35	13
G4025[a]	TS-64/F7599	8		Clinical[e] (S)	CDC; 6	16	3	5	5	3	4	35	13
G3964[a]	TS-3/L4704	20		Clinical; soft cheese (S)	CDC; 6, 24	16	3	5	5	7	5	29	17
G4008[a]	TS-47/L4705	20		Soft cheese (S)	CDC; 6, 24	16	3	5	5	7	5	29	17
G3966	TS-5/L5674	24	United Kingdom	Clinical (S)	CDC; 6	16	3	5	5	7	5	29	17
G3977	TS-16/L5089	24	United Kingdom	Clinical (S)	CDC; 6	16	3	5	5	7	5	29	17
G4007	TS-46/L6116	24	United Kingdom	Clinical (S)	CDC; 6	16	3	5	5	7	5	29	17
G4598			Italy	GE[f]; rice salad (E)	CDC; 46	15	3	5	4	4	4	33	14
G4599			Italy	GE[f]; rice salad (E)	CDC; 46	15	3	5	4	4	4	33	14
G4600			Italy	GE[f]; rice salad (E)	CDC; 46	15	3	5	4	4	4	33	14
G4601			Italy	GE[f]; rice salad (E)	CDC; 46	15	3	5	4	4	4	33	14
G4602			Italy	GE[f]; rice salad (E)	CDC; 46	15	3	5	4	4	4	33	14
G6003			1994/IL	GE[f]; chocolate milk (E)	CDC; 16	16	3	5	5	6	5	25	17
G6004			1994/IL	GE[f]; chocolate milk (E)	CDC; 16	16	3	5	5	6	5	25	17
G6005			1994/IL	GE[f]; chocolate milk (E)	CDC; 16	16	3	5	5	6	5	25	17
H8393[a]			1994/IL	GE[f]; chocolate milk (E)	CDC; 16	16	3	5	5	6	5	25	17
G6006[a]			1994/IL	GE[f]; chocolate milk (E)	CDC; 16	16	3	5	5	6	5	25	17
G6054			1994/IL	GE[f]; chocolate milk (E)	CDC; 16	16	3	5	5	6	5	25	17
G6055[a]			1994/IL	GE[f]; chocolate milk (E)	CDC; 16	16	3	5	5	6	5	25	17
G6060			1994/IL	GE[f]; chocolate milk (E)	CDC; 16	16	3	5	5	6	5	25	17
NC2003-123[a]	J2326		2003/NC	Clinical	NCSLPH	12	3	6	5	4	−2	33	17
NC2005-625	J3773		2005/NC	Clinical	NCSLPH	16	3	5	4	3	3	27	14
NCAg2006-1396-1400			2006/NC	Egg Salad	NCSLPH	16	3	5	4	1	4	33	17
NCAg2006-1429-1433			2006/NC	Egg Salad	NCSLPH	16	3	5	4	1	4	33	17
NCAg2006-1434-1438	J4334		2006/NC	Egg Salad	NCSLPH	16	3	5	4	1	4	33	17
NCAg2006-1477-1481	J4335		2006/NC	Macaroni salad	NCSLPH	16	3	5	4	1	4	33	17
NCAg2006-1482-1486	J4336		2006/NC	Sweet slaw	NCSLPH	16	3	5	4	1	4	33	17
Serotype 1/2c													
G3963[a]	TS-2/L4706	22		Clinical[d] (S)	CDC; 6, 38	19	3	5	4	6	2	19	13
G3969[a]	TS-8/L940	22		Clinical[d] (S)	CDC; 6, 38	19	3	5	4	6	2	19	13
G4018	TS-57/L4397	24	United Kingdom	Clinical (S)	CDC; 6	19	3	5	4	6	2	19	13
G4041	TS-80	24	United Kingdom	Clinical (S)	CDC; 6	19	3	5	4	6	2	19	13
NCSU2006-1			2006/NC	Clinical	NCSLPH	15	3	−2	4	6	2	23	13
Serotype 3b													
G3968[a]	TS-7/G0039	6		Clinical (S)	CDC; 6	16	3	5	5	6	5	29	17
G3986[a]	TS-25/G0141	6		Food (S)	CDC; 6	16	3	5	5	6	5	29	17
G4015	TS-54/G0145	6		Food (S)	CDC; 6	16	3	5	5	6	5	29	17
G4035	TS-74	6		Food (S)	CDC; 6	16	3	5	5	6	5	29	17
F6218			1988/CA	Clinical (S)	CDC; 6	15	4	7	5	1	3	33	18
H1852			1996	Clinical (S)	CDC	16	3	3	5	3	4	33	15
H3288				Clinical	CDC	16	3	7	3	5	4	19	17

Continued on following page

TABLE 1—*Continued*

Serotype and isolate name	Alternate name	Strain information			Source and/or reference	MLVA locus copy number							
		EG[b]	Date/location	Origin (type)[c]		Lm-2	Lm-8	Lm-10	Lm-11	Lm-3	Lm-15	Lm-23	Lm-32
Serotype 4?													
G3962	TS-1/L2772	24	United Kingdom	Clinical (S)	CDC; 6	15	3	3	5	1	5	23	17
Serotype 4b													
G3971	TS-10/F8353	2	1988–1990	Food (S)	CDC; 6, 43	15	3	3	5	1	5	23	17
G3983	TS-22/F7954	2	1988–1990	Clinical (S)	CDC; 6, 43	15	3	3	5	1	5	23	17
G3993	TS-32/F8070	2	1988–1990	Food (S)	CDC; 6, 43	15	3	3	5	1	5	23	17
G4033	TS-72	2	1988–1990	Food (S)	CDC; 6, 43	15	3	3	5	1	5	23	17
G3974[a]	TS-13/F7440	9		Clinical[e] (S)	CDC; 6	15	4	5	5	1	3	33	18
G3989[a]	TS-28/F7441	9		Clinical[e] (S)	CDC; 6	15	4	5	5	1	3	33	18
G3995[a]	TS-34/F7439	9		Clinical[e] (S)	CDC; 6	15	4	5	5	1	3	33	18
G3996[a]	TS-35/F5070	11	1983/MA	Clinical; milk (E)	CDC; 6, 26	15	3	3	5	1	5	23	17
G4003[a]	TS-42/F1092	11	1983/MA	Clinical; milk (E)	CDC; 6, 26	15	3	3	5	1	5	23	17
G4036	TS-75	11	1983/MA	Clinical; milk (E)	CDC; 6, 26	15	3	3	5	1	5	23	17
G3990[a]	TS-29/F2365	12	1985/LA	Mexican-style cheese (E)	CDC; 6, 37	15	4	5	6	1	3	33	18
H8394[a]		12	1985/LA	Mexican-style cheese (E)	CDC; 6, 37	15	4	5	6	1	3	33	18
G4002	TS-41/G3129	12	1985/LA	Clinical; Mexican-style cheese (E)	CDC; 6, 37	15	4	5	6	1	3	33	18
G4004[a]	TS-43/F4565	12	1985/LA	Clinical; Mexican-style cheese (E)	CDC; 6, 37	15	4	5	6	1	3	33	18
G4037	TS-76	12	1985/LA	Clinical; Mexican-style cheese (E)	CDC; 6, 37	15	4	5	6	1	3	33	18
G3988[a]	TS-27/L4738	13	1981/Nova Scotia	Clinical; coleslaw (E)	CDC; 6, 47	15	4	5	5	1	3	33	18
G4011[a]	TS-50/L4760	13	1981/Nova Scotia	Coleslaw (E)	CDC; 6, 47	15	4	5	5	1	3	33	18
G4040	TS-79	13	1981/Nova Scotia	Clinical; coleslaw (E)	CDC; 6, 47	15	4	5	5	1	3	33	18
G3992[a]	TS-31/L4491e	15	1976/France	Clinical (E)	CDC; 6, 12	15	4	5	5	1	3	33	18
G4030[a]	TS-69/L4491a	15	1976/France	Clinical (E)	CDC; 6, 12	15	4	5	5	1	3	33	18
G3982[a]	TS-21/L4486j	16	1987/Switzerland	Cheese (E)	CDC; 5, 6	15	4	5	5	1	3	33	18
G4016[a]	TS-55/L4486a	16	1987/Switzerland	Clinical; cheese (E)	CDC; 5, 6	15	4	5	5	1	3	33	18
G4021[a]	TS-60/4486b	16	1987/Switzerland	Clinical; cheese (E)	CDC; 5, 6	15	4	5	5	1	3	33	18
G4032	TS-71	16	1987/Switzerland	Clinical; cheese (E)	CDC; 5, 6	15	4	5	5	1	3	33	18
G3973[a]	TS-12/L2192	17	1988/United Kingdom	Clinical; goat cheese (S)	CDC; 1, 6	15	4	5	6	1	3	33	18
G4001[a]	TS-40/L2190a	17	1988/United Kingdom	Goat cheese (S)	CDC; 1, 6	15	4	5	6	1	3	33	18
G3980[a]	TS-19/L1323	18	England	Soft cheese (S)	CDC; 2, 6	15	4	5	5	1	3	33	18
G3998[a]	TS-37/L1327	18	England	Clinical; soft cheese (S)	CDC; 2, 6	15	4	5	5	1	3	33	18
G3999[a]	TS-38/L3306	19	1988–1990/United Kingdom	Clinical; pâté (E)	CDC; 6, 39	15	3	3	5	1	5	23	17
G4006[a]	TS-45/L3350	19	1988–1990/United Kingdom	Pâté (E)	CDC; 6, 39	15	3	3	5	1	5	23	17
G3970	TS-9/L4704	21		Alfalfa (S)	CDC; 6, 24	15	4	5	5	1	3	33	18
G4024	TS-63/L4706	21		Clinical; alfalfa (S)	CDC; 6, 24	15	4	5	5	1	3	33	18
G4034	TS-73	21		Clinical; alfalfa (S)	CDC; 6, 24	15	4	5	5	1	3	33	18
G4031	TS-70				CDC	15	3	3	5	1	5	23	17

