Listeria monocytogenes Serotype Identification by PCR
Monica K. Borucki and Douglas R. Call

A panel of 122 L. monocytogenes strains was assembled from human (n = 46), veterinary (n = 20), and environmental (n = 41) samples, as well as from samples from undetermined sources (n = 15). A majority of the strains were from the United States or Canada, and 10 were of European origin. Ten serotypes were included in the analysis, although the majority of the strains tested belonged to the four most common serotypes: 1/2a (n = 30), 1/2b (n = 20), 1/2c (n = 18), and 4b (n = 35).

Serotyping. Samples were serotyped with a commercially available serotyping kit (Denka Seiken Co., Tokyo, Japan) according to the manufacturer’s instructions, or they had been previously serotyped by a reference laboratory.

PCR primer design. Division-specific multiplex primers (Table 1) were designed on the basis of a divergent region of the L. monocytogenes genome that was previously identified by means of microarray analysis (4, 8). Microarray
probe 302 (GenBank accession no. BH170518) has high sequence similarity to an iron transport protein and was shown by microarray analysis to hybridize only to strains belonging to phylogenetic Divisions I and III; therefore, primers were designed on the basis of sequence differences that distinguish the two major divisions.

Strains identified as belonging in Division I or III were further subtyped by using primers designed to differentiate serotypes 4 and 1/2b (Table 1). These primers (hereinafter called GLT primers) were designed from a 1/2b serotype-specific region flanking the gltA-gltB cassette described by Lei et al. (18) (Table 1). Strains identified as serotype 4 were further serotyped for identification of Division III strains using the MAMA-C PCR primers as described by Jinneman and Hill (17). If strains tested positive with these primers, then the serotype was identified as 4a/c. Serovar 4 strains that tested negative were considered serotype 1/2c or 3c.

FlaA primers were considered serotype 1/2a or 3a. Strains testing negative were considered as 4a/c. Serovar 4 strains that tested negative were considered serotype 1/2c or 3c. Strains testing negative with both primer sets.

Primer set Forward primer sequence Reverse primer sequence Product size (bp) Anneal temp (°C) Specificity

<table>
<thead>
<tr>
<th>Primer set</th>
<th>Forward primer sequence</th>
<th>Reverse primer sequence</th>
<th>Product size (bp)</th>
<th>Anneal temp (°C)</th>
<th>Specificity</th>
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<tbody>
<tr>
<td>D1</td>
<td>CGATATTTTATCTACCTGTCA</td>
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<td>Division 1 or III</td>
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<tr>
<td>D2</td>
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<td>Division II</td>
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<tr>
<td>FlaA</td>
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<td>54</td>
<td>Serotypes 1/2a and 3a</td>
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<tr>
<td>GLT</td>
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<td>AATTAGGAATCAGCCTCTCT</td>
<td>483</td>
<td>45</td>
<td>Serotypes 1/2b and 3b</td>
</tr>
</tbody>
</table>

* PCR products run on a 2% agarose gel.

** PCR products run on a 1.2% agarose gel.

The underlined nucleotide is a mismatch that was introduced to increase primer specificity.

### RESULTS

The major phylogenetic division of 122 L. monocytogenes strains was determined by multiplex PCR carried out with primers designed from a region of an iron transport protein (GenBank accession no. BH170518) (Table 1) (Fig. 1). Ninety-nine percent (121 of 122) of strains tested with primer pairs D1 and D2 were typeable, and 98% (118 of 121) of the strains were placed in the correct phylogenetic division (Table 2). Overall, the sensitivities were 100 and 95%, and the specificities were 96 and 100% for the D1 and D2 primer sets, respectively. Listeria innocua (ATCC 51742) and Listeria ivanovii (ATCC 70402) isolates were also tested with the D1 and D2 multiplex primers, with L. innocua testing positive for Division I and L. ivanovii testing negative with both primer sets.

Strains testing positive with D1 primers (n = 69) were fur-
ther subtyped by using GLT primers designed from a 1/2b serotype-specific region flanking the gltA-gltB cassette (Table 1) (18). All 4b strains were negative (n = 35), and all 1/2b strains were positive (n = 20) when tested with these primers (Table 2, Fig. 1). The only 1/2a strain misclassified by the D1 and D2 primers was negative using the GLT primers, as were the 4a (n = 3), 4c (n = 6), and 4d (n = 1) strains. The 3c strain misclassified by the D1 and D2 primers tested positive when the GLT primers were used, as did the two 3b strains. Overall, 100% of strains correctly classified by the D1 and D2 primers as belonging in Division I or III were classified correctly with this primer set. Consequently, the sensitivity was 100% and the specificity was 100% for this primer set.

