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A novel identification scheme for genus *Mycobacterium*, *M. tuberculosis* complex, and seven mycobacteria species of human clinical impact

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Abstract Recently, the incidence of human mycobacterial infections due to species other than *M. tuberculosis* has increased worldwide. Since disease control depends on appropriate antimicrobial therapy, the precise identification of these species of clinical importance has become a major public health concern. Identification of mycobacteria has been hampered because of the lack of specific, rapid, and inexpensive methods. Therefore, we aimed at designing and validating a bacterial lysate-based polymerase chain reaction identification scheme. This scheme can classify clinical isolates into: (1) the genus *Mycobacterium*, (2) the *M. tuberculosis* complex, (3) the nontuberculous mycobacteria, and (4) the species *M. avium*, *M. intracellulare*, *M. abscessus*, *M. chelonae*, *M. fortuitum* and *M. bovis* of clinical importance, and *M. gordonae*, the most commonly encountered nonpathogenic species in clinical laboratories.

By using *M. fortuitum* and *M. avium* lysates as models, the method sensitivity was determined to be 372 pg of DNA. In a blind parallel comparison between our approach and conventional biochemical tests, both assays correctly categorized 75 patient's mycobacterial isolates. However, our approach only required 4–9 h for categorization compared with at least 15 days by conventional tests. Furthermore, our methodology could also detect *M. fortuitum* and *M. avium* from liquid cultures, after only 2 and 6 days, respectively, of incubation. Our new identification scheme is therefore sensitive, specific, rapid, and economic. Additionally, it can help to provide proper treatment to patients, to control these diseases, and to improve our knowledge of the epidemiology of mycobacteriosis, all urgently needed, particularly in developing countries.

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Mycobacterial diseases in humans can be caused by species belonging to the *M. tuberculosis* complex (MTC, which includes the species *M. tuberculosis*, *M. bovis*, *M. africanum*, *M. canetii*, and *M. microti*) and some nontuberculous mycobacteria (NTM) [1]. The HIV epidemic not only revealed that other mycobacteria other than *M. tuberculosis* can frequently cause pulmonary- [2, 3] and extrapulmonary-mycobacteriosis in HIV-infected patients [2, 4] but in immunocompetent subjects as well [2, 5]. For example, in some developing countries, *M. avium* complex (MAC) appears to be the second most common group causing pulmonary-mycobacteriosis after *M. tuberculosis* [2, 3, 5]. Other implicated species include *M. bovis*, *M. kansasii*, *M. fortuitum*, *M. abscessus* and *M. chelonae* [4–6], all of which present clinical features that may resemble those of

tuberculosis. Notably, antibiotic treatment for *M. tuberculosis* differs from that of other mycobacterial species [1, 5, 7], particularly for those in the NTM group. *M. gordonae* has become the most commonly encountered nonpathogenic species in clinical laboratories, which seems to be a nonpathogenic colonizing organism even among persons with local or general immune suppression [6]; thus, this also needs to be differentiated from other pathogenic species.

Unfortunately, in developing countries, most patients with a positive acid-fast smear are still treated for *M. tuberculosis*, since further mycobacterial identification is not pursued because it is lengthy and expensive. Regrettably, misidentifying mycobacteria could have serious consequences such as increasing the length of hospitalization, the risk of acquiring potentially lethal disseminated infections, and selecting for multidrug-resistant strains. Therefore, only correct identification of the *Mycobacterium* species will enable public health workers to provide the appropriate antimicrobial treatment to patients, which is of primary importance for infection control. Consequently, it is important to develop a specific, rapid, and inexpensive diagnostic method to identify and distinguish mycobacteria. Although several molecular diagnostic methods have been published [8–12], most of them only identify one or two species, mainly *M. tuberculosis* [13–15]. Those that identify several species are only commercially available (Cobas Amplicor MTB Test, Roche; Accuprobe Culture Identification Tests, Gen-Probe, San Diego, CA) or use sophisticated equipment [16, 17], making them inaccessible to developing countries or to health services with limited budgets. One of the major problems that remains for the design of a molecular-based test for mycobacteria is the lack of mycobacterial DNA conserved regions which can identify to the genus level or that distinguish among species. Therefore, based on conserved regions between mycobacteria that we [18, 19] and others [20] have characterized in prior studies, we designed and validated a PCR-based approach that uses mycobacterial lysates to identify the genus *Mycobacterium*, the MTC, and seven mycobacteria species: *M. bovis*, *M. fortuitum*, *M. chelonae*, *M. abscessus, M. intracellulare, M. avium* and *M. gordonae*.

