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Suspension Microarray with Dendrimer Signal Amplification Allows Direct and High-Throughput Subtyping of *Listeria monocytogenes* from Genomic DNA

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***Listeria monocytogenes* is a significant cause of food-borne disease and mortality; therefore, epidemiological investigations of this pathogen require subtyping methods that are rapid, discriminatory, and reproducible. Although conventional microarray subtyping analysis has been shown to be both high resolution and genetically informative, it is still relatively low throughput and technically challenging. Suspension microarray technology eliminates the technical issues associated with planar microarrays and allows high-throughput subtyping of *L. monocytogenes* strains. In this study, a suspension array assay using dendrimer signal amplification allowed rapid and accurate serovar identification of *L. monocytogenes* strains using genomic DNA as a target. The ability to subtype genomic DNA without PCR amplification allows probes to be designed for many different regions within the bacterial genome and should allow high-resolution subtyping not possible with multiplex PCR.**

Listeria monocytogenes is a gram-positive bacterial pathogen that causes significant morbidity and mortality. Human listeriosis may occur as widespread epidemics or, more commonly, as sporadic cases (18). It is likely, however, that a significant percentage of sporadic cases are unrecognized single-source outbreaks (19). Epidemiological investigation of epidemic and sporadic cases of listeriosis requires molecular characterization to allow the identification of specific subtypes.

Molecular subtyping techniques have identified two major phylogenetic divisions within the species, with division I including serotypes 1/2b, 3b, and 4b and division II consisting of serotypes 1/2a, 1/2c, 3a, and 3c (15, 16). A third division consisting of serotypes 4a, 4c, and a subset of 4b strains has also been described (4, 24). Although 13 serotypes of *L. monocytogenes* have been described (20), only three serotypes (1/2a, 1/2b, and 4b) are responsible for the vast majority of clinical cases (23).

L. monocytogenes subtypes are usually characterized by serotyping and then further subtyped using pulsed-field gel electrophoresis (10) or ribotyping. Due to the importance of *L. monocytogenes* epidemiology to human health, new subtyping technologies are constantly being developed in the hope of increasing the resolution, speed, and reproducibility of *L. monocytogenes* subtyping. The most recent examples of this are multilocus sequence subtyping (12, 17) and microarray genomic analysis (1, 5, 26). Recent studies have shown that a relatively simple genotyping microarray has subtyping resolution comparable to that of pulsed-field gel electrophoresis (AscI and ApaI digestion) and superior to that of multilocus sequence subtyping (six housekeeping genes) and ribotyping (3). *L. monocytogenes* subtyping using DNA microarrays is also genetically informative because it can identify the genes or alleles that characterize subtypes.

DNA microarrays are typically composed of DNA "probes" (nucleic acids of known sequence) that are bound to a solid substrate, such as a glass microarray slide (referred to as "planar microarrays"). Although planar-microarray analysis allows screening of a large percentage of the genome very rapidly and subsequent identification of subtype-specific sequences (1, 5, 26), the technology is currently labor-intensive and technically challenging.

Suspension microarrays are a novel technology that circumvents many of the technical challenges associated with planar microarrays. The suspension microarray system has recently been demonstrated to be a rapid, sensitive, and specific assay for detection of bacterial pathogens, including *L. monocytogenes* (8, 25). The Luminex (Austin, TX) suspension array is simply a transfer of the microarray format from a glass slide (planar microarray) to a high-throughput and efficient bead format ("suspension microarray"). With this type of assay, the DNA probes are attached to 5.6-μm polystyrene microspheres ("beads") containing an internal fluorescent dye. Each probe is assigned to a particular bead set containing a unique mixture of fluorescent dyes, or "spectral address." Bead sets coupled to the probes of interest are then mixed together in the wells of a 96-well microtiter plate, allowing many different probes to be analyzed simultaneously. Target DNA molecules are labeled with a different and spectrally distinct fluorescent dye and hybridized to the probes on the beads. Beads with the hybridized targets are then separated and quantified using a two-laser flow cytometer. The unique internal color of the bead is read by one laser and serves to identify which probe is present on

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TABLE 1. Nucleotide sequences of serotype-specific capture and detection probes[a]

