Journal of Microbiological Methods 80 (2010) 190–197

Contents lists available at ScienceDirect

Journal of Microbiological Methods

journal homepage: www.elsevier.com/locate/jmicmeth

hsp65 PCR-restriction analysis (PRA) with capillary electrophoresis in comparison to three other methods for identification of *Mycobacterium* species

Anna Sajduda [1], Anandi Martin, Françoise Portaels, Juan Carlos Palomino *

Mycobacteriology Unit, Department of Microbiology, Institute of Tropical Medicine, Nationalestraat 155, B-2000 Antwerp, Belgium

ARTICLE INFO

Article history:
Received 10 November 2009
Received in revised form 15 December 2009
Accepted 16 December 2009
Available online 4 January 2010

Keywords:
Capillary electrophoresis
hsp65
Non-tuberculous mycobacteria
PRA
Species identification

ABSTRACT

We developed a scheme for rapid identification of *Mycobacterium* species using an automated fluorescence capillary electrophoresis instrument. A 441-bp region of the *hsp65* gene was examined using PCR-restriction analysis (PRA). The assay was initially evaluated on 38 reference strains. The observed sizes of restriction fragments were consistently smaller than the real sizes for each of the species as deduced from the sequence analysis (mean variance = 7 bp). Nevertheless, the obtained PRA patterns were highly reproducible and resulted in correct species identifications. A blind test was then successfully performed on 64 test isolates previously characterized by conventional biochemical methods, a commercial INNO-LiPA Mycobacteria assay and/or sequence determination of the 5′ end of 16 S rRNA gene. A total of 14 of 64 isolates were erroneously identified by conventional methods (78% accuracy). In contrast, PRA performed very well in comparison with the LiPA (89% concordance) and especially with DNA sequencing (93.3% of concordant results). Also, PRA identified seven isolates representing five previously unreported *hsp65* alleles. We conclude that *hsp65* PRA based on automated capillary electrophoresis is a rapid, simple and reliable method for identification of mycobacteria.

1. Introduction

The genus *Mycobacterium* contains more than 100 species, including organisms that cause serious human and animal diseases (Tortoli, 2003). Identification of mycobacteria up to the species level is necessary for application of adequate drug therapy and to address epidemiological questions. Conventional identification techniques based on the cultural and biochemical characteristics of acid-fast isolates have been the most commonly used methods for the determination of mycobacterial species, but these procedures are time-consuming and sometimes fail to produce a precise identification.

During the last decade, nucleic acid sequence-based identification tests have been developed, and commercially available systems such as AccuProbe (Gen-Probe, San Diego, CA) and INNO-LiPA Mycobacteria (LiPA; Innogenetics, Ghent, Belgium) are important new acquisitions for the diagnostic laboratory. These assays are highly specific and sensitive, but characterize a limited number of species and their costs remain high (Scarparo et al., 2001; Tortoli et al., 2003). The most sensitive and accurate, but still expensive and technically demanding, procedure for identification of a large number of mycobacterial species is sequencing of a fragment of conserved genes, of which the 16 S rDNA analysis is now regarded as the "gold standard" (Cloud et al., 2002). In the last years, several studies have been performed using other sequences such as *recA* (Blackwood et al., 2000), *rpoB* (Kim et al., 1999), 16 S–23 S internal transcribed spacer (ITS, (Mohamed et al., 2005)), *sod* (Zolg and Philippi-Schultz, 1994), *gyrB* (Kasai et al., 2000) or *hsp65* (Häfner et al., 2004; Senna et al., 2008), and a combination of sequences of several genes that gives the possibility of increasing discriminatory power (Devulder et al., 2005).

Restriction enzyme analysis of *Mycobacterium*-specific PCR products generates mostly species-specific DNA patterns, and provides a comparatively cheap alternative over sequencing regions of the 16 S rRNA gene. One such approach, analysis of a region of the gene coding for the 65-kDa heat shock protein (*hsp65*) by PCR-restriction analysis (PRA), was described by Telenti et al., 1993. This method is based on the amplification of a 441-bp fragment of the *hsp65* gene present in all mycobacteria, followed by digestion of the PCR product with the restriction enzymes BstEII and HaeIII. By combining both restriction patterns a species assignment is possible based on the comparison with patterns described in published algorithms (Telenti et al., 1993; Devallois et al., 1997; Taylor et al., 1997; Brunello et al., 2001; Chimara et al., 2008) or available from an Internet database (http://app.chuv.ch/prasite). PRA has been used for diagnostic (Taylor et al., 1997; Wong et al., 2003; Chimara et al., 2008) and taxonomic purposes (Ferdinand et al., 2004), and characterization of isolates with novel characteristics (da Silva Rocha et al., 2002).

The aim of this study was to evaluate the *hsp65* PRA with an automated capillary electrophoresis in identification of mycobacteria isolated from clinical and environmental sources in a reference

* Corresponding author. Tel.: +32 3 247 6334; fax: +32 3 247 6333.
E-mail address: palomino@itg.be (J.C. Palomino).
[1] Present address: Department of Genetics of Microorganisms, University of Łódź, Banacha 12/16, 90-237 Łódź, Poland.

0167-7012/$ – see front matter © 2010 Elsevier B.V. All rights reserved.
doi:10.1016/j.mimet.2009.12.012

laboratory, and its comparison with conventional and molecular methods (LiPA and 16 S rDNA sequencing).

2. Materials and methods

2.1. Study strains

Thirty-eight *Mycobacterium* reference strains (Table 1) were investigated to standardize PRA in our laboratory. Thereafter, the method was used in a blind test to determine the species of 64 cultured isolates from human ($n=29$), animal ($n=22$) and environmental ($n=5$) origin (the sources of 8 isolates were unknown). All the isolates had been previously submitted to classical biochemical identification procedures. Thirty-six (56%) strains were also identified by the INNO-LiPA Mycobacteria v2 assay (Innogenetics, Ghent, Belgium), and 26 (41%) strains were subjected to sequencing of the hypervariable region of PCR-amplified 16 S rDNA. Selection of test species for this study was based on both clinical significance and difficulties with identification by conventional biochemical tests.

2.2. DNA preparation

Crude DNA preparations were obtained from Löwenstein–Jensen medium cultures by suspending a loopful of each strain in 400 µl of TET buffer (10 mM Tris–HCl, 1 mM EDTA, 1% Triton X-100) and boiling once for 10 min. Each sample was then centrifuged at 12,000×*g* for 5 min to remove cell debris. The supernatant containing the extracted DNA was transferred to a clean microcentrifuge tube and frozen at −20 °C for at least 18 h. The supernatant was used as a template for amplification.

Table 1
Reference *Mycobacterium* strains used in the present study.

