Multiplexed typing of *Mycobacterium avium* subsp. *paratuberculosis* Type I, II and III by Luminex xMAP suspension array

Running title: Multiplexed typing of *Mycobacterium a. paratuberculosis*

Michele Gastaldelli¹, Elisabetta Stefani¹, Antonia Anna Lettini² and Nicola Pozzato¹* 

¹Istituto Zooprofilattico Sperimentale delle Venezie - Sezione di Verona, Via San Giacomo 5, 37135 Verona, Italy. 

²OIE, National Reference Laboratory for Salmonella, Istituto Zooprofilattico Sperimentale delle Venezie, Viale dell’Università 10, 35020 Legnaro, Italy. 

*Corresponding author. Mailing address: Istituto Zooprofilattico Sperimentale delle Venezie - Sezione di Verona, Via San Giacomo 5, 37135 Verona, Italy. Phone: +39045500285. Fax. +39045582811. E-mail: npozzato@izsvenezie.it
Abstract

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 (M. a. paratuberculosis) is an important 21 animal pathogen, widely disseminated in the environment and responsible for paratuberculosis (Johne’s disease), a chronic inflammation of the gastrointestinal tract that mainly affects livestock and wild ruminants (4, 10). M. a. paratuberculosis strains can be classified into three groups: type I (sheep, S), type II (cattle, C) and type III (intermediate, I) (6, 15, 16). Over the last few years, various molecular-typing techniques have been proposed for typing of M. a. 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 DNA-gyrase B (gyrB) gene that allow discriminating all three types of M. a. paratuberculosis (3). Besides, the authors developed a PCR-restriction enzyme analysis (PCR-REA) of gyrB locus 1626 capable of differentiating types I/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. a. paratuberculosis in a single session. The method relies on PCR-mediated amplification and biotinylation of two gyrB fragments each carrying a distinct SNP (1353 and 1626, respectively; see 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-phycoerythrin 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. a.* paratuberculosis strains, recovered from four different European countries and five different hosts (see Table 3). All the Italian strains were isolated in our laboratory, the others were delivered to us as bacterial cultures or purified genomic DNA. DNA extraction and purification from bacterial broths was carried out using the High Pure PCR Template Preparation Kit (Roche, Germany), according to the manufacturer’s instructions. All *M. a. paratuberculosis* samples were pre-analyzed by IS1311-based PCR-REA (18), which allows discriminating type II from type I/III strains (Table 3). In order to amplify two DNA fragments carrying respectively the SNPs at position 1353 and 1626 of *gyrB*, we designed two pairs of primers (F1+R1 and F2+R2; Table 2) and set up a duplex PCR reaction. PCR was carried out in 50 µl containing 1 µM primer F1 and R1, 400 nM primer F2 and R2, 2 mM MgCl2 (Applied Biosystems, Roche, Foster City, USA), 200 µM dNTPs (Applied Biosystems), 5 µl Buffer 10X (Applied Biosystems), 2.5 U AmpliTaq GoldTM (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 4 SNP-specific NH2-probes (Table 2) via a carbodiimide reaction, and coupling was confirmed via titration with biotinylated anti-probe oligonucleotides as previously published (1). Coupled microspheres were stored in TE buffer at 4°C in the dark. The samples were then analyzed in duplicate with TE as negative control. Briefly, 5 µl of each amplification mixture were 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 4950 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 6000 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-PlexTM 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. 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 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 T was replaced by C in the polymorphic locus under analysis. In Fig. 1 we plotted ΔNMFI values of all samples as bi-dimensional coordinates. It is clearly visible the SNP-dependent distribution of the samples to form 3 distinct groups, corresponding to the 3 different types of M. a. 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. a. paratuberculosis transmission and improve paratuberculosis control programs in livestock (14). In the recent past, several M. a. paratuberculosis typing techniques have been implemented to
improve and simplify traditional biochemical and molecular methods. In particular, the
discriminatory power of SNPs has 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. a. paratuberculosis* types. Our approach is based on
PCR amplification of 2 *gyrB* fragments carrying type-specific polymorphisms revealed by
hybridisation 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
that 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 high-throughput and to
have high discriminatory power. Like PCR-REA, it needs a low amount of bacterial DNA and
has the added advantage of differentiating all three *M. a. 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 to
the detection of other polymorphisms by multiplexing the initial PCR and adding the desired
probes. This way, subtyping of *M. a. 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. a.
paratuberculosis*. This technology could provide the basis for further subtyping protocols for *M.
a. paratuberculosis* genome discrimination.

