

# Rapid detection and differentiation of mycobacterial species using a multiplex PCR system

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## ABSTRACT

**Introduction:** The early diagnosis of mycobacterial infections is a critical step for initiating treatment and curing the patient. Molecular analytical methods have led to considerable improvements in the speed and accuracy of mycobacteria detection. **Methods:** The purpose of this study was to evaluate a multiplex polymerase chain reaction system using mycobacterial strains as an auxiliary tool in the differential diagnosis of tuberculosis and diseases caused by nontuberculous mycobacteria (NTM). **Results:** Forty mycobacterial strains isolated from pulmonary and extrapulmonary origin specimens from 37 patients diagnosed with tuberculosis were processed. Using phenotypic and biochemical characteristics of the 40 mycobacteria isolated in LJ medium, 57.5% (n=23) were characterized as the *Mycobacterium tuberculosis* complex (MTBC) and 20% (n=8) as nontuberculous mycobacteria (NTM), with 22.5% (n=9) of the results being inconclusive. When the results of the phenotypic and biochemical tests in 30 strains of mycobacteria were compared with the results of the multiplex PCR, there was 100% concordance in the identification of the MTBC and NTM species, respectively. A total of 32.5% (n=13) of the samples in multiplex PCR exhibited a molecular pattern consistent with NTM, thus disagreeing with the final diagnosis from the attending physician. **Conclusions:** Multiplex PCR can be used as a differential method for determining TB infections caused by NTM a valuable tool in reducing the time necessary to make clinical diagnoses and begin treatment. It is also useful for identifying species that were previously not identifiable using conventional biochemical and phenotypic techniques.

**Keywords:** *Mycobacterium tuberculosis* complex. Nontuberculous mycobacteria. Diagnosis. Polymerase chain reaction. Multiplex.

## INTRODUCTION

The ability of nontuberculous mycobacteria (NTM) to cause disease is clearly described in the literature, and its importance is increasing progressively, with an increasing amount of various species being cultured and isolated in laboratories<sup>1-3</sup>.

In Brazil, the NTM species most frequently associated with lung disease are *Mycobacterium kasasii* and *Mycobacterium avium*. Other species, such as *Mycobacterium xenopi*, *Mycobacterium malmoense*, *Mycobacterium lentiflavum*, *Mycobacterium abscessus* and *Mycobacterium szulgai*, have occasionally been isolated in cultures<sup>4,5</sup>. In 2011 the National Agency for Sanitary Surveillance (ANVISA) published the number of cases of infections by rapidly growing mycobacteria (RGMs) obtained in the last eleven years in Brazil.

Data also suggest that this disease occurs in subjects undergoing invasive procedures, mostly surgical cases

guided by video, where the instruments are subjected to high levels of sterilization in glutaraldehyde solution. From 1998 to 2009, there were 2,520 reported cases of post-surgical infections related to rapidly growing mycobacteria, including *Mycobacterium fortuitum*, *Mycobacterium chelonae*, *M. abscessus* and *Mycobacterium massiliense*, which were distributed predominantly in private hospitals throughout the country. Cases have been reported in 23 states of Brazil, with 97.8% of the cases concentrated within 10 states<sup>6</sup>. The diseases caused by NTMs are not compulsorily notifiable, except where the infection occurred during surgical procedures; however, several studies in Brazil have noted isolated strains of nontuberculous mycobacteria in pulmonary and extrapulmonary clinical samples from patients with suspected tuberculosis<sup>4,7,8</sup>.

The differential diagnosis between tuberculosis (TB) and diseases caused by NTMs is of great importance because the epidemiology, treatment and prognosis are different. Conventional diagnostics present numerous difficulties as clinical symptoms are generally similar and non-specific smear testing has limited sensitivity and cannot differentiate the species of mycobacteria.

