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Genotyping based on thermal denaturation of amplification products identifies species of the *Mycobacterium tuberculosis* complex

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Abstract

Purpose. To develop a fast and inexpensive genotyping assay to identify the *Mycobacterium tuberculosis* complex (MTC) species most prevalent in human tuberculosis (TB), based on the thermal denaturation profiles of PCR products from mycobacterial 16S rDNA and three MTC genomic regions of difference (RD).

Methodology. Genotypes were determined by the presence and thermal denaturation profiles of the amplicons generated in the 'preliminary' PCR mixture (16S rDNA), followed by those of the simultaneous D1 (RD9+, RD1−) and D2 (RD4+, RD4−) PCR mixtures. The 16S rDNA profile identifies the genus *Mycobacterium*; the absence of any additional RD profile identifies *Mycobacterium* non-tuberculous (MNT) strains; additional RD4+ and RD9+ profiles without RD1− identify *M. tuberculosis*; an additional RD4+ profile *per se* identifies *M. africanum*; an additional RD4− profile *per se* identifies *Mycobaterium bovis*; additional RD1− and RD4− profiles identify *M. bovis* BCG.

Results. Genotypes of a panel with 44 mycobacterial strains coincided in 16 *MB* and five non-MTC strains; in the remaining 23 MTC strains, 17 *MTB* and five *MA* concordant genotypes and one discordant *MB* genotype were resolved. The genotypes of 13 human and bovine MTC isolates coincided in all four *MB* and eight of the nine *MTB* isolates.

Conclusion. Sensitivity, specificity and positive and negative predictive values of the method are 100 % for the genus *Mycobacterium*, which resolves *MB*, *MTB* and *MA* genotypes. Species/genotype agreement is 97.7 % for the panel and 92.3 % for the MTC isolates. This method may be advantageously used to identify the most prevalent MTC species in humans.

INTRODUCTION

Tuberculosis (TB) is the ninth most common global cause of death, and the most common by a single infectious agent, especially in developing countries [1]. Most deaths from TB could be prevented with early diagnosis and appropriate treatment [1].

Mycobacterium tuberculosis (*MTB*) and *Mycobacterium bovis* (*MB*) are the major species of the *Mycobacterium tuberculosis* complex (MTC) causing active TB, followed by *Mycobacterium africanum* [2]. *M. tuberculosis* is transmitted from human to human, and *M. bovis* mainly from bovines to humans through contact with infected animals or consumption of contaminated meat and dairy products [3, 4].

In Mexico the median prevalence of human TB by *M. bovis* is 7.6 % [5]. In patients in Mexico City, *M. bovis* is associated with 26.6 % of all TB forms and 16.6 % of the pulmonary form [6]. TB is usually diagnosed by microscopy and culture, mostly in patients experiencing treatment failure [5], and only a small number of Mexican laboratories are able to identify MTC species [7].

MTC species can be differentiated by thermal denaturation assays containing DNA intercalating agents, which are

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Keywords: *Mycobacterium tuberculosis*; *Mycobacterium bovis*; *Mycobacterium africanum*; *Mycobacterium tuberculosis* complex; EvaGreen; thermal denaturation.

Abbreviations: Ct, threshold cycle in real time PCR assays; D1, real time-PCR mixture for RD1- and RD9+ amplicons; D2, real time-PCR mixture for RD4+ and RD4- amplicons; InDRE, Mexican Institute for Epidemiological Diagnosis and Reference; MA, *Mycobacterium africanum*; MB, *Mycobacterium bovis*; MNT, *Mycobacterium* non-tuberculosis; MTB, *Mycobacterium tuberculosis*; MTC, *Mycobacterium tuberculosis* Complex; NTC, PCR mixtures without DNA used as negative controls; RD, regions of difference in MTC genomes; TB, human tuberculosis; Tm, melting temperature of the amplicons analysed.

Four supplementary tables and two supplementary figures are available with the online version of this article.

faster and less laborious than endpoint PCR and spoligotyping [8]. Deletions of the genomic regions of difference, RD1, RD4 and RD9, are prominent among the markers that distinguish *M. tuberculosis* from *M. bovis* and other MTC species [9].

In this paper we describe a fast, reliable and easily interpreted genotyping method based on a uniplex thermal denaturation assay for mycobacterial 16S rDNA, followed by two simultaneous RD1−/RD9+, RD4+/RD4− duplex assays that distinguish *M. tuberculosis*, *M. bovis*, *M. africanum*, and *Mycobacterium*-non tuberculous (MNT) strains.

METHODS

Reference strains, InDRE panel and MTC isolates

DNA and lysates from three reference strains, *M. tuberculosis* H37Rv, *M. bovis* AN5 and *M. bovis* BCG str. Mexico, were used as positive controls. The panel from Instituto de Diagnóstico y Referencia Epidemiológicos (InDRE) of the Mexican Health Secretariat consisted of DNA samples from 50 *Mycobacterium* strains (45 MTC and five non-MTC) that had been identified by spoligotyping [10]. *MTB* and *MB* DNA from MTC isolates of 10 human infections and three bovine infections had previously been identified by spoligotyping in our laboratory [11].

Oligonucleotide design

New oligonucleotides required to generate the desired amplicons were designed with the Premier 5 program (www.premierbiosoft.com) using the genome sequences of *M. tuberculosis* H37Rv (NC_018143.2), *M. bovis* subsp. *bovis* (LT708304.1) and *M. bovis* BCG str. Mexico (CP002095.1).

The 16S F/16S R oligonucleotide pairs generated the 16S rDNA amplicon—characteristic of the genus *Mycobacterium*—and the RD9 F/RD9 R pair generated the RD9 amplicon. The reverse oligonucleotides, RD4+ R and RD4 R, mixed with the PB-RD4 F oligonucleotide from Pinsky and Banaei [9] formed the PB-RD4 F/RD4+ R pair to generate the RD4+ amplicon, and the PB-RD4 formed the F/RD4− R pair to generate the RD4− amplicon. The PB-RD1 F/PB-RD1 R pair from Pinsky and Banaei [9] was used to generate the RD1 amplicon (Table 1).

Tm values of the amplicons were calculated using the University of Utah algorithm (www.dna.utah.edu/umelt/umelt.html).

Constructs with the amplicons of interest

Control plasmids carrying inserts from the 16S rDNA (445 bp), RD4+ (402 bp) or RD9 (361 bp) were constructed with amplicons generated from *M. tuberculosis* H37Rv DNA, and the amplicons RD4- (172 bp) or RD1- (222 bp) from *M. bovis* BCG str. Mexico DNA (Table 2).

