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Gene Scanning of an Internalin B Gene Fragment Using High-Resolution Melting Curve Analysis as a Tool for Rapid Typing of *Listeria monocytogenes*

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The ability to accurately track *Listeria monocytogenes* strains involved in outbreaks is essential for control and prevention of listeriosis. Because current typing techniques are time-consuming, cost-intensive, technically demanding, and difficult to standardize, we developed a rapid and cost-effective method for typing of *L. monocytogenes*. In all, 172 clinical *L. monocytogenes* isolates and 20 isolates from culture collections were typed by high-resolution melting (HRM) curve analysis of a specific locus of the internalin B gene (*inlB*). All obtained HRM curve profiles were verified by sequence analysis. The 192 tested *L. monocytogenes* isolates yielded 15 specific HRM curve profiles. Sequence analysis revealed that these 15 HRM curve profiles correspond to 18 distinct *inlB* sequence types. The HRM curve profiles obtained correlated with the five phylogenetic groups I.1, I.2, II.1, II.2, and III. Thus, HRM curve analysis constitutes an inexpensive assay and represents an improvement in typing relative to classical serotyping or multiplex PCR typing protocols. This method provides a rapid and powerful screening tool for simultaneous preliminary typing of up to 384 samples in approximately 2 hours. *(J Mol Diagn 2011, 13:57–63; DOI: 10.1016/j.jmoldx.2010.11.002)*

Listeria monocytogenes, the causative agent of listeriosis, is a facultative intracellular pathogen of humans and animals. It is widespread in the environment and has the ability to survive and grow under extreme conditions. Patients with listeriosis show symptoms such as gastroenteritis, encephalitis, meningitis, and septicemia. The high case-fatality proportion of approximately 20% to 30% makes *L. monocytogenes* an important human pathogen.[1–3]

In listeriosis outbreaks and for epidemiological investigations, a fast and accurate protocol for subtyping *L. monocytogenes* is essential.[4] In outbreak situations the *L. monocytogenes* serotyping scheme based on somatic (O) and flagellar (H) antigens[5] has limited value for tracking isolates. Of the 13 serotypes of *L. monocytogenes*, only a small fraction (serotypes 4b, 1/2a, and 1/2b) account for more than 96% of reported human listeriosis cases in Austria.[2] Insufficient reproducibility of serotyping,[6] relatively low discriminatory power, and antigen sharing among serotypes all impede the value of serotyping in outbreak investigations and so demand more accurate molecular-based typing solutions.[7] Research toward development of molecular typing protocols based on virulence gene analysis, ribotyping, and microarray analysis revealed that *L. monocytogenes* comprises five phylogenetic groups (PG).[8–11] Recently developed multiplex PCR serotyping methods allow a differentiation of isolates on the PG level.[12,13] Phylogenetic groups have been correlated with serotypes: PG I.1 with serotype 1/2a, 3a; I.2 with 1/2c, 3c; II.1 with 4b, 4d, 4e; II.2 with 1/2b, 3b, 7; and III with 4a, 4c.[10] In cases of outbreak, the discriminatory power of multiplex PCR serotyping methods is insufficient, and it is necessary to type isolates by pulsed-field gel electrophoresis (PFGE), which is the current gold standard for *L. monocytogenes* typing. The PFGE method is time-consuming, however, and is difficult to standardize, which hampers interlaboratory exchange and comparison of typing results.[14]

Typing methods by DNA sequence analysis and single nucleotide polymorphism (SNP) detection appear more promising for fast and accurate strain typing. Recently developed multilocus sequence typing and multivirulence locus sequence typing protocols are accurate and highly discriminative procedures for subtyping of *L. monocytogenes* strains.[14–17] The improved discriminatory power of multivirulence locus sequence typing, compared with multilocus sequence typing and PFGE, was demonstrated by subtyping of genetically diverse *L. monocytogenes* isolates.[18,19]

For rapid identification and typing of isolates in routine diagnostics, a PCR-based typing method targeting a sin-

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gle genetic region would be preferable in terms of cost, simplicity, turnaround time, and potential for standardization,[20] because both multilocus sequence typing and multivirulence locus sequence typing still represent time-consuming and cost-intensive approaches.

High-resolution analysis of DNA melting curves represents a new, simple, rapid, and precise mutation detection method applicable to genotyping.[21–23] The specificity of SNP detection using high-resolution melting (HRM) curve technology is comparable to DNA sequencing and surpasses specific probe-dependent classical PCR genotyping methods. Gene scanning by HRM allows the detection of unknown sequence alterations within the amplification product, in addition to known mutations.[21] The potential of HRM curve technology to detect diverse mutations in a single, simple PCR step makes the method very powerful for SNP-based typing applications.

