Rapid Identification and Differentiation of *Mycobacterium avium* Subspecies *paratuberculosis* Types by Use of Real-Time PCR and High-Resolution Melt Analysis of the MAP1506 Locus^{▽†}

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High-resolution melt (HRM) analysis can identify sequence polymorphisms by comparing the melting curves of amplicons generated by real-time PCR amplification. We describe the application of this technique to identify *Mycobacterium avium* subspecies *paratuberculosis* types I, II, and III. The HRM approach was based on type-specific nucleotide sequences in MAP1506, a member of the PPE (proline-proline-glutamic acid) gene family.

Mycobacterium avium subspecies *paratuberculosis* is a slow-growing mycobacterium that can infect a variety of hosts (18). Three types or clusters have been described: type I (“sheep” or “S” type), type II (“cattle” or “C” type), and type III (“intermediate” or “I”). This clustering has been accomplished with IS900 restriction fragment length polymorphism (RFLP) (5, 7, 14, 15), pulsed-field gel electrophoresis (PFGE) (6, 21), analysis of sequences of *gyrA*, *gyrB*, and *inhA* genes (1, 4, 14), denaturing gradient gel electrophoresis (DGGE) of MAP1506 (10), PCR sequencing of *recF* (22), comparative genomic hybridization comparison (CGH) (3), and single nucleotide polymorphisms (SNPs) in the IS900 (2).

High-resolution melt analysis (HRM) is a novel molecular technique based on the generation of melting curves after PCR amplification, using the fluorescent signal of a saturating dye with high affinity for DNA (23). The differences obtained in the melting curves are based on the length, base sequence, and especially the GC content. Previously, HRM analysis has been used for the detection of SNPs in the *rpoB* gene in *Mycobacterium tuberculosis* (11, 16).

PPE proteins (motifs proline-proline-glutamic acid) constitute a polymorphic protein family that is restricted to mycobacteria. One of the members of this family, MACPPE23 (MAP1506) contains *M. avium* subspecies *paratuberculosis* type-specific SNPs in its sequence (12). Previously, these SNPs were used as a target to type *M. avium* subspecies *paratuberculosis* isolates by DGGE (10).

Therefore, the objective of this study was to demonstrate the usefulness of the combination of real-time PCR and analysis of melting curves, a novel and rapid method to discriminate among *M. avium* subspecies *paratuberculosis* types I, II and III, based on previously reported SNPs in MAP1506.

For this purpose, we examined 47 *M. avium* subspecies *paratuberculosis* isolates recovered from 10 countries and different hosts (Table 1). Every isolate was categorized as type I ($n = 6$), II ($n = 31$), or type III ($n = 10$) based on prior data obtained from PFGE analysis (6), IS900 RFLP (5, 15), DGGE (10), restriction enzyme analysis PCR (PCR-REA) of *gyrB* genes, and the IS900 sequence (2, 4). The method was developed using 30 *M. avium* subspecies *paratuberculosis* isolates whose MAP1506 locus was sequenced in a previous study (10). Additionally, isolates with unknown sequences ($n = 17$) were used to validate the technique. Also, three MAC reference strains other than *M. avium* subspecies *paratuberculosis* were included: *M. avium* subspecies *avium* ATCC 25291, *M. avium* subspecies *hominissuis* 104, and *M. avium* subspecies *silvaticum* ATCC 49884 (Table 1).

Genomic DNA was extracted with the DNeasy blood and tissue kit (Qiagen, Mississauga, Ontario, Canada) and analyzed with a spectrophotometer (Nanodrop 1000; Thermo Scientific). Then, the 5' end of MAP1506 locus was amplified with forward (5'-GTGGGGTGGATGAGTACGAC-3') and reverse (5'-TGAGCAGGAACCAGATCTCC-3') primers (University Core DNA Services, University of Calgary, Alberta, Canada), with a resulting amplicon of 499 bp. The 20- μ l final reaction mixture contained 2 μ l of genomic DNA (minimum concentration of 1.2 ng/ μ l), 10 μ l of the SsoFast EvaGreen supermix (Bio-Rad, Mississauga, Ontario, Canada), and 5 pmol of each primer. Each *M. avium* subspecies *paratuberculosis* isolate and MAC strain was tested in triplicate. All reactions were performed in low 96-well clear multiplate PCR plates (Bio-Rad) and sealed with Microseal “B” film (Bio-Rad). Real-time PCR and HRM analysis were carried out in a CFX96 real-time PCR detection system (Bio-Rad). The reaction conditions were as follows: 98.0°C for 2 min, then 40 cycles at 98.0°C for 5 s, and annealing/extension at 55.0°C for 10 s. Afterwards, PCR products were heated to 95.0°C for 1 min and then cooled down to 70.0°C for 1 min. Finally, the melt curve was generated by measuring fluorescence during a temperature increase from 70.0°C to 95.0°C (with 0.2°C/10-s increments).