Material and methods

Bacterial strains

The following mycobacterial strains were used to develop the PCRs assays: *M. tuberculosis* (H37Rv, CDC1551), *M. bovis* (AN5), and *M. fortuitum* (ATCC 6841). For *M. abscessus*, *M. chelonae*, *M. gordonae*, *M. avium*, and *M.*

intracellulare, clinical isolates biochemically [21] or genetically (Accuprobe Culture Identification Tests 2835, 2840 and 2850; Gen-Probe, San Diego, CA) characterized were used. All clinical isolates were further characterized by confirming their identities by the automatic sequencing of their 16S rRNA V2 region [20]. *Nocardia* sp., *Streptomyces coelicolor*, and enterotoxigenic *Escherichia coli* (ETEC) H10407 lysates were used as negative controls.

Biochemical identification

Mycobacterial cultures were identified using the gold standard method, grown on Lowenstein-Jensen slants, and were subjected to biochemical tests including the following: pyrazinamidase, arylsulfatase, growth at 42°C, Tween hydrolysis, heat-stable (68°C) catalase, semiquantitative catalase, niacin production, nitrate reduction, urease, growth on 5% NaCl, and MacConkey agar without crystal violet as described in standard publications [21].

Preparation of mycobacterial lysates

In order to obtain lysates that provided a good quality DNA for PCR assays, a mycobacteria loopful from a Lowenstein-Jensen slant was resuspended in 100 μL of deionised water (Milli-Q System, Millipore), boiled for different time periods (5, 10, 15, and 20 min) placed in ice (10 min), centrifuged at room temperature (13,600×g, 5 min) and supernatants (bacterial lysates) used for PCR. Since 5 min boiling was enough to provide a good quality lysate for PCR assays, we used this time interval to perform all PCRs.

PCR assays

Three separate PCR assays were performed: PCR 1, PCR 2, and PCR 3. In all cases, each PCR tube contained 22 μL of reaction mix, which comprised in final concentration: 20 mM Tris-HCl pH 8.4, 50 mM KCl, 1.5 mM MgCl$_2$, 200 μM each of dATP, dCTP, dGTP, and dTTP (Roche Diagnostics GmbH, Mannheim, Germany), 0.04U/μL Ampli*Taq* polymerase (Perkin-Elmer Corporation, Foster City, California, USA), 3 μL of bacterial lysate, and a mix of 6, 2, and 4 primers for PCR 1, 2, and 3, respectively (Table 1). In addition, for PCR 1, 10% DMSO (Sigma-Aldrich Corp., St. Louis, Missouri, USA) was added to the reaction mix. All PCRs were subjected to the same program: 36 cycles (95°C/30 s, 55°C /1 min, and 72°C/2 min) followed by a final extension step at 72°C/5 min in a thermocycler Perkin-Elmer 2400. PCR products (5 μL) were visualized by electrophoresis in a 2% agarose gel at 80 volts for 75 minutes and stained with ethidium bromide; all bands gave the expected molecular sizes (Fig. 2a–c).

Table 1 Description of the three PCR assays used to identify genus *Mycobacterium*, MTC, and seven mycobacterial species