Li2[a]	19115/ A52868/ A52869			ATCC	15	3	3	5	1	5	23	17
H2444		1996	Clinical (S)	CDC	12	3	5	5	1	3	19	17
H3396		1997	Clinical (S)	CDC	12	3	5	5	1	3	19	17
H6383		1996	Clinical (S)	CDC	12	3	5	5	1	3	19	17
H7550[a]		1998	Clinical; hot dogs (E)	CDC; 13	12	3	5	5	1	3	42	17
H7596[a]		1998	Hot dogs (E)	CDC; 13	12	3	5	5	1	3	42	17
H7762[a]		1998	Hot dogs (E)	CDC; 13	12	3	5	5	1	3	42	17
H7969[a]		1998	Hot dogs (E)	CDC; 13	12	3	5	5	1	3	42	17
J1735		2002	Clinical; deli meat (E)	CDC; 30	12	3	5	5	1	3	19	17
J1815		2002	Environmental; deli meat (E)	CDC; 30	12	3	5	5	1	3	19	17
J1925		2002	Deli meat (E)	CDC; 30	12	3	5	5	1	3	19	17
J1838		2002/NJ	Clinical (E)	CDC; 30	12	3	5	5	1	3	25	17
J2206		2003/NJ	Clinical (S)	CDC	12	3	5	5	1	3	19	17
J2213		2003/AZ	Clinical (S)	CDC	15	4	5	5	1	3	33	18
J2230		2003/MA	Clinical (S)	CDC	12	3	5	5	1	3	42	17
J2255		2003/GA	Clinical (S)	CDC	15	3	3	5	2	5	31	11
J2269		2003/GA	Clinical (S)	CDC	16	3	5	5	6	5	29	17
J2275		2003/PA	Clinical (S)	CDC	15	4	5	5	1	3	33	18
J2282		2003/MD	Clinical (S)	CDC	15	4	5	5	1	3	33	18
J2288		2003/TX	Clinical (S)	CDC	15	4	7	5	1	3	33	18
J2302		2003/CA	Clinical (S)	CDC	15	4	5	5	1	3	33	18
J2313		2003/TX	Clinical (S)	CDC	15	4	7	5	1	3	33	18
J2327		2003/MI	Clinical (S)	CDC	15	4	5	5	1	3	37	18
J2353		2003/IL	Clinical (S)	CDC	15	4	5	5	1	3	33	18
J2391		2003/TX	Clinical; Mexican-style cheese (S)	CDC	15	4	7	5	1	3	33	18
J2422		2003/RI	Clinical (S)	CDC	15	3	9	4	2	5	27	17
J2433			Mexican-style cheese	CDC	15	4	7	5	1	3	33	18
J2446		2003/OH	Clinical (S)	CDC	12	3	5	5	1	3	42	17
J2479		2003/MI	Clinical (S)	CDC	11	3	5	5	−2	2	34	−2
J2571		2003/KY	Clinical (S)	CDC	12	3	5	−2	−2	2	33	−2
J2584		2003/VT	Clinical (S)	CDC	15	4	5	5	1	3	33	18
J2621		2003/OR	Clinical (S)	CDC	15	3	5	5	2	3	15	17
J2685		2004/NY	Clinical (S)	CDC	12	3	5	5	1	3	19	17
J3006		2004/TX	Clinical (S)	CDC	12	3	5	5	1	3	19	17
J3033		2004/IL	Clinical (S)	CDC	12	3	5	5	1	3	19	17
J3200		2004/CT	Clinical (S)	CDC	12	3	5	5	1	3	25	17
J3238		2004/NY	Clinical (S)	CDC	12	3	5	5	1	3	25	17
J3558		2005/GA	Clinical (S)	CDC	15	4	5	5	1	3	33	18
NC2001-004[a]	J0247	2001/NC	Clinical	NCSLPH	16	3	5	5	1	4	33	17
NC2001-006[a]	J0246	2001/NC	Clinical	NCSLPH	16	3	5	5	1	4	33	17
NC2001-007[a]	J0245	2001/NC	Clinical	NCSLPH	15	3	3	5	3	4	25	17
NC2001-008[a]	J0244	2001/NC	Clinical	NCSLPH	15	3	3	5	3	4	25	17
NC2001-075[a]	J0835	2001/NC	Clinical	NCSLPH	11	3	5	5	−2	2	27	−2
NC2001-126[a]	J0833	2001/NC	Clinical	NCSLPH	12	3	5	5	1	3	34	17
NC2001-182[a]	J0927	2001/NC	Clinical	NCSLPH	15	4	5	5	1	3	33	14
NC2003-151[a]	J2339	2003/NC	Clinical	NCSLPH	11	3	5	5	−2	−2	36	−2
NC2003-173[a]	J2415	2003/NC	Clinical	NCSLPH	15	3	7	4	3	3	27	17
NC2003-196[a]	J2470	2003/NC	Clinical	NCSLPH	15	3	5	5	1	5	33	17
NC2003-289[a]	J2599	2003/NC	Clinical	NCSLPH	11	3	5	−2	−2	2	28	−2

Continued on following page

TABLE 1—*Continued*

Serotype and isolate name	Alternate name	Strain information			Source and/or reference	MLVA locus copy number							
		EG[b]	Date/location	Origin (type)[c]		Lm-2	Lm-8	Lm-10	Lm-11	Lm-3	Lm-15	Lm-23	Lm-32
NC2003-332[a]	J2638		2003/NC	Clinical	NCSLPH	15	4	5	5	1	3	33	18
NC2004-271-273[a]	J2932		2004/NC	Clinical[e]	NCSLPH	16	3	5	5	1	4	33	17
NC2004-287[a]	J2933		2004/NC	Clinical	NCSLPH	15	4	5	5	1	3	33	18
NC2004-445-446[a]	J3061		2004/NC	Clinical[d]	NCSLPH	16	3	5	5	1	4	33	17
NC2004-471	J3112		2004/NC	Clinical	NCSLPH	15	3	7	5	2	4	33	18
NC2004-643	J3219		2004/NC	Clinical	NCSLPH	15	3	7	5	1	3	33	17
NC2005-062	J3294		2004/NC	Clinical	NCSLPH	15	3	5	5	1	3	15	21
NC2005-446	J3552		2005/NC	Clinical	NCSLPH	15	4	5	5	1	3	33	18
NC2005-490	J3590		2005/NC	Clinical	NCSLPH	12	3	5	5	1	3	40	17
NC2005-681	J3825		2005/NC	Clinical	NCSLPH	15	4	5	5	1	3	33	18
NC2006-296	J3982		2006/NC	Clinical	NCSLPH	15	3	3	5	3	4	25	17
NCSU-34-2b			2003/NC	Processing plant	S. Kathariou; 22	12	3	5	5	1	3	40	17
NCSU-34-6a			2003/NC	Processing plant	S. Kathariou	12	3	5	5	1	3	25	17
Serotype 4bx													
G4012[a]	TS-51/L3334	19	1988–1990/United Kindgom	Pâté (E)	CDC; 6, 39	15	3	3	5	1	5	23	17
G4026[a]	TS-65/L3238	19	1988–1990/United Kindgom	Clinical; pâté (E)	CDC; 6, 39	15	3	3	5	1	5	23	17
Serotype 4c													
NC2006-612	J4245	21	2006/NC	Clinical	NCSLPH	11	3	5	−2	−2	2	30	−2
Serotype 4d													
G3987[a]	TS-26/L4742	13	1981/Nova Scotia	Clinical; coleslaw (E)	CDC; 6, 47	15	4	5	5	1	3	33	18

[a] Seventy nine isolates used to determine subtyping ability.
[b] EG are based on a WHO study (6).
[c] S, sporadic; E, epidemic. If the food vehicle is known, it is given after the semicolon.
[d] Multiple isolates from the same patient.
[e] Grouped isolates from mothers and babies.
[f] GE, gastroenteritis outbreak.