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providing *M. a. paratuberculosis* DNA and isolates. We also thank M.C. Dalla Pozza for her
References


18. **Whittington, R., I. Marsh, E. Choy, and D. Cousins.** 1998. Polymorphisms in IS1311, an insertion sequence common to *Mycobacterium avium* and *M. avium* subsp. *paratuberculosis*, can be used to distinguish between and within these species. Mol Cell Probes 12:349-58.
TABLE 1. SNPs in loci 1353 and 1626 of gene gyrB that allow discrimination among 181 Mycobacterium avium subsp. paratuberculosis types I, II and III.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Base position</th>
<th>SNP</th>
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<tr>
<td></td>
<td></td>
<td>Type I</td>
</tr>
<tr>
<td>gyrB</td>
<td>1353</td>
<td>C</td>
</tr>
<tr>
<td></td>
<td>1626</td>
<td>C</td>
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</table>

* The position is referred to the gyrB gene of *M. avium* subsp. *paratuberculosis* K-10 (Accession No. NC_002944).

TABLE 2. Primers and probes used in this study.

<table>
<thead>
<tr>
<th>Primer/probe</th>
<th>Sequence (5′–3′)</th>
<th>5′ modification</th>
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<tr>
<td>F1</td>
<td>5′-CAAGGCCGCGAGAGTTGGTG-3′</td>
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</tr>
<tr>
<td>R1</td>
<td>5′-GAAGGATGGCCTGGAAACATCG-3′</td>
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<tr>
<td>F2</td>
<td>5′-ACCACAAGATCGGTGTTGATGTC-3′</td>
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<tr>
<td>R2</td>
<td>5′-GTGCCCGTGTTTCGATAGC-3′</td>
<td>biotin</td>
</tr>
<tr>
<td>1353_C</td>
<td>5′-GTCGACCCTTCTCGGACAGT-3′</td>
<td>NH2-C12'</td>
</tr>
<tr>
<td>1353_T</td>
<td>5′-GTCGACCCTTCTCGGACAGT-3′</td>
<td>NH2-C12'</td>
</tr>
<tr>
<td>1626_C</td>
<td>5′-GCCAGCATCTCGAGCAGT-3′</td>
<td>NH2-C12'</td>
</tr>
<tr>
<td>1626_T</td>
<td>5′-GCCAGCATCTCGAGCAGT-3′</td>
<td>NH2-C12'</td>
</tr>
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TABLE 3. Characteristics of *Mycobacterium avium* subsp. *paratuberculosis* isolates and summarized results of liquid phase array analysis.

<table>
<thead>
<tr>
<th>Geographic origin</th>
<th>Host</th>
<th>Number of isolates</th>
<th>IS1311 PCR-REA type</th>
<th>gyrB SNP profilea</th>
<th>gyrB type</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1353 nt</td>
<td>1626 nt</td>
</tr>
<tr>
<td>Italy</td>
<td>Cattle</td>
<td>30</td>
<td>II</td>
<td>T C</td>
<td>II</td>
</tr>
<tr>
<td></td>
<td>Red deer</td>
<td>29</td>
<td>II</td>
<td>T C</td>
<td>II</td>
</tr>
<tr>
<td></td>
<td>White-tailed deer</td>
<td>1</td>
<td>II</td>
<td>T C</td>
<td>II</td>
</tr>
<tr>
<td>Germany</td>
<td>Cattle</td>
<td>5</td>
<td>II</td>
<td>T C</td>
<td>II</td>
</tr>
<tr>
<td></td>
<td>Sheep</td>
<td>2</td>
<td>I/III</td>
<td>C T</td>
<td>III</td>
</tr>
<tr>
<td>Spain</td>
<td>Goat</td>
<td>1</td>
<td>I/III</td>
<td>C T</td>
<td>III</td>
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<tr>
<td></td>
<td>Sheep</td>
<td>4</td>
<td>I/III</td>
<td>C T</td>
<td>III</td>
</tr>
<tr>
<td>UK</td>
<td>Sheep</td>
<td>9</td>
<td>I/III</td>
<td>C C</td>
<td>I</td>
</tr>
</tbody>
</table>

*a* gyrB SNP profile as revealed by liquid phase array analysis and sequencing.

Figure 1

FIG. 1. Polymorphism-dependent distribution of *Mycobacterium avium* subsp. *paratuberculosis* strains based on ∆NMFI values, as revealed by liquid phase array analysis. ∆NMFI values are expressed as logarithmic units and referred to the 1353 (∆NMFI 1353) and 1626 (∆NMFI 1626) gyrB loci. Error bars represent the Standard Deviations.