Serovar	Capture probe	Detection probe	ORF	Reference(s)
1/2a(3a)	TAGCAGCACCTgTAGCAGTT	TGATCTAGTAATTTGATGTTGTTGTAGTTATTTTTCG	FlaA	2, 6, 16
1/2c(3c)	TAGCAGCACCTaTAGCAGTT	TGATCTAGTAATTTGATGTTGTTGTAGTTATTTTTCG	FlaA	2, 6, 16
1/2b(3b)	CAAGTATTAGGAGTTATAAG	GGGATTATTAATACAATCTGGCTTTATGGATTTTTCG	Serotype-specific gene cassette	11
Div I 4b	CATCCCTTACTTTGGACGTG	TCGTAATTTATCCCTTTTTGTTTGCTTACATTTTTCG	ORF2110	7

[a] The single base difference that confers serotype specificity on the 1/2a(3a) and 1/2c(3c) capture probes is shown in lowercase. The seven additional nucleotides on the 3′ end of each detection probe (underlined) serve as a bridging sequence and are required for ligation of the detection probe to the dendrimer arms. ORF, open reading frame.

the bead. The second laser measures the fluorescent signal of the reporter dye present on the labeled target DNA and allows one to assess the strength of the hybridization between the target DNA and the probe. Because this technology allows up to 100 different probes to be analyzed in a single well of a 96-well plate, it promises to make microarray subtyping faster and less expensive.

The established suspension array protocol requires that relatively short PCR products be used as targets (8). Although this is not a significant limitation for subtyping assays that sample limited regions of the genome, high-resolution subtyping is likely to require that many different regions of the genome be analyzed. Because an unamplified genomic-DNA target alone does not provide adequate signal intensity (M. K. Borucki and J. Reynolds, unpublished data), DNA dendrimers were employed to increase target labeling by approximately 100-fold (21). In this study, we describe a novel method for rapid and high-throughput subtyping of *L. monocytogenes* strains that, unlike multiplex PCR, can readily be expanded to include many more probe sequences and thus permit increased subtyping resolution.

MATERIALS AND METHODS

Bacterial strains. A panel of 50 *L. monocytogenes* strains was assembled from human ($n = 29$), veterinary ($n = 8$), environmental ($n = 11$), and undetermined ($n = 2$) sources. The strains were provided by the Centers for Disease Control and Prevention, Atlanta, Ga. ($n = 3$); International Life Sciences Institute, *L. monocytogenes* strain collection (http://www.foodscience.cornell.edu/wiedmann/listeriadbase.htm) ($n = 28$); the U.S. Food and Drug Administration, Bothel, Wash. ($n = 5$); and the U.S. Department of Agriculture ($n = 5$), Washington State Department of Health ($n = 7$), Health Canada ($n = 1$), and American Type Culture Collection ($n = 1$). Representatives of the three phylogenetic divisions, as well as eight serotypes, were included in the analysis, although the majority of the strains tested belonged to the four most common serotypes: 1/2a ($n = 11$), 1/2b ($n = 8$), 1/2c ($n = 4$), and 4b ($n = 20$).

Serotyping. Denka Seiken *Listeria* antisera (Tokyo, Japan) were obtained from Accurate Scientific (Westbury, NY). Serotyping was performed according to the manufacturer's recommendations or by using these reagents in an enzyme-linked immunosorbent assay format (14).

DNA preparation. Individual colonies were picked from Oxford Agar Base plates (Remel, Lenexa, KS) and used to inoculate culture tubes containing 5 ml of brain heart infusion broth (Remel), and cultures were incubated at 30°C without shaking for 18 to 20 h. Genomic DNA was isolated using a DNeasy tissue kit (QIAGEN) using the manufacturer's protocol for gram-positive bacteria modified by adding freshly prepared lysozyme (20 mg/ml) to the extraction buffer for each batch of genomic isolations. Three milliliters of culture was processed for each isolate by running two digested pellets through a single column with a final elution volume of 200 μl (two pooled 100-μl elution steps). The DNA was then sheared for 90 s using a cup-horn sonicator (sonicator-ultrasonic processor; Misonix Inc., Farmingdale, NY) tuned to 55% continuous power. Fragmented DNA was quantified using a spectrophotometer.