PCR number	Primers sequence (5′– 3′) [reference]	Primer concentrations in mix	Mycobacteria amplified region	Amplicon size in base pairs	Identified
1 (multiplex PCR)	RAC-1: TCGATGATCACCGAGAACGTGTTC [19]	5 pmol	16S rRNA	934	Genus *Mycobacterium* (e.g. all the MTC species and *M. gordonae*)
	RAC-8:CACTGGTGCCTCCCGTAGG [19]			1,100	*M. fortuitum*
				1,200	*M. chelonae*
				1,300	*M. abscessus*
	G1:CCCTACGCAGACTGACAGCAA [22]	5 pmol	*M. gordonae* antigen 85A	407	*M. gordonae*
	G3:AGCCACCGACGAACCCC [22]				
	Y277-32F: ACATGTACGAGAGACGGCATGAG [23]	10 pmol	RD4 (region of difference 4)	1,031	All species belonging to the MTC, except *M. bovis*
	Y277-32R: AATCCAACACGCAGCAACCAG [23]				
2 (single PCR)	MTB-F: CGGGTATGCTGTTAGGCGACG [11]	10 pmol	16S rRNA	488	MTC
	MTB-R:CCACCACAAGACATGCATG [11]				
3 (multiplex PCR)	RAC-1: TCGATGATCACCGAGAACGTGTTC [18]	5 pmol	16S rRNA	1,000	*M. avium/M. intracellulare*
	RAC-8:CACTGGTGCCTCCCGTAGG [18]				
	RAC-1: TCGATGATCACCGAGAACGTGTTC [19]	5 pmol	16S rRNA	900	*M. avium*
	Mycav-R: ACCAGAAGACATGCGTCTTG [24]				
	Mycint-F:CCTTTAGGCGCATGTCTTTA [24]	5 pmol	16S rRNA	176	*M. intracellulare*
	RAC-8:CACTGGTGCCTCCCGTAGG [19]				

MTC: *M. tuberculosis* complex

Methodology sensitivity

Briefly, lysates of *M. fortuitum* and *M. avium* were prepared, serial dilutions were made (1:10, 1:100, 1:500 and 1:1000), and DNA concentrations determined spectrophotometrically at 260 nm. Then each dilution was tested by our developed methodology, PCR products were analyzed by electrophoresis on 2% agarose gel, and the assay sensitivity was established (DNA concentration in the last dilution that produced a visible band).

Characterization of the three PCR assays using bacterial strains

The PCR assays were characterized (Table 2) by using bacterial lysates of the following: (1) biochemically or genetically characterized reference strains and clinical isolates for each of the seven species detected by our assay, (2) mycobacterial reference strains and well characterized clinical isolates not detected until species by our methodology, and (3) bacteria that do not belong to the genus *Mycobacterium*.

Confirmation of the species identity

PCR products, obtained from at least one patient's clinical isolate of each of the seven *Mycobacterium* species, identified by our methodology were cloned directly into pBluescript KS+ (Stratagene, La Jolla, California, USA). Purified plasmids from selected colonies were screened for the correct insert by digestion with endonucleases XhoI (New England Biolabs, Inc. Beverly, Massachusetts, USA) and

Table 2 Characterization of the three developed PCR assays using 66 bacterial strains

Group MTC, NTM and species identified (number of strains tested for each species)	Amplicon(s) identified in base pairs in each PCR		
	PCR 1	PCR 2	PCR 3
MTC/*M. tuberculosis* (12)	934 and 1,031	488	934
MTC/*M. africanum* (1)	934 and 1,031	488	934
MTC/*M. canettii* (1)	934 and 1,031	488	934
MTC/*M. bovis* (8)	934	488	934
NTM/*M. gordonae* (7)	934 and 407	None	934
NTM/*M. kansasii* (2)	934	None	934
NTM/*M. ulcerans* (1)	934	None	934
NTM/*M. celatum* (3)	934	None	934
NTM/*M. avium* (6)	1,000	None	1,000 and 900
NTM/*M. intracellulare* (6)	1,000	None	1,000 and 176
NTM/*M. scrofulaceum* (1)	1,000	None	1,000
NTM/*M. marinum* (2)	1,000	None	1,000
NTM/*M. simiae* (1)	1,000	None	1,000
NTM/*M. fortuitum* (4)	1,100	None	1,100
NTM/*M. chelonae* (4)	1,200	None	1,200
NTM/*M. abscessus* (4)	1,300	None	1,300
Nocardia sp. (1)	None	None	None
Streptomyces coelicolor (1)	None	None	None
Enterotoxigenic *E. coli* (1)	None	None	None

MTC: *M. tuberculosis* complex, *NTM*: nontuberculous Mycobacteria

BamHI (New England Biolabs, Inc., Beverly, Massachusetts, USA) and analyzed by agarose gel electrophoresis. Both strands of all inserts (from 2 clones) were automatically sequenced using the M13 and the corresponding reverse primers using the Big Dye Terminator Ready reaction Kit (PerKin-Elmer, Inc., Wellesley, Massachusetts, USA) and analyzed by ABI PRISM 310 Genetic Analyzer System (Perkin-Elmer). Finally all sequences obtained were compared by BLAST.

