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A Targeted Multilocus Genotyping Assay for Lineage, Serogroup, and Epidemic Clone Typing of *Listeria monocytogenes*▽†

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A 30-probe assay was developed for simultaneous classification of *Listeria monocytogenes* isolates by lineage (I to IV), major serogroup (4b, 1/2b, 1/2a, and 1/2c), and epidemic clone (EC) type (ECI, ECIa, ECII, and ECIII). The assay was designed to facilitate rapid strain characterization and the integration of subtype data into risk-based inspection programs.

Listeria monocytogenes is a facultative intracellular pathogen that can cause serious invasive illness (listeriosis) in humans and other animals. *L. monocytogenes* is responsible for over 25% of food-borne-disease-related deaths attributable to known pathogens and is a leading cause of food recalls due to microbial adulteration (12, 21). However, not all *L. monocytogenes* subtypes contribute equally to human illness, and substantial differences in the ecologies and virulence attributes of different *L. monocytogenes* subtypes have been identified (9, 13, 14, 23, 24, 33, 35, 36). Among the four major evolutionary lineages of *L. monocytogenes*, only lineages I and II are commonly isolated from contaminated food and human listeriosis patients (19, 27, 29, 33). Lineage I strains are overrepresented among human listeriosis isolates, particularly those associated with epidemic outbreaks, whereas lineage II strains are overrepresented in foods and the environment (13, 14, 24). Lineage III strains account for approximately 1% of human listeriosis cases but are common among animal listeriosis isolates and appear to be a host-adapted group that is poorly adapted to food-processing environments (6, 34–36). The ecological and virulence attributes of lineage IV are poorly understood, as this lineage is rare and was only recently described based on a small number of strains (19, 26, 29, 33).

L. monocytogenes is differentiated into 13 serotypes; however, four major serogroups (4b, 1/2b, 1/2a, and 1/2c) from within lineages I and II account for more than 98% of human and food isolates (16, 31). Serogroups refer to evolutionary complexes typified by a predominant serotype but which include very rare serotypes that represent minor evolutionary variants (7, 9, 33). Phylogenetic analyses have indicated that rare serotypes may have evolved recently, or even multiple times, from one of the major serotypes (9), and numerous molecular methods fail to discriminate minor serotypes as independent groups (1, 4, 7, 9, 18, 22, 33, 38, 39). Serotyping is one of the most common methods for *L. monocytogenes* subtyping, and serogroup classifications are a useful component of strain characterization because ecotype divisions appear largely congruent with serogroup distinctions (16, 34). Serogroup 4b strains are of particular public health concern because contamination with these strains appears to increase the probability that a ready-to-eat (RTE) food will be implicated in listeriosis (16, 28). Serogroup 4b strains account for approximately 40% of sporadic listeriosis and also are responsible for the majority of listeriosis outbreaks despite being relatively rare contaminants of food products (9, 13, 17, 30, 34). In addition, serogroup 4b strains are associated with more severe clinical presentations and higher mortality rates than other serogroups (11, 16, 20, 31, 34). Serogroups 1/2a and 1/2b are overrepresented among food isolates but also contribute significantly to human listeriosis, whereas serogroup 1/2c rarely causes human illness and may pose a lower risk of listeriosis for humans (16). Serogroup-specific differences in association with human listeriosis are consistent with the prevalence of virulence-attenuating mutations in *inlA* within these serogroups (32, 34); however, a number of additional factors likely contribute to these differences.

Four previously described epidemic clones (ECs; ECI, ECIa, ECII, and ECIII) of *L. monocytogenes* have been implicated in numerous listeriosis outbreaks and have contributed significantly to sporadic illness (15, 34). ECI, ECIa, and ECII are distinct groups within serogroup 4b that were each responsible for repeated outbreaks of listeriosis in the United States and Europe. ECIII is a lineage II clone of serotype 1/2a that persisted in the same processing facility for more than a decade prior to causing a multistate outbreak linked to contaminated turkey (15, 25). While there has been speculation that epidemic clones possess unique adaptations that explain their frequent involvement in listeriosis outbreaks (9, 34, 37), it is not clear that epidemic clones are more virulent than other strains with the same serotype. However, contamination of RTE food with EC strains would be cause for increased concern due to the previous involvement of these clones in major outbreaks of listeriosis (16).