Species	Strain[a]
M. tuberculosis	ITM M8613
M. africanum	ITM 01-12
M. microti	ITM 99-2426
M. abscessus	ATCC 19977
M. avium	ITM 96-1091
M. avium s. *silvaticum*	ITM 2668
M. celatum	ITM 95-143
M. chelonae	ATCC 35752
M. chitae	ATCC 19627
M. duvalii	NCTC 348
M. fallax	IPP 301585
M. flavescens	ATCC 14474
M. fortuitum	ITM 97-461
M. frederiksbergense	ITM 04-361
M. genavense	ITM 96-0109
M. haemophilum	ITM 3065
M. interjectum	ITM 96-116
M. intermedium	ITM 96-117
M. intracellulare	ITM 4199
M. kansasii	ATCC 12478
M. lentiflavum	ITM 96-190
M. malmoense	ITM 96-1635
M. mucogenicum	ITM 98-1288
M. nebraskiae	ITM 03-2889
M. neoaurum	ITM 98-1357
M. nonchromogenicum	ATCC 19530
M. parafortuitum	ATCC 25808
M. peregrinum	ATCC 14467
M. phlei	NCTC 8151
M. scrofulaceum	CIPT 140220031
M. smegmatis	ATCC 607
M. szulgai	CIPT 14024003
M. terrae	CIPT 14032001
M. triplex	ITM 97-966
M. triviale	ATCC 23292
M. ulcerans	NCTC 10417
M. vaccae	ATCC 15483
M. xenopi	ITM 9741

[a] ATCC, American Type Culture Collection; NCTC, National Collection of Type Cultures; CIPT, Collection Instituut Pasteur de Paris-Tuberculose; IPP, Instituut Pasteur de Paris; and ITM, Institute of Tropical Medicine, Antwerp, Belgium.

2.3. DNA amplifications

Ten µl of DNA extract was used to amplify a 441-bp fragment of the *hsp65* gene with primers Tb11 (5′-ACCAACGATGGTGTGTCCAT-3′) and Tb12 (5′-CTTGTCGAACCGC ATACCCT-3′) (Telenti et al., 1993). The PCR reaction mixture (50 µl) contained 50 mM KCl, 10 mM Tris–HCl (pH 8.6), 1.65 mM $MgCl_2$, 0.1% Triton X-100, 160 µM each deoxynucleotide triphosphate, 20 pM of each primer, and 1 U of AmpliTaq DNA polymerase (Roche Molecular Systems, Brussels, Belgium) under mineral oil. PCR amplification was performed using the following protocol: initial denaturation at 95 °C for 5 min, 45 cycles of denaturation at 94 °C for 1 min, annealing at 60 °C for 1 min, extension at 72 °C for 1 min, and a final extension at 72 °C for 7 min.

Primers P7 (5′-CATGCAAGTCGAACGGAAAGG-3′) and P16 (5′-CGAACAACG CGACAAACCA-3′) were used in the amplification of about 500-bp 5′ end fragment of the 16 S rRNA gene. Two µl of crude bacterial lysate was used as a template in PCR together with the remaining reaction components listed above. The PCR conditions consisted of initial denaturation at 94 °C for 5 min followed by 40 cycles of denaturation at 94 °C for 45 s, annealing at 58 °C for 45 s, and extension at 72 °C for 45 s with additional 10 min of elongation step after the last cycle.

The presence of amplified products was confirmed by agarose gel electrophoresis.

2.4. Restriction enzyme analysis and interpretation of hsp65 PRA patterns

Fifteen µl of PCR product was digested with 10 U of BstEII or HaeIII (Promega, Madison, WI) in a total reaction volume of 25 µl, according to the manufacturer's instructions. BstEII digestion was incubated at 60 °C under mineral oil and HaeIII digestion at 37 °C for 4 h.

Digestion products were electrophoresed on both a 3% agarose gel prepared in 1×Tris-borate EDTA (TBE) and sized with 50- and 25-bp ladders (Promega), and by using the Agilent 2100 bioanalyzer (Agilent Technologies, Waldbronn, Germany) for more accurate determination of fragment sizes. For the latter purpose, aliquots of restriction digests were ethanol precipitated and 1-µl sample was then electrophoresed and analyzed using the DNA 1000 LabChip® kit according to the manufacturer's instructions. Samples were combined in disposable labchip wells with a sieving polymer (gel matrix) containing fluorescent dye and internal size markers, vortex mixed, and electrophoresed. Twelve samples and a molecular size ladder in individual wells moved through the microchannels, and were then injected into a separation channel where components were electrophoretically separated and detected by fluorescence. The bioanalyzer system software displayed data as both simulated bands on gel images (Fig. 1) and peaks in electrophoregrams. Results also included band migration time (s), size (bp), concentration (ng/µl), corrected peak area, and molarity (nM/l) of DNA fragments.

The observed PRA patterns were compared to the patterns reported in publications (Telenti et al., 1993; Devallois et al., 1997; Taylor et al., 1997; Brunello et al., 2001; Chimara et al., 2008) and on the PRA site or calculated from *hsp65* gene sequences deposited in the National Center for Biotechnology Information GenBank database (http://www.ncbi.nlm.nih.gov).

2.5. DNA sequence analysis

For those isolates for which conventional and PRA methods gave discordant or inconclusive results, sequences of about 500-bp hypervariable region of 16 S rRNA or *hsp65* gene amplicons were determined with the primers used for PCR amplifications. The PCR products were