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TABLE 1. Panel of *Mycobacterium avium* subspecies *paratuberculosis* isolates and other members of the *Mycobacterium avium* complex analyzed by real-time PCR and HRM analysis

Isolate ^a	MAC member ^b	Host	Location	MAP1506 SNP profile ^c			
				293 bp	328 bp	344 bp	411 bp
6756	<i>M. avium</i> subsp. <i>paratuberculosis</i> type I	Sheep	New Zealand	A	T	G	G
6759	<i>M. avium</i> subsp. <i>paratuberculosis</i> type I	Sheep	New Zealand	A	T	G	G
P133/79	<i>M. avium</i> subsp. <i>paratuberculosis</i> type I	Sheep	Denmark (Faroe Islands)	A	T	G	G
6601	<i>M. avium</i> subsp. <i>paratuberculosis</i> type II	Cattle	Australia	T	G	G	T
87/8880	<i>M. avium</i> subsp. <i>paratuberculosis</i> type II	Cattle	Australia	T	G	G	T
7428	<i>M. avium</i> subsp. <i>paratuberculosis</i> type II	Cattle	New Zealand	T	G	G	T
6772	<i>M. avium</i> subsp. <i>paratuberculosis</i> type II	Goat	New Zealand	T	G	G	T
5979	<i>M. avium</i> subsp. <i>paratuberculosis</i> type II	Deer	New Zealand	T	G	G	T
Bison Neiker	<i>M. avium</i> subsp. <i>paratuberculosis</i> type II	Bison	Spain	T	G	G	T
834	<i>M. avium</i> subsp. <i>paratuberculosis</i> type II	Cattle	Spain	T	G	G	T
808	<i>M. avium</i> subsp. <i>paratuberculosis</i> type II	Cattle	Spain	T	G	G	T
791	<i>M. avium</i> subsp. <i>paratuberculosis</i> type II	Cattle	Spain	T	G	G	T
Neiker 116	<i>M. avium</i> subsp. <i>paratuberculosis</i> type II	Cattle	Spain	T	G	G	T
TC 1613	<i>M. avium</i> subsp. <i>paratuberculosis</i> type II	Cattle	United States	T	G	G	T
D0624010	<i>M. avium</i> subsp. <i>paratuberculosis</i> type II	Cattle	Canada	T	G	G	T
R0635848	<i>M. avium</i> subsp. <i>paratuberculosis</i> type II	Cattle	Canada	T	G	G	T
R0629132	<i>M. avium</i> subsp. <i>paratuberculosis</i> type II	Cattle	Canada	T	G	G	T
JD143	<i>M. avium</i> subsp. <i>paratuberculosis</i> type II	Sheep	Scotland	T	G	G	T
JD18	<i>M. avium</i> subsp. <i>paratuberculosis</i> type II	Cattle	Scotland	T	G	G	T
571	<i>M. avium</i> subsp. <i>paratuberculosis</i> type II	Hare	Scotland	T	G	G	T
M212/04	<i>M. avium</i> subsp. <i>paratuberculosis</i> type II	Deer	Czech Republic	T	G	G	T
9354	<i>M. avium</i> subsp. <i>paratuberculosis</i> type II	Cattle	United States	T	G	G	T
Ovicap18	<i>M. avium</i> subsp. <i>paratuberculosis</i> type III	Goat	Spain	A	T	A	G
311	<i>M. avium</i> subsp. <i>paratuberculosis</i> type III	Goat	Spain	A	T	A	G