Validation of the identification scheme developed

A blind study was performed using 75 patient's clinical isolates provided by the Clinical Microbiology Laboratory at the National Institute of Nutrition in Mexico City. All isolates were lysed, and subjected to the PCR assays as described above.

Mycobacterial species identification from liquid cultures using the newly developed methodology

Briefly, 100 μL of each *M. fortuitum* and *M. avium* cultures adjusted to a 0.5 McFarland turbidity standard were inoculated in both Lowenstein-Jensen slants for biochemical characterization and liquid medium containing Middlebrook 7H9, enriched with OADC (oleic acid, albumin, dextrose and catalase; Becton Dickinson, Sparks, Maryland, USA), to obtain lysates for PCR. Each day 1 mL was taken from liquid culture, lysed, and tested in the PCR assays. Solid

slants were visually verified for mycobacterial growth until biomass was enough to carry out traditional tests.

Results

Characterization of the three developed PCR assays using bacterial isolates

A total of 66 bacterial strains were analyzed in each of the three PCRs assays (PCR 1, 2, 3) developed for this study. These included six reference strains (four *Mycobacterium* and one enterotoxigenic *Escherichia coli*) and 61 well defined clinical isolates (59 *Mycobacterium spp*., one *Nocardia spp*., and one *Streptomyces coelicolor*). As shown in Table 2, the analysis revealed that in PCR 1 three strains did not belong to genus *Mycobacterium*. Sixty-three strains belonged to the genus *Mycobacterium*, of which 14 were MTC, and 19 *Mycobacterium* were further identified to the species level: four as *M. abscessus*, four as *M. chelonae*, four as *M. fortuitum*, and seven as *M. gordonae*. In PCR 2 three strains did not yield any band, 41 of the 63 *Mycobacterium* strains were NTM, and 22 belonged to the MTC; eight of these were identified as *M. bovis*. In PCR 3 it was confirmed, as in PCR1, that three strains were indeed not genus *Mycobacterium* and 63 strains were *Mycobacterium*. In addition, 12 of the 63 strains were identified to the species level: six were *M. avium* and six were *M. intracellulare*.

Development of a mycobacteria identification scheme

As shown in Table 2, using a combination of our 3 PCR assays enabled us to clearly identify and characterize 63 mycobacterial strains and to discriminate these from non-mycobacterium species. Based on these results, we designed a diagnostic approach consisting of three steps (Fig. 1).

Step 1 A multiplex PCR that establishes whether an isolate belongs to the genus *Mycobacterium*, the MTC group (except for *M. bovis*), or to the species *M. abscessus*, *M. chelonae*, *M. fortuitum* and *M. gordonae*. By using RAC-1 and RAC-8 primers [11], we determined that an isolate was genus *Mycobacterium*. As shown in Table 2, most mycobacterial species yielded a band of 934 bp, as MTC species (e.g. Fig. 2a, lanes 6–7) or a band of 1000 bp, as *M. avium* and *M. intracellulare* (Fig. 2a, lanes 8–9). Notably, *M. abscessus* (1300 bp), *M. chelonae* (1200 bp), and *M. fortuitum* (1100 bp) produced a specific size band, thus allowing its identification (Fig. 2a, lanes 2–4). Since *M. gordonae* yielded the 934 bp band like other mycobacterial species, it was further characterized by a specific set of primers for this species, G1 and G3 [22], that produced a band of 407 bp (Fig. 2a, lane 5). Finally, primers Y277-32F and Y277-32R [23] helped us to determine whether an isolate belongs to the MTC, as most of these species, except for *M. bovis*, produced a 1031 bp band in addition to the 934bp band previously described (Fig. 2a, lanes 6–7).

Step 2 If the mycobacterial isolate did not belong to any of the above species and produced a band of 934 bp, it could be further characterized in a single PCR by using MTB-F and MTB-R primers [11], which specifically identifies all MTC species, including *M. bovis*. Therefore, if the isolate produces a characteristic 488 bp band (Fig. 2b), this will indicate that it is *M. bovis* (Fig. 2b, lane 3), given that it was not classified as the other MTC species in step 1. However, if a band is not observed, indicates that the isolate belongs to the NTM group.