For many years, the identification of mycobacteria was undertaken by gathering phenotypic results and biochemical test results of isolated species in culture medium<sup>9,10</sup>. In the last 20 or 30 years, with the increasing need to identify a greater number of species and also for early diagnosis, new methods

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have been developed to enable rapid and accurate identification of mycobacterial species<sup>10-12</sup>. In Brazil, the PRA-*hsp65* technique proposed by Telenti et al.<sup>13</sup>, and Devallois et al.<sup>14</sup> has enabled the identification of several species of NTM, the results of which correlated well with those from biochemical identification<sup>13-17</sup>. Beyond the molecular techniques, the sequencing of the specific genes *rpoB* and *hsp65* has become the gold standard for identifying mycobacteria<sup>18,19</sup>. Because of its high discriminatory power, this tool has been used by several groups in epidemiological studies to identify the species involved in human diseases and in outbreaks, and also for taxonomic reclassification<sup>20,21</sup>.

Multiplex polymerase chain reaction (PCR) is a fast tool that allows the simultaneous amplification of more than one sequence of target deoxyribonucleic acid (DNA) in a single reaction, saving time and reagents<sup>22-24</sup>. This system, which amplifies two or three different targets, can differentiate *M. tuberculosis* from the NTMs<sup>25,26</sup>. This molecular approach is used in identifying and differentiating microbes because it is able to supply a simple fingerprint of certain bacterial groups when compared to the standard profiles of referent strains. The correct choice of target sequences in the genome is one of the key criteria for detecting and identifying mycobacteria by PCR<sup>27</sup>.

Faced with the need to differentiate tuberculosis from other mycobacteria, the aim of this study was to evaluate a system based on multiplex PCR, already optimized by Poroca et al.<sup>26</sup>, for identifying mycobacterial species isolated from different clinical samples from patients diagnosed with pulmonary and extrapulmonary tuberculosis. This test, in addition to contributing to a rapid and accurate differential diagnosis between tuberculosis and other mycobacteria, will certainly assist in the early and appropriate therapeutic management of the patient.

## METHODS

### Study population

Thirty-seven male and female patients over the age of 12 years with pulmonary and extrapulmonary tuberculosis diagnoses, who were referred to public clinics in the metropolitan region of Recife of northeastern Brazil between March 2008 and December 2009, were chosen for this study. The types of biological samples collected were chosen based on the different clinical forms of the disease. The diagnosis of tuberculosis was made by the attending physician in accordance with the standards of the American Thoracic Society (ATS), in 2007, based on the isolation of *Mycobacterium* in culture from different samples and visualization of acid fast bacilli (AFB) in the smear microscopy<sup>28</sup>.

### Biological specimens

Forty pulmonary and extrapulmonary samples were analyzed, including 26 (65%) samples of pulmonary origin and 14 (35%) samples of extrapulmonary origin, according to the following distribution: 23 (57.5%) from sputum, 3 (7.5%) from a bronchoalveolar lavage (BAL), 8 (20%) from urine, 1 (2.5%)

from skin lesion aspiration, 3 (7.5%) from pleural fluid (PL), 1 (2.5%) from a node biopsy and 1 (2.5%) from a bone biopsy. Clinical specimens were analyzed by bacilloscopy, culture, multiplex PCR and polymerase chain reaction restriction enzyme analysis (PRA-*hsp65*) to identify and differentiate mycobacterial species as described below.

### Smear microscopy

The presence of acid fast bacilli in different clinical specimens was determined by Ziehl-Neelsen staining (Stewart, 1953) in the Public Health Laboratory of Recife (Unified Health System - SUS), in accordance with the guidelines of the Brazilian Ministry of Health<sup>29</sup>.

### Specimen processing

In total, of 1-5 ml of various clinical specimens (except for the sterile samples collected) was processed using the modified Petroff method in 4% NaOH<sup>29,30</sup>.

### Culture

Cultures were performed in a Lowenstein-Jensen medium<sup>29,31</sup> and mycobacterium species were identified using the following biochemical tests: selective inhibition by para-nitrobenzoic (PNB) acid and thiophene-2-carboxylic hydrazide (TCH) acid, niacin accumulation and heat-stable catalase at 68°C<sup>29</sup>. The cultures analyzed using the biochemical tests in this study were considered as references for comparison with the results obtained by multiplex PCR and PRA-*hsp65*.

