Multiplexed Typing of Mycobacterium avium subsp. paratuberculosis Types I, II, and III by Luminex xMAP Suspension Array

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Differentiation among types I, II, and III is the primary step in typing Mycobacterium avium subsp. paratuberculosis. We propose an innovative approach based on detection of gyrase B (gyrB) gene polymorphisms by suspension array technology, with high discriminatory power and high-throughput potential.

Mycobacterium avium subsp. paratuberculosis is an important animal pathogen that is widely disseminated in the environment and responsible for paratuberculosis (Johne’s disease), a chronic inflammation of the gastrointestinal tract that affects mainly livestock and wild ruminants (4, 10). M. avium subsp. paratuberculosis strains can be classified into three groups: type I (sheep), type II (cattle), and type III (intermediate) (6, 15, 16). Over the last few years, various molecular typing techniques have been proposed for the typing of M. avium subsp. paratuberculosis (for a review, see Motiwala et al., 2006 [13]). Some of them take advantage of the discriminatory power of single nucleotide polymorphisms (SNPs) disseminated in the genome (12). In particular, Castellanos et al. identified SNPs in the gyrase B (gyrB) gene that allow discriminating all three types of M. avium subsp. paratuberculosis (3). The authors also developed a PCR restriction enzyme analysis (PCR-REA) of gyrB locus 1626 that is capable of differentiating types I and II from type III.

In this study, we propose a novel approach based on analysis of two gyrB loci that allow identifying all three types of M. avium subsp. paratuberculosis in a single session. The method relies on PCR-mediated amplification and biotinylation of two gyrB fragments, each carrying a distinct SNP (at positions 1353 and 1626, respectively [Table 1]) and on amplicon analysis via liquid-phase microarray Luminex xMAP technology. This implies the use of microsphere sets coupled to probes that recognize and bind the amplicon carrying the specific SNP (if present). Each microsphere set is characterized by a distinct spectral address given by the combination of red and infrared fluorophores within the spheres. Once bound, the target DNA molecules are fluorescently tagged with streptavidin–R-phycocerythrin, and the beads are individually analyzed with a red laser that recognizes the microsphere set and a green laser that provides a quantitative readout of the bound target (8). Thanks to its simple, high-throughput, multiplex architecture, this technology has already gained a solid reputation in the field of bacterial genotyping (5, 9, 11).

In order to verify the robustness of the method, we analyzed 81 different M. avium subsp. paratuberculosis strains, recovered from four different European countries and five different hosts (see Table 3). All the Italian strains were isolated in our laboratory, and the others were delivered to us as bacterial cultures or purified genomic DNA. DNA extraction and purification from bacterial broths were carried out using the High Pure PCR template preparation kit (Roche, Germany) according to the manufacturer’s instructions. All M. avium subsp. paratuberculosis samples were preanalyzed by IS1311-based PCR-REA (18), which allows discriminating type II from type I/III strains (see Table 3). In order to amplify two DNA fragments carrying the SNPs at positions 1353 and 1626 of gyrB, we designed two pairs of primers (F1 plus R1 and F2 plus R2 [Table 2]) and set up a duplex PCR. PCR was carried out in 50 μl containing 1 μM primers F1 and R1, 400 nM primers F2 and R2, 2 mM MgCl2 (Applied Biosystems, Roche, Foster City, CA), 200 μM deoxynucleoside triphosphate (dNTP; Applied Biosystems), 5 μl buffer 10× (Applied Biosystems), 2.5 U AmpliTaq Gold (Applied Biosystems), and about 20 ng DNA. The PCR thermal profile consisted of 94°C for 10 min, 35 cycles of 1 min at 94°C, 30 s at 60°C, and 30 s at 72°C, and 10 min at 72°C. The amplification of the two products of 176 and 110 bp was confirmed by electrophoresis in a 2% agarose-ethidium bromide gel and visualization under UV light (data not shown). Four distinct sets of COOH microspheres (Bio-Rad Laboratories, Hercules, CA) were coupled to the four SNP-specific NH2 probes (Table 2) via a carbodiimide reaction, and coupling was

<table>
<thead>
<tr>
<th>SNP</th>
<th>Type I</th>
<th>Type II</th>
<th>Type III</th>
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<tbody>
<tr>
<td>1353</td>
<td>C</td>
<td>T</td>
<td>C</td>
</tr>
<tr>
<td>1626</td>
<td>C</td>
<td>C</td>
<td>T</td>
</tr>
</tbody>
</table>

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* Position refers to the loci of the gyrB gene of M. avium subsp. paratuberculosis K-10 (accession no. NC_002944).

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confirmed via titration with biotinylated anti-probe oligonucleotides as previously published (1). Coupled microspheres were stored in Tris-EDTA (TE) buffer at 4°C in the dark. The samples were then analyzed in duplicate with TE as the negative control. Briefly, 5 μl of each amplification mixture was added to 45 μl of hybridization solution (3 M tetramethylammonium chloride, 50 mM Tris-HCl [pH 8.0], 4 mM EDTA [pH 8.0], 0.1% Sarkosyl) containing 4,950 microspheres of each probe-coupled set. Reaction mixtures were denatured at 95°C for 5 min, hybridized at 60°C for 30 min, and then centrifuged at 6,000 g for 3 min. After removing the supernatant, the bead-associated amplicons were labeled with fluorescent reporter, as previously described (1). Finally, 100 beads of each set per sample were analyzed with a Luminex 100 analyzer (Bio-Plex 200 System Instrument, Bio-Rad) and Bio-Plex Manager software (version 4.1.1), which expressed the results as median fluorescence intensity (MFI) values.