Step 3 If a mycobacterial isolate yielded a band of 1000 bp in step 1, it could be further characterized by a multiplex PCR, that in addition to RAC-1 and RAC-8 primers for genus *Mycobacterium*, now includes Mycav-R and Mycint-F primers [24], which will identify *M. avium* if a band of 900 bp is produced (Fig. 2c, lane 2) and *M. intracellulare* if a 176 bp band is observed (Fig. 2c, lane 3).

Methodology sensitivity

The sensitivity of the amplification procedure was investigated with *M. fortuitum* and *M. avium* as model systems. By using serial dilutions of mycobacterial lysates it was determined that the lowest lysate dilution that yielded a detectable band in an agarose gel for both species contained 372 pg of DNA.

Confirmation of species identity

The percentages of identity between the insert DNA sequences and those in the Genbank (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi) for the seven species identified by our method (*M. avium*, *M. intracellulare*, *M. abscessus*, *M. chelonae*, *M. fortuitum*, *M. gordonae*, and *M. bovis*) were from 88% to 99%, thus confirming the species identities.

Fig. 1 Three step identification scheme for genus *Mycobacterium*, MTC group, and seven mycobacteria species: *M. bovis*, *M. fortuitum*, *M. chelonae*, *M. abscessus*, *M. avium*, *M. intracellulare*, and *M. gordonae*
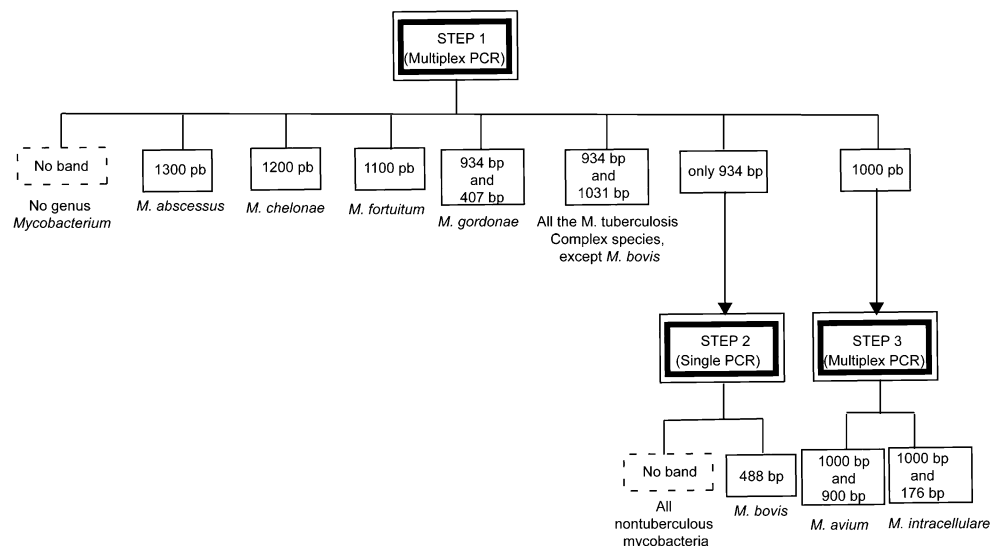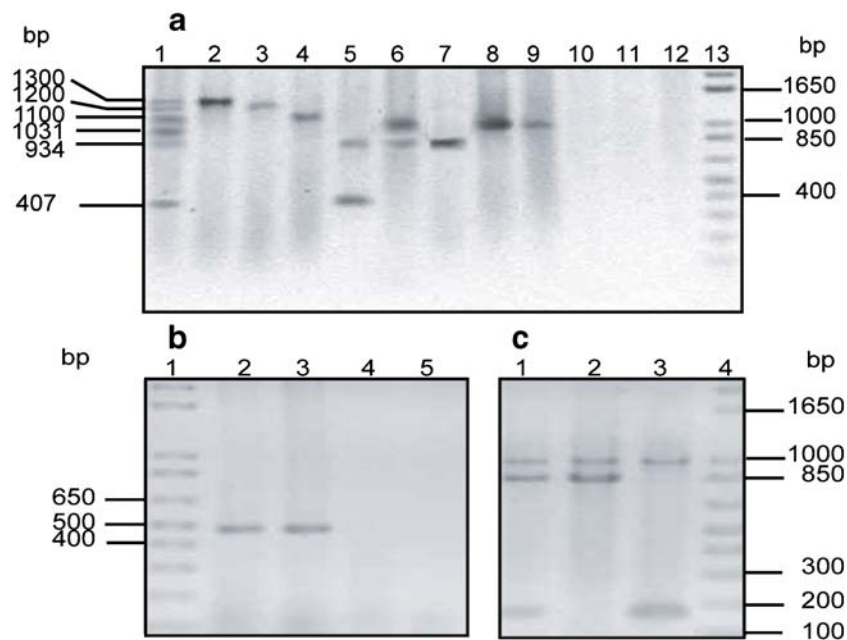
Fig. 2 Amplifying products obtained using bacteria lysates in each step of the identification scheme. **a** Step 1. Lane 1, lysates mix from different mycobacterial species; lane 2, *M. abscessus*; lane 3, *M. chelonae*; lane 4, *M. fortuitum*; lane 5, *M. gordonae*; lane 6, *M. tuberculosis*; lane 7, *M. bovis*; lane 8, *M. avium*; lane 9, *M. intracellulare*; lane 10, *Nocardia* spp.; lane 11, *Streptomyces coelicolor*; lane 12, ETEC. **b** Step 2. Lane 2, *M. tuberculosis*; lane 3, *M. bovis*; lane 4, *M. avium*; lane 5, *M, fortuitum*. **c** Step 3. Lane 1, lysates mix from *M. avium* and *M. intracellulare*; lane 2, *M. avium*; lane 3, *M. intracellulare*. Lanes 13a, 1b, and 4c are 1 Kb plus DNA ladder molecular weight markers in base pairs (bp)