269ov	<i>M. avium</i> subsp. <i>paratuberculosis</i> type III	Sheep	Spain	A	T	A	G
86/45	<i>M. avium</i> subsp. <i>paratuberculosis</i> type III	Sheep	Canada	A	T	A	G
85/14	<i>M. avium</i> subsp. <i>paratuberculosis</i> type III	Sheep	Canada	A	T	A	G
P465	<i>M. avium</i> subsp. <i>paratuberculosis</i> type III	Sheep	Iceland	A	T	A	G
M214/04	<i>M. avium</i> subsp. <i>paratuberculosis</i> type III	Deer	Czech Republic	A	T	A	G
M173/04	<i>M. avium</i> subsp. <i>paratuberculosis</i> type III	Deer	Netherlands	A	T	A	G
21P	<i>M. avium</i> subsp. <i>paratuberculosis</i> type I	Sheep	Denmark (Faroe Islands)	ND	ND	ND	ND
213G	<i>M. avium</i> subsp. <i>paratuberculosis</i> type I	Sheep	Scotland	ND	ND	ND	ND
235G	<i>M. avium</i> subsp. <i>paratuberculosis</i> type I	Sheep	Scotland	ND	ND	ND	ND
MI07/2713	<i>M. avium</i> subsp. <i>paratuberculosis</i> type II	Cattle	Spain	ND	ND	ND	ND
MI07/2721	<i>M. avium</i> subsp. <i>paratuberculosis</i> type II	Cattle	Spain	ND	ND	ND	ND
MI07/02728	<i>M. avium</i> subsp. <i>paratuberculosis</i> type II	Cattle	Spain	ND	ND	ND	ND
MI07/02731	<i>M. avium</i> subsp. <i>paratuberculosis</i> type II	Cattle	Spain	ND	ND	ND	ND
MI05/02948-2	<i>M. avium</i> subsp. <i>paratuberculosis</i> type II	Cattle	Spain	ND	ND	ND	ND
MI06/00304-2	<i>M. avium</i> subsp. <i>paratuberculosis</i> type II	Bullfighting cattle	Spain	ND	ND	ND	ND
940	<i>M. avium</i> subsp. <i>paratuberculosis</i> type II	Bullfighting cattle	Spain	ND	ND	ND	ND
MI05/03723-2	<i>M. avium</i> subsp. <i>paratuberculosis</i> type II	Goat	Spain	ND	ND	ND	ND
574	<i>M. avium</i> subsp. <i>paratuberculosis</i> type II	Goat	Spain	ND	ND	ND	ND
MI05/00483-2	<i>M. avium</i> subsp. <i>paratuberculosis</i> type II	Goat	Spain	ND	ND	ND	ND
MI05/00484-2	<i>M. avium</i> subsp. <i>paratuberculosis</i> type II	Goat	Spain	ND	ND	ND	ND
CAM20	<i>M. avium</i> subsp. <i>paratuberculosis</i> type II	Goat	Spain	ND	ND	ND	ND
CAM 86	<i>M. avium</i> subsp. <i>paratuberculosis</i> type III	Goat	Spain	ND	ND	ND	ND
733	<i>M. avium</i> subsp. <i>paratuberculosis</i> type III	Bullfighting cattle	Spain	ND	ND	ND	ND
K-10 ^d	<i>M. avium</i> subsp. <i>paratuberculosis</i> type II	Cattle	United States	T	G	G	T
104 ^d	<i>M. avium</i> subsp. <i>hominissuis</i>	Human	United States	A	T	G	G
ATCC 25291 ^d	<i>M. avium</i> subsp. <i>avium</i>	Chicken	ATCC	A	T	G	G
ATCC 49884	<i>M. avium</i> subsp. <i>silvaticum</i>	Wood pigeon	France				