Each amplicon was purified with the Wizard SV Gel and PCR Clean-Up System kit (Promega, Madison, USA) and ligated to the pGEM T-Easy Vector (Promega, Madison, USA). Constructs with different inserts were then used to obtain *Escherichia coli* TOP10 or JM109 transformant clones.

Inserts from each construct were shown to be amplifiable, liberated with *Eco*RI and automatically sequenced with the Sanger and Coulson method [12].

PCR mixtures and denaturation patterns

Amplification mixtures were prepared in a PCR hood (UV PCR Systems, Upland, USA). DNA samples, constructs and lysates were handled in a level 2 biosafety cabinet (Labconco, Kansas City, USA).

Uniplex PCR mixtures of 20 µl contained dNTPs (200 nM each), 2 mM $MgCl_2$, 0.1 U μl^{-1} Taq DNA polymerase (Invitrogen, Waltham, USA), EvaGreen fluorescent DNA stain 1× (Jena Bioscience, Jena, Germany) and 4.8 ng of genomic DNA (from *M. tuberculosis* H37Rv, *M. bovis* AN5 or *M. bovis* BCG str. Mexico) or 3.4–3.7 pg of the pDZ101, pHE102, pDZ205, pDZ107, pDZ108 or pDZ110 plasmids carrying the inserts of interest. Mixtures lacking DNA (NTC) were used as negative controls.

PCR amplification was performed in the RotorGene 6000 (Corbett Life Science, Concord, Australia) with the following programme: (1) initial denaturation at 95 °C for 5 min;

Table 1. Oligonucleotides used

Oligonucleotides					Reference
Pair	Name	Sequence (5′→ 3′)	% GC	Tm °C	
16S	16S F	TGGGCAGTAACTGACGCTGAG	57.1	59.3	This work
	16S R	ACCTTCCTCCGAGTTGACC	57.9	56.6	This work
RD9	RD9 F	ATCAGGGTGGAGCCGATACCA	57.1	60.2	This work
	RD9 R	GCTTCCCCGGTTCGTCTGG	68.4	61.0	This work
RD1	PB-RD1 F	GGATTTGACGTCGTGCTTCT	50.0	55.4	Pinsky and Banaei [9]
	PB-RD1 R	TTCAACGGGTTACTGCGAAT	45.0	54.6	Pinsky and Banaei [9]
RD4-	PB-RD4 F	AGAAGCGCAACACTCTTGGA	50.0	56.9	Pinsky and Banaei [9]
	RD4- R	ACCCCAAAAGGAGCACCATC	55.0	57.8	This work
RD4+	PB-RD4 F	AGAAGCGCAACACTCTTGGA	50.0	56.9	Pinsky and Banaei [9]
	RD4+ R	TGTGGCTATGGGGCTCTACTG	57.1	58.7	This work

Table 2. Amplicons expected and location of oligonucleotide pairs in the corresponding genomes

Oligonucleotides		Amplicons expected		
Pair	**Component**	**Name**	**Size**	**Location in the genome**
16S	16 S F/16 S R	rDNA 16S	445 bp	*M. tuberculosis* H37 Rv
				Nucleotides 472582–1473026 (GenBank GI:561108321)
RD1	PB-RD1 F/PB-RD1 R	RD1−	222 bp	*M. bovis* BCG *str.* Mexico
				Nucleotides 4298541–4298762 (GenBank CP002095.1)
RD9	RD9 F/RD9 R	RD9+	361 bp	*M. tuberculosis* H37 Rv
				Nucleotides 2329898–2330258 (GenBank GI:561108321)
RD4+	PB-RD4 F/RD4+ R	RD4+	402 bp	*M. tuberculosis* H37 Rv
				Nucleotides 1696001–1696402 (GenBank GI:561108321)
RD4-	PB-RD4 F/RD4- R	RD4−	172 bp	*M. bovis* BCG *str.* Mexico
				Nucleotides 1691347–1691518 (GenBank GI:356592064)

(2) denaturation at 95 °C for 15 s; (3) annealing at 60 °C for 30 s; and (4) extension at 72 °C for 50 s. Fluorescence was registered during the extension cycles and PCR products were separated by electrophoresis in 2 % agarose gels run with sodium borate buffer [13]. Amplicon sizes were determined with QuantityOne software (BioRad, Hercules, USA) by interpolation with DNA markers of known size.

The optimal concentration of each oligonucleotide pair was determined by comparing the threshold cycle (Ct) values of mixtures prepared with or without DNA and the height of the amplicon thermal denaturation peaks. Preliminary uniplex and duplex D1 or D2 mixtures with optimal oligonucleotide concentrations were amplified for 30 cycles in the RotorGene 6000.

The following conditions were used to determine the melting temperature (Tm) after amplification: (1) temperature range 80 to 99 °C; (2) 90 s for first step pre-denaturation conditioning; (3) 1 °C increase in each step; and (4) 10 s waiting time between steps. Amplification and thermal denaturation curves were analysed with Rotor-Gene 6000 software version 1.7. Average (mean) and standard deviation (SD) values, and the corresponding variation coefficients [VC=100(SD/mean)], were calculated from the analysis of at least three duplicate samples.

Validation and testing of genotyping method

The genotype of each DNA sample was inferred from the thermal denaturation pattern of the amplicons generated in preliminary, D1 and D2 mixtures (Fig. 1). PCR mixtures with 10 ng of DNA from *M. tuberculosis* H37Rv or *M. bovis* BCG str. Mexico were used as positive controls; one mixture without DNA (NTC) and another with 10 ng of human DNA were used as negative controls.

Agreement of the genotypes with the identity assigned to the InDRE panel strains served to validate the method, and to determine the positive and negative predictive values of the preliminary reaction.

After validation, the genotyping method was tested on 13 MTC strains isolated from human and bovine tuberculosis cases that had been diagnosed in our laboratory by spoligotyping.

RESULTS

Characterization of cloned amplicons

In mixtures with the five oligonucleotide pairs used (Table 1), the amplicons of interest from reference strains *M. tuberculosis* H37Rv and *M. bovis* BCG str. Mexico were obtained, purified and cloned in pGEM T-Easy. All inserts were of the expected size (Table 2) and their sequences were 98.8–100 % identical to those of the reference strains (Table 3).

The Tm values of the amplicons generated from the inserts (Table S1, available in the online version of this article) were 90.5 °C for 16S rDNA (Fig. 2), 94.2 °C for RD9+, 92.7 °C for RD1− (Fig. 3), 91.8 °C for RD4+ and 90.5 °C for RD4− (Fig. 4).