Validation of the developed identification scheme using clinical isolates

A blind study of our proposed identification scheme was conducted on 75 patient's clinical isolates. As shown in Table 3, all 75 clinical bacterial isolates were genus *Mycobacterium*, while seven belonged to the MTC, of which two were *M. bovis*. The remaining 68 isolates belonged to the NTM of which 49 were identified to the species level: 17 as *M. fortuitum*, 17 as *M. avium*, six as *M. chelonae*, four as *M. intracellulare*, three as *M. gordonae*, and two as *M. abscessus*. All results were confirmed by conventional tests. This study also revealed that in step 1 most mycobacterial species yielded bands of 934 bp or 1000 bp for the genus level, that in step 2 the species belonging to the MTC or NTM were clearly distinguished, and that by using the three-step identification approach it was possible to identify seven mycobacterial species.

Mycobacterial species identification from liquid cultures using the newly developed identification scheme

We identified *M. fortuitum* and *M. avium* from liquid cultures after 2 days and 6 days of incubation, respectively, compared to 21 and 30 days, respectively, with conventional tests. This included transfer and growth in solid medium of each mycobacteria and previously described biochemical and genetic tests.

Discussion

The emergence of NTM diseases is not yet clear but could be the consequence of various factors such as the HIV pandemic, the establishment of NTM surveillance systems and, more recently, a sharp increase of diabetes mellitus, another immunosuppressive disease that has reached epidemic proportions [25]. For instance, in Mexico it is estimated that 8% of the population is affected by diabetes mellitus [26], and their risk for tuberculosis increases 6.8-fold compared with healthy individuals [27]. Conceivably, infections by other mycobacteria might also increase in these patients worldwide.

Currently, the NTM remains the most challenging mycobacterial group to be identified, and it continues to gain clinical significance worldwide [4–6]. Therefore, based on our experience both in developing clinical microbiology diagnostic techniques and characterizing mycobacteria 16S rRNA and *mur*A regions [11, 28, 29], we aimed at developing a mycobacteria lysate PCR-based identification scheme. This new technique establishes whether an isolate belongs to the genus *Mycobacterium*, distinguishes between MTC and NTM species, and identifies seven species of clinical importance including *M. bovis* and six NTM species (*M. fortuitum*, *M. chelonae*, *M. abscessus*, *M. gordonae*, *M. avium*, and *M. intracellulare*). Most PCR methods described to date depend on DNA purification methods (which are expensive and time