Among the outbreak strains of serotype 4b, two strain subtypes continually reemerge. These are known as epidemic clone I (ECI) and epidemic clone II (ECII). These clones have been responsible for many outbreaks within North America and Europe (23, 34, 54). Due to the clonal nature of *L. monocytogenes*, novel subtyping methods are required to accurately discriminate among these common strain types. The current method, PFGE, is very labor-intensive and somewhat subjective. PFGE also relies on computer-based band marking, which can be inaccurate and requires manual interpretation by trained personnel to identify clusters. In practice, even minor deviations in experimental conditions can produce pronounced differences in patterns. Significant differences in patterns can also be attributed to the presence of mobile genetic elements. Other methods developed to subtype *L. monocytogenes* strains include multilocus sequence typing (15, 44, 56) and suspension microarray analysis based on specific genes or single-nucleotide polymorphisms (8, 9, 21). These methods tend to be technically demanding and expensive although the data are portable and nonsubjective. Since these methods are based on DNA sequences, they are genetically relevant.

Multiple-locus variable-number tandem-repeat (VNTR) analysis (MLVA) is a proven, rapid, and highly discriminatory subtyping method for agents such as *Bacillus anthracis*, *Francisella tularensis*, and *Escherichia coli* (25, 28, 35, 36). This type of analysis has been successful because bacteria have highly variable repeated elements throughout their genomes. VNTRs are short segments of DNA that have hypervariable copy numbers. It is thought that the variation in copy number is due to slipped-strand mispairing during DNA polymerase mediated duplications or possibly due to recombination (51). Despite mutations that may occur within the tandem repeat, the unit length remains relatively constant while the copy number varies. The tandem repeats are in stable regions of the genome, and they are not likely to be associated with mobile genetic elements, such as plasmids. The difference in copy numbers at specific loci is used to measure relatedness of strains in this subtyping scheme. To date, only limited information is available on MLVA applications with *L. monocytogenes*. In a recently published study, six loci were employed to subtype 45 isolates. Most of the isolates included were serotype 1/2a; strains of serotypes 1/2b and 4b were not sufficiently represented in this study (40). The current research was aimed at the development of MLVA for *L. monocytogenes* strains that can be implemented in PulseNet laboratories to replace or complement existing protocols.

MATERIALS AND METHODS

Bacterial strains and nucleic acid extraction. A total of 193 *L. monocytogenes* isolates were acquired from the culture collections at the CDC (Atlanta, GA), North Carolina State University (Raleigh, NC), North Carolina State Laboratory of Public Health ([NCSLPH] Raleigh, NC) (clinical specimens from 2001 to 2006), and the American Type Culture Collection (Manassas, VA) (Table 1). Many of the strains included have been described previously in the World Health Organization (WHO) international multicenter *L. monocytogenes* subtyping study as well as other publications (6, 10, 27).

Each isolate was streaked for isolation and grown on 5% sheep blood agar (BBL blood agar base [infusion agar]; BD, Franklin Lakes, NJ) at 35°C overnight. Cultures were preserved using a Microbank cryo-preservation system (Pro-Lab Diagnostic, Austin, TX), per the manufacturer's directions, and stored at −70°C. Multiple methods for nucleic acid preparation were used. A loopful (using a sterile calibrated 1-μl inoculating loop) of pure bacterial growth was used for all methods. The MLVA protocol utilized the "boil prep" method (28, 35). Briefly, the bacteria were suspended in 100 μl of sterile nuclease-free H_2O (Amresco, Solon, OH); the suspension was boiled at 95 to 100°C for 10 min and immediately chilled on ice to aid in cell lysis. Cell suspensions were centrifuged at 8,000 × *g* for 10 min to separate cellular debris. The clarified supernatant was used in the PCR. During initial development and for nucleic acid sequencing, DNA was extracted using a DNeasy Tissue kit (Qiagen, Valencia, CA) or MagNA Pure LC DNA Isolation III kit (Roche, Indianapolis, IN) per the manufacturer's directions for gram-positive organisms. Extracted DNA was stored at −20°C.

PFGE. All PFGE was performed using PulseNet standardized procedures with Asc1 and Apa1 restriction enzymes (www.cdc.gov/pulsenet/protocols.htm) (32). Many of the PFGE patterns were downloaded from the PulseNet international database. The PFGE patterns that could not be acquired from the PulseNet database were analyzed by NCSLPH's PulseNet laboratory. Analysis was performed using BioNumerics (Applied Maths, Austin, TX) cluster analysis. The average from experiments was used to cluster the similarity matrix determined by each single enzyme analysis (AscI and ApaI) using the Dice coefficient with a 1.5 tolerance and the unweighted pair group method with arithmetic mean (UPGMA).

Genome analysis and primer design. The two fully sequenced *L. monocytogenes* genomes, EGDe (accession number AL591824) (29) and F2365 (AE017262) (41), were used for analysis of tandem repeats. The two genomes were initially scanned individually using the Tandem Repeat Finder program (http://tandem.bu.edu/trf/trf.html) (4). The Genomes, Polymorphism and Minisatellites strain comparison page in the Microorganisms Tandem Repeat Database(http://minisatellites.u-psud.fr/) was then used to scan and compare both genomes (18, 19, 52). Primers were designed utilizing Primer3 software (http://frodo.wi.mit.edu/) (45). Each primer was designed in the flanking region of the tandem repeat to produce a fragment size no larger than 600 bp. Primers were synthesized by Proligo (Boulder, CO) and Integrated DNA Technologies (Coralville, IA). For fragment analysis, the forward primers were labeled with one of three WellRed dye-labeled phosphoramidites (D2, D3, and D4) and purified by high-performance liquid chromatography.

PCR amplification and fragment analysis. Initial analysis of each locus was performed utilizing QuantiTect Sybr Green PCR (Qiagen, Valencia, CA) per the manufacturer's instructions on an iQ iCycler (Bio-Rad, Hercules, CA) with 0.3 μM unlabeled primer and 2 μl of DNA in 25-μl reaction mixtures; the PCR program consisted of 35 cycles, annealing at 50°C, and melt curve analysis. Standard PCR was performed per the manufacturer's directions using HotStar *Taq* polymerase (Qiagen, Valencia, CA), 0.3 μM of each primer, and 2 μl of DNA in 25-μl reaction mixtures; the PCR program consisted of 35 cycles and annealing at 50°C. Initial multiplexed PCR used a Multiplex PCR kit (Qiagen, Valencia, CA) per the manufacturer's instructions with 0.3 μM of each labeled primer (Table 2) and 2 μl of DNA in 25-μl reaction mixtures; the PCR program consisted of 35 cycles and annealing at 50°C. The finalized protocol consisted of two multiplexed PCRs (R1 and R2), each with four primer sets (concentrations are given in Table 2) using 1.5 U of Platinum *Taq* DNA polymerase, 2 mM $MgCl_2$, 0.2 mM of the deoxynucleoside triphosphates, 1× PCR buffer, PCR-grade water (Invitrogen, Carlsbad, CA), and 1 μl of DNA in 10-μl reaction mixtures. The cycling conditions used were as follows: 95°C for 5 min and 35 cycles of 94°C for 20 s, 50°C for 20 s, and 72°C for 20 s, followed by one cycle of 72°C for 5 min and an indefinite hold at 4°C on an MJF Tetrad (Bio-Rad, Hercules, CA) (28).