As a result of the *L. monocytogenes* subtype-specific differences in ecology, virulence, and association with human illness, molecular subtyping technologies have the potential to inform

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TABLE 1. Primers used in multiplex amplification for the TMLGT assay

Amplicon	Position[a]	Gene(s)	Primer	Sequence (5′–3′)[b]
INLa	455381–456505	*inlA*	inl2-a1	GTCCTTGATAGTCTACTG
			inl2–a2	ACCAAATTAGTAATCTAGCAC
INLb	457726–458752	*inlB*	inl-f1d	GAATTRTTTAGYCAAGAATGT
			inlb-r	CTACCGGRACTTTATAGTAYG
LMO	325116–326096	lmo0298–lmo0300	lmo-a1	AAGGCTTACAAGATGGCT
			lmo1a-1r	AAATAATAYGTGATACCGAC
VGCa	205366–206622	*plcA*, *hly*	plca-f	CTCATCGTATCRTGTGTACC
			hly-r	TCTGGAAGGTCKTGTAGGTTC
VGCb	208447–209465	*mpl*	ra_mpl-f	GTGGAYAGAACTCATAAAGG
			ra_mpl-r	ACTCCCTCCTYGTGATASGCT
VGCc	209728–211239	*actA*	vgc1a-2f	TTCMATRCCAGCAGAACG
			vgc1a-2r	GCAGACCTAATAGCAATGTTG

[a] Corresponding nucleotide positions in the complete genome sequence of *L. monocytogenes* strain EGD-e (GenBank accession number NC_003210).
[b] See IUPAC codes for definition of degenerate bases.

assessments of relative risk and to improve risk-based inspection programs. The objective of the present study was to develop a single assay for rapid and accurate classification of *L. monocytogenes* isolates by lineage, major serogroup, and epidemic clone in order to facilitate strain characterization and the integration of subtype data into inspection programs that are based on assessment of relative risk.

A database of more than 5.3 Mb of comparative DNA sequences from 238 *L. monocytogenes* isolates (9, 33–35) was scanned for single nucleotide polymorphisms that could be used to differentiate lineages, major serogroups, and epidemic clones via a targeted multilocus genotyping (TMLGT) approach. The acronym TMLGT is used to distinguish this approach from previously published multilocus genotyping (MLGT) assays that were lineage specific and designed for haplotype discrimination (9, 33). To provide for simultaneous interrogation of the selected polymorphisms via TMLGT, six genomic regions (Table 1) were coamplified in a multiplex PCR. While the previous MLGT assays were based on three lineage-specific multiplexes and required prior identification of lineage identity, TMLGT was designed to target variation across all of the lineages simultaneously and is based on a unique set of amplicons. PCR was performed in 50-μl volumes with 1× High Fidelity PCR buffer (Invitrogen Life Technologies), 2 mM $MgSO_4$, 100 μM deoxynucleoside triphosphate (dNTP), 300 nM primer, 1.5 U Platinum *Taq* high-fidelity DNA polymerase (Invitrogen Life Technologies), and 100 ng of genomic DNA. PCR consisted of an initial denaturation of 90 s at 96°C, followed by 40 cycles of 30 s at 94°C, 30 s at 50°C, and 90 s at 68°C. Amplification products were purified using Montage PCR cleanup filter plates (Millipore) and served as a template for allele-specific primer extension (ASPE) reactions utilizing subtype-specific probes.

