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ORIGINAL ARTICLE

Rapid identification of *Mycobacterium tuberculosis* in cultures by molecular and immunological methods

Identificação rápida de *Mycobacterium tuberculosis* em culturas por método molecular e método imunológico

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Abstract

Objective: The study aimed to evaluate molecular and immunological methods and to propose a workflow using them for tuberculosis (TB) diagnosis routine. **Methods**: A cross-sectional retrospective study was performed, including 121 liquid cultures from a TB laboratory located in the extreme south of Brazil. All cultures were positive for *Mycobacterium tuberculosis* complex (MTBC) by in-house Polymerase Chain Reaction (PCR) using DNA extracted by the CTAB method (PCR-CTAB) for *IS6110* detection. These cultures were subjected to faster tests than this one, the immunological MPT64 assay and the PCR using DNA extracted by thermal lysis method (PCR-TL), and these were evaluated for MTBC identification using PCR-CTAB as a reference method. **Results**: The sensitivity of MPT64 assay and PCR-TL to identify MTBC in positive cultures by PCR-CTAB were 73.6% (89/121) and 98.3% (119/121), respectively. We proposed a workflow based on the use of MPT64 assay in liquid cultures suggestive of MTBC, and in case of a negative result, we suggest the performance of PCR-TL. The PCR-CTAB is suggested only if faster tests are negative. **Conclusions**: Methods capable of confirming MTBC in cultures should continue to be standardized, tested, and optimized to meet the ideal requirements of simplicity, quickness, and effectiveness. The molecular and immunological methods evaluated have differences in the execution and detection of MTBC in cultures, but they are rapid tools for laboratory TB diagnosis.

Keywords: Cetrimonium Bromide; Diagnostic Tests; Mycobacteria; MPT64 Protein; Polymerase Chain Reaction.

Resumo

Objetivos: O estudo objetivou avaliar métodos molecular e imunológico e propor um fluxo de trabalho utilizando-os para a rotina de diagnóstico da tuberculose (TB). **Métodos**: Foi realizado um estudo transversal retrospectivo, incluindo 121 culturas líquidas de um laboratório de TB localizado no extremo sul do Brasil. Todas as culturas foram positivas para o complexo *Mycobacterium tuberculosis* (CMTB) por Reação em Cadeia da Polimerase (PCR) *in-house* para detecção do *IS6110*, usando DNA extraído pelo método CTAB (PCR-CTAB). Essas culturas foram submetidas a testes mais rápidos que este, o ensaio imunológico MPT64 e a PCR com DNA extraído pelo método de lise térmica (PCR-LT), e estas foram avaliadas para identificação de CMTB usando PCR-CTAB como método de referência. **Resultados**: A sensibilidade do ensaio MPT64 e da PCR-LT para identificar o CMTB em culturas positivas pela PCR-CTAB foi de 73,6% (89/121) e 98,3% (119/121), respectivamente. Propusemos um fluxo de trabalho baseado no uso do ensaio MPT64 em culturas líquidas sugestivas de CMTB e, em caso de resultado negativo, sugerimos a realização de PCR-LT. Sugere-se a PCR-CTAB apenas se os testes mais rápidos forem negativos. **Conclusões:** Os métodos capazes de confirmar o CMTB em culturas devem continuar sendo padronizados, testados e otimizados para atender aos requisitos ideais de simplicidade, rapidez e eficácia. Os métodos molecular e imunológico avaliados apresentam diferenças na execução e detecção do CMTB em culturas, mas são ferramentas rápidas para o diagnóstico laboratorial da TB.

Palavras-chave: Brometo de Cetrimônio; Testes diagnósticos; Micobactérias; Proteína MPT64; Reação em Cadeia da Polimerase.

INTRODUCTION

Tuberculosis (TB), an infectious disease caused by *Mycobacterium tuberculosis*, remains a serious public health problem, with approximately 10 million new cases and 1.514 million deaths (including 214 thousand deaths among patients with HIV/AIDS), estimated in 2020. Aiming for the global TB epidemic to end, the End TB Strategy proposes a 95% reduction in TB deaths and a 90% reduction in TB incidence until 2035, compared with 2015. In this sense, it is recognized that rapid and accurate diagnosis of all forms of TB is essential to disease control[1].