Genotyping algorithm

The algorithm used to determine the identity of unknown samples (Fig. 1) was based on the genotype inferred from the thermal denaturation patterns given by the Tm values of the amplicons generated in the preliminary mixture for the 16S rDNA amplicon, the D1 duplex mixture for RD9+ and RD1− amplicons (ΔTm~1.4 °C) and the D2 duplex mixture for RD4+ and RD4− amplicons (ΔTm~1.5 °C).

All samples had to be tested in the preliminary mixture, but only those testing positive were then simultaneously analysed in D1 and D2 mixtures (Fig. 1).

The presence of the 16S rDNA amplicon indicated that the sample belongs to the genus *Mycobacterium*; its absence would indicate either that it does not belong to the genus *Mycobacterium* or that its DNA is insufficient or degraded and would end the process.

The presence of the 16S rDNA amplicon in the absence of D1 and D2 amplicons classified samples as MNT. The presence of RD9+ and RD4+ amplicons in the absence of RD1− identified *M. tuberculosis*, whereas the presence of the RD4+ amplicon and the absence of RD9+ identified *M. africanum*. On the other hand, the presence of the RD4−

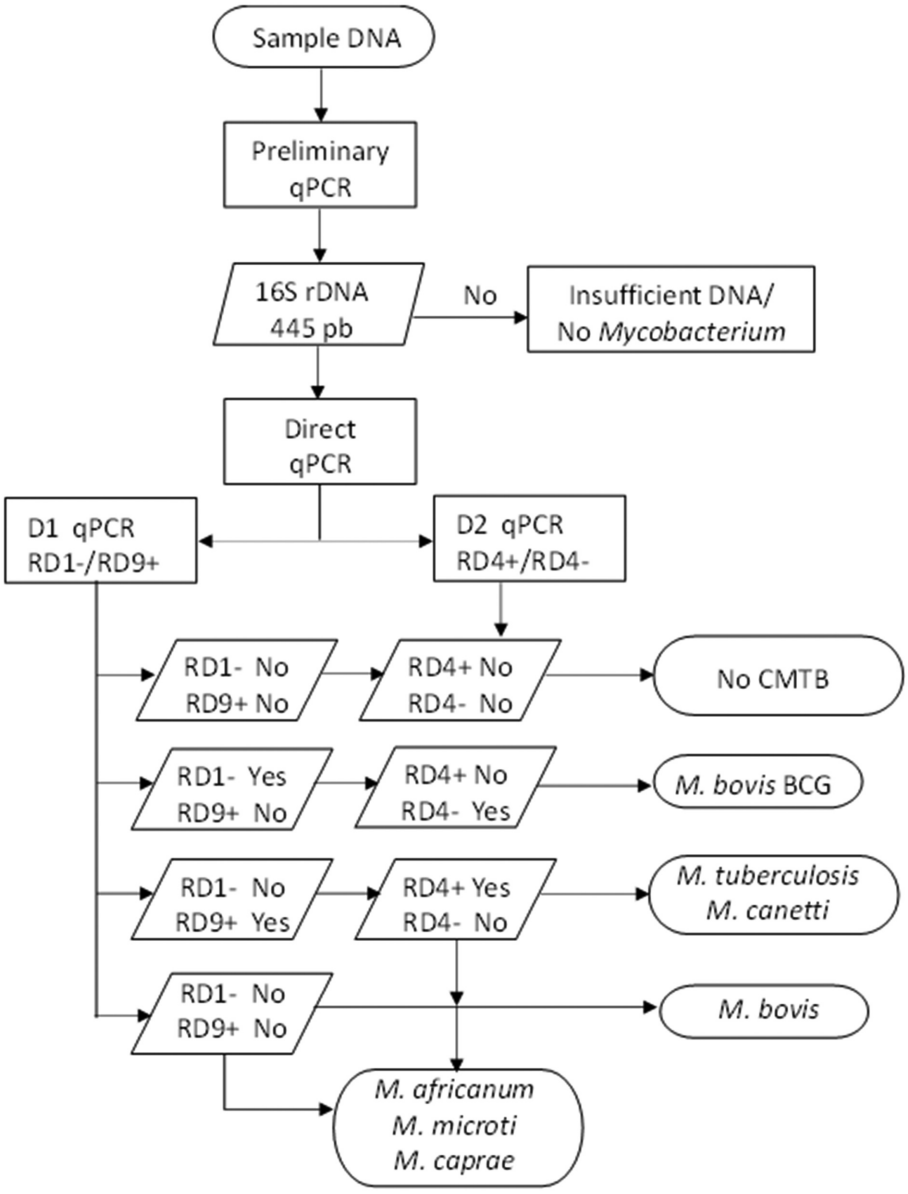

Fig. 1. Algorithm used for the analysis of suspected human and bovine tuberculosis samples. Preliminary PCR, uniplex assay for the 16S rDNA amplicon which identifies the genus *Mycobacterium*. D1 qPCR and D2 qPCR, simultaneous duplex assays for the amplicon pairs RD1−/RD9+ and RD4+/RD4−, respectively, to determine MTC genotype based on the absence/presence of the RD amplicons evaluated.

amplicon alone identified *M. bovis*, whereas the presence of both RD1− and RD4− amplicons identified *M. bovis* BCG.

Optimization of oligonucleotide concentrations

Excessive amounts of non-specific amplification products in the initial assay mixtures prevented adequate resolution of the thermal denaturation patterns, a problem that was resolved by optimizing the oligonucleotide concentration.

The following concentrations of oligonucleotide pairs prevented the generation of non-specific products and led to the resolution of their thermal denaturation patterns: in the preliminary mixture, 100 nM; in the D1 mixture, 250 nM for the RD9+ pair and 300 nM for the RD1− pair; in the D2 mixture, 200 nM for both RD4+ and RD4− pairs (Table S2).

Genotypes of the InDRE panel strains

The initially blind identity of the 50 mycobacterial strains in the InDRE panel was compared to that obtained through genotyping. Six panel samples were excluded because no amplicons were generated in either the preliminary mixture or the D1 and D2 mixtures. The 44 *Mycobacterium* panel samples included consisted of 39 MTC strains (23 *MTB* and 16 *MB*) and the five MNT strains from different species (Table S3, Fig. 5).