ASPE was performed in multiplex reactions including 30 probes, with each lineage (I to IV), major serogroup (4b, 1/2b, 1/2a, and 1/2c), and epidemic clone (ECI, ECIa, ECII, and ECIII) targeted by two different probes (Table 2). In addition, positive-control probes were included to confirm the presence of each amplicon in the multiplex PCR. As serogroups and epidemic clones are nested within a particular lineage, probes for these groups were designed to be specific within the appropriate lineage and values for these probes were evaluated only for isolates of the appropriate lineage. For example, serogroup 1/2a probes were evaluated only for isolates that were positive for lineage II probes. ASPE probes were designed with a unique 5′ sequence tag specific to individual sets of *x*MAP fluorescent polystyrene microspheres (Luminex Corporation) used to sort extension products. Extension and hybridization reactions were performed as described previously (9) except microspheres were twice pelleted by centrifugation (4 min at 2,250 × *g*) and resuspended in 75 μl 1× TM buffer prior to being pelleted and resuspended in 100 μl 1× TM buffer containing 2 μg/ml streptavidin–*R*-phycoerythrin (Invitrogen Life Technologies). Samples were incubated for 15 min at 37°C prior to detecting the microsphere complexes with a Luminex 100 flow cytometer (Luminex Corporation). The median fluorescence intensity (MFI) from biotinylated extension products attached to 100 microspheres was measured for each probe. The average MFI from three template-free control samples was also determined and subtracted from the raw MFI of each sample to account for background fluorescence. Probe performance was initially evaluated via the index of discrimination (ID) as described by Ducey et al. (9), and probes with ID values less than 2.0 were redesigned.

Validation of the TMLGT assay was performed using 906 *L. monocytogenes* isolates for which the lineage, major serogroup, and epidemic clone type had been determined independently (see Table S1 in the supplemental material). A subset of 92 isolates, including at least five isolates from each lineage, serogroup, and epidemic clone type, was used to evaluate the discriminatory power of subtype-specific probes and the repeatability of the assay (see Table S1). Two independent runs of the 30-probe TMLGT assay produced identical results for these 92 isolates. In addition, genotypes matched expectations for all isolate/probe combinations, and the fluorescence intensities for positive genotypes (those targeted by a particular probe) were 3.8 to 58.3 (mean, 18.5) times as high as background values for isolates with negative genotypes (those not targeted by a particular probe) (Table 2). The performances of individual probes also were assessed in terms of sensitivity and specificity, where sensitivity is defined as the percentage of positive samples that produced positive results and specificity indicates the percentage of negative samples that produce negative results (5). Based on results from all 906 isolates analyzed by TMLGT, probe sensitivity was at least 98.6% and 23 of the 24 subtype-specific probes exhibited