Oligonucleotide probe design. Capture probes used to detect the genomic-DNA target were designed using serovar-specific sequences obtained from GenBank (Table 1). A 30-mer detection probe was designed from sequences immediately adjacent to the capture probe sequences. An additional 7-nucleotide "bridging" sequence was added to the 3′ end of the detection probe (5′-TTT TTC G-3′) to allow enzymatic ligation of the probe to the single-stranded peripheral DNA "arms" of the dendrimers (a proprietary process performed by Genisphere, Inc., Hatfield, PA). All probes were checked for secondary structure using Oligo 6.0 (Molecular Biology Insights, Inc., Cascade, CO).

Microsphere preparation. XMap Multi-Analyte COOH Microspheres were purchased from Luminex Corporation (Austin, TX). These microspheres are internally labeled with florescent dyes and contain surface carboxyl groups for covalent attachment of amine-modified oligonucleotide probes ("capture probes"). The capture probe oligonucleotides were modified with a 5′ unilink and a C-6 linker arm (Oligos Etc., Wilsonville, OR). The amine-modified oligonucleotides were linked to the microspheres as described previously (8) with an estimated yield of 30,000 coupled microspheres per μl.

Hybridization. Target-probe hybridization was a three-step process. First, each genomic-DNA sample was hybridized to the capture probes coupled to the microspheres. Secondly, the detection probe-dendrimers were hybridized to the microsphere-DNA complex, and finally, strepto-avidin (SA)–phycoerythin was bound to the dendrimer-DNA-microsphere complex. Each strain was assayed in triplicate with each well containing a mixture of all probe-coupled microsphere sets (multiplex reactions), and the experiment was performed twice.

Genomic-DNA hybridization. Sonicated DNA was adjusted to 75 ng/μl in Tris-EDTA after quantification, and 110 μl of DNA from each strain was placed in individual 1.5-ml tubes. A no-target control tube received 110 μl Tris-EDTA. Two hundred twenty microliters of 1.5× TMAC hybridization solution (4.5 M tetramethylammonium chloride, 0.15% Sarkosyl, 75.0 mM Tris-HCl, pH 8.0, 6.0 mM EDTA, pH 8.0) containing ca. 3,300 beads of each probe-coupled microsphere set was added to each tube (total volume, 330 μl). The target DNA and probes were vortexed and aliquoted into a deep-well PCR tray (Axygen Scientific, Union City, CA) at three wells per tube and 100 μl per well. The tray was covered with a silicone sealing mat, placed in a 96-well format thermocycler, and denatured at 95°C for 5 min, followed by a 3-h hybridization at 50°C. One hundred microliters of 1× TMAC prewarmed to 50°C was added to each well, and the solution was mixed by pipetting it gently three times with a multichannel pipette. The tray was centrifuged in a swing bucket table top centrifuge (Eppendorf Centrifuge 5810-R) at 3,000 rpm (1,750 × *g*) for 3 min at room temperature in order to pellet the microspheres. The supernatant was removed using a multichannel pipette, leaving 10 to 20 μl supernatant above the pellet to prevent aspiration of the microspheres. A second wash with 100 μl 1× TMAC followed.

Detection probe-dendrimer hybridization. After the supernatant was removed, 50 μl of the detection probe-dendrimer mix was added to each well, mixed gently, and hybridized for 1 hour at 50°C. The detection probe-dendrimer mix was composed of 1 μl of each detection probe-dendrimer (20 ng/μl) covalently labeled with 850 to 900 biotins and suspended in a final concentration of 1× TMAC. After the probe-dendrimer hybridization, a wash using 100 μl of 1× TMAC was performed, and the supernatant was removed.

SA-phycoerythin binding. Fifty microliters of SA-phycoerythin (10 μg/ml in 1× TMAC) was added to each well, and the plate was incubated at 50°C for 30 min. Beads were pelleted and washed once as described above. Finally, 60 μl of prewarmed (50°C) 1× TMAC was mixed into each well, and the reactions were analyzed at 50°C using a Bio-Rad Bio-Plex instrument (Luminex xMAP technology marketed by Bio-Rad, Hercules, CA). The Bio-Plex was adjusted to read 50 of each microsphere-capture probe, while all other settings were left in the default mode.