The bacteriological TB diagnosis is essential for the correct management of the TB patient, including the selection of appropriate therapeutic measures. For this, culture is the reference method[1], and the World Health Organization (WHO) recommends the use of liquid culture systems in the diagnostic routine since 2007. Liquid culture, when compared to solid culture, is more sensitive and requires less time to obtain bacterial growth, being an important tool for the diagnosis of paucibacillary and multidrug-resistant TB. However, an additional test remains necessary to confirm the bacterial species that grow in the culture[2].

Molecular methods have been widely used for the identification

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of *M. tuberculosis* complex (MTBC) isolates, and are often based on the detection of the *IS6110* insertion element by Polymerase Chain Reaction (PCR). However, although *IS6110* amplification is related to good accuracy in the diagnosis of TB, the sensitivity and specificity of in-house PCR are variable according to how the methodology is conducted and having as critical determinants the isolation and purity of the DNA used[3,4].

On the other hand, since the rapid specie identification in cultures is preconized by WHO[1], commercial assays such as immunological tests are being introduced into the TB diagnostic routine. These tests can identify MTBC isolates by detecting the 24 kDa MPT64 antigen, a protein secreted by viable MTBC cells. Immunological tests for detecting the MPT64 antigen (MPT64 assay) have the advantage of being simple to perform and release rapid and easy-to-interpret results[5,6].

In this sense, the study aimed to evaluate an MPT64 assay and an in-house PCR using DNA extracted by a simple extraction method, for rapid identification of liquid cultures positive for MTBC. In addition, we proposed a workflow using these methods for TB diagnosis routine in a setting with a high TB burden.

METHODS

Study design

A cross-sectional retrospective study was performed, including 121 liquid cultures by BACTEC™ MGIT™ 320 (Becton, Dickinson and Company, USA) positive for MTBC. The cultures were from samples of patients with TB received from June 2017 to September 2018, at a TB laboratory located in the extreme south of Brazil. This laboratory performs the TB diagnostic service in a reference hospital for the care of patients living with HIV/AIDS in a municipality with a high TB incidence (77.6 new cases per 100,000 inhabitants in 2018 versus the national average of 36.9 new cases per 100,000 inhabitants in the same year)[7,8].

All cultures were positive for MTBC by detecting the *IS6110* using DNA extracted by the CTAB (cetrimonium bromide) method (PCR-CTAB)[9], a method that is efficient for the extraction of mycobacterial DNA. This method was considered the reference method of this study. These cultures were subjected to faster tests than this one for the identification of MTBC, such as the MPT64 assay (SD Bioline TB Ag MPT64 Rapid, Standard Diagnostics Inc, South Korea), and PCR for detection of *IS6110* using DNA extracted by thermal lysis method (PCR-TL)[10]. The results obtained for MPT64 assay and PCR-TL were tabulated in an Excel spreadsheet (Microsoft, USA) and compared to PCR-CTAB. The sensitivity of the methods to detect MTBC in liquid cultures was determined using PCR-CTAB as the gold standard.

Experimental activities

Reference method

DNA extraction by the CTAB method was performed in two steps. In the first step, bacterial inactivation and DNA extraction by thermal lysis were conducted as described by Kabir et al.[10], with some modifications. Bacterial liquid culture (1 mL) was centrifuged for 5 minutes at 1,677 x g. Subsequently, the supernatant was discarded, and the pellet was resuspended in 1x TE (10mM Tris-Cl, 1mM EDTA, pH 8.0), vortexed, and incubated at 85°C for 30 minutes for bacterial inactivation. A new centrifugation step was performed at 1,677 x g for 5 minutes to collect the supernatant containing the DNA.