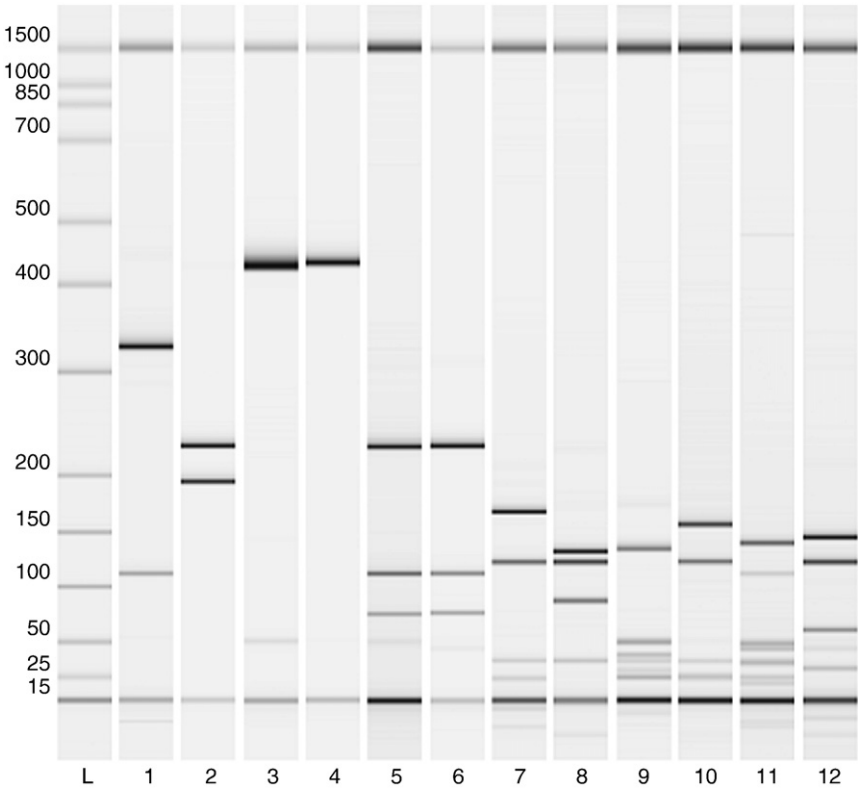

Fig. 1. Electrophoretic gel image of *hsp65* PRA patterns resulting from BstEII (lanes 1–6) and HaeIII (lanes 7–12) digestion obtained with the use of bioanalyzer system. Lanes: L, DNA ladder; 1 and 7, *M. terrae* CIPT 14032001; 2 and 8, *M. scrofulaceum* CIPT 140220031; 3 and 9, *M. flavescens* ATCC 14474; 4 and 10, *M. triviale* ATCC 23292; 5 and 11, *M. nonchromogenicum* ATCC 19530; 6 and 12, *M. tuberculosis* ITM M8613. The 15- and 1500-bp bands in all lanes represent lower and upper internal size markers.

sequenced by VIB Genetic Service Facility, University of Antwerp, Wilrijk, Belgium with the use of dye terminator reactions followed by electrophoresis on an automated ABI Prism 377 DNA sequencer (Applied Biosystems, Foster City, CA). For analysis of 16 S rDNA and *hsp65* sequencing data, the results obtained in this investigation were compared to known sequences in the GenBank database, and interpreted using the BlastN algorithm (available on http://www.ncbi.nlm.nih.gov/BLAST/). The 16 S rDNA sequences were also matched against entries in the RIDOM (Ribosomal Differentiation of Medical Microorganisms) database (http://www.ridom-rdna.de/) by online analysis.

3. Results

3.1. Application of hsp65 PRA to reference strains

A total of 38 *Mycobacterium* species were subjected to *hsp65* PRA with automated fluorescence capillary electrophoresis instrument (Agilent 2100 bioanalyzer). The fragment sizes estimated by the bioanalyzer system and deduced by sequence analysis of the reference *Mycobacterium* species are summarized in Table 2. Restriction fragments shorter than 50 bp, in contrast to 60 bp in the original protocol by Telenti et al. (1993), were not considered to avoid confusion with primer and primer–dimer bands. Moreover, fragments of 50–60 bp in length were shown crucial for definitive identification of some species (Brunello et al., 2001) and these were easily resolved by the method used. An electrophoretic gel image for six strains is shown in Fig. 1 and resembled patterns observed after 12.5-μl samples of digests were electrophoresed in 3% agarose gels.

Fragment sizes obtained by capillary electrophoresis differed from those determined by DNA sequence analysis in a range of 2 bp greater to 19 bp smaller, and the average variance was −7 bp (Table 2). Each reference strain was evaluated at least twice, and fragment sizes varied by no more than 2 bp on any successive run. In a practical sense, the image analysis for differentiating the PRA patterns could be programmed to accept the variations in fragment sizes within these limits. The mean size and standard deviation (SD) of the total of 47 real fragments were also analyzed. For the estimated mean sizes in comparison with the corresponding real sizes, 36 (77%) fragments differed by ≤10 bp and 11 (23%) fragments differed by >10 bp. In terms of the SD, 46 (98%) fragments yielded a reproducible pattern with the variations of 5 bp or less, whereas that of only 1 (2%) fragment was equal to 7 bp. Although fragment sizes determined by the instrument were consistently smaller than the predicted sizes, these provided highly reproducible and sufficiently polymorphic patterns necessary for the differentiation of 35 out of 38 species among the reference strains available (one pattern was common to *M. tuberculosis*, *M. africanum* and *M. microti* [*M. tuberculosis* complex, MTC, species] and the other one was shared by *M. avium* and *M. avium* ssp. *silvaticum*).

3.2. Evaluation of hsp65 PRA on test strains and comparison with other methods

To evaluate the usefulness of the developed bioanalyzer system-based method for the identification of mycobacterial isolates, we blind tested 64 cultured isolates from various origins. Strains with PRA patterns not matching any of those included in our diagnostic scheme were identified on the basis of their agarose electrophoretic profiles using published algorithms (Telenti et al., 1993; Devallois et al., 1997; Taylor et al., 1997; Brunello et al., 2001; Chimara et al., 2008) and the PRA site.

The results of identification of the study isolates using all four methods are summarized in Table 3. Among the 64 isolates analyzed, conventional identification assigned a species or complex/group to 34 (53%) and 26 (41%) isolates, respectively. For two (3%) isolates biochemical and phenotypic methods were not able to match a species

Table 2
Restriction fragments of *hsp65* of 38 reference *Mycobacterium* species obtained using the Agilent 2100 Bioanalyzer.