Table 3. Sequences of inserts carried by the constructs

Insert	Sequence (5′→3′)
16S rDNA (445 bp)	TGGGCAGTAACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAACAGGATTAGATACCCTG GTAGTCCACGCCGTAAACGGTGGGTACTAGGTGTGGGTTTCCTTCCTTGGGATCCGTGCC GTAGCTAACGCATTAAGTACCCCGCCTGGGGAGTACGGCCGCAAGGCTAAAACTCAAAGG AATTGACGGGGGCCCGCACAAGCGGCGGAGCATGTGGATTAATTCGATGCAACGCGAAGA ACCTTACCTGGGTTTGACATGCACAGGACGCGTCTAGAGATAGGCGTTCCCTTGTGGCCT GTGTGCAGGTGGTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCG CAACGAGCGCAACCCTTGTCTCATGTTGCCAGCACGTAATGGTGGGGACTCGTGAGAGAC TGCCGGGGTCAACTCGGAGGAAGGT
RD1− (226 bp)	GGATTTGACGTCGTGCTTCTGGTCGACGATTGGCACATCCAGCCGCCCGGATCCAGCATC TGTCTGGCATAGCTGCCCGTCGGCCTGGTAATACTCATCCCCTACTGCCCTCCCCAAACC GCCAGATCGCCTCGCGGATCACCGTCCGGTTGGCCTCCGGCATTTCACGCCGGCTCGGCC GCTGGATCCACCCCGCGCCGGTATTCGCAGTAACCCGTTGAA
RD9 (361 bp)	ATCAGGGTGGAGCCGATACCATGCAACAACGGGTCGCCGCTGGCAACCACGTGTAGGTCA GCCCCATCCGGTGACAGGCCTTGCACCGCGGGCAGCATCGGCGTCGGCCACTCCCAGCGC TCGGCGGTGACGGTATCGTCGAGCAGGGCAAGTTGCCGTTTCGAGCCGTAAATTACTGTG GCCCTGCGCAATTCGGAGCGAGAATGCTCGGAGAGACCGGTCATGCCGTCGGCGCCGATC CCGACAACGATGATCATCGGCGCCGCTCTCCCCCGCAAGCGGGCGGTACCCCCACCGCAT CGCTGCGCTCTGCATCGTCGCGGATCATCGCGGCATCCTGCGCCAGACGAACCGGGGAAGC
RD4+ (402 bp)	TGTGGCTATGGGGCTCTACTGGACTACCTAAAATGCGCGTGGCTTCAAAACTGATTACAC CGGCATCGACGTCTCCCCCGAAATGGTGCGCGCGGCCGCTCTACGTTTCGAAGGTCGGGC GAACGCAGACTTCATCTGCGCGGCGCGCATAGATCGGGAGGCGGACTATAGCGTCGCGAG TGGAATATTCAATGTTCGTCTGAAATCGTTGGACACGGAATGGTGCGCTCACATCGAAGC GACGCTCGACATGCTGAATGCCGCGAGTCGCCGTGGCTTCTCTTTTAATTGCCTGACATC TTATTCCGATGCACCAAAGATGCGCGACGACCTGTACTATGCTGACCCATGCGCCCTATT TGATCTCTGCAAGCGCAGGTACTCCAAGAGTGTTGCGCTTCT
RD4− (172 bp)	ACCCCAAAAGGAGCACCATCGTCCACCTTAGTGGGGACGCTACTACGGCACGGCGCGCCC CGTAGCGTTACTGAGAAATTGCTGAAAAATGGCTATTGACCAGCTAAGATATCCGGTACG CCCGCGCCGCGGAGAGCGCCGTTGTAGGCCAGTCCAAGAGTGTTGCGCTTCT

All panel samples included generated 16S rDNA amplicons with Tm values of 90.8±0.36 °C. In D1 mixtures, 17 were RD9+ positive with Tm values of 93.7±0.76 °C, and none was RD1− positive. In D2 mixtures, 22 were RD4+ positive with Tm values of 91.6±0.25 °C and 17 were RD4− positive with Tm values of 89.6±0.31 °C (Table S4). The amplicon with the greatest variation in Tm was RD9+ (CV=0.81 %) (Table S4).

The genotypes of the panel strains included corresponded to MTC and MNT. In the MTC group, 17 corresponded to *MTB* (73.9 %) and five to *MA* (21.7 %). Only one strain assigned to *MTB* had a discordant *MB* genotype (4.3 %). There was absolute genotype concordance in all 16 strains assigned to *MB* and the five assigned to MNT (Fig. 5, Table 4).

The sensitivity, specificity and positive and negative predictive values of the genotyping method were 100 % for the genus *Mycobacterium*.

Genotypes of MTC strains isolated from human and bovine infections

The sizes expected for all the amplicons generated in the positive controls and samples were confirmed by agarose gel electrophoresis (Fig. S1).

The 13 MTC strains isolated from human and bovine infections generated 16S rDNA (445 pb) amplicons with Tm values of 90.5 °C. In D1 mixtures, eight out of the 10 strains from human infections generated RD9 (361 bp) amplicons with Tm values of 94.3 °C (Fig. S2a). In D2 mixtures, eight out of 10 strains from human infections generated RD4+ (402 bp) amplicons with Tm values of 91.7 °C (Fig. S2b). One strain from a human infection and three from bovine infections generated RD4− (172 bp) amplicons with Tm values of 90.5 °C (Fig. S2c).

The genotypes of the 13 MTC strains (10 isolated from human pulmonary TB cases and three from bovine TB cases) were in agreement in 12 cases (92.3 %): eight of the nine isolates assigned to *MTB* (88.9 %) and all four of those assigned to *MB* (100 %) (Fig. 6, Table 5).

DISCUSSION

Thermal denaturation of PCR products in the presence of EvaGreen used in this work to differentiate *M. tuberculosis* and *M. bovis* using DNA or culture lysates is based on a uniplex preliminary mixture followed by two simultaneous duplex mixtures for four amplicons of three genomic regions of difference. The preliminary assay