^a In boldface type are the isolates (also with their correspondent technical replicates) for which the melting curves are presented in Fig. 1.

^b *M. avium* subsp. *paratuberculosis* isolates were classified into types I, II, or III based on prior data obtained from PFGE analysis (6), IS900 RFLP (5, 15), DGGE (10), PCR-REA on the *gyrB* gene (4), and the IS900 sequence (2).

^c MAP1506 locus sequence data from first 30 isolates obtained from a previous study (10). The rest of the *M. avium* subsp. *paratuberculosis* isolates included in the HRM study had unknown sequences for this MAP1506 locus. ND, not determined.

^d Reference strains of MAC included in the study. MAP1506 sequences were obtained from the report by Mackenzie et al. (12).

Results were analyzed with Bio-Rad CFX Manager software v.1 and then with Bio-Rad Precision Melt Analysis software. The clustering of melting curves is based on regions of the melting curve corresponding to the premelting, melting, and postmelting regions. The pre- and postmelting regions were optimized to attain the best clustering (premelting region, 89.0

to 90.0°C; postmelting region, 93.0 to 94.0°C). The obtained melting curves were normalized to relative values of 100% to 0% to eliminate the differences in background fluorescence (Fig. 1A). To allow comparison and melting curve differentiation between *M. avium* subspecies *paratuberculosis* types I and III, curve differences were magnified by subtracting each curve

FIG. 1. Normalized difference curves (A) and the normalized and temperature-shifted difference curves (B) of the amplified product of MAP1506 locus of the three different *Mycobacterium avium* subspecies *paratuberculosis* types as obtained with the Precision Melt Analysis software. The type I, II, and III isolates are presented in green, red, and blue, respectively. The isolates included in this graph in triplicate are indicated in boldface type in Table 1. Each individual line represents a single replicate.

from the type II melting curve, which was chosen as the baseline. Also, in the case of the three MAC strains tested, melting curves were normalized against the *M. avium* subspecies *paratuberculosis* K-10 (type II) melting curve. The normalized melting curves were also compared to the temperature-shifted curve along the temperature axis (Fig. 1B). Prior to Precision Melt Analysis, all the differences observed in the melting profiles were considered significant if the amplification reactions occurred in amplification cycle values prior to 30 cycles and if the samples showed equal PCR efficiencies and plateau fluorescence.

Previous sequence comparison of the MAP1506 locus of type II isolates with respect to type III isolates revealed four SNPs at positions 293, 328, 344, and 411 (Table 1). Type III isolates also differed from type I isolates only in one SNP at bp 344 (Table 1). Accordingly, three distinct melting profiles were observed for each of the *M. avium* subspecies *paratuberculosis* types included in the analysis (Fig. 1A and B). In these results, *M. avium* subspecies *paratuberculosis* isolates with unknown MAP1506 sequences ($n = 17$) matched with the melting profiles of the corresponding types (I, II, or III) of the known sequences ($n = 30$). Considering the varied panel of *M. avium* subspecies *paratuberculosis* isolates tested, we could assume

the stability of this locus. The lack of extra clusters would indicate that apart from the specific SNPs, the target sequence is conserved. Regarding the three MAC reference strains tested, the preliminary results obtained with HRM analysis were in agreement with former *in silico* comparison of the MAP1506 sequences in *M. avium* subspecies *avium* and *M. avium* subspecies *hominissuis* (12). Accordingly, HRM analysis distinguished only *M. avium* subspecies *paratuberculosis* types II and III from a cluster containing the MAC strains and *M. avium* subspecies *paratuberculosis* type I isolates (see Fig. S1 in the supplemental material).

M. avium subspecies *paratuberculosis* is difficult to grow, and traditional identification and typing techniques (5, 6, 8, 9, 15, 20, 21) are time-consuming and require high DNA quantity—limiting factors for some *M. avium* subspecies *paratuberculosis* strains, especially those with the most slow-growing phenotypes. In this report, we present a novel technique based on real-time PCR principles plus the use of HRM analysis, capable of typing isolates in just 20 min after the amplification reaction. HRM analysis has been described as a genotyping method of low cost and simplicity that provides rapid results (17) and does not need further separation steps, such as gel

electrophoresis (24). The limiting factor of this technique is that the precision and further analysis of the melting profiles are dependent on the presence of an initial DNA template of sufficient quality. Previous studies have established that cycle values over 40 should be considered carefully due to possible inaccurate melting data (13), and also the amount of salt carryover in the DNA template can influence the thermodynamics of the melting transition (19). Therefore, one of the requirements is that DNA samples in the same assay must have been extracted using the same protocol to avoid possible biased results and misinterpretations. For the results described in this report, only the *M. avium* subspecies *paratuberculosis* isolates for which the three technical replicates amplified were considered, thus ensuring that the reliability of the results was not compromised due to insufficient DNA concentration and quality. For each of the isolates included, the three technical replicates clustered into the same melting profile, confirming the reproducibility of the technique.

To conclude, we have described an HRM technique based on the MAP1506 locus, capable of detecting the differences in the sequences among the *M. avium* subspecies *paratuberculosis* types with 100% specificity. However, the number of tested isolates was relatively limited, and a more extensive panel of *M. avium* subspecies *paratuberculosis* isolates should be tested.

This technique could also be applied to short sequence repeat (SSR) locus analysis, thus avoiding the sequencing step. In addition, HRM analysis will find more applications in *M. avium* subspecies *paratuberculosis* typing with the sequencing of new *M. avium* subspecies *paratuberculosis* strains, as new SNPs with a higher index of discrimination are discovered.

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