Species (type)	Fragment sizes (bp)[a]	
	BstEII	HaeIII
M. triviale	427 (441)	157 (168), 123 (127)
M. vaccae	422 (441)	154 (161), 77 (87), 53[b] (59), 53[b] (58)
M. lentiflavum (1)	426 (441)	132 (145), 121 (127)
M. parafortuitum (1)	422 (441)	141 (145), 77 (87), 49[b] (59), 49[b] (58)
M. flavescens (1)	423 (441)	135 (139), 50 (58), 40 (51)
M. duvalii	427 (441)	128 (135), 120 (128), 54 (65)
M. szulgai	429 (441)	122 (127), 93 (103), 59 (69)
M. nebraskense	425 (441)	125 (127), 95 (103), 51 (59)
M. fallax	316 (325), 112 (116)	176 (187), 140 (145)
M. terrae (1)	321 (325), 112 (116)	168 (181), 123 (126)
M. triplex	322 (325), 112 (116)	134 (145), 123 (127), 42 (51)
M. chitae	312 (325), 113 (116)	133 (139), 77 (87), 54 (58)
M. genavense (1)	317 (325), 112 (116)	123 (127), 94 (103)
M. chelonae	308 (310), 127 (131)	193 (197), 52 (60), 47 (58), 39 (54)
M. haemophilum	302 (310), 125 (131)	155 (161), 102[b] (112), 102[b] (111)
M. frederiksbergense	301 (310), 113 (116)	161 (172), 154 (161)
M. neoaurum	304 (310), 111 (116)	161 (172), 136 (139)
M. mucogenicum (3)	297 (310), 112 (116)	134 (139), 77 (87), 48 (58)
M. peregrinum (1)	224 (231), 198 (210)	136[b] (146), 136[b] (139), 98 (98), 54 (52)
M. intermedium	228 (231), 194 (210)	133 (145), 123[b] (129), 123[b] (127)
M. scrofulaceum	229 (231), 195 (210)	133 (145), 123 (127), 95 (95)
M. interjectum (1)	226 (231), 193 (210)	132 (145), 122 (127)
M. ulcerans (1)	228 (231), 193 (210)	133 (145), 103 (105), 68 (78)
M. abscessus (1)	224 (231), 196 (210)	140 (145), 60 (69), 48 (58), 41 (52)
M. phlei	228 (231), 196 (210)	133 (139), 74 (81), 49 (58), 38 (51)
M. kansasii (1)	226 (231), 193 (210)	122 (127), 93 (103), 69 (78)
M. avium (1)	226 (231), 195 (212)	121 (127), 92 (103)
M. avium s. *silvaticum*	226 (231), 195 (212)	123 (127), 94 (103)
M. smegmatis	226 (231), 125 (131), 77 (79)	141 (154), 115 (123), 49 (58)
M. celatum (2)	228 (231), 125 (131), 77 (79)	122 (127), 94 (103), 70 (78)
M. intracellulare (1)	228 (231), 110 (116), 88 (94)	132 (145), 121 (127), 49 (57)
M. malmoense (1)	229 (231), 110 (116), 87 (94)	131 (145), 102 (105), 68 (78)
M. xenopi	226 (231), 111 (119), 75 (79)	153 (161), 93 (104), 48 (59)
M. tuberculosis complex	229 (231), 113 (116), 77 (79)	146 (152), 123 (127), 61 (69)
M. fortuitum (1)	227 (231), 113 (116), 77 (79)	139 (145), 115 (123), 49[b] (58), 49[b] (52)
M. nonchromogenicum (1)	228 (231), 113 (116), 75 (79)	140 (145), 50 (58), 46 (54)

[a] Restriction fragments that were ≥50 bp. Fragment sizes derived from DNA sequence analysis are in parentheses.
[b] Co-migrating fragments.

while the remaining two (3%) isolates were identified as not belonging to genus *Mycobacterium*.

For 35 (97%) of the 36 isolates tested also by the LiPA assay, both methods gave the same species or complex identification (Table 3). However, LiPA was able to further differentiate 4 isolates phenotypically identified as MAC into *M. avium* ($n=2$) and *M. intracellulare* ($n=2$). On the other hand, neither members of MTC nor *M. marinum* and *M. ulcerans* could be resolved by differences in the 16 S–23 S rDNA spacer region which is the target in LiPA. They also share identical sequence of their 16 S rRNA genes. Only one (3%) isolate that had been identified by conventional methods as *M. gastri* was identified by LiPA as *M. kansasii*. In contrast to the 16 S rDNA sequencing, these two closely related species could be differentiated by the ITS region analysis.

Out of the 26 study strains that had been previously subjected to the sequence determination of 16 S rRNA gene, 24 (92%) isolates yielded concordant results for phenotypic identification and molecular test(s) (Table 3). These included 3 isolates of *M. chelonae*/*M. abscessus* that could not be differentiated by DNA analysis of their 16 S rDNA sequences. On the other hand, DNA sequencing enabled further discrimination of 4 MAC isolates into *M. intracellulare* ($n=2$) and *M. chimaera* ($n=2$). Among the two (8%) discrepant results, one isolate was identified by conventional testing as a member of MAIS group while based on the 16 S rDNA sequence it was *M. frederiksbergense*. The remaining discordant isolate was assigned *M. scrofulaceum* by phenotypic methods and the LiPA assay, but genetic sequencing verified it as closely related species *M. parascrofulaceum* (Table 3). The cross-reaction of the *M. scrofulaceum* LiPA probe with the species *M. parascrofulaceum* is documented in literature; this is surprising, as these species differ in the nucleotide sequence of their ITSs (Tortoli et al., 2005).

Phenotypic/biochemical identification together with the two molecular methods was used for comparison with PRA results (Table 3). The PRA results were in excellent agreement (100%) with those of 16 S rDNA sequencing, which is the most popular molecular target for identification purposes. Moreover, PRA enabled differentiation *M. abscessus* from *M. chelonae* and *M. kansasii* from *M. gastri* that the genetic sequencing could not achieve, although it did not discriminate between *M. intracellulare* 1 and *M. chimaera*. Also, PRA performed very well compared to the results of LiPA (89% concordance). The one (3%) discordant isolate was identified as *M. scrofulaceum* by conventional testing and LiPA, but DNA sequencing and PRA assigned it *M. parascrofulaceum* proving the latter method more reliable. For the three (8%) remaining isolates, identified by the LiPA assay as *M. malmoense*, PRA patterns were unreported so far. The lowest level of concordance, 48 out of 64 isolates tested (75%), was observed between PRA and conventional identification (Table 3). PRA allowed identification of 53 (83%) and 2 (3%) isolates to the species level or MTC, respectively. For 9 (14%) isolates studied, PRA patterns did not match those in available databases. Seven (11%) isolates gave discordant results, three of which were resolved by DNA sequencing (MAIS vs. *M. frederiksbergense*, and *M. scrofulaceum* vs. *M. parascrofulaceum*) or the LiPA assay (*M. gastri* vs. *M. kansasii*), thus confirming PRA identification (Table 3).

