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Simultaneous Detection of *Mycobacterium tuberculosis* and *Mycobacterium avium* Complex by a Multiplex PCR

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HIGHLIGHTS

- A multiplex PCR to identifying mycobacteria of medical interest was evaluated.
- *Mycobacterium tuberculosis* can be differentiated from other mycobacteria with the assay.
- The assay showed high sensitivity and specificity in detecting the *M. avium* complex.

Abstract: Infection disease caused by nontuberculous mycobacteria (NTM) have been increasingly reported and often manifest with the same symptoms of tuberculosis (TB). The identification of the causative agent is fundamental for the determination of an appropriate therapy, since each species of mycobacteria requires a specific treatment. Thus, rapid and accurate tests to identify mycobacteria of medical interest, such as *Mycobacterium tuberculosis* complex (MTBC) and *Mycobacterium avium* complex (MAC), are necessary in the clinical routine. The present study evaluated an in-house multiplex PCR to detect, in reference and clinical strains, the genus *Mycobacterium* and also *M. tuberculosis* and MAC. To identify the *Mycobacterium* genus, *M. tuberculosis* and MAC, it was amplified a fragment of *hsp65* gene, *esat-6* gene, and the internal transcribed spacer between the 16S and 23S rRNA genes, respectively. In total, 87 mycobacteria strains were used, being 10 reference and 77 clinical strains, previously identified as MTBC (n = 66), *M. avium* (n = 8) or other NTM specie (n = 13). The *hsp65* gene fragment was amplified for all mycobacteria strains evaluated (87/87). This multiplex PCR presented sensitivity of 100% and specificity of 95.2% for *M. tuberculosis* detection, and sensitivity of 100% and specificity of 100% for MAC detection. The multiplex PCR evaluated is an important tool for the differentiation between *M. tuberculosis* and NTM, as well as for the identification of MAC, a complex composed by species with high prevalence in the world.

Keywords: *esat-6*; molecular identification; nontuberculous mycobacteria; tuberculosis; *Mycobacterium avium.*

INTRODUCTION

Genus *Mycobacterium* is constituted for more than 180 species, some of these are responsible for serious diseases in humans [1]. Tuberculosis (TB), caused by *Mycobacterium tuberculosis*, the most important specie of the *M. tuberculosis* complex (MTBC), remains a global health problem, affecting millions of people every year [2]. Additionally, infection disease caused by nontuberculous mycobacteria (NTM), like the species of *Mycobacterium avium* complex (MAC), have been increasingly reported and can cause lung diseases, with clinical manifestations similar to TB [3,4].

MAC infections and *M. tuberculosis* are often reported in patients living with HIV/AIDS, and appropriate clinical management of this population should be immediate, since mycobacterial infections accelerate HIV disease progression [3,5]. In this sense, the differentiation of *M. tuberculosis* and *M. avium* complex from NTM is important, especially to increase the success of the treatment, since this is specie-specific [3].

In recent years, commercial and in-house molecular methods, based on polymerase chain reaction (PCR), have been proposed for the identification of mycobacterial infections. In this way, the amplification of DNA by PCR became a widely used technique, with multiple targets available. The insertion element IS*6110*, described by Thierry and coauthors [6], is the most commonly used target for identification of MTBC [7,8], however other targets are being investigated for the diagnosis of TB, such as the *esat-6* gene, used to identify *M. tuberculosis* [9]. For the differentiation between mycobacteria, the internal transcribed spacer (ITS), between the 16S and 23S rRNA genes, has been suggested as potential target [10]; in addition, the 65-kDa heat shock protein gene (*hsp65*), present in all mycobacteria, is one of the main targets used for the identification of mycobacteria [11].

In this context, studies have been proposing in-house PCR to identify mycobacteria species of medical interest, with the intention of introducing these methodologies in the diagnostic routine [12]. The present study evaluated a previously described in-house multiplex PCR [12] to detect the genus *Mycobacterium*, *M. tuberculosis* and MAC in reference and clinical strains.

MATERIAL AND METHODS

Study design

In total, 87 mycobacteria strains were used, being 10 reference strains and 77 clinical strains of MTBC and NTM. The strains were stored in the bacterial collection of Mycobacteria Laboratory, at Federal University of Rio Grande, Rio Grande do Sul, Brazil. The clinical strains included in this study were positive in liquid culture in BACTEC™ MGIT™ (BD - Becton, Dickinson and Company) and previously identified. The MTBC strains were identified by conventional PCR for detection of a 245 base pairs (bp) fragment of IS*6110* [13], and the NTM strains were identified by partial sequencing of genes *hsp65* and *rpoB* [14].

Mycobacteria strains

The reference strains used in the study were of the species: *M. tuberculosis* (H37Rv), *M. avium* (03057HC), *M. abscessus* (ATCC 19975), *M. peregrinum* (ATCC 14467), *M. chelonae* (ATCC 946), *M. gordonae* (ATCC 14470), *M. fortuitum* (ATCC 35931), *M. malmoense* (ATCC 295711), *M. marinum* (ATCC 927), and *M. xenopi* (ATCC 19156); and the clinical strains belonged to the following species: MTBC (n = 65), *M. avium* (n = 7), *M. abscessus* (n = 3), and *M. massiliense* (n = 2).