### DNA extraction

DNA was extracted and purified from reference strains of *Mycobacterium tuberculosis* (H37Rv). In all, 40 species of mycobacteria were isolated on a Lowenstein-Jensen medium from clinical samples obtained from 37 patients diagnosed with pulmonary and extrapulmonary tuberculosis using the conventional method described by Sambrook et al. using a mixture of solvents and phenol chloroform<sup>32</sup>.

### Polymerase chain reaction and restriction enzyme analysis

The polymerase chain reaction and restriction enzyme analysis (PRA-*hsp65*) were performed using the techniques described in Telenti et al.<sup>13</sup> The identification was determined by comparing the sizes of the fragments with the algorithm described in the PRASITE site (<http://app.chuv.ch/prasite/index.html>)<sup>17</sup>.

### Multiplex polymerase chain reaction

In multiplex PCR, there are 3 pairs of primers, 1 pair for each target. The gene encoding the antigen of *Mycobacterium tuberculosis* 65KDa, the *dnaJ* gene and the insertion element IS6110 were used in a single reaction with a mixture containing 10mM Tris-HCl (pH 8.3); 50mM KCl; 2.5mM MgCl<sub>2</sub>; 2mM dNTP; 2.5U of Taq DNA Polymerase (Invitrogen); and 20pmol, 50pmol and 10pmol of each oligonucleotide pair, respectively. A total of 2µL of DNA at a concentration of 20ng/µL for each strain of mycobacteria was added to the reaction mixture for

a final volume of 50 $\mu$ L. These reaction parameters are in accordance with the conditions described by Poroca et al.<sup>26</sup>.

### Statistical analysis

The value of Kappa was determined using SPSS statistical software for Windows (version 18; SPSS, Chicago, IL).

### Ethical considerations

The research was approved by the Ethics Committee of the Aggeu Magalhães Research Center (CPqAM) and the respective ethics committees of the participating hospitals under protocol No. 39/2009.

## RESULTS

### Clinical, epidemiological and laboratory findings

Of the 37 patients diagnosed with tuberculosis, 48.7% (n=18) of the cases originated from the ward, and 59.5% (n=22) were males aged 12 to 64 years (median 39 years). The clinical results showed that 70.3% (n=26) of the patients had pulmonary tuberculosis. The main clinical symptoms found were weight loss (90%), cough (80%) and fever (62.5%). Among the patients' pre-existing diseases, 24.3% (n=9) were infected with the human immunodeficiency virus (HIV) virus and 13.5% (n=5) previously had tuberculosis. In laboratory tests, 82% had chest X-rays and 21.7% had the Mantoux tuberculin skin test. A smear of lung samples was taken from 73% (n=19) of the patients; 17 were sputum samples and 2 were bronchoalveolar lavages, and 94.4% (n=18) of the specimens lung specimens were positive for The presence of acid fast bacilli in different clinical specimens was determined by Ziehl-Neelsen staining (Stewart, 1953) in the Public Health Laboratory of Recife (Unified Health System - SUS), in accordance with the guidelines of the Brazilian Ministry of Health. Mycobacterial strains were isolated in Lowenstein-Jensen culture medium for the 40 biological specimens analyzed (Table 1).

### Identification of the mycobacterial species by culture and biochemical testing (reference test)

With respect to the reference test, of the 40 mycobacterial strains isolated by culture from the specimens obtained from the 37 patients diagnosed with tuberculosis, 57.5% (n=23) of the strains were identified as belonging to the *M. tuberculosis* complex, 20% (n=8) of the strains were identified as non-tuberculous mycobacteria (NTM) and 22.5% (n=9) of the results were inconclusive.

