High-resolution melting for analysis of short sequence repeats in *Mycobacterium avium* subsp. *paratuberculosis*

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**Abstract**

Analysis of micro- and minisatellite loci is widely used in sub-typing of *Mycobacterium avium* subsp. *paratuberculosis*. Microsatellite (short sequence repeat, SSR) loci have shown highest discriminatory power, but direct sequencing of amplicons is required for correct assignment of the repeat number. We developed an alternative method to sequencing, focusing on the SSR8 locus (constituted by GGT triplets from three to six repeats). The approach is based on asymmetric quantitative PCR, followed by high-resolution melting analysis with unlabelled probes (UP-HRM). Data showed perfect concordance between direct sequencing and UP-HRM, which is faster, simpler and more cost effective.

**Introduction**

*Mycobacterium avium* subsp. *paratuberculosis* (Map) is the causative agent of paratuberculosis in ruminants and other species. This bacterium is characterized by a very slow growth rate and limited genomic diversity (Stevenson et al., 2009). Numerous methods have been proposed to sub-type Map strains, such as multiplex PCR for IS900 integration loci, IS900 restriction fragment length polymorphism, amplified fragment length polymorphism, pulsed field gel electrophoresis and genotyping microarrays (Motiwala et al., 2006; Pribylova et al., 2009; Stevenson et al., 2009). However, methods based on micro- and minisatellite analyses are the most widely used techniques in this regard (Motiwala et al., 2006; Thibault et al., 2007) because of their relative simplicity and efficacy. Short sequence repeat (SSR) loci, particularly SSR1, SSR2, SSR8 and SSR9, showed highest allelic diversity, making these loci very useful for the evaluation of discriminatory indices (Amonsin et al., 2004; Thibault et al., 2008). However, at present, the only method available for the scanning of these loci is direct sequencing, which requires expensive systems and dedicated facilities.

To overcome this problem, we developed a new alternative approach to sequencing, for the identification of SSR loci repeat number. We focused on the SSR8 locus, which comprises GGT triplets. So far, four alleles (ranging from three to six repeats) have been described for this locus (Amonsin et al., 2004; Ghadiali et al., 2004; Motiwala et al., 2006; Thibault et al., 2008).

The new method is based on an asymmetric quantitative PCR (qPCR), followed by high-resolution melting (HRM) analysis with unlabelled probes (hereafter UP-HRM) (Zhou et al., 2004). The efficiency and specificity of the asymmetric PCR reaction was improved by designing primers according to the linear-after-the-exponential PCR (LATE-PCR) strategy (Pierce et al., 2005), whereas to avoid any elongation during the amplification, the unlabelled probe was blocked at its 3’ end. The shortness of the probe (32 bp) enhanced the ability of HRM analysis to differentiate between sequences with very similar melting temperature ($T_m$), allowing clear identification of typical $T_m$ values for every single allele.
Materials and methods

Map strains were collected at the Italian National Reference Centre for Paratuberculosis. Briefly, DNA was extracted by suspending one colony of Map in 100 µL of PCR-grade water. After the addition of 300 mg of acid-washed glass beads (Sigma-Aldrich, Milan, Italy), the mixture was homogenized in a Tissue Lyser (Qiagen, Milan, Italy) and DNA was extracted with QIAamp DNA mini kit (Qiagen), following the manufacturer’s procedures. The DNA was spectrophotometrically quantified and then diluted in elution buffer. For one sample, containing the allele with three repeats, culture was not possible and the DNA was extracted directly from the intestine of a diseased sheep, positive to IS900 PCR. Briefly, 25 mg of frozen intestinal mucosa was manually minced and homogenized in a Tissue Lyser in the presence of acid-washed glass beads. The mixture was digested with 10 mg mL⁻¹ lysozyme (Roche, Monza, Italy) for 30 min at 37 °C, followed by incubation with protease K for 30 min at 56 °C. The DNA was then purified with QIAamp DNA mini kit.