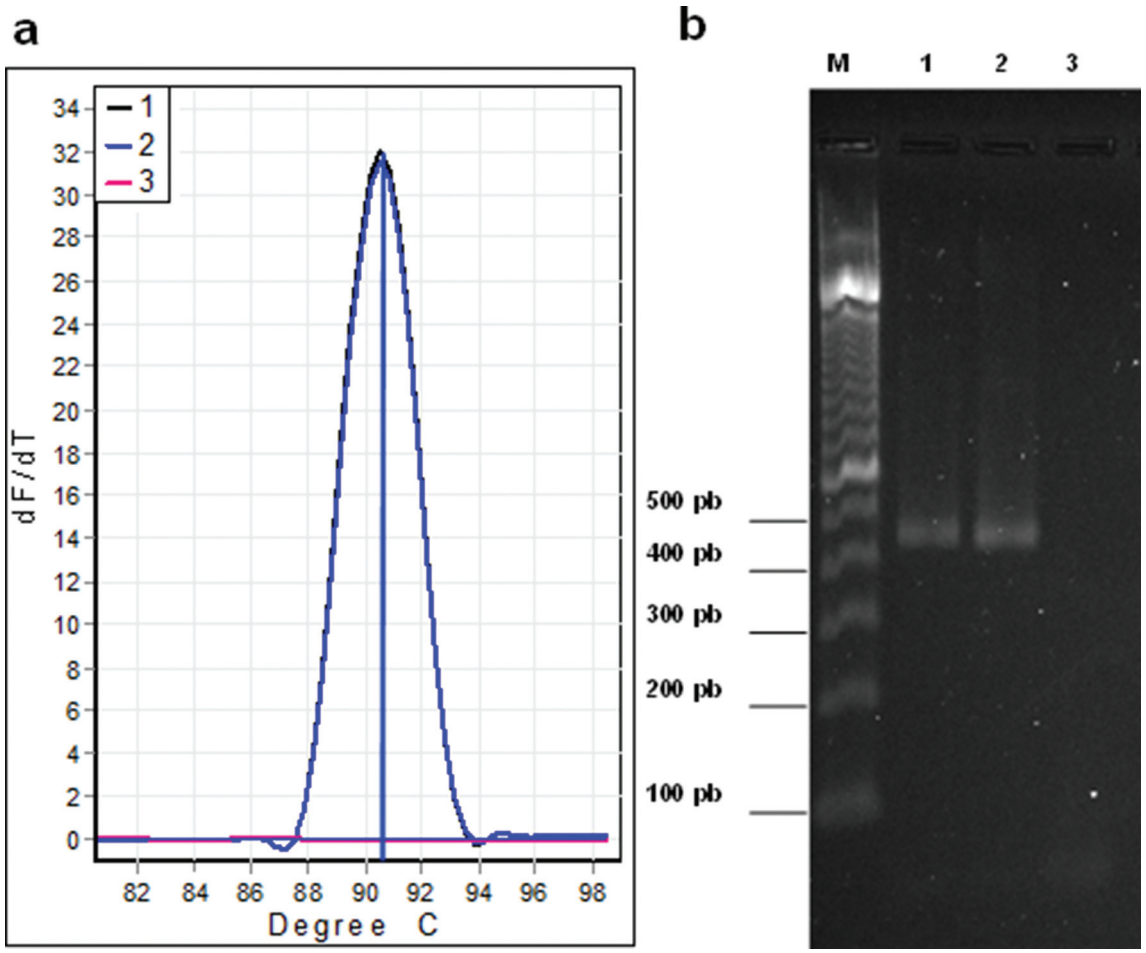

Fig. 2. Thermal denaturation profiles and electropherogram of the 16S rDNA reference amplicons. PCR mixtures for the 16S rDNA amplicons (445 bp). (a) Denaturation patterns of 16S rDNA amplicons. Black, mixture with *M. tuberculosis* H37Rv DNA (Tm=90.5 °C). Blue, mixture with pDZ101 DNA (Tm=90.5 °C). Pink, NTC mixture (without DNA). (b) Electropherogram of the 16S rDNA amplicons. M, 100 bp ladder. Lane 1, mixture with *M. tuberculosis* H37Rv DNA. Lane 2, mixture with pDZ101 DNA. Lane 3, NTC mixture (without DNA).

requires under two hours to detect the 16S rDNA amplicon characteristic of the genus *Mycobacterium*; positive samples are then assayed simultaneously in the D1 duplex mixture for RD9+ and RD1− amplicons and the D2 duplex mixture for RD4+ and RD4− amplicons which differentiate *M. tuberculosis* from *M. bovis* in under two hours.

Identification of the major *Mycobacterium* species causing human TB demands simple, fast and easy-to-interpret techniques. In the presence of DNA-intercalating fluorochromes, PCR products can be differentiated by their thermal denaturation profiles which are dependent on their length, GC content and sequence [14]. In mixtures with EvaGreen, thermal denaturation peaks of amplification products are sharper and better separated than with SYBR Green [14, 15], and optimization of the amplification conditions improves the efficiency and minimizes the generation of spurious products [16].

Spoligotyping to determine MTC lineages, families and species is widely used in epidemiology [17] ,but has the disadvantage of being laborious and subject to errors in identifying the spacers in homemade membranes and during data transcription to digital format [18]. On the other hand, GeneXpert *MTB*/RIF, a PCR system recommended by WHO and based on cartridges with probes for a specialized thermocycler [19], determines rifampicin resistance but does not distinguish *M. tuberculosis* from *M. bovis* [20]; its main advantage is that the process of nucleic acid extraction and purification incorporated in the cartridges avoids contamination by inadequate samples or reagent manipulation [21].

To set up the preliminary and duplex mixtures of the genotyping method based on thermal denaturation, we used nine oligonucleotides with annealing temperatures close to 60 °C and verified that all oligonucleotide pairs in uniplex PCR mixtures generated amplicons of the expected sizes and Tm values.