### Evaluation of multiplex PCR using mycobacterial species

Of the 40 mycobacterial isolates, 27 (67.5%) showed the molecular pattern for *M. tuberculosis* with the simultaneous amplification of the three targets IS6110 (541bp), *dnaJ* (365bp) and *hsp65* (165bp). In 13 of the samples (32.5%), amplification of a fragment sized 20 to 40 base pairs less than 165bp was observed, which characterized these samples as NTM, according to Poroca et al.<sup>26</sup> (Figure 1).

TABLE 1 - Clinical, epidemiological and laboratory characteristics of the 37 patients studied.

Characteristic	Pulmonary TB		Extrapulmonary TB	
	n	%	n	%
Out-patients	10	27.0	9	24.4
Ward	16	43.2	2	5.5
Sex				
male	17	45.9	5	13.5
female	9	24.3	6	16.2
Pre-existing disease				
HIV	1	2.7	5	13.5
TB re-treatment	4	10.8	1	2.7
Laboratory exams				
smear	19	73.0	0	0.0
culture	26	65.0	14	35.0

HIV: human immunodeficiency virus; TB: tuberculosis.

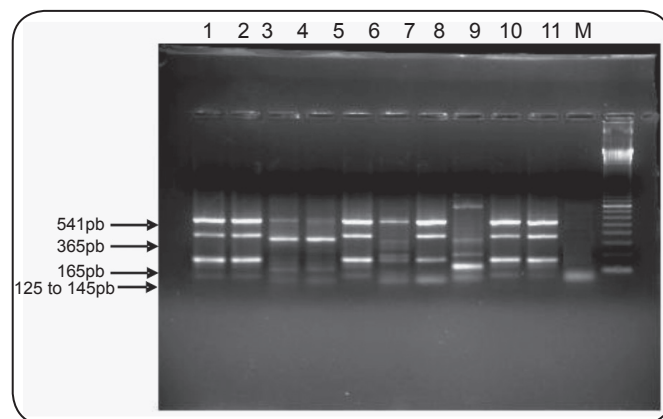


FIGURE 1 - Multiplex PCR of mycobacterial isolates from patients diagnosed with tuberculosis. Bands 1, 2, 5 and 7 represent the mycobacterial species displaying the molecular pattern of *M. tuberculosis*; Bands 3, 4, 6 and 8 represent the mycobacterial species displaying the molecular pattern of NTM; Bands 9 and 10 represent genomic DNA for *M. tuberculosis* (H37Rv); Band 11 represents a negative control for the reaction; Band M represents a 50bp DNA ladder (New England BioLabs, Hitchin, Hertfordshire, UK).

### Species identification using restriction enzyme analysis

When the mycobacterial species were identified by analyzing the banding pattern obtained by the PRA-*hsp65* technique, 29 (72.5%) of the species were identified as the *Mycobacterium tuberculosis* complex; 4 (10%) of the species as *Mycobacterium fortuitum* 2; one (2.5%) of the species as *Mycobacterium abscessus* 2, *Mycobacterium bolletii*/*Mycobacterium massiliense*; one (2.5%) of the species as *Mycobacterium gastritis* 2/*M. kansasii* 6 and one (2.5%) of the species as *Mycobacterium parmense* type 1, whereas 4 (10%) of the results were inconclusive. None of the results indicated the presence of the *M. avium* complex or



*M. intracellulare* among the strains isolated via culture from the pulmonary and extrapulmonary tuberculosis samples.

### Results of the multiplex PCR and PRA-*hsp65* with strains whose results were inconclusive by the reference test (culture and biochemical testing)

In this study, 9 (22.5%) of the mycobacteria samples isolated in culture from the clinical specimens of patients diagnosed with tuberculosis could not be identified by culture and biochemical tests; however, 5 (55.5%) of these strains were identified as *M. tuberculosis* by multiplex PCR, and 4 (44.4%) were identified as NTM. When using the PRA-*hsp65*, 6 (66.6%) strains were identified as an *M. tuberculosis* complex, and 3 (33.3%) could not be identified by PRA-*hsp65*. The diagnosis of tuberculosis in these patients was performed by the medical health service based on smear and/or clinical symptoms.