Fragments were sized by combining 1 μl of a 1:60 dilution of the PCR product with 20 μl of deionized formamide (sample loading solution) and 0.08 μl of DNA size standard 600 (Beckman Coulter, Fullerton, CA) (9, 28). Fragment analysis was performed on a Beckman Coulter CEQ 8000 genetic analyzer (Beckman Coulter, Fullerton, CA) using the Frag-Test method, and fragments were analyzed with default fragment analysis parameters edited to reflect the DNA size standard 600 and quartic model (28). Estimated fragment size, peak height, and dye for each isolate were exported in comma-delimited format (.cvs) and imported in BioNumerics (Applied Maths, Austin, TX). Customized scripts were developed by Applied Maths (Austin, TX) and are available at www.applied-maths.com. These scripts are used to import the fragment sizes (VNTRimport_v3) and to calculate copy numbers (VNTRcalc). Null alleles were coded as negative. UPGMA cluster analysis of copy number was performed with a categorical multistate coefficient.

Sequence verification. The loci and flanking regions were amplified with HotStar *Taq* Polymerase (Qiagen, Valencia, CA) as described above. The PCR products were purified using the QIAquick PCR Purification kit (Qiagen, Valencia, CA). Cycle sequencing was performed per the manufacturer's directions

TABLE 2. MLVA Primers for *L. monocytogenes*

PCR and VNTR locus	Dye[a]	Forward primer (5′–3′)	Reverse primer (5′–3′)	Final conc (μM)
R1				
Lm-2	D4	CGTATTGTGCGCCAGAAGTA	CAGCAACGCAACAACAAACAG	0.1
Lm-8	D2	ACGCGCAATACTATAAAGGGTGTC	AGAAAAAGCGGAAGCAGATAAGAA	0.2
Lm-10	D3	CAGATATCGATACGATTGAC	CAGTTAGTATTTCCAACGTC	0.35
Lm-11	D3	GAATAAAATGCTAGATGTGG	CCGATTCAAAAATAGTAAAC	0.15
R2				
Lm-3	D4	CAAACCGAGATGGTGTAGCA	TGGTTTTGATGGATCAACTGG	0.05
Lm-15	D2	GGACTTAACGAATACAAAAG	GCTGTTACAAGTAAAACTGG	0.15
Lm-23	D4	TATTTACGGAAAAGACGTAG	CGTAACTGTCCTACCATTAG	0.1
Lm-32	D3	AAAGCTTTGCCAGTGCAAGT	TTGTGACTTGGCACTTCTGG	0.25

[a] WellRed phosphoramidite dye label attached to the 5′ end of forward primer.

with the CEQ DTCS (dye terminator cycle sequencing) Quick Start kit (Beckman Coulter, Fullerton, CA) using 0.2 μM primer and 25 to 100 fmol of template DNA. Unincorporated dye terminators were removed with the DyeEx 2.0 spin kit (Qiagen, Valencia, CA) using an additional wash step of 300 μl of sterile nuclease-free sequence grade water (Amresco, Solon, OH) prior to the application of the sample or by using Clean Seq (Agencourt Biosciences, Beverly, MA) per the manufacturer's instructions. All samples were run on the CEQ 8000 genetic analyzer (Beckman Coulter, Fullerton, CA) using the LFR-1 method and default sequence analysis parameters.

Stability and reproducibility determinations. Two *L. monocytogenes* strains, EGDe and Li2 (ATCC, Manassas, VA), were chosen for the stability study. Each was passaged on sheep blood agar plates 45 times, approximately 3 times per week. Samples were boiled as described previously at each passage. Ten evenly spaced isolates were tested by MLVA to determine the stability of each locus.

Reproducibility of the assay was determined by testing 100 isolates via the two multiplexed PCRs. This analysis was performed in triplicate on three different runs by two technicians.

RESULTS AND DISCUSSION

Identification of suitable loci. A total of 75 tandem repeats were identified within the two fully sequenced genomes of *L. monocytogenes* (EGDe and F2365). Forty-three of these repeated elements that were under 600 bp in total length and had a unit length of 3 to 21 bp (Table 3) were analyzed. Initial screening of the loci was performed in a pilot study with a panel of 10 isolates consisting of two *L. monocytogenes* isolates, representing each of the following serotypes: 1/2a, 1/2b, 1/2c, 3b, and 4b. This pilot study of all loci was performed using Sybr green PCR with unlabeled primers for rapid and inexpensive preliminary screening. To ensure accurate amplification, primers where redesigned as needed.

The Genomes, Polymorphism and Minisatellites website allowed us to compare the tandem repeats found within the two fully sequenced genomes. Some of the tandem repeats found individually in each of the genomes were, in fact, the same or overlapping. This was seen with Lm-8, Lm-9, and Lm-36; Lm-6, Lm-7, Lm-23, and Lm-35; and Lm-26 and Lm-37. The 37 remaining loci were tested on an expanded panel of 79 isolates (Table 1) with labeled primers using capillary electrophoresis fragment analysis to determine subtyping ability. The tandem repeats that did not produce accurate and reproducible results within the serotypes causing the majority of human illness (1/2a, 1/2b, and 4b) were eliminated from the study (Table 3). Lm-5, Lm-29, and Lm-34 were eliminated for their failure to amplify serotypes 1/2b, 3b, and 4b (Table 3).

In order to verify that the size determined by fragment analysis was indeed due to a change in repeat number and not to other genetic events, such as insertions and deletions, sequence analysis of both DNA strands was performed. For each locus, two isolates of each different allele size identified were sequenced. When necessary, primers were redesigned to provide more consistent sequencing results (data not shown). Sequence analysis showed that Lm-1, Lm-4, Lm-20, Lm-25, Lm-27, and Lm-40 had variability in the flanking regions that could not be avoided even with primer redesign. The variability included insertions and deletions that produced a change in fragment size that was not due to variations in repeat number. Since the sequence variability is not related to the tandem repeat, these loci were eliminated from this study. Loci that had very low diversity or did not affect subtyping ability were also eliminated (Table 3). Thus, a total of eight loci (Lm-2, Lm-3, Lm-8, Lm-10, Lm-11, Lm-15, Lm-23, and Lm-32) remained in this study.

Two of these loci, Lm-3 and Lm-10, were identified independently by Murphy et al. (LM-TR-1 and LM-TR-4) (40). In our study we also independently identified the other four loci described previously (40). These four loci were not chosen for inclusion in our *L. monocytogenes* MLVA for several reasons. Lm-5 (LM-TR-6) did not amplify serotypes 1/2b, 3b, and 4b, while Lm-26 (LM-TR-2) was found to have very low diversity. Genomic analysis showed that the other two loci (LM-TR-3 and LM-TR-5) overlapped. These tandem repeats were equivalent to our Lm-4, which was eliminated from the study due to sequence variability in the flanking region, possibly due to the overlapping repeat and not to a difference in copy number within the tandem repeat.

Assay development. Eight of the remaining tandem repeats provided adequate diversity and were thoroughly evaluated with all 193 isolates (Table 1) for their ability to subtype these strains into epidemiologically significant clusters. Partial repeats were seen in Lm-10 and Lm-23. In all cases, these resulted in half of a repeat. For the purpose of analysis in BioNumerics, the allele size was changed from 12 to 6 and 6 to 3, respectively, to account for these half-repeats. In some instances no amplification was observed at a particular locus. Lack of amplification (a null allele) could be due to mutation