DNA extraction

Total DNA was obtained by heat treatment [11]. The mycobacteria colonies cultivated in Ogawa-Kudoh medium were suspended in 600 µL of TE (10 mM Tris-1 mM EDTA, pH 7.4). The bacterial suspension was inactivated at 85°C for 30 min, and centrifuged at 5000 rpm for 5 min. The supernatant was collected and stored at -20ºC until use.

Multiplex PCR

To identify the genus *Mycobacterium*, TB11 (5'-ACCAACGATGGTGTGTCCAT-3') and TB12 (5'-CTTGTCGAACCGCATACCCT-3') primers were used to amplify a 441 bp fragment of *hsp65* gene [15]. To identify *M. tuberculosis*, ESAT-6 F (5'-GCGGATCCCATGACAGAGCAGCAGTGGA-3') and ESAT-6 R (5'-CCAAGCTTCCTATGCGAACATCCCAGTGACG-3') primers were used to amplify a 320 bp fragment of *esat-6* gene (6 kDa early secretory antigenic target gene) [9]. To identify MAC, ITS F (5'-CCCTGAGACAACACTCGGTC-3') and ITS R (5'-ATTACACATTTCGATGAACGC-3') primers were used to

amplify a 144 bp fragment of internal transcribed spacer (ITS) of this complex, between the 16S and 23S rRNA genes [10].

The multiplex PCR assay was performed as described by Sankar and coauthors [12], with some modifications in PCR MIX according to previous standardization of reagents to optimize their use in laboratory routine. Briefly, the PCR reaction was set up for 25 µL final volume and contained 2 µL of DNA, ultra pure water, PCR buffer (200 mM Tris-HCl [pH 8,4], 500 mM KCl), dimethyl sulfoxide, $MgCl_2$, 7,5 pmole of ESAT-6 primers, 17,5 pmole of ITS primers, 12,5 pmole of TB11 and TB12 primers, deoxynucleosides triphosphate and 1 U of T*aq* DNA polymerase (Invitrogen, EUA). The PCR cycling conditions were 10 minutes at 95°C, followed by 30 cycles of denaturation, annealing and extension at 95°C, 59°C and 72°C, respectively, for 1 minute each, with a final step at 72°C for 10 minutes. To validate the PCR performed, in all reactions were used a negative control (without DNA) and two positive controls (one containing genomic DNA of a reference strain of *M. tuberculosis* and other of a reference strain of *M. avium*). The PCR products were subjected to electrophoresis on 1.5% agarose gel, stained with ethidium bromide 0.001 mg/mL and visualized in UV light. The results obtained by multiplex PCR were compared with previous identification. Sensitivity and specificity of multiplex PCR for *M. tuberculosis* and MAC detection were determined.

Ethical aspects

The present study did not require approval by the Research Ethics Committee of the Federal University of Rio Grande, according to the committee's regulations.

RESULTS

In our study, the *hsp65* gene fragment was amplified for all mycobacteria strains evaluated (87/87). Additionally, the amplification of *esat-6* gene and ITS fragments was observed in all MTBC (66/66) and MAC (8/8) strains, respectively. On the other hand, all strains from other NTM specie (13/13) were negative for ITS, but the reference strain of *M. marinum* was positive for *esat-6* (Figure 1). These results obtained can be visualized in Table 1. This multiplex PCR presented sensitivity of 100% and specificity of 95.2% for *M. tuberculosis* detection, and sensitivity and specificity of 100% for MAC detection.