3.3. Discordant or inconclusive identification resolution

The 13 isolates with discrepant or inconclusive results between conventional and *hsp65* PRA identification methods were subjected to 16 S rDNA sequence analysis using the RIDOM database tool and BlastN search against the GenBank database (Table 4). In 1 of the 4 discordant cases, DNA sequencing confirmed identification result of PRA (*M. heckershornense* vs. *M. xenopi*). For another isolate, conventionally identified as *M. flavescens*, DNA sequencing also seemed to support its PRA identification as *M. gordonae* showing, however, a number of mismatches in the best matching sequences derived from both the RIDOM ($n=3$) and GenBank ($n=4$) databases. For 2 other isolates (not assigned a given *Mycobacterium* species by conventional methods) 16 S rDNA sequencing gave conflicting results. One of these isolates (identified as *M. lentiflavum* by PRA) showed 5 mismatches to the top score sequences of *M. lentiflavum* as well as *M. simiae*

Table 3
Summary of results of identification of 64 isolates by different methods.[a]

Conventional identification	*hsp65* PRA	LiPA	16 S rDNA sequencing
M. chelonae group (10)	*M. abscessus* 1 (2)	*M. chelonae* complex (7)	*M. chelonae/abscessus* (3)
	M. abscessus 2 (2)	NT (3)	NT (7)
	M. chelonae (6)		
M. avium complex (6)	*M. intracellulare* 1/*chimaera*[b] (4)	*M. intracellulare* (2)	*M. intracellulare* (2)
	M. avium 1 (2)	*M. avium* (2)	*M. chimaera* (2)
		NT (2)	NT (2)
M. fortuitum group (5)	*M. peregrinum* 1 (3)	*M. fortuitum* complex (1)	*M. peregrinum* (1)
	M. peregrinum 2 (1)	NT (4)	NT (4)
	M. fortuitum 1 (1)		
M. gordonae (5)	*M. gordonae*[c] (3)	*M. gordonae* (1)	*M. gordonae* (3)
	M. gordonae 3 (1)	NT (4)	NT (2)
	No matching pattern (1)		
M. malmoense (4)	*M. malmoense* 1 (1)	*M. malmoense* (4)	*M. malmoense* (1)
	No matching patterns (3)		NT (3)
M. kansasii (4)	*M. kansasii* 1 (1)	*M. kansasii* (4)	*M. kansasii/gastri* (1)
	M. kansasii 2 (1)		NT (3)
	M. kansasii 4 (1)		
	M. kansasii 5 (1)		
M. simiae (4)	*M. simiae* 1 (2)	*M. simiae* (3)	*M. simiae* (1)
	M. simiae 6 (2)	NT (1)	NT (3)
M. xenopi (4)	*M. xenopi* (3)	*M. xenopi* (3)	*M. xenopi* (1)
	M. heckershornense (1)	NT (1)	NT (3)
MAIS (4)	*M. frederiksbergense* (1)	NT (4)	*M. frederiksbergense* (1)
	No matching patterns (3)		NT (3)
M. marinum (2)	*M. marinum/ulcerans* 1 (2)	*M. marinum/ulcerans* (1)	NT (2)
		NT (1)	
M. ulcerans (2)	*M. ulcerans* 1/*marinum* (2)	*M. ulcerans/marinum* (1)	*M. ulcerans/marinum* (1)
		NT (1)	NT (1)
Mycobacterium sp. (2)	*M. avium* 1 (1)	NT (2)	NT (2)
	M. lentiflavum 3 (1)		
Non-*Mycobacterium* (2)	No matching patterns (2)	NT (2)	NT (2)
M. bovis (1)	*M. tuberculosis* complex	*M. tuberculosis* complex	*M. tuberculosis* complex
M. celatum (1)	*M. celatum* 1	*M. celatum*	*M. celatum*
M. flavescens (1)	*M. gordonae* 5	NT	NT
M. gastri (1)	*M. kansasii* 5	*M. kansasii*	*M. kansasii/gastri*
M. genavense (1)	*M. genavense* 1	*M. genavense*	*M. genavense*
M. haemophilum (1)	*M. haemophilum*	*M. haemophilum*	*M. haemophilum*
M. microti (1)	*M. tuberculosis* complex	*M. tuberculosis* complex	*M. tuberculosis* complex
M. scrofulaceum (1)	*M. parascrofulaceum* 1	*M. scrofulaceum*	*M. parascrofulaceum*
M. szulgai (1)	*M. szulgai*	NT	*M. szulgai*
M. terrae group (1)	*M. nonchromogenicum* 1	NT	*M. nonchromogenicum*

NT, not tested; and MAIS, *M. avium–M. intracellulare–M. scrofulaceum*.
[a] Number of isolates tested is given in parentheses.
[b] *M. chimaera* is former sequevar MAC-A.
[c] *hsp65* PRA pattern previously described for one *M. gordonae* isolate (da Silva Rocha et al., 2002).

deposited in the RIDOM and only 1 mismatch to the GenBank derived sequence of *Mycobacterium* IWGMT 90093. In the remaining discordant case (assigned *M. avium* by PRA), DNA sequencing revealed *M. paraffinicum* as species identification in the RIDOM database. *M. paraffinicum* is regarded as a not validly published species (Turenne et al., 2001) with a sequence of the 16 S rRNA gene 99.3% identical to the one of *M. scrofulaceum*. BlastN search performed against the GenBank database yielded, however, 100% match to the sequences of 3 *Mycobacterium* sp. strains: NLA 000800224, 01-636, and 01-627 (Table 4).

Nine strains could not be identified on the basis of *hsp65* PRA. Following sequencing of 16 S rDNA amplicons and species assignment, new PRA types for the following species or subspecies were identified (Table 4): *M. gordonae* (2 types in 2 strains), *M. interjectum* (1 type in 1 strain), and *M. avium* ssp. *paratuberculosis/silvaticum/avium/hominissuis* (1 type in 1 strain; identical to the new type of *M. interjectum*). For 3 other strains, that produced 2 new PRA patterns, the analysis of 16 S rDNA sequence gave conflicting results. Their sequences yielded a 100% match to the GenBank deposited sequence of *Mycobacterium* IWGMT 90093 while showing 4 mismatches to the best matching sequences of *M. lentiflavum* and *M. simiae* derived from the RIDOM database. The remaining 2 strains showing different new PRA patterns could not be identified on the basis of their 16 S rDNA sequence since they failed to produce detectable PCR products. In these cases, sequencing the PCR-amplified 441 bp of the *hsp65* gene and BlastN database search revealed 98% match to the corresponding sequence of *Tsukamurella paurometabola*, an acid-fast organism from the genus phylogenetically related to *Mycobacterium* (Stanley et al., 2006). Both strains were isolated from insects (*Appasus* and *Naucoris* spp.) in Benin.

4. Discussion

The conventional identification of mycobacteria by culture and biochemical characteristics is time-consuming because biochemical tests rely on bacterial growth. In addition, intraspecies variation makes interpretation of the results more difficult, leading to misidentification. A number of DNA-based methods for differentiation of mycobacteria have therefore been proposed. Although most of them still have limitations, these methods have been shown to have more advantages, such as rapid and accurate identification result. A comparatively simple PCR-based technique yielding good results is the *hsp65* PRA. A large number of *hsp65* PRA patterns based on gel analysis have been published so far and the number is still growing (Telenti et al., 1993; Devallois et al., 1997; Taylor et al., 1997; Brunello et al., 2001; da Silva Rocha et al., 2002; Ferdinand et al., 2004; Häfner et al., 2004; Chimara et al., 2008). Also, some modifications to the original method

Table 4
Evaluation of discrepant and inconclusive *Mycobacterium* identification results between conventional and *hsp65* PRA tests using 16 S rDNA sequence analysis with the RIDOM and GenBank BlastN database tools.