The aim of the present study was the development of a rapid typing method based on polymorphisms of the internalin B gene (*inlB*).[24–26] Preliminary sequence analysis of various virulence genes (*prfA*, *inlB*, *inlC*, *dal*, *clpP*, *lisR*, *inlA*, *actA*), as well as housekeeping genes (*abcZ*, *dat*, *ldh*, *sod*, *cat*, *dapE*, *pgm*, *bglA*, *lhkA*) on an arbitrary selection of isolates revealed that *inlB* showed the highest genetic variability (unpublished data). This observation indicated that *inlB* could be a promising candidate for HRM curve-based typing.

Materials and Methods

Microorganisms

One hundred seventy-two clinical isolates were provided by the Austrian Reference Center for *Listeria*, isolated from human cases from 2004 to 2009. Twenty reference strains of serotypes 1/2a (DSMZ 20600), 1/2b (DSMZ 19094, CIP 105449), 1/2c (CIP 103573, ATCC 19112), 3b (CIP 80.10, CIP 78.35), 3c (CIP 78.36), 4a (ATCC 19114, CIP 105457, CIP 61.4), 4b (LMG 23189, LMG 23905, ATCC 13932, LMG 23356, DSMZ 15675), 4d (CIP 105458, ATCC 19117), 4e (ATCC 19118), and 7 (CIP 78.43) were purchased from culture collections, as follows: American Type Culture Collection (ATCC, Manassas, VA), the German Collection of Microorganisms and Cell Cultures [Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ), Braunschweig, Germany], the Collection of the Laboratory for Microbiology and Microbial Genetics [Laboratorium voor Microbiologie en Microbiele Genetica (LMG), Gent, Belgium], and the Collection of the Pasteur Institute [Collection de l'Institut Pasteur (CIP), Paris, France]. Isolates of serotypes 4c and 4ab were not available and therefore were not tested.

Genomic bacterial DNA (gDNA) was extracted from bacterial cells grown overnight at 37°C on RAPID'*L.mono* agar (Bio-Rad, Vienna, Austria) using the GenElute bacterial genomic DNA kit (Sigma-Aldrich, St. Louis, MO) according to the manufacturer's instructions. Purified gDNA was quantified spectrophotometrically at 260 nm and the gDNA quality was assessed by the 260/280 ratio.[27]

HRM Analysis

A 500-bp fragment located in the virulence gene internalin B (*inlB*) was amplified for subsequent HRM analysis using the forward primer *inlB*-forward (5′-CATGGGAGAGTAACCCAACC-3′) and the reverse primer *inlB*-reverse (5′-GCGGTAACCCCTTTGTCATA-3′).[17]

Both PCR and HRM were performed in a single run on a LightCycler 480 instrument (Roche Diagnostics, Penzberg, Germany) in a reaction mix containing 10 ng of genomic DNA, 0.25 pmol of each primer, and 3 mmol/L $MgCl_2$ in the LightCycler 480 high-resolution melting master mix containing ResoLight dye (Roche Diagnostics) with PCR-grade water adjusted to a final volume of 10 μL. Reaction conditions included an activation step at 95°C for 10 minutes followed by 50 cycles at 95°C for 10 seconds, 60°C for 10 seconds, and 72°C for 40 seconds. Before HRM, the amplification products were heated to 95°C for 1 minute and then cooled to 40°C for 1 minute. The HRM was performed over a range from 60°C to 95°C, rising at 1°C/s with 25 acquisitions per 1°C step. All reactions were performed in quadruplicate using an epMotion workstation (Eppendorf, Hamburg, Germany) for automatic sample preparation in 384-well microtiter plates (LC plate; Roche Diagnostics, Vienna, Austria).

For generating HRM curves, LC480 gene scanning software version 1.5 was used with manual settings for sensitivity to 0.30, for temperature shift to threshold four, and a pre-melt normalization range from 70°C to 78°C and a postmelt normalization range from 85°C to 90°C. Difference plots were generated by selecting a negative control, converting the melting profile to a horizontal line, and normalizing the melting profiles of the other samples against this sample. The HRM was performed only on amplification products that reached the plateau phase.

Sequencing

For all samples, *inlB* was subsequently sequenced from position 934 to 1433. The PCR products for sequencing were amplified with primers *inlB*-F and *inlB*-R containing a respective M13 sequence attached to the 5′ end. Sequence analysis was performed using a SequiTherm Excel II DNA sequencing kit (Epicentre Biotechnologies, Madison, WI) with fluorescent-labeled primers M13 universal (5′-TGTAAAACGACGGCCAGT) and M13 reverse (5′-CAGGAAACAGCTATGACC) (MWG-Biotech, Ebersberg, Germany) on a Li-Cor 4300 automated DNA sequencer v2.0 (LI-COR Bioscience, Lincoln, NE), according to the manufacturer's instructions. All sequences obtained were assembled, edited and compared using a Li-Cor AlignIR software package to determine sequence variations.

Results

Gene scanning of a 500-bp amplification product of *inlB* on all 192 *L. monocytogenes* isolates yielded 15 distinct HRM curve profiles (Figure 1). Subsequent sequence analysis of the amplification products revealed that these 15 specific HRM curve profiles originated from 18 different *inlB* sequence types (*inlB* STs) (Table 1).