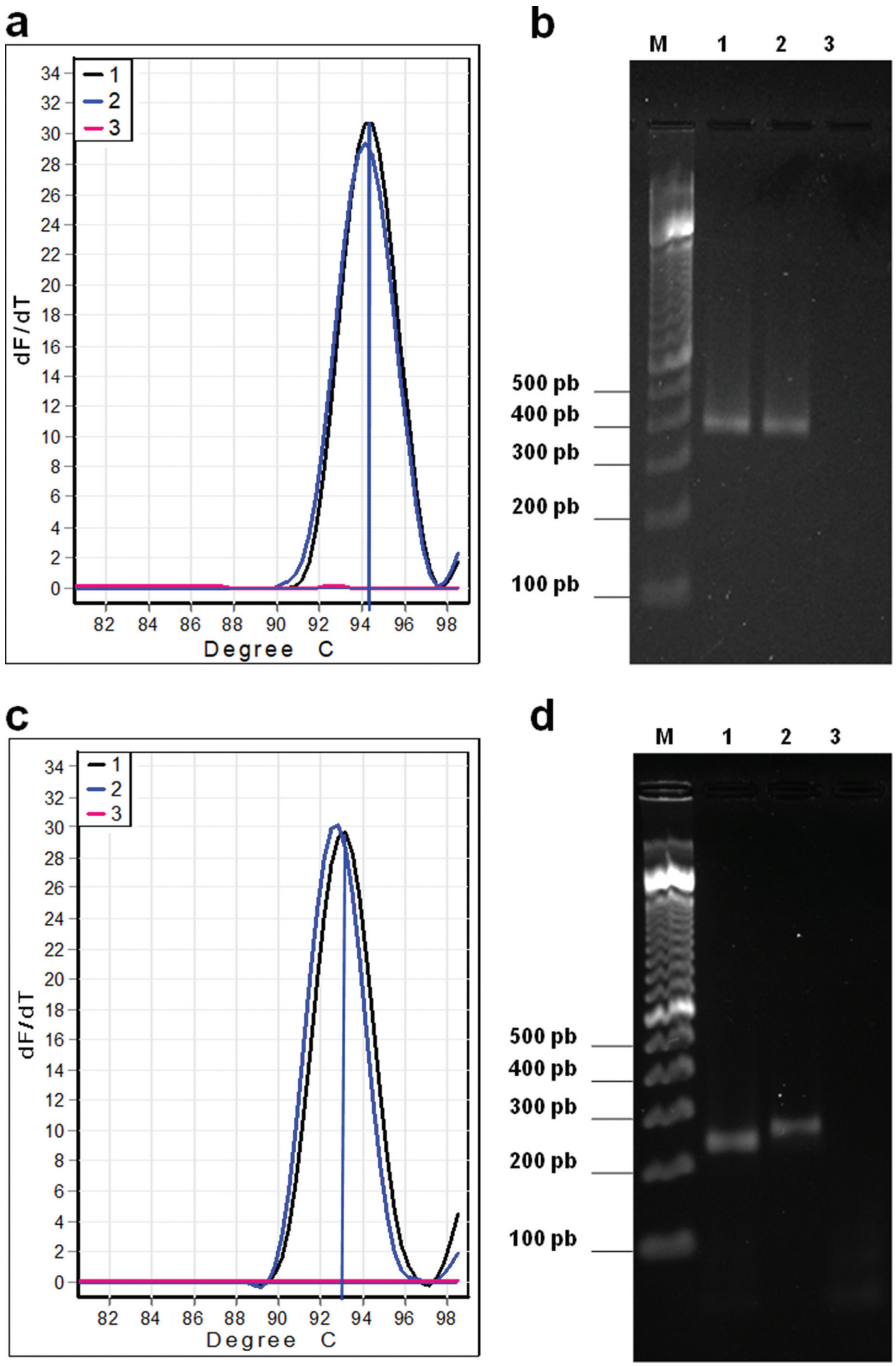

Fig. 3. Thermal denaturation profiles and electropherograms of the RD9+ and RD1− reference amplicons. PCR mixtures for RD9+ and RD1− amplicons. (a) RD9+ amplicon denaturation. Black, mixture with *M. tuberculosis* H37Rv DNA (Tm=94.3 °C). Blue, mixture with pHE102 DNA (Tm=94.2 °C). Pink, NTC mixture (without DNA). (b) RD9+ amplicon electropherogram. M, 100 bp ladder. Lane 1, mixture with *M. tuberculosis* H37Rv DNA. Lane 2, mixture with pHE102 DNA. Lane 3, NTC mixture (without DNA). (c) RD1− amplicon denaturation. Black, mixture with *M. bovis* BCG DNA (Tm=93.0 °C). Blue, mixture with pDZ205 DNA (Tm=92.7 °C). Pink, NTC mixture (without DNA). (d) RD1− amplicon electropherogram. M, 100 bp ladder. Lane 1, mixture with *M. bovis* BCG DNA. Lane 2, mixture with pDZ205 DNA. Lane 3, NTC mixture (without DNA).

With the 16S F/16S R oligonucleotide pair in the preliminary mixture, 16S rDNA of the genus *Mycobacterium* is detected, as shown by other authors [22]. The D1 mixture detects the RD9 and RD1− regions; RD9+ and RD1 amplicons have different sizes and Tm values yielding the expected patterns for *M. tuberculosis* (RD9+ without