### Comparison of the reference test with multiplex PCR and PRA-*hsp65*

The results of the multiplex PCR and PRA-*hsp65* were compared to the results of the reference test (culture and biochemical assays) and are summarized in **Table 2**. The correlation calculation between the two tests was conducted using the results from 30 strains identified by culture and biochemical tests, as these results were not considered inconclusive in the reference tests and PRA-*hsp65*.

TABLE 2 - Concordance between the results of multiplex PCR and PRA-*hsp65* with the biochemical and phenotypic tests considered for 30 different isolates of mycobacteria differentiated by phenotypic and biochemical tests.

Biochemical and phenotypic tests	Multiplex PCR	PRA- <i>hsp65</i>
MTBC	23	23
NTM	7	7
Total	30	30
Kappa	1	1

MTBC: *Mycobacterium tuberculosis* complex; NTM: non-tuberculous mycobacteria. PCR: polymerase chain reaction; PRA-*hsp65*: polymerase chain reaction and restriction enzyme analysis.

### Data from subjects who had mycobacteria identified as NTM by multiplex PCR and PRA-*hsp65*

We found that 7 (17.5%) strains of mycobacteria were identified as NTM using multiplex PCR and PRA-*hsp65* in 6 individuals who were diagnosed with pulmonary or extrapulmonary tuberculosis. Among those individuals, only one subject had two different isolates derived from clinical urine (non-sterile) and pleural fluid (sterile) specimens. The other 5 patients had only one species of NTM isolated in culture from the clinical urine, sputum or BAL specimens. None of these cases were diagnosed as an NTM infection.

## DISCUSSION

Advances in molecular biology techniques and the information provided by sequencing the complete genome of *M. tuberculosis* prompted the development of new tools for the rapid diagnosis of tuberculosis, including the differentiation of *M. tuberculosis* from other mycobacteria<sup>33,34</sup>. Multiplex PCR has the ability to amplify different targets simultaneously and has been used to detect and identify mycobacteria from the *Mycobacterium tuberculosis* complex and nontuberculous mycobacteria<sup>26,27,35,36</sup>.

In this study, when the multiplex PCR was compared with the reference tests (cultures and phenotypic and biochemical tests) to identify 40 mycobacterial isolates, a significant agreement was observed: 22 (100%) of the 37 cases with a final diagnoses of pulmonary or extrapulmonary tuberculosis presented species of the *Mycobacterium tuberculosis* complex (MTBC) using the reference tests. Multiplex PCR identified the presence of the *M. tuberculosis* complex in 27 (67.5%) of the cases. In 13 (32.5%) of the cases, multiplex PCR showed a molecular pattern consistent with an NTM, thus disagreeing with the final diagnosis made by the attending physician. It is important to be cautious when making a differential diagnosis between tuberculosis and other mycobacteria because the clinical symptoms and X-ray images may be similar. In Pernambuco in 2010, approximately 26.7% of the patients were treated without bacteriological confirmation of pulmonary tuberculosis; the diagnosis was based only on clinical and X-ray findings, which are often inconclusive and provide inferior data relative to those obtained in this study<sup>37</sup>.

The results of the PRA-*hsp65* technique, when compared with the reference tests, showed the same degree of agreement obtained with the multiplex PCR for the *Mycobacterium tuberculosis* complex and NTM. It is notable that the multiplex PCR, because of its ability to amplify three different, specific targets in the same reaction, has the ability to identify the *Mycobacterium tuberculosis* complex and differentiate it from the species *M. tuberculosis*, *M. bovis* and *M. avium*<sup>26,27</sup>. In contrast, although PRA-*hsp65* does not differentiate between the species of the *Mycobacterium tuberculosis* complex, it is able to identify most of the NTM species<sup>5,38</sup>. This tool is recommended when the culture and phenotypic and biochemical tests suggest the presence of NTM species, but it is costly and time-intensive<sup>5</sup>.

