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Short Communication

Identification of *Mycobacterium tuberculosis* complex by polymerase chain reaction of Exact Tandem Repeat-D fragment from mycobacterial cultures

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ABSTRACT

This study evaluated an in-house polymerase chain reaction (PCR) for rapid identification of the *Mycobacterium tuberculosis* complex (MTBC) using the MTBC-specific Exact Tandem Repeat D (ETR-D) as the amplification target. In a prospective study, 801 clinical isolates identified as MTBC and 15 nontuberculous mycobacteria were analyzed. Mycobacterial DNA was extracted from automated broth cultures or from egg-based media. The amplification of the ETR-D showed to a sensitivity of 99.6% and a specificity of 100% for the correct identification of MTBC; improved extractions protocols led to 100% sensitivity. The main utility of this technique is the simplicity, rapidity, low cost and accuracy.

Introduction

In Uruguay, tuberculosis (TB) is still a health problem. The notified annual incidence is 24 × 100,000 inhabitants, and it has remained stable in the last 10 years. The isolation of other opportunistic nontuberculous mycobacteria (NTM) as etiological agents of mycobacteriosis is restricted to the HIV/AIDS population.

To improve the control of tuberculosis, TB control programs must have access to rapid and accurate laboratory diagnosis [1]. New tools to improve the laboratory diagnosis of TB have been developed in the last years [1]. Automated broth culture (ABC) systems are now able to detect mycobacterial growth within 1–3 weeks while solid media take 3–8 weeks [2]. Moreover, nucleic acid amplification (NAA) assays for identification of mycobacteria improve sensitivity and time-to-diagnosis compared with phenotypic identification. Sequence analysis of the Exact Tandem Repeat-D (ETR-D; synonym MIRU04) was postulated as a rapid method for the identification of members of the *Mycobacterium tuberculosis* complex (MTBC) [3].

The aim of this study was to evaluate the ETR-D region as a PCR target to detect and differentiate members of MTBC from NTM.

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Methods

Study design

The study was carried out with samples obtained from July 2010 to May 2012 by the National TB Reference Center of Uruguay. In total, 816 positive cultures obtained from patients with clinical and radiological suspected TB, or other mycobacterial disease, were included. To determine sensitivity and specificity, a positive culture on Löwenstein–Jensen medium was used as the gold standard for the presence of TB. Phenotypic and genotyping characterization was performed using previously described methods [4,5].

Strains

Two hundred and forty-six strains recovered from ABC isolates (biopsies, exudates, and punction fluid samples) were evaluated using the MB-BacT TB system (Biomerieux®), and 570 strains recovered from sputum cultured in egg-based media (Löwenstein–Jensen and Ogawa).

Phenotypic and genotypic identification

Phenotypic characterization was performed using previously described methods [4,5], and complementary genotyping identification, if it was needed, was made using the hsp65 PCR-restriction fragment length polymorphism analysis as described by Telenti [6] and Devallois [7]; 801 strains were identified as members of MTBC (797 *M. tuberculosis* and 4 *M. bovis*). The other 15 strains were identified as NTM (*M. avium*: 7; *M. intracellulare st18*: 2; *M. kansasii*: 2; *M. fortuitum*: 2; *M. abscessus*: 2).

Also tested were the reference strains: *M. tuberculosis* H37Rv (ATCC 27294), *M. avium* (ATCC 25291), *M. kansasii* (ATCC 12478), *M. scrofulaceum* (ATCC 19981), *M. bovis* (AN5 Rotterdam) and *M. abscessus*, *M. fortuitum*, *M. terrae*, and *M. bovis*-BCG, from the National Reference Culture Collection of Uruguay (NRCCU) as controls. *M. africanum* and other rare members of the complex not encountered in Uruguay were not included in this work.

DNA extraction

Five milliliters of positive ABC fluid or 1 ml suspension from a loop-full of bacterial colonies were centrifuged at 4000*g* × 30 min; the pellet was re-suspended in 0.5 ml of purified sterile water and heated (95 °C, 30 min); after cooling, the sample was frozen and thawed three times (protocol from [8] with minor modifications).

PCR conditions

The reaction mix contained 1 pmol of previously described primers [3], 1 Unit of Taq DNA polymerase (Fermentas, Germany), 1× reaction buffer and 0.25 mM of dNTPs. The reaction conditions involved an initial step at 95 °C for 3 min, 35 cycles at 95 °C for 30 s, at 60 °C for 20 s and 72 °C for 30 s, with 3 min at 72 °C for the final extension. The amplified products were electrophoresed in 2% agarose and visualized with ethidium bromide. Parallel partial *rpoB* PCR amplification [9] was used as a control of inhibition. Cross-contamination was not a problem, since all negative controls remained negative throughout the study.