Samples testing negative with the GLT primers were further subtyped by using the previously described MAMA-C primers (17) for inclusion in Division III. All serotype 4a samples (n = 3), 4c samples (n = 6), 91% (32 of 35) of 4b samples, and the 4d sample were serotyped correctly with these primers (Table 2). The 1/2a strain misclassified by the D1 and D2 primers tested positive. Overall, the sensitivity was 100%, and the specificity was 92% for this primer set.

Samples classified by multiplex PCR as belonging in Division II (n = 52) were further subtyped with primers designed to be specific for serotype 1/2a based on the flad4 gene (10, 25). One hundred percent of serotype 1/2a (29 of 29) and 3a (4 of 4) strains tested positive with these primers, as did 6% (1 of 18) of the 1/2c strains (Table 2). Ninety-four percent of the 1/2c strains and the single 3c strain tested negative. The sensitivity was 100% and the specificity was 95% for this primer set.

A concordance analysis was conducted given the null hypothesis that serotype and PCR assays produce similar results. In this case, it was assumed that slide agglutination serotype classification represented the gold standard so that discrepancies between the two methods were attributable to the PCR assay. A McNemar test for paired observations with a discontinuity adjustment was applied for each category (e.g., Division I or II) or serotype (e.g., 1/2b) relevant to the primer set being tested. In all cases, classification of strains into division or serotype using PCR primers was concordant with classification based on conventional serotyping (McNemar test; P ≥ 0.25).

To verify that the assay results were reproducible, all 122 strains were tested in three separate experiments with similar results (range, 98 to 100% reproducibility). A subset of 10 strains was retested independently by a different technician not associated with the project, and reproducibility was 100%.

**DISCUSSION**

Although the genetic basis for serotype identification is not well defined, genetic analyses indicate that the evolution of somatic and flagellar antigens has paralleled that of many other genes (8, 11, 30). Therefore, we hypothesized that serotyping PCR primers may be designed as an alternative to the slide agglutination method of serotyping. Primers were designed by using genomic regions that had been previously identified as diverse by sequence analysis, monoclonal antibody binding, and/or microarray analysis (4, 8, 18, 25). In particular, microarray subtyping has been previously used to identify regions of the genome that vary between phylogenetic division and/or serotype (4, 8). One probe with sequence similarity to an iron transport protein was identified as being unique to Division I, and this information has allowed the design of division-specific multiplex primers.

Three other probes encoding different proteins involved in cell wall synthesis were identified by microarray (8; M. K. Borucki, unpublished data) and sequenced. Four sets of primers were tested for the ability to distinguish between serotypes within Division I. Results from all four primer sets were comparable to those obtained with the GLT primers (data not shown). However, due to the fact that the gltA-gltB region has been shown to be serotype specific by the use of monoclonal antibodies, we felt that these primers were the most biologically relevant.

In conclusion, PCR primers accurately identified both phylogenetic divisions and serotypes [1/2a(3a), 1/2b, 1/2c(3c), 4b(d,e), and 4a/c]. A panel of 122 _L. monocytogenes_ strains was tested; the sensitivity of the primer sets ranged from 95 to 100%, and specificity ranged from 92 to 100%. For isolates of the four major serotypes (1/2a, 1/2b, 1/2c and 4b), results of the PCR method agreed with those of the slide agglutination method for 97 (29 of 30), 100 (20 of 20), 94 (17 of 18), and 91% (32 of 35) of strains, respectively. Of the primers sets, the MAMA-C primer set was the least accurate (Table 2). This is not surprising, as these primers were designed to differentiate according to division rather than serotype. Additionally, one of the three 4b strains (strain FSL-J1-158) identified using the MAMA-C primers as belonging to Division III has also been classified as such by sequence analysis (7).

PCR is routinely performed in most diagnostic and research laboratories, and these primers should greatly increase the ease of _L. monocytogenes_ serotyping. Future research will focus on the development of PCR primers that distinguish the rare serotypes as well as refining the assay into a more efficient multiplex format.

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**REFERENCES**