TABLE 3. All loci examined

VNTR locus (Lm)	Reason for exclusion[a]	Identification in strain EGDe[b]		Identification in strain F2365[b]		Comment (reference)
		Location (nt)	Locus tag (lmo)	Location (nt)	Locus tag (LMOf2365)	
1	VFR	159184–159239	0159	168482–168437	0174	
2	IU	619313–619457	0582	626060–626186	0611	
3	IU	881561–881690	0842	881681–881756	0859	LM-TR-1 (40)
4	VFR	1171166–1171425	1136	1150533–1150708	1144	LM-TR-3, LM-TR-5 (40)
5	FAS	1315205–1315275	1289	ND	ND	LM-TR-6 (40)
8	IU	2016006–2016236	1941	1997109–1997354	1970	Overlap with Lm-9, Lm-36
10	IU	ND	ND	234028–234127	0231	LM-TR-4 (40)
11	IU	344972–345002	0320	358075–358150	0338	
12	LD	426092–426116	NC 0402/043	N/D	ND	
13	LD	ND	ND	473899–473987	0450	
14	LD	498894–499103	0460	515502–515689	0495	
15	IU	668815–668859	0627	675532–675588	0656	
16	LD	ND	ND	4B661176–661207	0643	
17	LD	912002–912032	0872	ND	ND	
18	LD	1084362–1084389	NC 1055/1056	1085094–1085121	NC 1076/1077	
19	LD	1109362–1109397	1077	ND	ND	
20	VFR	1163213–1163250	1129	ND	ND	
21	LD	1674419–1674448	1632 (*trpG*)	1652787–1652816	1654	
22	LD	1821786–1821825	1752	ND	ND	
23	IU	ND	ND	1849586–1849694	NC 1825/1826	Overlap with Lm-6, Lm-7, Lm-35
24	LD	2229537–2229561	2144	ND	ND	
25	MB	2390349–2390507	2312	ND	ND	
26	LD	2169162–2169208	NC 2089/2090	2157801–2157867	NC 2121/2122	Overlap with Lm-37, LM-TR-2 (40)
27	VFR	ND	ND	2407116–2407160	2347	
28	LD	ND	ND	386518–386530	NC 0361/0362	
29	FAS	ND	ND	510256–510273	0492	
30	LD	ND	ND	512407–512437	NC 0493/0494	
31	LD	907377–907434	0866	907466–907491	0884	
32	IU	1317290–1317361	1290	1297917–1298018	1307	
33	LD	1318139–1318186	1291	1298796–1298855	1308	
34	FAS	1706118–1706142	NC 1656/1657	1683878–1683950	NC 1677/1678	
38	LD in 4b	2682706–2682722	2778	2631575–2631585	NC 2655/2567	
39	LD in 4b	161183–161252	0160	170379–170475	0175	
40	VFR	173499–173536	0175	178838–178893	0186	
41	SNI	361132–361199	0333	376005–376063	0350	
42	SNI	1748203–1748237	NC 1682/1683	1729244–1729287	NC 1706/1707	
43	SNI	2264184–2264205	2178	2259418–2259445	2211	

[a] IU, locus in use; LD, low diversity; VFR, variability in flanking region; SNI, subtyping ability not improved; FAS, failed to amplify serotypes 1/2b, 3b, and 4b; MB, multiple band/peak products.
[b] nt, nucleotide; NC, noncoding region, located between locus tags; ND, not detected by tandem-repeat database.

at the primer site resulting in no PCR product. Since a null allele is different from a locus having zero repeats, it is denoted as −2 by BioNumerics (Table 4). Null alleles were observed in Lm-3, Lm-11, and Lm-32.

A multiplexed PCR protocol was developed for these eight loci consisting of two reactions with four loci in each (Fig. 1). This assay was made to be concordant with the protocols previously developed for PulseNet laboratories to subtype *E. coli* and *Salmonella enterica* serotype Typhimurium (9, 28). To determine subtyping capabilities, the complete panel of 193 isolates consisting of both known outbreak and sporadic strains and including isolates from each of the serotypes of interest was analyzed (Table 1). The Simpson's index of diversity (49) for the eight tandem repeats ranged from 33.8% to 84.5% (Table 4). Based on the complete panel of isolates and the 54 unique MLVA profiles they produced, the calculated Simpson's index of diversity for the assay is 94%.

The stability of each locus was evaluated to determine the effect of laboratory passage. All fragment sizes varied by less than ±1 bp (0.02 bp to 0.88 bp), indicating that these loci are stable during routine laboratory manipulation. The multiplexed MLVA was shown to be reproducible by determining the copy number for each locus on a panel of 100 isolates. Although the fragment sizes showed slight variation (less than ±1 bp), the copy number was determined to be 100% reproducible (data not shown).

Comparative effectiveness of MLVA and select other methods. Copy numbers as determined by BioNumerics of all 193 isolates are shown in Table 1. A panel of 123 isolates (Table 1) was analyzed by this multiplexed MLVA and compared to PFGE (AscI and ApaI). Cluster analysis of these isolates reveals that MLVA efficiently separates isolates of genomic division 1 (lineage II) (serotypes 1/2a and 1/2c) from those of genomic division 2 (lineage I) (serotypes 1/2b, 3b, 4b, and a single isolate of 4d) (Fig. 2). Only a single clinical isolate of serotype 4c was available and was not included in the cluster analysis; however, the MLVA type is indicated in Table 1. The clear differentiation between the major genomic divisions (lineages) was in agreement with similar findings from numerous

TABLE 4. *L. monocytogenes* VNTR Loci

VNTR locus	Unit length (bp)	Allele range (copy no.)	Offset (bp)[c]	Repeat	Diversity (%)[d]	Locus tag	Protein description or function
Lm-2	6	11–20	294	TTGTAT	68.8	lmo0582	P60 extracellular protein; invasion associated protein
Lm-3	9	1–9 (−2)[b]	203	TAAAACCTA	56.8	lmo0842	Cell wall surface anchor family protein
Lm-8	15	3–4	187	CAGCTTTCTCAGCAG	33.8	lmo1941	*lysM* domain protein
Lm-10	12 (6)[a]	3–9	315	GAAGAACCAAAA	29.4	lmo0220	ATP-dependent metalloprotease FtsH
Lm-11	12	1–6 (−2)[b]	103	TTGCTTGTTTTTG	60.4	lmo0320	Cell wall surface anchor family protein
Lm-15	12	1–7	321	CAAAAGATACAC	74.6	lmo0627	Cell wall surface anchor family protein
Lm-23	6 (3)[a]	15–42	164	CATCGG	84.5	lmo1799	Putative peptidoglycan bound protein (LPXTG motif)
Lm-32	6	13–21 (−2)[b]	84	AACACC	74.2	lmo1290	Hypothetical protein

[a] Half-unit lengths used to compensate for partial alleles observed.
[b] Null alleles, interpreted by the BioNumerics program as a copy number of −2, are possible at this locus.
[c] Size of the fragment with no tandem repeats.
[d] Simpson's index of diversity (based on 193 isolates tested) is calculated as follows: $[1 - \Sigma(\text{allele frequency}^2)] \times 100$, where the allele frequency is the number of times a particular allele appears/total number of strains tested.

other subtyping approaches (for a review, see references 8, 10, 17, 44, 55, and 56).

Comparisons of MLVA and PFGE techniques can be quite difficult since they evaluate different types of genetic events. While PFGE relies on changes in the restriction enzyme site, MLVA relies on copy number changes of tandem repeats. Both are successful in grouping closely related and differentiating unrelated *L. monocytogenes* strains. PFGE and MLVA comparisons were performed by separating the isolates into groups based on serotype.

Although the MLVA and PFGE techniques clustered this diverse set of isolates differently, the results were very similar. Seven unique profiles were produced for 32 1/2a and 1/2c isolates examined by MLVA (Fig. 3A), while PFGE (AscI and ApaI) (Fig. 3B) produced 11 unique profiles based on cluster analysis in BioNumerics. These different profiles were in some cases due to one- or two-band differences. For instance, several strains (Fig. 3B, filled circles) exhibited a one-band difference in the ApaI pattern. The strains with this profile included isolate J0161 from the 2001 multistate outbreak as well as food,