Table 3 Validation of the identification scheme using 75 clinical isolates

Group MTC, NTM and species identified (number of strains tested for each species)	Amplicon(s) identified in base pairs in each step		
	Step 1	Step 2[a]	Step 3[b]
MTC/*M. tuberculosis* (5)	934 and 1,031	488	
MTC/*M. bovis* (2)	934	488	
NTM/*M. gordonae* (3)	934 and 407	None	
NTM/*M. kansasii* (2)	934	None	
NTM/*M. terrae* (1)	934	None	
NTM/NI (2)	934	None	
NTM/*M. avium* (17)	1,000		1,000 and 900
NTM/*M. intracellulare* (4)	1,000		1,000 and 176
NTM/*M. scrofulaceum* (1)	1,000		1,000
NTM/*M. vaccae* (3)	1,000		1,000
NTM/*M. marinum* (2)	1,000		1,000
NTM/*M. lentiflavum* (1)	1,000		1,000
NTM/MAC (6)	1,000		1,000
NTM/NI (1)	1,000		1,000
NTM/*M. fortuitum* (17)	1,100		
NTM/*M. chelonae* (6)	1,200		
NTM/*M. abscessus* (2)	1,300		

MTC: *M. tuberculosis* complex, *NTM*: nontuberculous Mycobacteria, *NI*: species not identified, *MAC*: *M. avium* complex
[a] Only species which yielded a band of 934 bp in step 1 were analyzed
[b] Only species which yielded a band of 1,000 bp in step 1 were analyzed

consuming) and only identify the genus *Mycobacterium* [8], or the MTC group [13–15], or one [22, 30, 31] to two [9, 10, 24] mycobacteria species, and some methods in addition to PCR use restriction enzyme analysis, which is expensive and time consuming [32]. Recently, even the elegant and precise pyrosequencing technique has been proposed for identification of several *Mycobacterium* species [17]. However, it has the limitations of being a good technique for analyzing shorter DNA sequences (20–50 nucleotides) but not longer DNA sequences, and that, in addition to PCR, it requires other expensive reagents and equipment not available in resource-limited settings.

Therefore, our approach using mycobacterial lysates and targeting the 16S rRNA and other specific mycobacterial regions, significantly reduced time, labour, and cost associated with mycobacteria identification, compared to other detection methods. It was estimated that the cost of our method per sample was of $25.00 USD, compared to $75.00 USD for conventional phenotypic tests (gold standard), $48.00 USD for other techniques using the 16S rRNA region [12], and $72.00 USD for commercial PCR kits, which are only available for *M. avium*, *M. intracellulare* and MTC (Cobas Amplicor, Applied Science Roche). Also, our identification scheme can specifically amplify discrete DNA fragments in samples where the target sequences are present in only picogram quantities (372 pg); thus, this highly sensitive technique could be potentially useful to identify these pathogens in clinical samples. Furthermore, we demonstrated that our technique

was able to quickly identify *M. fortuitum* and *M. avium* from liquid cultures (in 2 and 6 days, respectively), compared to at least 21 days by the gold standard tests, further reducing identification time and costs.

The main limitation of our identification scheme is the number of mycobacterial species that were distinguished. For instance, those not distinguished included *M. kansasii*, which is considered second only to the MAC as a cause of NTM lung disease in several countries [6, 33], and *M. terrae*, which is the species most commonly associated with tenosynovitis of the upper extremity following trauma [6, 34]. However, the scheme allows for the introduction of specific primers in any of the steps for development of precise identification of any given mycobacteria species and for the identification of mutations associated with antibiotic resistance.

It is important to mention that the new microscopy-observation drug-susceptibility assay (MODS) for the diagnosis of TB has become a milestone technique for recognizing tuberculosis, multidrug-resistance tuberculosis [35], and tuberculous meningitis [36]. A study conducted in Peru [35] revealed that a single MODS culture of sputum offers more rapid, sensitive, and inexpensive ($2 USD per sample) detection of tuberculosis and multidrug-resistance tuberculosis than the existing gold standard methods. This provides, for the first time, an excellent diagnostic technique that can be used in resource-limited settings and developing countries. However, MODS application for the detection of other mycobacteria species other than *M.*

tuberculosis, to the best of our knowledge, has yet to be determined, because this technique relies on the characteristic cord formation of *M. tuberculosis* in liquid medium that distinguishes this species from other mycobacteria.

Hopefully, our new approach may help to optimize mycobacteriosis treatment, reduce days of hospitalization, and enhance our understanding of the epidemiology of pulmonary- and extrapulmonary-mycobacteriosis. All of these factors will lead to improvements in disease control.

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