Positive results were called when the MFI values were at least 1.5 times higher than the TE MFI values. In order to standardize results among samples, we subtracted the TE MFI from each MFI value, thus obtaining the sample net median fluorescence intensity (NMFI) values. Finally, we calculated ΔNMFI 1353 and ΔNMFI 1626 values by subtracting the NMFI values given by probes 1353_C and 1626_C from the ones given by probes 1353_T and 1626_T, respectively. This way, positive ΔNMFI values indicated the presence of T, while negative ΔNMFI values implied that T was replaced by C in the polymorphic locus under analysis. In Fig. 1, we plotted ΔNMFI values of all samples as bidimensional coordinates. It is clearly visible that the SNP-dependent distribution of the samples forms three distinct groups, corresponding to the three different types of M. avium subsp. paratuberculosis. The exactness of the distribution of each sample agreed with the typing profile obtained by IS1311 PCR-REA (Table 3) and was confirmed by direct sequencing of each amplicon (ABI 377 DNA sequencer, Applied Biosystems). The minimal relative distance between samples carrying different SNPs, and thus belonging to different types, was equal to 3 log units, which demonstrates the high discriminatory power of this method. Furthermore, as already observed by others (1, 7), the technique proved to be fast, allowing the typing of a panel of 96-well-plate PCR products in about 2 h.

Molecular typing applications represent powerful tools to identify sources of M. avium subsp. paratuberculosis transmission and improve paratuberculosis control programs in livestock (14). In the recent past, several M. avium subsp. paratuberculosis typing techniques have been implemented to improve and simplify traditional biochemical and molecular methods. In particular, the discriminatory power of SNPs has

### TABLE 2. Primers and probes used in this study

<table>
<thead>
<tr>
<th>Primer/probe</th>
<th>Sequence (5’ to 3’)</th>
<th>5’ modification</th>
</tr>
</thead>
<tbody>
<tr>
<td>F1</td>
<td>5’-CAAGGCCGAGAGATTGTG-3’</td>
<td></td>
</tr>
<tr>
<td>R1</td>
<td>5’-GAAGGATGGCCGGAATAATCG-3’</td>
<td>Biotin</td>
</tr>
<tr>
<td>F2</td>
<td>5’-ACCACAAGATCGTTGTGATGG-3’</td>
<td></td>
</tr>
<tr>
<td>R2</td>
<td>5’-GTCGCGGTGTTATCGACGC-3’</td>
<td>Biotin</td>
</tr>
<tr>
<td>1353_C</td>
<td>5’-GTCGACCGACCCCGCGAGAAGT-3’</td>
<td>NH2-C12</td>
</tr>
<tr>
<td>1353_T</td>
<td>5’-GTCGACCGATCGCGAAGT-3’</td>
<td>NH2-C12</td>
</tr>
<tr>
<td>1626_C</td>
<td>5’-GCCAGACATTCGACGCTG-3’</td>
<td>NH2-C12</td>
</tr>
<tr>
<td>1626_T</td>
<td>5’-GCCAGACATTCGACGCTG-3’</td>
<td>NH2-C12</td>
</tr>
</tbody>
</table>

*Bolding represents SNP-specific nucleotides.*

### FIG. 1. Polymorphism-dependent distribution of *Mycobacterium avium* subsp. *paratuberculosis* strains based on ΔNMFI values, as revealed by liquid-phase array analysis. ΔNMFI values are expressed as logarithmic units and referred to the 1353 (ΔNMFI 1353) and 1626 (ΔNMFI 1626) *gyrB* loci. Error bars represent the standard deviations.
been used in PCR-REA (3, 17, 18) and, more recently, high-resolution melt (HRM) analysis (2). The present study evaluates the potential of suspension array technology in effectively discriminating M. avium subsp. paratuberculosis types. Our approach is based on PCR amplification of two gyrB fragments carrying type-specific polymorphisms revealed by hybridization with bead-coupled probes in a liquid system. The detection of a single amplicon carrying both SNPs proved not to be possible because of the high GC content of this fragment, which led to the formation of stable secondary structures hiding the polymorphic site at nucleotide 1353 (data not shown). Overall, this method was found to be fast and to have a high throughput and high discriminatory power. Like PCR-REA, it needs a small amount of bacterial DNA and has the added advantage of differentiating all three M. avium subsp. paratuberculosis types in a single session and returning numeric results which are convenient for statistical analysis. Furthermore, the ability of the Luminex technology to test up to 100 analytes in a single reaction translates into a high implementation potential. For example, the analysis of gyrB SNPs could be combined with the detection of other polymorphisms by multiplexing the initial PCR and adding the desired probes. This way, subtyping of M. avium subsp. paratuberculosis strains could be easily achieved.

In conclusion, we have described a method that allows the simultaneous, powerful, and reliable analysis of multiple SNPs for differentiation among type I, II, and III strains of M. avium subsp. paratuberculosis. This technology could provide the basis for further subtyping protocols for M. avium subsp. paratuberculosis genome discrimination.

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REFERENCES