Primers and probe were designed with reference to the Map K10 genome sequence (GenBank accession no. AE016958) with Beacon Designer 7.60 (Premier Biosoft International) and then modified according to LATE-PCR strategy. The \( T_m \) of the primers and probe was also checked by different software packages (1.5-ITECH; Idaho Technology Inc., Salt Lake City, UT), the only software able to evaluate the presence of dimethyl sulfoxide (DMSO) in the mix, available at http://www.idahotech.com/Support/TmUtilitySoftware/SupportForm-TmUtility. html; OLIGO CALC 3.26, available at http://www.basic.northwestern.edu/biotools/oligocalc.html (Kibbe, 2007); and OLIGO ANALYZER 3.1; Integrated DNA Technologies, Inc., http://eu.idtdna.com/analyzer/Applications/OligoAnalyzer/.

The concentrations of the primers and probe were: 50 nM for the limiting primer (forward), 500 nM for the excess primer (reverse) and 500 nM for the probe. According to the LATE-PCR strategy, the \( T_m \) of the limiting primer was 5 °C higher than that of the excess primer. Primers and probe sequences were: forward, 5′-CGGGTGCGCGAGGCTGGTCG-3′; reverse, 5′-CGCTCC TCGGGCATCTGC-3′; probe, 5′-GAGGGCGCGGTGGTGGT GTGGTGTGGTGGCGCA-3′. The probe was synthesized with the longest triplet repeat number already described (six GGT triplets, in bold type) and was blocked with a C6-amino group at the 3′-end. Eight and four flanking nucleotides were included to facilitate the suitable match with the single strand DNA generated during the asymmetric amplification. For PCR reactions, 10 ng of DNA was amplified on a StepOne Plus system (Applied Biosystems, Milan, Italy) in a final volume of 25 µL. The mix contained 1× LCGreen® Plus (Idaho Technology Inc.), 0.2 mM dNTPs (EuroClone, Pero, Italy), 3 mM Mg²⁺, 5% DMSO and 0.5 U of Hot-start Taq Polymerase (EuroClone). Cycle conditions were: initial denaturation at 95 °C for 3 min, then 50 cycles of 15 s denaturation at 96 °C and 30 s annealing/extension at 67 °C. At the end of the qPCR reaction, samples were heated to 95 °C for 15 s, followed by 1 min at 60 °C. They were then gradually heated from 60 to 95 °C according to the instrument default parameters and the fluorescence was recovered. Initially, the fluorescence was recorded for each 0.1 or 0.3 °C step (10 and 3.3 times per 1 °C respectively) and no significant differences were observed between the two procedures. Accordingly, the method was then validated recording fluorescence at each 0.3 °C step. \( T_m \) was directly measured by the internal software (StepOne Software v2.1; Applied Biosystems). Data were exported and processed according to mathematical algorithms for high-resolution DNA melting analysis (Palais & Wittwer, 2009). Briefly, the background was evaluated and removed to the negative derivative of the fluorescence data. The results obtained were then normalized and smoothed with the ‘running average’ method. Graphs were generated with SIGMA PLOT 5.0 (SSI, CA).

To develop the method, we used three different Map strains, carrying three, four and five repeats; unfortunately, we did not have any strains containing the allele with six repeats in our collection, so we used a synthetic single strand DNA amplicon holding six triplets. For this, 1 µg of the reverse single strand DNA (Eurofins MWG, Ebersberg, Germany) was copied in the presence of forward primer. The synthetic double strand DNA was then diluted to 10 ng prior to PCR.

The number of triplet repeats for all strains was confirmed by sequencing with ABI Prism 3100 Avant Sequencer (Applied Biosystems), according to Amonsin et al. (2004). The sequences were analysed using the SEQUENOM Module within the Lasergene Package (DNA Star, Madison, WI).

Results and discussion

Representative results of HRM analysis are shown in Fig. 1, as derivative melting curves after normalization and exponential background removal. Two melting domains for each sample were observed: one relative to the amplicon homoduplex product (DNA double strand) and another one relative to the heteroduplex single strand DNA/probe. According to the LATE-PCR strategy, the homoduplex products were generated during the first cycles of amplification, whereas the single strand DNA was generated during the late cycles (data not shown).
This single strand DNA can match with the probe and generate the heteroduplex single strand DNA/probe.