Conventional identification	*hsp65* PRA	16 S rDNA sequence analysis result[a]			
		RIDOM	mm	GenBank	mm
M. flavescens (1)	*M. gordonae* 5	*M. gordonae*	3	*M. gordonae*	4
M. xenopi (1)	*M. heckershornense*	*M. heckershornense*	0	*M. heckershornense*	2
Mycobacterium sp. (2)	*M. lentiflavum* 3 (1)	*M. lentiflavum/M. simiae*	5	*Mycobacterium* IWGMT 90093	1
	M. avium 1 (1)	*M. paraffinicum*[b]	2	*Mycobacterium* sp. NLA 000800224/01-636/01-627	0
MAIS (3)	New pattern 1 [431-122/93] (1)	*M. gordonae*	0	*M. gordonae*	0
	New pattern 2 [226/196-121/93/72] (1)	*M. gordonae*	0	*M. gordonae*	0
	New pattern 3 [227/110/90-122/102/49] (1)	*M. interjectum*	2	*M. interjectum*	1
M. gordonae (1)	New pattern 3 [230/111/91-123/102/50]	*M. avium* s. *avium/paratuberculosis/silvaticum*	0	*M. avium* s. *avium/paratuberculosis/silvaticum/hominissuis*	0
M. malmoense (3)[c]	New pattern 4 [430-124/95/54] (2)	*M. lentiflavum/M. simiae* (3)	4	*Mycobacterium* IWGMT 90093 (3)	0
	New pattern 5 [428-124/50] (1)				
Non-*Mycobacterium* (2)	New pattern 6 [429-153/133/97/79] (1)	NT (2)		NT (2)	
	New pattern 7 [426-132/96/53] (1)				

The data for the identification by 16 S rDNA sequencing are provided (the matching sequence of the RIDOM database as well as the matching sequence of the GenBank/EMBL/DDBJ/PDB database are given) along with the number of mismatches (mm) observed for the best matching sequence available in both public databases. Number of isolates is given in parentheses. Fragment sizes after digestion (BstEII–HaeIII), obtained with the use of the Agilent 2100 bioanalyzer, are provided in brackets (fragments below the size of 50 bp are omitted).

[a] NT, not tested because of the lack of detectable amplification product.

[b] Nomenclatural status of *M. paraffinicum* is not established and taxonomical status not accepted. It has to be regarded as a synonym for *M. scrofulaceum* (Turenne et al., 2001).

[c] The same result of identification obtained with LiPA assay.

by Telenti et al. (1993) have been described. These include e.g. separation of restriction digests by 10% polyacrylamide gel electrophoresis (Brunello et al., 2001), use of a novel 644-bp region of the *hsp65* gene (Kim et al., 2005), nested-PRA (Wu et al., 2008), duplex PCR (Kim et al., 2008) or PRA with capillary electrophoresis (Hernandez et al., 1999; Ho et al., 2004; Chang et al., 2007). Especially the latter approach offers the advantage of accuracy and rapidity in identification process. However, laboratories planning to adopt *hsp65* PRA are suggested to construct customized strain collections and own databases.

In this study, therefore, we aimed to construct our own diagnostic scheme and evaluate PRA with automated capillary electrophoresis for identification of mycobacteria originating from various sources in comparison with three other methods. We evaluated PRA on both reference strains and test isolates. The results obtained using the automated fluorescence capillary electrophoresis instrument agreed with the results obtained from the same samples using conventional agarose electrophoresis. The 38 reference strains representing 38 *Mycobacterium* species were correctly identified by PRA despite errors in results that varied from predicted sizes by an average of 7 bp. The sizes of fragments generated only by HaeIII digestion have been analyzed by in-house capillary electrophoresis (Chang et al., 2007). In that study, the authors concluded that 96% of ≥40-bp fragments differed from the real size by <5 bp. In our study, among 36 HaeIII digestion fragments that were ≥50 bp, 29 (81%) differed from the real size by ≤10 bp. However, the strains and their number used for comparison were different between the two studies (12 vs. 38 in the present study) that could affect the estimates. On the other hand, the reproducibility of patterns was higher in our study (a reproducible pattern with SDs that were ≤2 bp for 100% of 47 fragments analyzed). Moreover, in comparison with the study by Chang et al. (2007) our method enabled simultaneous analysis of 12 (instead of only 1) samples together with internal size markers in addition to the external molecular size ladder. Compared to the study by Hernandez et al. (1999) the method described here does not require any capillary coating process or the use of expensive fluorescence-labeled primers. These advantages make this method able to meet the aim of lower cost for the rapid identification of *Mycobacterium* species.

Data obtained on the reference strains have enabled our design of interpretative table for identification of 35 *Mycobacterium* species or PRA subtypes of species. We used that elaborated diagnostic scheme to test 64 cultured isolates from both clinical and environmental specimens. For the vast majority (94%) of isolates, phenotypic methods provided a species or complex/group identification. However, for a fifth of these isolates the phenotypic identification proved incorrect upon further analysis by DNA-based methods, including commercial LiPA assay, partial sequencing of the 16 S rRNA gene, and/or *hsp65* PRA. Similar discrepancies between conventional and molecular methods have been observed in other studies (Mohamed et al., 2005; Wu et al., 2007; Chimara et al., 2008). The mistakes of conventional identification may be caused by bacterial aberrance or by misreading of the results from a series of biochemistry tests. Also, the increasing number of newly defined mycobacterial species and the "difficult-to-identify" variants of known species represent a significant challenge for conventional approaches (Levi et al., 2003).

In contrast, PRA performed very well in comparison with the LiPA assay (89% concordance) and especially with 16 S rDNA sequencing (93.3% of concordant results). LiPA test enables identification of 16 species (Tortoli et al., 2003), while our bioanalyzer system-based PRA currently identifies 35 different *Mycobacterium* species and this number will likely increase in the future as new reference strains are included. In comparison with DNA sequencing, our PRA method does not require so costly equipment and specially trained technicians to conduct the analysis. Also, PRA provides some advantages when identifying non-tuberculous mycobacteria (NTM). Some species or complexes which cannot be discriminated by sequencing of the 16 S rDNA can indeed be differentiated by PRA that was shown also in the present study for *M. abscessus/M. chelonae* complex and *M. kansasii/M. gastri*.