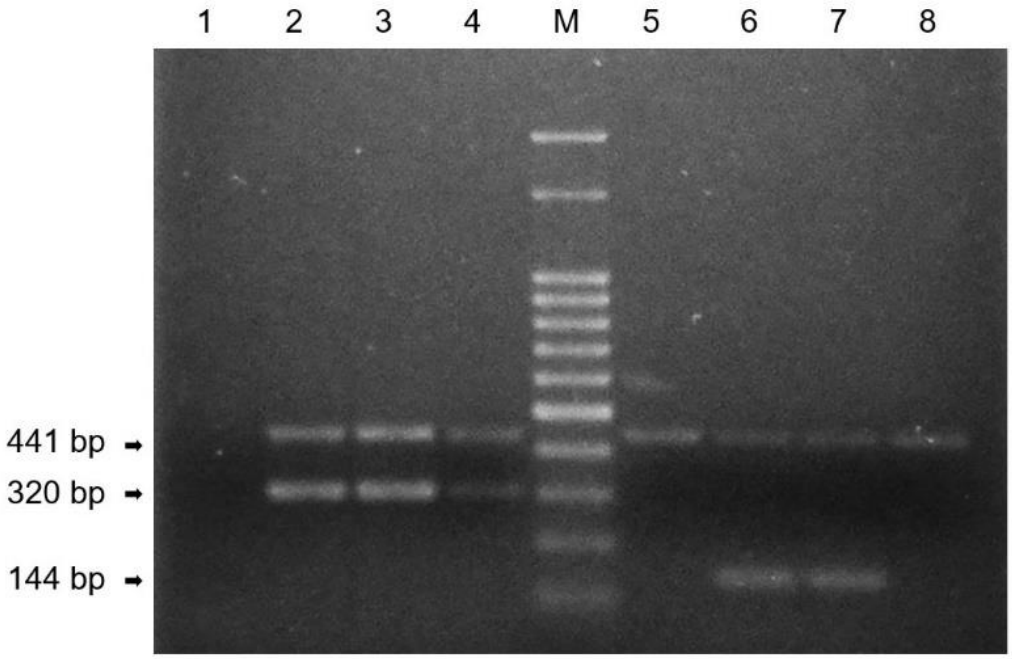

Figure 1. Agarose gel electrophoresis representing the amplification of *hsp65* (441 bp), *esat-6* (320 bp) and ITS (144 bp) fragments. Column 1: negative control. Column 2: *M. tuberculosis* (H37Rv). Column 3: *M. tuberculosis* (clinical strain). Column 4: *M. marinum* (ATCC 927). Column 5: *M. abscessus* (ATCC 19975). Column 6: *M. avium* (03057HC). Column 7: *M. avium* (clinical strain). Column 8: *M. chelonae* (ATCC 946). Column M: 100 bp marker.

Table 1. Amplification of *hsp65*, *esat-6* and ITS fragments in reference and clinical strains.

Mycobacterial species	Strains number	Target amplicon		
		hsp65	***esat-6***	**ITS**
Reference strains				
M. tuberculosis H37Rv	1	+	+	-
M. avium 03057HC	1	+	-	+
M. abscessus ATCC 19975	1	+	-	-
M. peregrinum ATCC 14467	1	+	-	-
M. chelonae ATCC 946	1	+	-	-
M. gordonae ATCC 14470	1	+	-	-
M. fortuitum ATCC 35931	1	+	-	-
M. malmoense ATCC 29571	1	+	-	-
M. marinum ATCC 927	1	+	+	-
M. xenopi ATCC 19156	1	+	-	-
Clinical strains				
M. tuberculosis complex	65	+	+	-
M. avium	7	+	-	+
M. abscessus	3	+	-	-
M. massiliense	2	+	-	-

(+) amplified fragment, (-) non-amplified fragment

DISCUSSION

Commercial PCR-based assays have been developed and are available for the diagnosis of mycobacterial infections. However, most of these tests are expensive and laborious, which implies in its difficult implementation in several places [16]. The nucleic acid amplification by conventional PCR, a cheaper alternative for mycobacteria identification, is the most frequently available, and the multiplex PCR is enabled to identify multiple targets and, consequently, to detect more than one microorganism in a single tube [12]. The method evaluated is simple to perform and does not require a more advanced molecular biology infrastructure, as it is a conventional in-house PCR assay. In addition, it can generate results in one day, since the PCR and electrophoresis steps (duration of approximately 4 hours) do not need to be repeated for each target investigated.

The multiplex PCR evaluated in this study was proposed by Sankar and coauthors [12], but it was not evaluated for MAC. For Sankar and coauthors [12], the in-house PCR was effective to detect the genus *Mycobacterium* in 85.4% (135/158) of clinical strains, but all samples were from patients with TB. Of these strains, 95.5% (129/135) were classified as *M. tuberculosis* and 4.4% (6/135) as NTM. Our results showed that the multiplex PCR is an effective assay for the identification of mycobacterial strains, being able to differentiate strains of *M. tuberculosis* and MAC with high sensitivity (100% and 100%, respectively) and specificity (95.2% and 100%, respectively).

In our study we found one limitation for this multiplex PCR. We observed that the multiplex PCR was effective to identify *M. tuberculosis* in clinical strains. However, just like us, Singh and coauthors [9] found

that *esat-6* was present in *M. marinum*, generating a false positive result for TB. Despite this, the frequency of isolation of *M. marinum* in laboratories is low, and the infection caused by this mycobacteria is often related to aquatic exposure [17,18]. In addition, the most frequent manifestation of *M. marinum* infection is cutaneous lesion [17], differing clinically of the manifestations of TB. Cases of cutaneous TB are rare, representing 1 to 2% of all TB cases [19]. Thus, it is emphasized the importance of to associate microbiological diagnosis with clinical findings for the correct identification of mycobacterial infection [3].

The multiplex PCR evaluated in the current study is an important tool for the differentiation of *M. tuberculosis* and NTM, as well as for the identification of MAC. The rapid and accurate diagnosis of infections caused by mycobacteria, allows a better management of the patient, besides allowing the choice of the appropriate therapeutic regimen, since the treatment is different according to the etiological agent of the disease. Considering a wide variety of NTM species that cause infections in humans, the present multiplex PCR can be expanded in the future for the detection of other mycobacteria of medical interest through the addition of different molecular targets. In addition, despite being a conventional PCR, the assay evaluated shows promise to be adapted for real-time PCR in laboratories that have appropriate infrastructure.

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Conflicts of Interest: The authors declare no conflict of interest.

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