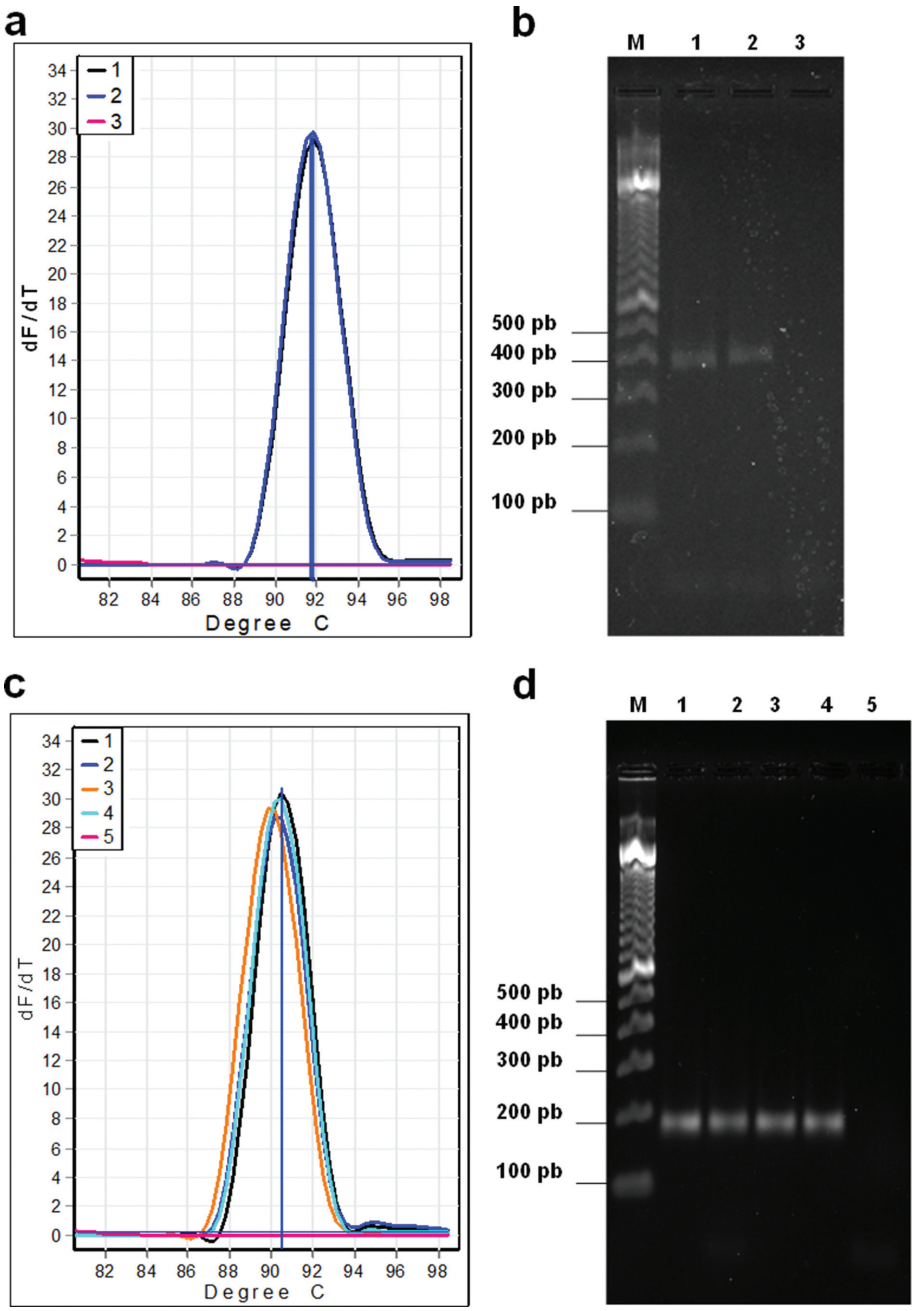

Fig. 4. Thermal denaturation profiles and electropherograms of the RD4+ and RD4− reference amplicons. Uniplex PCR mixtures for RD4+ and RD4− amplicons. (a) RD4+ amplicon denaturation. Black, mixture with *M. tuberculosis* H37Rv DNA (Tm=91.8 °C). Blue, mixture with pDZ110 DNA (Tm=91.8 °C). Pink, NTC mixture (without DNA). (b) RD4+ amplicon electropherogram. M, 100 bp ladder. Lane 1, mixture with *M. tuberculosis* H37Rv DNA. Lane 2, mixture with pDZ110 DNA. Lane 3, NTC mixture (without DNA). (c) RD4− amplicon denaturation. Black, mixture with *M. bovis* BCG DNA (Tm=90.5 °C). Blue, mixture with *M. bovis* AN5 DNA (Tm=90.3 °C). Orange, mixture with pDZ107 DNA (Tm=90.1 °C). Aquamarine, mixture with pDZ108 DNA (Tm=90.3 °C). Pink, NTC mixture (without DNA). (d) RD4− amplicon electropherogram. M, 100 bp ladder. Lane 1, mixture with *M. bovis* BCG DNA. Lane 2, mixture with *M. bovis* AN5 DNA. Lane 3, pDZ107 DNA. Lane 4, pDZ108 DNA. Lane 5, NTC (without DNA).

RD1−), *M. bovis* BCG (RD1− without RD9+) and *M. bovis* (without RD9+ and RD1−) [23]. The D2 mixture with the reverse oligonucleotides designed by us, together with the PB-RD4+ F from Pinsky and Banaei [9], generates two amplicons more than 100 bp in length distinguished by their different sizes and Tm values to determine the presence/

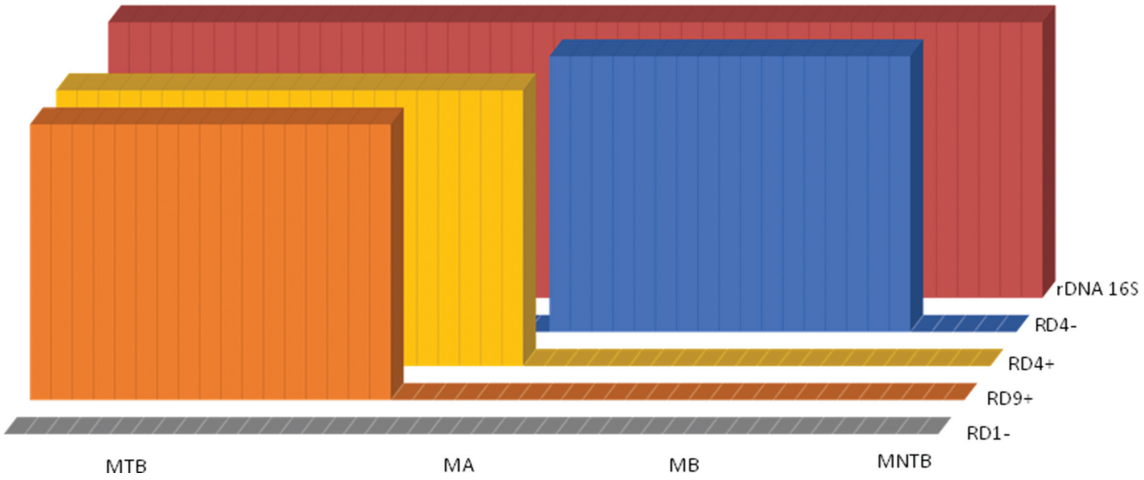

Fig. 5. Genotypes inferred from thermal denaturation patterns of the 44 panel samples included. *MA*, *M. africanum* (*n*=5). *MB*, *M. bovis* (*n*=17). *MTB*, *M. tuberculosis* (*n*=17). MNT, *Mycobacterium* non-tuberculous (*n*=5).

absence of the RD4 region in *M. tuberculosis*, *M. bovis* and *M. bovis* BCG genomes.

To ensure the specificity of our method we cloned and sequenced the amplicons of each genomic region of interest, unlike other authors who relied only on the electrophoretic migration pattern of the amplification products [9, 24]. The 16S rDNA, RD9+, and RD4+ control amplicons were generated from *M. tuberculosis* H37Rv, the RD1− from *M. bovis* BCG str. Mexico, and the RD4− from *M. bovis* AN5 and *M. bovis* BCG str. Mexico. Constructs were obtained by inserting those amplicons into pGEM vectors, and *E. coli* transformant clones were obtained. All the inserts were liberated by *Eco* RI restriction, were of the expected sizes and their sequences shared 99–100 % identity with those of the reference strains (Table 3).

Optimal oligonucleotide concentration avoids the generation of spurious amplification products, especially in multiplex PCR mixtures [16]. Optimal oligonucleotide concentrations for each amplicon minimized the presence of non-specific products in our uniplex and duplex mixtures.

Differences in Tm value, dependent on the GC content, length and sequence of the amplification products, are exploited to identify different amplicons present in the same PCR mixture and to differentiate these from non-specific amplification products [14]. Preliminary, D1 and D2 mixtures devoid of DNA, with optimized oligonucleotide content and incubated for 30 cycles, did not generate detectable non-specific products. On the other hand, the values of thermal denaturation peaks in mixtures containing control and sample DNA clearly differentiated the RD9+ (Tm=94.2 °C) and RD1− (Tm=93.0 °C) amplicons in D1 mixtures (ΔTm=1.2 °C), as well as the RD4+ (Tm=91.8 °C) and RD4− (Tm=90.5 °C) amplicons in D2 mixtures (ΔTm=1.3 °C).