PRA has proven similarly effective in other studies. In the study by Chimara et al. (2008) this method correctly identified over 90% of

434 NTM isolates from clinical specimens. They also reported 13 new PRA patterns found in 30 (6.9%) isolates, most of which represented species typically considered non-pathogens. Häfner et al. (2004) used 16 S rDNA sequencing to analyze 126 isolates randomly selected from a larger collection. PRA correctly identified 120 (95.2%) of these isolates. They also sequenced 10 additional isolates from the larger collection that gave PRA profiles not previously reported. All these isolates represented environmental species rarely associated with clinically significant disease. The authors emphasized the need of combining different methods for accurate identification of mycobacteria since one method may fail to identify correctly all strains tested. They also reported a number of conflicts which were observed when applying *hsp65* PRA, *hsp65* sequencing and 16 S rDNA sequencing in order to identify NTM strains. Indeed, also in this study we encountered similar problems while applying sequencing of 16 S rDNA to resolve discrepant and inconclusive results between PRA and conventional methods.

Among 64 test isolates examined in this study, 9 (14.1%) yielded 7 PRA patterns not previously reported. Eight of these isolates were cultured from animal specimens originating from Benin (fish, $n=3$; insect, $n=2$; and shrimp, $n=1$) and the USA (armadillo, $n=2$). Only one isolate with new PRA profile was cultured from patient's swab in Benin. Clinical correlation was not available and this isolate may reflect colonization by environmental organism, especially that the same pattern was identified in another isolate from an American armadillo. One of the 7 new PRA patterns was common for *M. interjectum* and 4 subspecies of *M. avium*. This finding illustrates a disadvantage related to PRA, which for some species produces identical or highly similar patterns making their differentiation very difficult or not possible. Two other new profiles were represented by 2 *M. gordonae* isolates. Some NTM appear highly polymorphic with respect to their *hsp65* PRA pattern. To our knowledge not less than 14 different patterns are currently available for *M. gordonae* in the literature including the two described here (da Silva Rocha et al., 2002; Häfner et al., 2004; Chimara et al., 2008; and the citation herein), some of them showing only minor differences. Of interest, 3 other *M. gordonae* isolates investigated here matched PRA pattern observed in a single isolate in Brazil (da Silva Rocha et al., 2002) indicating a worldwide distribution of given allelic variants.

It appears that PRA based on the automated fluorescence capillary electrophoresis offers several important advantages that could be useful in both identification of *Mycobacterium* species and strain typing (e.g. MIRU-VNTR analyses (Sajduda et al., 2006)). This new technology is fast (12 samples on each disposable labchip are analyzed within 30 min; similar instruments with capability expanded to 96 or more samples being analyzed simultaneously have lately been introduced that reduces turnaround times as well as cost per sample). It is easy (a minimum of technical skill is required), sufficiently accurate, reproducible, and it generates low waste. Also, it is cost effective (the volumes of reagents may be reduced because only 1-μl sample is evaluated) and versatile (it is complemented with kits for dsDNA, RNA, protein, and flow cytometric analyses). Despite the relatively high cost of equipment, which might limit its usefulness in developing countries and/or areas with high prevalence of mycobacterial infections, the bioanalyzer system appears a suitable format for performance of PCR-based identification as well as typing analyses.

Acknowledgements

We acknowledge Pim de Rijk for technical assistance.

This work was supported by INCO-CA project no. ICA4-CT-2001-10087 from the European Commission and by the Fund for Scientific Research of Flanders (Brussels, Belgium, grant G.0471.03 N). A. S. was supported by a NATO Science Fellowship at the Institute of Tropical Medicine, Antwerp, Belgium.

References

Blackwood, K.S., He, C., Gunton, J., Turenne, C.Y., Wolfe, J., Kabani, A.M., 2000. Evaluation of *recA* sequences for identification of *Mycobacterium* species. J. Clin. Microbiol. 38, 2846–2852.

Brunello, F., Ligozzi, M., Cristelli, E., Bonora, S., Tortoli, E., Fontana, R., 2001. Identification of 54 mycobacterial species by PCR-restriction fragment length polymorphism analysis of the *hsp65* gene. J. Clin. Microbiol. 39, 2799–2806.

Chang, P.-L., Hsieh, W.-S., Chiang, C.-L., Tuohy, M.-J., Hall, G.S., Procop, G.W., Chang, H.-T., Ho, H.-T., 2007. The *hsp65* gene patterns of less common *Mycobacterium* and *Nocardia* spp. by polymerase chain reaction–restriction fragment length polymorphism analysis with capillary electrophoresis. Diagn. Microbiol. Infect. Dis. 58, 315–323.

Chimara, E., Ferrazoli, L., Ueky, S.Y.M., Martins, M.C., Durham, A.M., Arbeit, R.D., Leão, S.C., 2008. Reliable identification of mycobacterial species by PCR-restriction enzyme analysis (PRA)-*hsp65* in a reference laboratory and elaboration of a sequence-based extended algorithm of PRA-*hsp65* patterns. BMC Microbiol. 8, 48.

Cloud, J.L., Neal, H., Rosenberry, R., Turenne, C.Y., Jama, M., Hillyard, D.R., Carroll, K.C., 2002. Identification of *Mycobacterium* spp. by using a commercial 16 S ribosomal DNA sequencing kit and additional sequencing libraries. J. Clin. Microbiol. 40, 400–406.

da Silva Rocha, A., Werneck Barreto, A.M., Dias Campos, C.E., Villas-Bôas da Silva, M., Fonseca, L., Saad, M.H., Degrave, W.M., Suffys, P.N., 2002. Novel allelic variants of mycobacteria isolated in Brazil as determined by PCR-restriction enzyme analysis of *hsp65*. J. Clin. Microbiol. 40, 4191–4196.

Devallois, A., Goh, K.S., Rastogi, N., 1997. Rapid identification of mycobacteria to species level by PCR-restriction fragment length polymorphism analysis of the *hsp65* gene and proposition of an algorithm to differentiate 34 mycobacterial species. J. Clin. Microbiol. 35, 2969–2973.

Devulder, G., Pérouse de Montclos, M., Flandrois, J.P., 2005. A multigene approach to phylogenetic analysis of the genus *Mycobacterium* as a model. Int. J. Syst. Evol. Microbiol. 55, 293–302.

Ferdinand, S., Legrand, E., Goh, K.S., Berchel, M., Mazzarelli, G., Sola, C., Tortoli, E., Rastogi, N., 2004. Taxonomic and phylogenetic status of non-tuberculous mycobacteria in Caribbean setting. Mol. Cell. Probes 18, 399–408.

Häfner, B., Haag, H., Geiss, H.K., Nolte, O., 2004. Different molecular methods for the identification of rarely isolated non-tuberculous mycobacteria and description of new *hsp*65 restriction fragment length polymorphism patterns. Mol. Cell. Probes 18, 59–65.