TABLE 2. TMLGT probes and probe performance data

Probe[b]	Target (*n*)[c]	Probe sequence[d]	ID[e]	Sensitivity (%)	Specificity (%)
VGCb-21	Lineage I (506)	AATCCTTTCTTTAATCTCAAATCAgcggaagcttgggaagcggtc	7.3	100	100
VGCa-94	Lineage I	CTTTCTATCTTTCTACTCAATAATcaacccgatgttcttcctgtc	51.7	100	100
VGCc-8	Lineage II (340)	AATCCTTTTACATTCATTACTTACattagctgattcgctttcct	14.1	100	100
INLb-51	Lineage II	TCATTTCAATCAATCATCAACAATagcgccaataaagctggc	21.9	100	100
VGCb-19	Lineage III (50)	TCAATCAATTACTTACTCAAATACccgctattaaaatgtactcca	31.0	100	100
VGCb-29	Lineage III	AATCTTACTACAAATCCTTTCTTTggtataccgctattaaaatgt	45.1	100	100
LMO-17	Lineage IV (10)	CTTTAATCCTTTATCACTTTATCAgaaccaaacaatgttattggt	11.8	100	100
VGCa-27	Lineage IV	CTTTTCAAATCAATACTCAACTTTttaacgacggtaacgtgccac	58.3	100	100
INLb-84	Serogroup 4b (213)	TCAACTAACTAATCATCTATCAATggtaaaaatatgcgaatattg	9.7	100	100
INLb-85	Serogroup 4b	ATACTACATCATAATCAAACATCActcgtgaacaagctttcc	5.5	100	100
INLb-16	Serogroup 1/2b (293)	AATCAATCTTCATTCAAATCATCAggtaaaaatatgcgtatctta	11.7	100	100
INLb-100	Serogroup 1/2b	CTATCTTTAAACTACAAATCTAACgtgaataagctatcggtctat	13.0	100	100
LMO-42	Serogroup 1/2a (268)	CTATCTTCATATTTCACTATAAACtggcgttgctgrctaagtttg	6.6	100	100
VGCb-40	Serogroup 1/2a	CTTTCTACATTATTCACAACATTAaatcaagcsgctcatatgaag	10.4	100	98.6
LMO-9	Serogroup 1/2c (72)	TAATCTTCTATATCAACATCTTACtttactggtgaaatggcg	13.5	100	100
VGCb-5	Serogroup 1/2c	CAATTCAAATCACAATAATCAATCaagattacgaatcgcttccac	20.8	98.6	100
LMO-10	ECI (111)	ATCATACATACATACAAATCTACAatgattaaaagtcagggaaag	19.0	100	100
LMO-28	ECI	CTACAAACAAACAAACATTATCAAaatcgaggcttacgaacgt	23.7	100	100
VGCc-80	ECIa (44)	CTAACTAACAATAATCTAACTAACactacaacgaaaacagcgc	10.7	100	100
VGCa-35	ECIa	CAATTTCATCATTCATTCATTTCAgttacttttatgtcgagt	9.2	100	100
LMO-12	ECII (35)	TACACTTTCTTTCTTTCTTTCTTTataccgattatttggacggtt	3.8	100	100
LMO-30	ECII	TTACCTTTATACCTTTCTTTTTACgacttgtagcagttgatttcaa	7.5	100	100
VGCc-45	ECIII (10)	TCATTTCACAATTCAATTACTCAActcttatttgcttttgttggtc	21.1	100	99.4
INLa-3	ECIII	TACACTTTATCAAATCTTACAATCgagcttaatgaaaatcagcta	17.0	100	99.4
INLa-1	INLa control	CTTTAATCTCAATCAATACAAATCagaagtggaagctgggaa	NA[a]	NA	NA
INLb-13	INLb control	CAATAAACTATACTTCTTCACTAAtgcacctaaacctccgac	NA	NA	NA
LMO-88	LMO control	TTACTTCACTTTCTATTTACAATCccgtttccttatgccaca	NA	NA	NA
VGCa-23	VGCa control	TTCAATCATTCAAATCTCAACTTTcaagycctaagacgccaatcg	NA	NA	NA
VGCb-25	VGCb control	CTTTTCAATTACTTCAAATCTTCAgcatgcgttagttcatgrcca	NA	NA	NA
VGCc-82	VGCc control	TACATACACTAATAACATACTCATgactgcatgctagaatctaag	NA	NA	NA

[a] NA, not applicable for positive amplicon control probes.
[b] Luminex microsphere sets (Luminex Corporation) used for hybridization reactions are indicated following the hyphen.
[c] *n*, number of isolates representing the target subtype among the 906 tested isolates.
[d] The 5′ sequence tag portions of extension probes are capitalized. See IUPAC codes for definitions of degenerate bases.
[e] ID, index of discrimination.

100% sensitivity (Table 2). The specificities for all probes were also greater than 98.6%, and 21 of the 24 subtype-specific probes exhibited 100% specificity (Table 2).