Data analysis. Fluorescent signals from each of the three triplicate wells were averaged for all strains and the no-target control, and the control background was subtracted from each averaged signal. Strains with background-subtracted

signals greater than twice that of the no-template control were considered positive. In the single case of ambiguous results [i.e., positive for both 1/2c(3c) and 1/2b(3b) probes], a subtype was assigned according to relative signal intensities.

Statistical analysis. GraphPad InStat version 3.05 (GraphPad Software, San Diego, CA) Pearson correlation coefficients were used to evaluate intra-assay reproducibility.

RESULTS AND DISCUSSION

The development of a Luminex non-PCR-based typing method using genomic DNA would be an important achievement because it would allow multiplex detection of sequence targets, greater automation potential, and a very flexible platform amenable to end user custom design and modification. Such a method would also be applicable to the detection of other genomic-DNA targets, including testing for other viral and bacterial genomes, and human genes (genetic diseases, HLA typing, comparative genomic hybridization, etc.).

To assess the utility of the Luminex suspension array system for subtyping *L. monocytogenes* strains, four probes were designed using genomic regions shown previously to be serovar specific (Table 1). Probe specificity was verified, first using complementary 20-mer biotin-labeled oligonucleotides as targets and then using 150- to 200-bp PCR product targets as described previously (8). Because high-resolution subtyping will require probes for numerous regions within the *L. monocytogenes* genome, unamplified genomic DNA was next tested as a target. Various concentrations of the target (250 to 6,000 ng/well) and microspheres (100 to 5,000/well) and various labeling methods (nick translation, random hexamer, and end labeling) were tested in order to increase the assay sensitivity sufficiently for use of the genomic-DNA target (Borucki et al., unpublished); however, we were unable to identify a successful protocol with an adequate signal-to-noise ratio. Therefore, dendrimer technology was used to increase the amount of label associated with each hybridized genomic target.

DNA dendrimers have been shown to provide significant signal amplification in a variety of assay formats, including DNA microarrays (9, 22). Dendritic nucleic acid signal amplification molecules, or 3DNA dendrimers (Genisphere, Hatfield, PA), are complex, highly branched molecules comprised of interconnected monomeric subunits of partially double-stranded DNA (13). Dendrimers are synthesized in a sequential manner, with each cycle of the synthesis adding another external layer of DNA molecules. The *Listeria*-specific detection probes are covalently ligated to the ends of the dendrimer branches and serve to bind the dendrimer structure to the complementary genomic DNA. In this assay, four-layer dendrimers, each with approximately 850 to 900 biotins per dendrimer, were used to amplify signal intensity and allow the direct detection of the genomic-DNA target (Fig. 1).

After the dendrimer signal amplification protocol was optimized using a small panel of *L. monocytogenes* strains, a larger panel of 50 strains was tested to assess the high-throughput capability, reproducibility, and accuracy of the assay. The four serovar-specific probes allowed 98% (49/50) of strains to be classified into four groups (Fig. 2 and Table 2). Although the 1/2a(3a) probe and the 1/2c(3c) probes differed by only a single nucleotide, these probes correctly grouped all 1/2a and 1/2c strains in both trials. Three serotype 3a strains were tested, with two strains classified as 1/2a(3a) serovar and one as 1/2b(3b). The 3c strain tested was positive for the 1/2c(3c) probe.