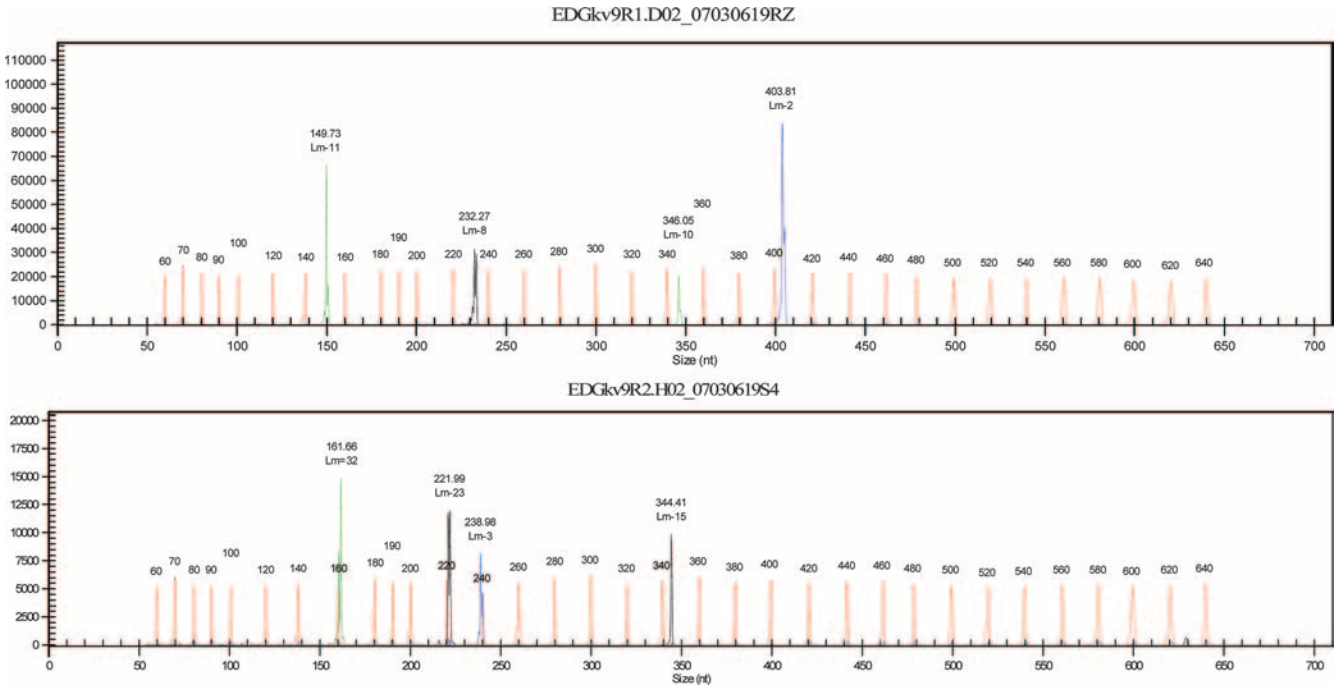

FIG. 1. *Listeria monocytogenes* MLVA. Two multiplex PCRs including four loci each were analyzed on the CEQ 8000 (Beckman Coulter). Red peaks, size standard 600; black peaks, Lm-8, Lm-15 and Lm-23; green peaks, Lm-10, Lm-11, and Lm-32; blue peaks, Lm-2 and Lm-3. Multiplex PCR R1 is shown in the top panel, and R2 is shown in the bottom panel. nt, nucleotide.

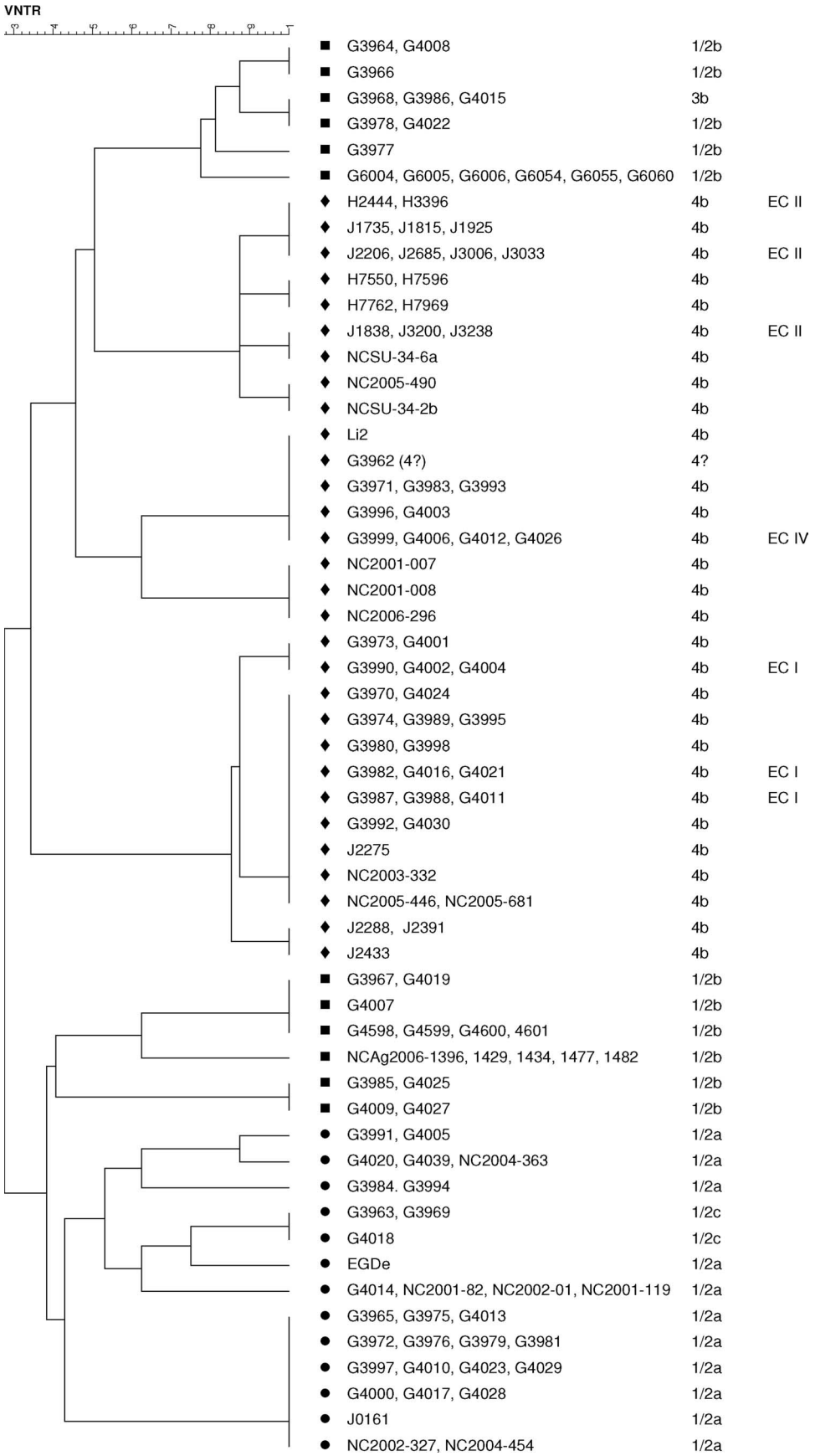

FIG. 2. Cluster analysis of 123 *L. monocytogenes* isolates based on MLVA type using the categorical coefficient and UPGMA. Genomic divisions are denoted as follows: ●, genomic division 1 (serotypes 1/2a and 1/2c); ■, genomic division 2 (serotypes 1/2b and 3b); ◆, genomic division 2 (serotype 4b). The EC group is indicated if known.