The second step was performed using a 100 µL aliquot of the supernatant resulting from thermal lysis extraction, as described by van Soolingen et al.[9]. Lysozyme (10 mg mL^{-1}) was initially added to the supernatant and incubated at 37°C for 1 hour. Subsequently, 10% (w/v) sodium dodecyl sulfate (SDS) + Proteinase K (10 mg mL^{-1}) was added, and a new incubation was performed at 65°C for 10 minutes. Following, 5M NaCl and CTAB-NaCl were added before further incubation at 65°C for 10 minutes. After the addition of chloroform:isoamyl alcohol (24:1), the suspension was centrifuged for 8 minutes at 9,660 x g, and after the addition of isopropanol, homogenization by inversion was performed to precipitate nucleic acids. The DNA was stored overnight at -20°C, and then centrifuged at 9,660 x g for 15 minutes. After discarding the supernatant, DNA was washed with 70% (v/v) alcohol. Finally, after 70% alcohol removal, the pellet was dried at room temperature and the DNA was resuspended in 1x TE and stored overnight to be used. For MTBC identification by PCR, we used the primers INS1 (5'-CGTGAGGGCATCGAGGTGGC-3') and INS2 (5'-GCGTAGGCGTCGGTGACAAA-3')[11], which amplify a fragment of 245 basis pairs (bp) of the insertion element *IS6110*.

In the PCR reaction, adapted from Hermans et al.[11], 2 µL of DNA was added to a mix containing Milli-Q water, PCR buffer (Invitrogen™), 1,6 mM $MgCl_2$ (Invitrogen™), dimethyl sulfoxide (DMSO), 0,5 µM each primer, 0,2 µM each deoxynucleotide (dGTP, dATP, dTTP, and dCTP) (LudwigBiotec®) and 1 U of *Taq* polymerase (Invitrogen™).

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. In each PCR performed, a negative control (without DNA) and a positive control containing the reference strain H37Rv DNA were used. Results were analyzed after electrophoresis in 1.5% (w/v) agarose gel, stained with ethidium bromide 0.001 mg mL^{-1}. Bands were viewed through UV radiation emission, using a 100 bp marker (Ludwig Biotec®) to define the size of the PCR products.

Evaluated methods

The MPT64 assay was done and interpreted as recommended by the manufacturer[12]. Results were read 15 minutes after sample application on the test.

In PCR-TL, DNA extraction was performed similarly to the first step of the CTAB DNA extraction method (without step two). Therefore, to perform the PCR assay, the remaining supernatant from thermal lysis containing the DNA was used. PCR reaction

and cycling conditions and electrophoresis conditions were the same between PCR-TL and PCR-CTAB.

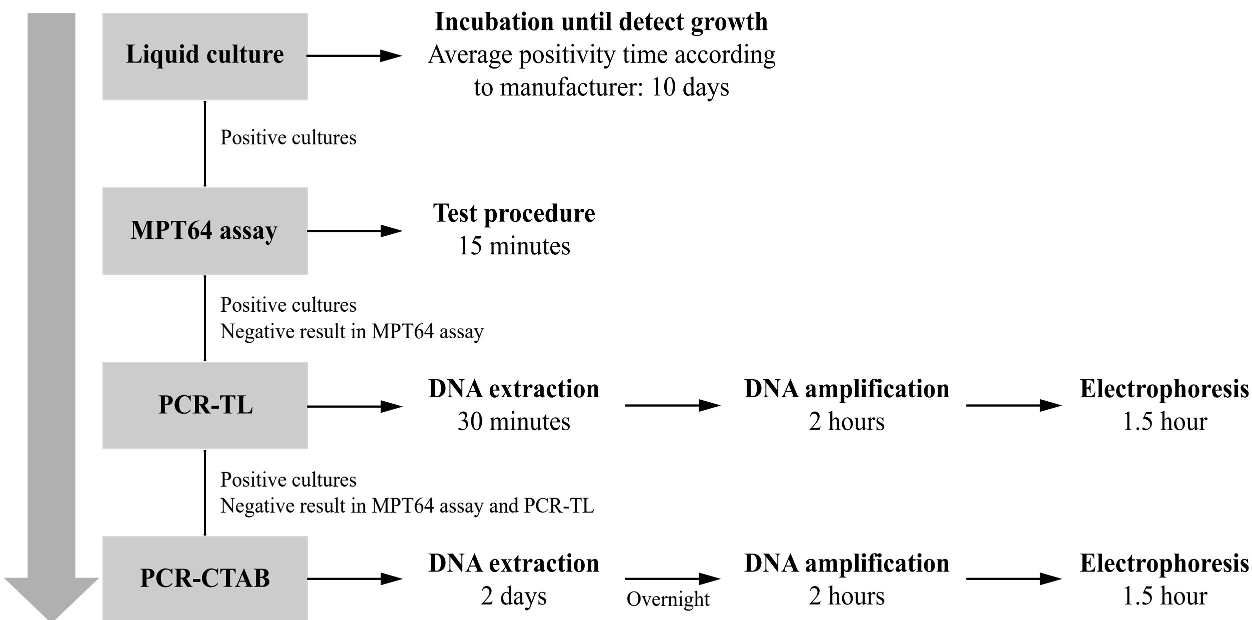
Ethical aspects

The study was conducted within the standards required by the Declaration of Helsinki and was approved by the Health Research Ethics Committee of the Federal University of Rio Grande, under the number 200/2019.