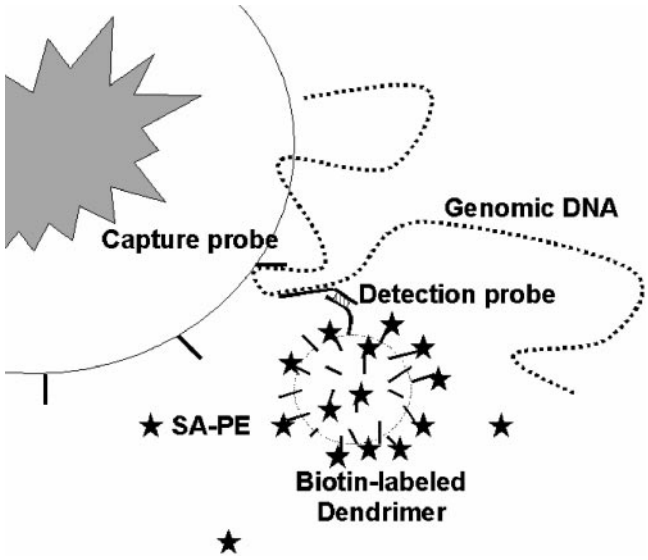

FIG. 1. Microsphere, probe, and genomic-DNA interaction and dendrimer signal amplification.

The 1/2b capture probe had lower than 50% GC content (Table 1), and this affected the probe sensitivity, with 1/2b strains yielding low signal for the 1/2b probe in 3 of the 16 assays. The 3b strain was positive for the 1/2b(3b) probe.

Serovar 4 strains are present in two of the three phylogenetic divisions, with most serotype 4b strains included in division I and with division III consisting of serotypes 4a, 4c, and 4b (4, 24). The 4b probe was specific for division I 4b strains and correctly identified all 18 strains in both trials. Two division III 4b strains (24) were included in the assay, and both were negative for the 4b probe, with one giving negative results for all probes and the other typing as 1/2b(3b) in trial 1 and 1/2a(3a) in trial 2. The 4a strain was positive for the 1/2b(3b) probe, and the 4c strain was positive for the 1/2a(3a) probe in trial 1 and negative for all probes in trial 2.

Interassay reproducibility was assessed by comparing the serogroup results from the two trials. Forty-three of the 50 strains (86%) gave the same result in both trials. Reproducibility was most affected by the 1/2b(3b) probe, as four of the seven results that differed between assays were due to a high signal-to-noise ratio for this probe. The three remaining samples that were not reproducible between trials were uncommon serotypes, including a 3a strain that was negative for all probes in one trial and positive for the 1/2a(3a) probe in the other trial, a division III 4b strain that typed once as 1/2b(3b) and once as 1/2a(3a), and a serotype 4c strain that typed first as 1/2a(3a) and was negative for the second trial.

Intra-assay reproducibility was assessed by preparing and testing biological replicates of a 1/2a strain in both trials. The replicate samples were positive for the 1/2a(3a) probe and negative for other probes in both trials. The average fluorescent signals of the replicates were compared for each probe, and the correlation coefficient was 0.99 ($P < 0.01$).

For a high-throughput assay to be useful to both research and public health laboratories, the cost of the assay and nec-