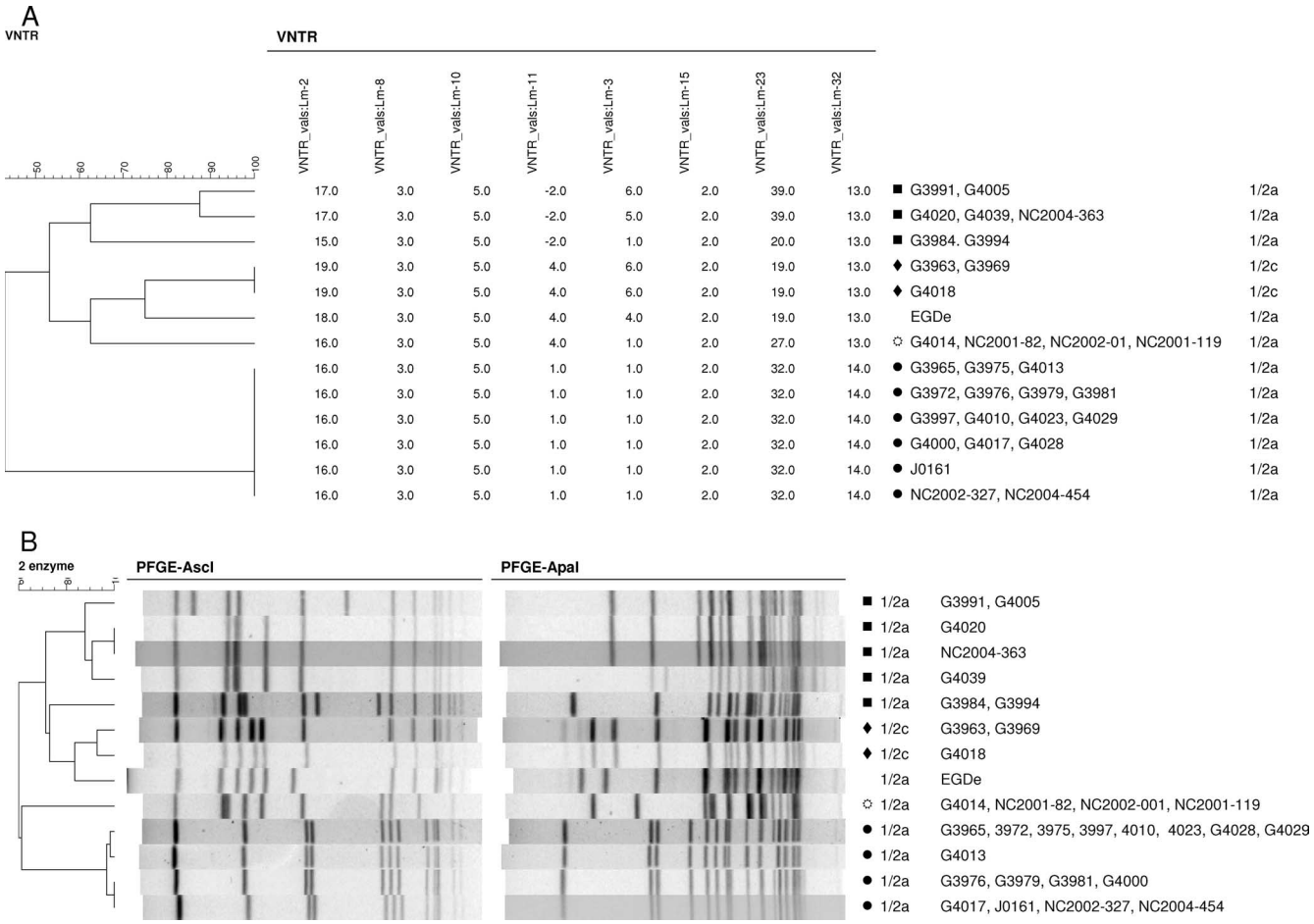

FIG. 3. Cluster analysis of 32 *L. monocytogenes* serotype 1/2a and 1/2c isolates. Symbols next to isolate names indicate similar isolates in MLVA and PFGE dendrograms. If isolates have indistinguishable patterns, then they are listed with one representative pattern. (A) Results of MLVA typing using the categorical coefficient and UPGMA. (B) Results of two-enzyme PFGE analysis (AscI and ApaI). The average from experiments was used to cluster the similarity matrix determined by single-enzyme analysis using the Dice coefficient with a 1.5 tolerance and UPGMA cluster.

clinical, and environmental isolates implicated in listeriosis cases from 1988 to 1990. Epidemiological studies have revealed that these isolates are associated with the same food processing plant and likely represent long-term contamination of that facility with the same strain (42). Lastly, this cluster also includes two clinical isolates from North Carolina (NC2002-327 and NC2004-454, isolated in 2002 and 2004, respectively) which were indistinguishable by PFGE, suggesting that this strain type continues to circulate in food.

Seven unique MLVA profiles and 12 unique PFGE (AscI and ApaI) profiles were detected among the 31 isolates of serotypes 1/2b and 3b, based on nine epidemiological groups and three unlinked isolates (Fig. 4). As described above, several PFGE profiles differed by only one to two bands. The isolates from the gastroenteritis outbreak in Italy (Table 1) were found to have identical MLVA and PFGE profiles. This MLVA profile was also found in three other isolates from the United States and the United Kingdom (Fig. 4, filled squares). The ApaI patterns for these isolates were also identical; however, differences were seen in the AscI pattern for the United Kingdom isolate. The chocolate milk outbreak isolates (Table 1; Fig. 4, indicated by an arrow) were also identical by both PFGE and MLVA.

Comparisons of 60 serotype 4b isolates showed the most significant differences between MLVA and PFGE (AscI and ApaI) (Fig. 5). Nine unique MLVA profiles and 26 unique PFGE (AscI and ApaI) profiles were produced based on cluster analysis of these isolates. The MLVA clearly separated the sporadic isolates from outbreak isolates. The MLVA was also able to group isolates by epidemic clone groups. Analyses based on unique genomic markers, gene cassettes, and single nucleotide polymorphisms have also found similarities between the strains associated with the epidemic clone groups (21, 23, 33, 34, 54–56). In efforts to increase the subtyping ability of this MLVA for *L. monocytogenes*, six loci (Lm-38 to Lm-43) were designed specifically for serotype 4b. Two of these loci had very low diversity, and one had variability in the flanking region. The remaining three loci did not affect the subtyping ability, clustering the 4b isolates identically as the panel of eight loci used in the multiplexed assay (Table 3).

Comparisons were made between the serotype 4b MLVA results and previously published work with multilocus genotyp-

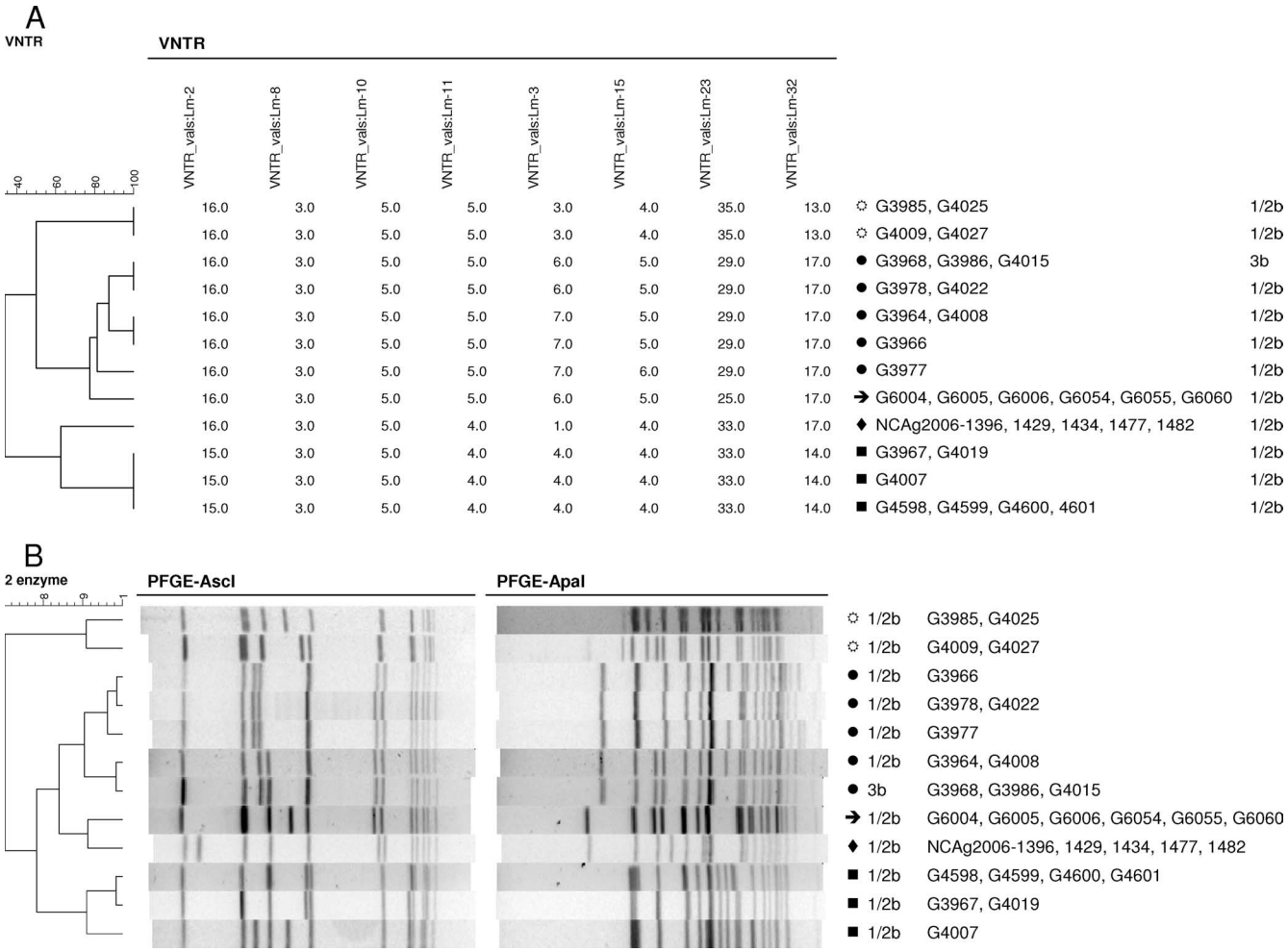

FIG. 4. Cluster analysis of 31 *L. monocytogenes* serotype 1/2b and 3b isolates. Symbols next to isolate names indicate similar isolates in MLVA and PFGE dendrograms. If isolates have indistinguishable patterns, then they are listed with one representative pattern. (A) Results of MLVA typing using the categorical coefficient and UPGMA. (B) Results of two-enzyme PFGE analysis (AscI and ApaI). The average from experiments was used to cluster the similarity matrix determined by single-enzyme analysis using the Dice coefficient with a 1.5 tolerance and UPGMA cluster.