All but three of the 906 isolates in the validation panel were fully and accurately typed relative to lineage, serogroup, and epidemic clone by using the TMLGT assay (typeability, 99.9%; accuracy of isolate assignment, 99.8%). One of the lineage II isolates, NRRL B-33880, could not be assigned to a serogroup based on the TMLGT results because this isolate was positive for one of the serogroup 1/2a probes (VGCb-40) and one of the serogroup 1/2c probes (LMO-9). This isolate was previously identified as a member of serogroup 1/2c based on mapping lineage-specific MLGT data onto a multilocus phylogeny (34) but produced a serogroup 1/2a-specific banding pattern (data not shown) with the multiplex PCR assay described by Doumith et al. (7). Similar strains, including the common laboratory strain EGD-e, were found to have genomes that are more similar to serogroup 1/2c strains than to strains from the 1/2a serogroup (8, 33) and likely represent intermediates in the evolution of the 1/2c clade from 1/2a ancestors. There is a poor correlation between genomic and antigenic variation for such isolates (34), consistent with the ambiguous results produced by application of the TMLGT assay to NRRL B-33880. The two other problematic isolates, NRRL B-33555 and NRRL B-33559, were accurately identified based on TMLGT data as lineage II isolates from the 1/2a serogroup. However, these two isolates were positive for both ECIII-specific probes in the TMLGT assay but have lineage-specific MLGT haplotypes (Lm2.46), indicating that they are representatives of a sister group closely related to ECIII (33).

In 2005, the Food Safety and Inspection Service (FSIS) implemented an approach to inspection that includes consideration of relative risk in order to determine *L. monocytogenes* sampling frequency among establishments that produce certain RTE products. This approach incorporates information on production volume, outgrowth potential in the product, steps taken to prevent postlethality contamination, and FSIS sampling history. However, *L. monocytogenes* subtype-specific variation in ecology and virulence indicates that information on the lineage, major serogroup, and epidemic clone identities of isolates could be used to inform assessments of relative risk and to improve inspection programs that are based on consideration of risk. Several PCR-based methods have been described for differentiation of various combinations of these subgroups (1–3, 5, 7, 10, 35, 37); however, these approaches have focused on a single subgroup or a smaller set of subgroups than is differentiated by TMLGT analysis. Although we previously developed a set of three MLGT assays that can be used to differentiate all of the major serogroups and epidemic clones of *L. monocytogenes* (9, 33, 34), those assays did not

include probes for lineage discrimination and require identification of the lineage prior to application of one of three unique sets of probes. In addition, the MLGT assays were designed to maximize strain discrimination, as opposed to subgroup identification, and require the use of at least twice as many probes as is needed for TMLGT analysis. MLGT data analysis is also more complicated than analysis of TMLGT data, and serogroup or epidemic clone type identification via MLGT requires phylogenetic analyses to place novel haplotypes within an established phylogenetic framework.

In the present study, we developed the first assay for simultaneous discrimination of the four lineages, the four major serogroups, and the four previously described epidemic clones of *L. monocytogenes*. The assay includes multiple markers for each of these subtype probes as well as control probes to ensure that negative probe data were not the result of amplification failure, providing a high degree of internal validation required for use in inspection programs that consider risk in making sampling decisions. In addition, the utility of the assay has been validated with a large and diverse panel of 906 isolates, including 567 isolates from FSIS surveillance of RTE products and processing facilities (see Table S1 in the supplemental material). Data produced by the TMLGT assay are amenable to high-throughput analysis, and a simple spreadsheet utility has been developed to semiautomate subtype identifications and to alert investigators to potentially conflicting probe data (available upon request). In addition to having a potential application in inspection programs, the TMLGT assay provides a rapid and accurate means of characterizing *L. monocytogenes* isolates from different environments, which would facilitate pathogen tracking and improve understanding of *L. monocytogenes* ecology.

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The mention of firm names or trade products does not imply that they are endorsed or recommended by the U.S. Department of Agriculture over other firms or similar products not mentioned.

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