Threshold cycle (Ct) values of our PCR mixtures with DNA from reference strains and constructs with inserts were 4.3–11.2 times lower than the corresponding mixtures lacking

Table 4. Concordance of genotypes with the identity assigned to the InDRE panel strains

Panel strains			Genotype				Concordance	
							Genus	Species
Identity*		*n* (%)	*MTB*	*MA*†	*MB*	MNT‡	*n* (%)	*n* (%)
CMTB	*MTB*	23 (52.3)	17	5	1	0	23 (100.0)	22§ (95.7)
	MB	16 (36.4)	0	0	16	0	16 (100.0)	16 (100.0)
MNT		5 (11.4)	0	0	0	5	5 (100.0)	5 (100.0)
Total		**44 (100.0)**	**17**	**5**	**17**	**5**	**44 (100.0)**	**43 (97.7)**

**MTB*, *M. tuberculosis*; *MB*, *M. bovis*; MNT, *Mycobacterium* non-MTC.

†*MA*, *M. africanum/M. caprae/M. microtii.*

‡These samples generated only the 16S rDNA amplicon.

§The sum of *MTB* species includes the 17 strains with *MTB* genotype and the five strains found to have *MA* genotype.

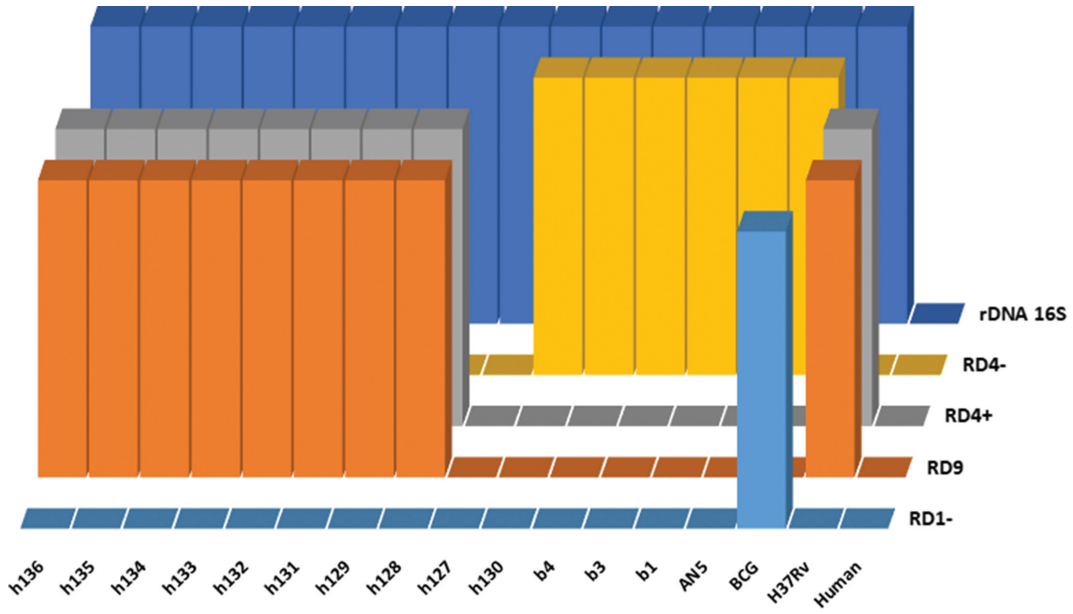

Fig. 6. Amplicons generated from human DNA, three reference strains and 13 human and bovine infections. No amplicon was generated from human DNA. 16S rDNA amplicon was generated from the DNA of 13 assayed isolates. RD9+ and RD4+ amplicons were generated from the DNA of eight human isolates. The RD4− amplicon was generated from the DNA of one human (h130) and three bovine (b1, b3, b4) isolates. The RD1− amplicon was only generated from the DNA of *M. bovis* BCG str. Mexico.

DNA. These values are similar to those found previously in our laboratory in human papillomavirus E6 oncogene assays [25].

All 44 InDRE panel strains included in this study (39 MTC and five MNT) corresponded 100 % to the sensitivity, specificity and positive and negative predictive values of the preliminary reaction in this novel method in identifying the genus *Mycobacterium*. Among the 23 InDRE panel strains assigned to *MTB*, the genotypes of 17 (73.9 %) corresponded to *MTB* and *MA* (*n*=5), and one to *MB* (4.3 %). The absolute correspondence between the identity of the 16 strains assigned to *MB* (100.0 %) and the *MA* genotype found by us in five of the panel strains originally assigned to *MTB* indicates that our thermal denaturation genotyping method has a higher resolution than spoligotyping in identifying the major MTC species causing TB in humans.

The genotypes derived from the denaturation patterns of the 13 MTC strains isolated from human and bovine infections coincided in eight out of the nine isolates previously assigned to *M. tuberculosis* (88.9 %), and all four assigned to *M. bovis* by spoligotyping, corresponding to 92.3 % global concordance.

Conclusions

Our genotyping method, based on thermal denaturation of the amplicons from 16S rDNA and three genomic regions of difference, distinguishes *M. tuberculosis*, *M. bovis* and *M. africanum*. It is less laborious, faster and cheaper than end-point PCR and spoligotyping, and may be used advantageously for

Table 5. Concordance of genotypes with the identity assigned to the 13 MTC strains from human and bovine infections

Strains		Genotypes*			Genus	Species
Identity	***n*** **(%)**	***MTB***	***MB***	**MNT**	***n*** **(%)**	***n*** **(%)**
MTB	9 (69.2)	8	0	1†	9 (100.0)	8 (88.9)
MB	4 (30.8)	0	4	0	4 (100.0)	4 (100.0)
Total	**13** (100.0)	**8**	**4**	**1**	**13** (100.0)	**12 (92.3)**

**MTB*, *M. tuberculosis; MB*, *M. bovis*; MNT, *Mycobacterium* non-tuberculous.

†This sample generated only the 16S rDNA amplicon.

diagnostic and epidemiologic studies in regions where there is a high prevalence of zoonotic tuberculosis.

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Conflicts of interest
The authors declare that there are no conflicts of interest.

Ethical statement
Research ethics committee approval was not required in Mexico for this study in developing a method that did not return results for clinical management.

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