ing (MLGT) (21) and multivirulence locus sequence typing (MVLST) (56). The ECI isolates grouped together in three main clusters, differing by only one locus, using MLVA (Fig. 5A, filled squares). The epidemic groups (EG) from the WHO multicenter study (6) are used here simply to denote epidemiological information (Table 1). EG-9, -13, -15, -16, -18, and -21 and EG-12 and -17 were identical by MLVA. PFGE clustering of EG-9, -15, -16, -17, and -18 showed identical AscI patterns and closely related ApaI patterns. EG-12 and EG-16 were identical, except for isolate G4021, which matched EG-9. PFGE did not cluster all isolates from EG-13 together nor did it cluster all isolates from EG-21 together (Fig. 5B, filled squares). MVLST clustered EG-12, -13, and -16 as identical to one another (56). MLGT clustered EG-12, -13, and -16 as similar although some differences were seen between the outbreaks (21). ECII isolates were not represented in the WHO multicenter study (6) since the ECII strain type was not identified until the multistate hotdog outbreak of 1998 to 1999 (13, 14, 23). ECII isolates grouped together into four clusters differing by one locus using MLVA (Fig. 5A, filled circles). As described previously, PFGE of these isolates with AscI produced two distinct patterns and PFGE with ApaI produced four closely related patterns (Fig. 5, filled circles) (31). These clusters correlated well with those identified by MLVA. The ECII isolates also clustered as identical by MVLST (15). In future studies, MLVA results could be more thoroughly compared to those of MVLST and MLGT by including additional isolates and serotypes.

In conclusion, this study details the development of an MLVA method for *L. monocytogenes* that consists of two multiplexed PCRs. The loci were selected based on their ability to subtype primarily serotypes 1/2a, 1/2b, and 4b. All of the loci selected have relatively small repeat units of 6 bp to 15 bp. The MLVA is a high-throughput, rapid assay with much improved data portability compared to PFGE. The most notable advantage of the MLVA is the lack of subjectivity. While PFGE relies on stringent adherence to subjective band-marking procedures, MLVA generates exact fragment sizes that are directly imported into BioNumerics. This allows the data transfer and interlaboratory comparison to be seamless. An added ben-

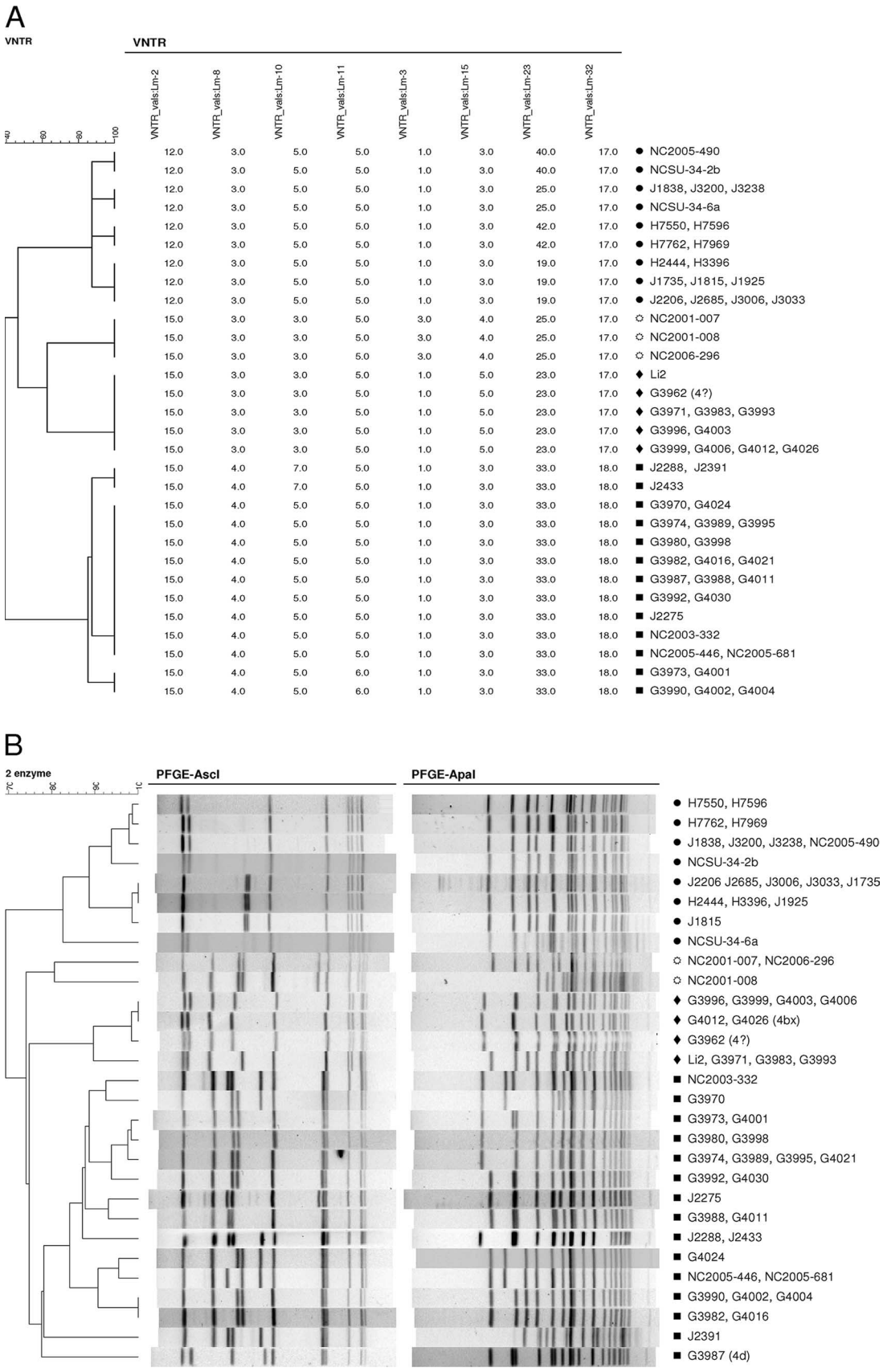

FIG. 5. Cluster analysis of 60 *L. monocytogenes* serotype 4b isolates. Symbols next to isolate names indicate similar isolates in MLVA and PFGE dendrograms. If isolates have indistinguishable patterns, then they are listed with one representative pattern. EC groups are indicated as follows: ■, ECI; ●, ECII. (A) Results of MLVA typing using the categorical coefficient and UPGMA. (B) Results of two-enzyme PFGE analysis (AscI and ApaI). The average from experiments was used to cluster the similarity matrix determined by single-enzyme analysis using the Dice coefficient with a 1.5 tolerance and UPGMA cluster.

efit for PulseNet laboratories is that different food-borne organisms (e.g., *E. coli*, *S. enterica* serotype Typhimurium, and *L. monocytogenes*) can be analyzed simultaneously using capillary gel electrophoresis. These advantages make MLVA an ideal choice for the next generation of food-borne subtyping tests in public health laboratories.

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AUTHOR'S CORRECTION

Multiple-Locus Variable-Number Tandem-Repeat Analysis as a Tool for Subtyping *Listeria monocytogenes* Strains

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Vol. 46, no. 4, p. 1435–1450. Page 1442, Table 2, line 9, column 2: "D4" should read "D2."
Page 1442, Table 2, line 9, column 3: "TATTTACGGAAAAGACGTAG" should read "TACGCCAGTTCCTCCGTTAG."
Page 1442, Table 2, line 9, column 4: "CGTAACTGTCCTACCATTAG" should read "TTGAAAGCTGGAGATGTTA TTCA."