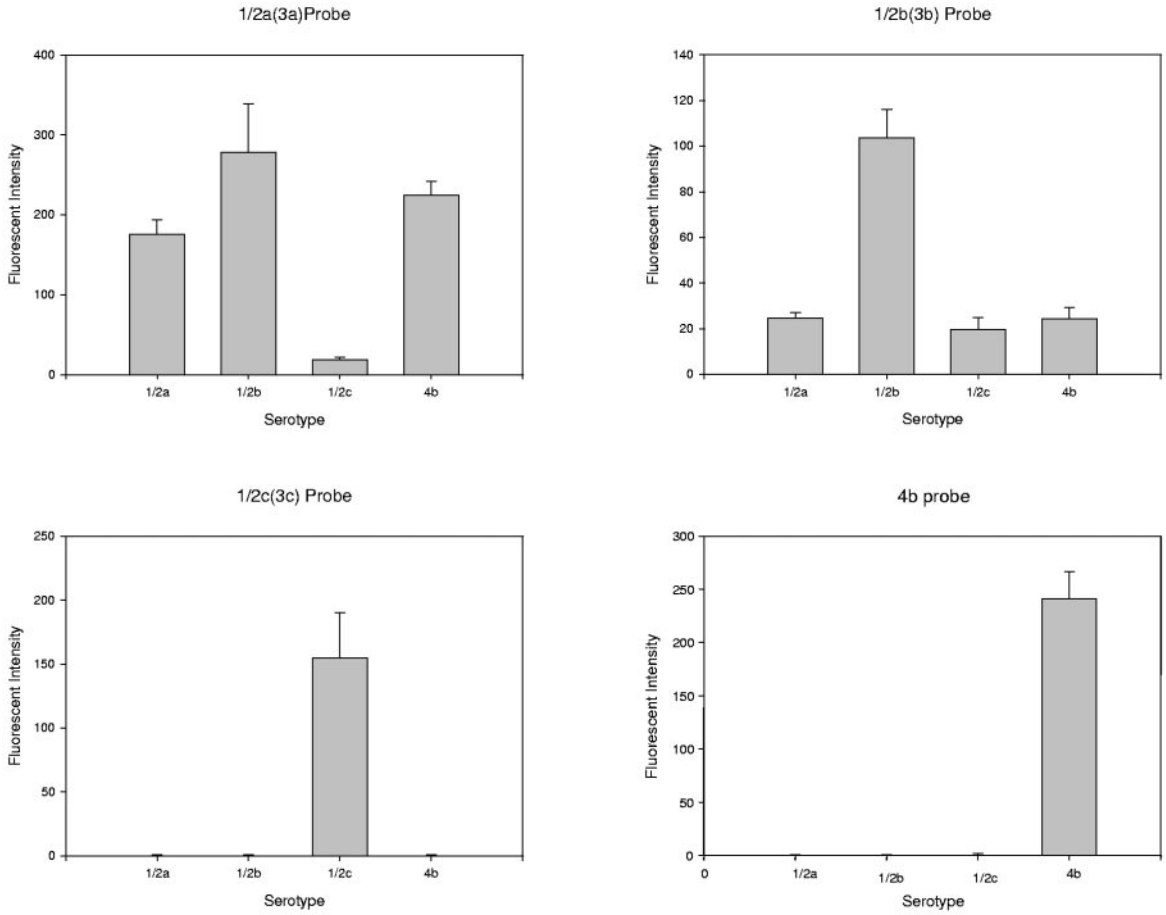

FIG. 2. Average fluorescent-signal intensities of common *L. monocytogenes* serotypes for each microarray probe. The error bars depict the standard errors.

essary instrumentation must be reasonable. In addition, an assay format that can accommodate analysis of a number of different organisms is preferable. These data illustrate the relative ease and efficiency of designing and implementing a suspension array for subtyping of genomic DNA. Based on our experience in developing this assay, we estimate the cost to be approximately $5.50 per isolate (for a four-probe assay with the sample tested in triplicate wells; the costs of DNA isolation and disposables are included). The amount of time required to subtype an isolate was less than 2 days. Although we used the Bio-Plex suspension array system to analyze the data, other flow cytometers may also be used for signal detection (9).

The development of a rapid, high-resolution, and genetically informative pathogen-subtyping technology is a necessary component of an effective infectious-disease surveillance system. Although planar-microarray subtyping analysis has been shown to be high resolution and genetically informative, it is still relatively low throughput and technically challenging. Suspension microarray technology eliminates many of the technical issues associated with planar-microarray analysis, including printing and washing artifacts, posthybridization image analysis, and data normalization. Thus, development of suspension subtyping arrays should facilitate standardization of microarray subtyping technology.

Future experiments will include design and testing of additional probes to improve *L. monocytogenes* suspension array subtyping resolution, including the addition of phylogenetic-division- and species-specific probes. Variable regions of the *L. monocytogenes* genome have already been identified using planar-microarray analysis (1, 3, 5). This information can be readily used for the design of additional suspension array probes to allow development of a rapid and high-resolution subtyping assay for *L. monocytogenes* isolates. Additionally, Dunbar et al. (8) have demonstrated the utility of the Luminex system for bacterial species identification using probes corresponding to the 23S rRNA gene, and similar probes can be added to this assay format.

TABLE 2. Microarray probe specificity for common *L. monocytogenes* serotypes

Strain and serotype	Probe specificity[a]			
	1/2a(3a)	1/2b(3b)	1/2c(3c)	4b
1/2a	+	−	−	−
1/2b	+	+	−	−
1/2c	−	−	+	−
4b	+	−	−	+

[a] +, positive result; −, negative result.

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