Hernandez, S.M., Morlock, G.P., Butler, W.R., Crawford, J.T., Cooksey, R.C., 1999. Identification of *Mycobacterium* species by PCR-restriction fragment length polymorphism analyses using fluorescence capillary electrophoresis. J. Clin. Microbiol. 37, 3688–3692.

Ho, H.-T., Chang, P.-L., Hung, C.-C., Chang, H.-T., 2004. Capillary electrophoretic restriction fragment length polymorphism patterns for the mycobacterial *hsp65* gene. J. Clin. Microbiol. 42, 3525–3531.

Kasai, H., Ezaki, T., Harayama, S., 2000. Differentiation of phylogenetically related slowly growing mycobacteria by their *gyrB* sequences. J. Clin. Microbiol. 38, 301–308.

Kim, B.-J., Lee, S.-H., Lyu, M.-A., Kim, S.-J., Bai, G.-H., Chae, G.-T., Kim, E.-C., Cha, C.-Y., Kook, Y.-H., 1999. Identification of mycobacterial species by comparative sequence analysis of the RNA polymerase gene (*rpoB*). J. Clin. Microbiol. 37, 1714–1720.

Kim, H., Kim, S.-H., Shim, T.-S., Kim, M.-n., Bai, G.-H., Park, Y.-G., Lee, S.-H., Cha, C.-Y., Kook, Y.-H., Kim, B.-J., 2005. PCR restriction fragment length polymorphism analysis (PRA)-algorithm targeting 644 bp heat shock protein 65 (*hsp65*) gene for differentiation of *Mycobacterium* spp. J. Microbiol. Methods 62, 199–209.

Kim, B.-J., Park, J.-H., Lee, S.-A., Kim, H., Cha, C.-Y., Kook, Y.-H., Kim, E.-C., Joo, S.I., Lee, J.S., Yim, J.J., 2008. Differentiation of mycobacteria in sputa by duplex polymerase chain reaction for mycobacterial *hsp65* gene. Diagn. Microbiol. Infect. Dis. 62, 193–198.

Levi, M.H., Bartell, J., Gandolfo, L., Smole, S.C., Costa, S.F., Weiss, L.M., Johnson, L.K., Osterhout, G., Herbst, L.H., 2003. Characterization of *Mycobacterium montefiorense* sp. nov., a novel pathogenic *Mycobacterium* from Moray eels that is related to *Mycobacterium triplex*. J. Clin. Microbiol. 41, 2147–2152.

Mohamed, A.M., Kuyper, D.J., Iwen, P.C., Ali, H.H., Bastola, D.R., Hinrichs, S.H., 2005. Computational approach involving use of the internal transcribed spacer 1 region for identification of *Mycobacterium* species. J. Clin. Microbiol. 43, 3811–3817.

Sajduda, A., Dziadek, J., Kotłowski, R., Portaels, F., 2006. Evaluation of multiple genetic markers for typing drug-resistant *Mycobacterium tuberculosis* strains from Poland. Diagn. Microbiol. Infect. Dis. 55, 59–64.

Scarparo, C., Piccoli, P., Rigon, A., Ruggiero, G., Nista, D., Piersimoni, C., 2001. Direct identification of mycobacteria from MB/BacT Alert 3D bottles: comparative evaluation of two commercial probe assays. J. Clin. Microbiol. 39, 3222–3227.

Senna, S.G., Battilana, J., Costa, J.C., Silva, M.G., Duarte, R.S., Fonseca, L.S., Suffys, P.N., Bogo, M.R., 2008. Sequencing of *hsp65* gene for identification of *Mycobacterium* species isolated from environmental and clinical sources in Rio de Janeiro, Brazil. J. Clin. Microbiol. 46, 3822–3825.

Stanley, T., Crothers, L., McCalmont, M., Xu, J., Millar, B.C., Goldsmith, C.E., Moore, J.E., 2006. The potential misidentification of *Tsukamurella pulmonis* as an atypical *Mycobacterium* species: a cautionary tale. J. Med. Microbiol. 55, 475–478.

Taylor, T.B., Patterson, C., Hale, Y., Safranek, W.W., 1997. Routine use of PCR-restriction fragment length polymorphism analysis for identification of mycobacteria growing in liquid media. J. Clin. Microbiol. 35, 79–85.

Telenti, A., Marchesi, F., Balz, M., Bally, F., Böttger, E.C., Bodmer, T., 1993. Rapid identification of mycobacteria to the species level by polymerase chain reaction and restriction enzyme analysis. J. Clin. Microbiol. 31, 175–178.

Tortoli, E., 2003. Impact of genotypic studies on mycobacterial taxonomy: the new mycobacteria of the 1990s. Clin. Microbiol. Rev. 16, 319–354.

Tortoli, E., Mariottini, A., Mazzarelli, G., 2003. Evaluation of INNO-LiPA MYCOBACTERIA v2: improved reverse hybridization multiple DNA probe assay for mycobacterial identification. J. Clin. Microbiol. 41, 4418–4420.

Tortoli, E., Chianura, L., Fabbro, L., Marittini, A., Martin-Casabona, N., Mazzarelli, G., Ruusso, C., Spinelli, M., 2005. Infections due to the newly described species *Mycobacterium parascrofulaceum*. J. Clin. Microbiol. 43, 4286–4287.

Turenne, C.Y., Tschetter, L., Wolfe, J., Kabani, A., 2001. Necessity of quality-controlled 16 S rRNA gene sequence databases: identifying non-tuberculous *Mycobacterium* species. J. Clin. Microbiol. 39, 3637–3648.

Wong, D.A., Yip, P.C.W., Tse, D.L.W., Tung, V.W.N., Cheung, D.T.L., Kam, K.M., 2003. Routine use of a simple low-cost genotypic assay for the identification of mycobacteria in a high throughput laboratory. Diagn. Microbiol. Infect. Dis. 47, 421–426.

Wu, X., Zhang, J., Liang, J., Lu, Y., Li, H., Li, C., Yue, J., Zhang, L., Liu, Z., 2007. Comparison of three methods for rapid identification of mycobacterial clinical isolates to the species level. J. Clin. Microbiol. 45, 1898–1903.

Wu, T.-L., Chia, J.-H., Kuo, A.-J., Su, L.-H., Wu, T.-S., Lai, H.-C., 2008. Rapid identification of mycobacteria from smear-positive sputum samples by nested PCR-restriction fragment length polymorphism analysis. J. Clin. Microbiol. 46, 3591–3594.

Zolg, J.W., Philippi-Schultz, S., 1994. The superoxide dismutase gene, a target for detection and identification of mycobacteria by PCR. J. Clin. Microbiol. 32, 2801–2812.