As shown in Fig. 1, analysis of homoduplex amplicons did not allow any differentiation between the various alleles. However, it did reveal approximately three degrees among the adjacent alleles, allowing an unbiased identification.

In silico analysis using the software mentioned above revealed that $T_m$ decreased theoretically by 1 °C for every triplet depletion, a difference between two adjacent alleles ($\Delta T_m$) smaller than that found in our analysis. An explanation for this discrepancy could be the formation of loops when the probe (six repeats) matches the DNA single strand of the alleles carrying three, four and five repeats. These secondary structures could contribute to further destabilize the heteroduplex, causing a larger $\Delta T_m$ among two adjacent alleles with respect to that theoretically evaluated by the software (see Supporting Information, Fig. S1). Moreover, a reliable estimation of the $T_m$ between single strand DNA and probe was possible only for the allele with six triplets (i.e. estimated to be 80.7 °C using 1.5-ITECH software).

These data were confirmed by the analysis of two strains carrying three repeats, 20 with four repeats and 20 with five repeats (Table 1). The allele with three repeats was less frequent than those with four and five repeats, but we were not able to check the method with a sample carrying the allele with six repeats because of its rarity among Map strains. In fact, despite the multitude of studies that have analysed the SSR8 locus, this rare allele has been described in only five strains (isolated in the USA from different host species) (Amonsin et al., 2004; Ghadiali et al., 2004; Harris et al., 2006; Thibault et al., 2008). Moreover, as PCR is an in vitro assay, the use of synthetic DNA should not interfere with the reaction.

Perfect concordance was observed between our approach and the results of the direct sequencing ($K = 1$), and low SDs confirmed the precision of the method.

As with many other Mycobacteria, Map is characterized by a genome very rich in GC (Li et al., 2005) and this feature could make it difficult to design appropriate primers for the amplification of specific targets. However, the design of the primers according to the LATE-PCR strategy allowed us to overcome this problem.

Erali & Wittwer (2010) showed that full-amplicon HRM analysis performed with specific HRM instruments allowed the identification of various single nucleotide polymorphisms, even those belonging to class 4 (A → T), which showed a difference in $T_m$ near 0.25 °C. As previously shown (Zhou et al., 2004), the use of short unlabelled probe directly in the PCR reaction mix enhanced the differences between each variant and allowed an unbiased identification of the polymorphism present.

The method proposed here is robust and reproducible and in comparison with direct sequencing, its results are more cost effective (£1.5 for each sample vs. £8–10) and faster (3 h to obtain a final result vs. 4 h). Moreover, it is a closed-tube technique requiring only a qPCR system, minimizing contamination risks. Finally, as HRM analysis is not destructive, and is compatible with sequencing techniques, it potentially allows new alleles or mutations inside the probe-matching site (peaks with unexpected $T_m$) to be found.

**Table 1.** Tm values (expressed as mean ± SD), number of strains tested and delta Tm between two adjacent allele

<table>
<thead>
<tr>
<th>No. of repeats</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tm ± SD (°C)</td>
<td>69.4 ± 0.3</td>
<td>72.8 ± 0.1</td>
<td>75.7 ± 0.1</td>
<td>79.4 ± 0.0</td>
</tr>
<tr>
<td>No. of tested strains</td>
<td>2</td>
<td>20</td>
<td>20</td>
<td>1</td>
</tr>
<tr>
<td>$\Delta T_m$ ± SD (°C)</td>
<td>–</td>
<td>3.4 ± 0.4</td>
<td>2.9 ± 0.2</td>
<td>3.7 ± 0.1</td>
</tr>
</tbody>
</table>

*Synthetic amplicon.
To the best of our knowledge, this is the first article suggesting the application of HRM analysis in the analysis of short repeat number. Further studies should investigate the usefulness of the method proposed for the identification of mononucleotide SSR loci, such as SSR1 and SSR2.

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References


Supporting information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Melting temperatures of the different alleles matched to the probe.

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