During this study, 3 patients diagnosed with pulmonary tuberculosis were beginning their second dose of anti-TB treatment when the species of mycobacteria was identified as NTM by multiplex PCR. In 2 cases, only 1 isolation from a lung sample was obtained, and in one of the cases, the species was identified as *M. gastritis* 2/*M. kansasii* 6 using PRA-*hsp65*. The presence of NTM in a single sample from a non-sterile source requires careful investigation, including requests for new samples to eliminate the possibility of contamination or transitory colonization<sup>11,39</sup>. In this study, no patients were diagnosed by the health service professionals as infected with NTM; therefore, there is a need for patient follow-up using a

new collection of biological specimens, as the diagnosis may be suspect. The present study showed that, despite the high prevalence of tuberculosis in Brazil, the presence of NTM should strongly be considered prior to growing mycobacterial cultures in sterile clinical samples as part of the investigation when patients have symptoms such as an undefined fever.

The findings in this study suggest the need to investigate the presence of NTM in diagnosed lung diseases, following from the recommendations of other studies, which showed that NTM species can be detected in cases where TB is being re-treated<sup>7,8</sup>. The most frequently isolated species belong to the *M. avium* (MAC) complex and *M. kansasii*<sup>40,41</sup>. Ueki et al.<sup>11</sup> demonstrated that the most prevalent species in the lung compartment was *M. kansasii* and MAC in disseminated diseases<sup>11</sup>. For the treatment of pulmonary NTM infections, laboratory support is needed both to identify species and to determine the *in vitro* profile of resistance to antimicrobial agents. *M. kansasii* remains the most easily treatable form of pulmonary infections; there is a strong correlation between the *in vitro* susceptibility and the *in vivo* response to rifampicin, macrolides and fluoroquinolones. The greatest risk is the emergence of drug-resistant strains, similar to what is observed with *M. tuberculosis*<sup>42</sup>, when patients fail to follow the correct treatment regimen.

Among the cases diagnosed as extrapulmonary tuberculosis, we isolated the same species of mycobacteria (*M. fortuitum* 2) in Lowenstein Jensen (LJ) culture medium from samples of urine and pleural fluid, where the bacteriological diagnosis for NTM can be defined according to ATS criteria because the pleural fluid is considered a sterile sample<sup>28</sup>. The *M. fortuitum* 2 species were identified by phenotypic and biochemical tests, multiplex PCR and PRA-*hsp65*. *M. fortuitum* is a rapidly growing mycobacterium. Although its pathogenic potential is very low, it is often acquired in hospitals and can infect immunocompromised patients. One patient was HIV-positive and died as soon as he began treatment for tuberculosis, which indicates the increasing clinical importance of identifying mycobacterial species<sup>43,44</sup>. Several studies have demonstrate the importance of *M. fortuitum* infections in immunocompromised patients and found that the most prevalent species in these cases include the *Mycobacterium avium* complex, *Mycobacterium kansasii* and *Mycobacterium fortuitum*<sup>10,11,45</sup>.

It can therefore be concluded that multiplex PCR has the ability to identify and differentiate species of *M. tuberculosis* and NTMs and thus can be used as an auxiliary tool in the differential diagnosis of tuberculosis and diseases caused by NTMs. However, multiplex PCR should not be used as a sole laboratory method; it is important that the differential diagnosis be based on the joint analysis of various parameters using PRA-*hsp65* tests and the sequencing of specific genes that may identify the NTM species.

The speed and accuracy of multiplex PCR to differentiate between the *M. tuberculosis* complex and NTMs enables it to be used as an alternative to phenotypic and biochemical tests when identifying species of the *M. tuberculosis* complex and when screening to determine the NTM species present by sequencing specific genes.

Thus, molecular methods (multiplex PCR, PRA-*hsp65* and sequencing of specific genes) can support diagnoses based on clinical determinations regarding mycobacterial infections, and the implementation of these methods, particularly in reference to services for those patients with tuberculosis (TB), Multidrug-resistant tuberculosis (MDR- TB) and Human Immunodeficiency Virus/ Acquired Immunodeficiency Syndrome (HIV/AIDS), reduces the possibility of an inadequate diagnosis and treatment.

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## CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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