Sequence analysis

ETR-D amplicons were sequenced with BigDyeTerminator chemistry (Applied Biosystems, USA) in ABI3130 Genetic Analyzer (Applied Biosystems, USA) and analyzed in SequenceScanner v1.0 (Applied Biosystems, USA).

Results

ETR-D PCR results are shown in Table 1. The sensitivity of the ETR-D assay was 99.6% and specificity was 100%, with a positive predictive value (PPV) of 100% and a negative predictive value (NPV) of 88%.

The three isolates with discrepant results were re-analyzed with a fresh DNA extraction, and these proved ETR-D positive. All the different lengths of ETR-D amplicons were sequenced to confirm the numbers and lengths of repeats (Table 2). Four isolates phenotypically identified as *M. bovis* showed PCR fragment lengths (680 bp) similar to those observed in *M. bovis* reference strains.

Seven strains with a fragment length of 824 bp and two strains with fragment lengths of 528 bp were identified as *M. tuberculosis* (data included in the total 798 strains).

Discussion

ETR-D PCR is a fast and accurate method for preliminary identification of positive mycobacterial cultures, with a sensitivity and specificity of 99.6% and 100%, respectively, and a PPV of 100% and an NPV of 88% (calculations according to [10]). Three of 801 (0.003%) MTBC isolates showed false negative results in the first amplification. Because a negative result indicates NTM infection, it may be recommended to repeat amplification (although *rpoB* control works) to prevent false negative results, especially if isolates resemble *M. tuberculosis* complex bacteria, phenotypically. The assay's performance could be enhanced with the optimization of the DNA extraction process and the use of an internal control of amplification with similar efficiencies as the ETR-D amplicon.

Of the 801 clinical MTBC isolates, 797 (98%) corresponded to *M. tuberculosis*, based on ETR-D fragment length, four isolates identified as *M. bovis* showed a distinct fragment length (680 bp), allowing the researchers to suspect the presence of *M. bovis* as an etiological agent, an added advantage.

Djelouadji et al. [3] reported more heterogeneity in the length of ETR-D. These differences could be explained by limited genotypic diversity of MTBC in Uruguay, where Latin-American Mediterranean, S and T genotypes make up 84% of all isolates [11].

This assay is easy to perform, less expensive than commercially available NAA assays or immunochromatographic tests and permits differentiation between MTBC and NTM within hours. The average turnaround time was 10 days for PCR-ETR-D faster than phenotypic identification.

Table 1 – ETR-D PCR results of 816 strains analyzed.

Phenotypic/genotypic identification	ETR-D positive	ETR-D negative	Total
MTBC[a]	798	3	801
NTM[b]	0	15	15
Total	798	18	816

Sensitivity: 99.6%; Specificity: 100%; PPV: 100%; NPV: 88.3%.

[a] MTBC = *M. tuberculosis*: 794; *M. bovis*: 4.

[b] NTM = *M. avium*: 7; *M. intracellulare st18*: 2; *M. kansasii*: 2; *M. fortuitum*: 2; *M. abscessus*: 2.

Table 2 – Length distribution of the ETR-D amplicon. The number of repeats was confirmed by sequencing ETR-D representative amplicons.

Length in bp	No. of repeats	Clinical isolates	Percentage
608 ± 6	3; 2x77b, 1x53 bp	788	98.12
824 ± 12	6; 5x77 bp, 1x53 bp	7	0.88
672 ± 6	4; 3x77 bp, 1x53 bp	4	0.75
528 ± 6	2; 1x77 bp, 1x53 bp	2	0.25
Total		801	100.0
Reference Strains			
631	3; 3x77 bp	H37Rv (ATCC 27294)	
555	2; 2x77 bp	*M. bovis*-BCG (NCCU)	
671	4; 3x77b, 1x53 bp	*M. bovis* (AN5 Rott.)	

Many in-house assays for identification of MTBC use the IS*6110* element as amplification target [12–14]. However, false-positive results have been reported in some NTM, probably related with primers design [11]. Also, false negative results have been described in *M. tuberculosis* strains devoid of IS*6110*, isolated from East Asian patients [14], so, this technique could be useful for this geographic region.

In conclusion, the ETR-D PCR allows in one step the accurate detection of MTBC discriminating from NTM, and the ability to suspect *M. bovis* as a TB etiological agent.

Conflict of interest

The authors declare that they have no conflict of interest.

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