RESULTS

The sensitivity of MPT64 assay and PCR-TL to identify MTBC in positive cultures by PCR-CTAB were 73.6% (89/121) and 98.3% (119/121), respectively. In addition, these methods have different periods for the release of results: while the MPT64 assay can deliver results in less than an hour, the results obtained by PCR-TL and PCR-CTAB can be visualized, after electrophoresis, in 24 and 72 hours, respectively. Therefore, the proposed workflow suggests the use of the MPT64 assay in positive cultures, and in case of negative results by the MPT64 assay, associated with the availability of molecular biology reagents and equipment, the performance of PCR-TL is recommended. The PCR-CTAB is suggested only if faster tests are negative, associated with clinical and imaging findings suggestive of TB (Figure 1).

Figure 1. Suggested workflow for identifying MTBC through the association of methods in positive cultures, including the approximate time to perform each method. Hands-on time is not included. PCR-TL = PCR using DNA extracted by thermal lysis. PCR-CTAB = PCR using DNA extracted by the CTAB method.



DISCUSSION

Culture remains an essential tool for TB diagnosis and for drug susceptibility profile identification[1]. However, despite being a sensitive method for the diagnosis of TB, it allows the growth of other microorganisms different from MTBC, such as non-tuberculous mycobacteria. Thus, it is necessary to identify the growth species by additional assays[2], such as the MPT64 and PCR assays evaluated.

The PCR-CTAB, used in this study as a reference method, is a more laborious and time-consuming method. However, this method uses reagents and steps that can reduce the possible presence of PCR inhibitors, increasing the sensitivity of PCR[13]. Compared to this method, the PCR-TL showed higher detection of MTBC than the MPT64 assay.

About PCR-TL, this method identified a different number of MTBC isolates, when compared to PCR-CTAB. Both methods presented the same PCR steps but differed in terms of the DNA extraction method. Mycobacterial DNA extraction methods should lyse the bacillus cell wall and remove organic and non-organic molecules that may impair DNA amplification, in addition to minimizing the loss of DNA and keeping it intact throughout the process[3].

The presence of proteins and polysaccharides in template DNA, for example, may inhibit DNA polymerase activity, interfering in the reaction. Thus, lysozyme and SDS act on the lysis of the mycobacterial cell wall. CTAB forms a complex with polysaccharides and proteins that have not been degraded by proteinase K; this complex is eliminated after the addition of chloroform:isoamino alcohol[14]. It is noteworthy that, although the various steps of the protocol are important in DNA

purification. PCR-TL requires fewer steps and reagents, making it a simpler, faster, and lower-cost method when compared to PCR-CTAB.

Regarding the MPT64 assays, these have already been shown to be efficient in the diagnosis of TB[5]. Compared to PCR, the MPT64 assay is easier to perform and does not require special instruments and advanced technical knowledge[5]. Moreover, it is faster and costs approximately US$ 1.4, for each test[15]. Molecular assays require complex laboratory infrastructure, including different rooms for the preparation of the MIX and for handling the DNA to avoid contamination, in addition to several equipment and supplies for the steps of DNA extraction, amplification and electrophoresis, which make them expensive.

In our study, the MPT64 identified fewer MTBC isolates than PCR assays. False-negative results obtained by the test have already been observed in other studies[6,16]. A factor that may influence the performance of the MPT64 assay is the concentration of inoculum used for the test, which must contain at least 10^5 colony-forming units/mL for MTBC identification[17]. Also, *mpt64* gene mutations were frequently reported in isolates that have false negative results for MTBC, leading to the production of an incomplete protein[18].

Since the release of false negative results difficult the proper clinical management of the TB patient, a study conducted by Nerurkar et al., in which the MPT64 assay was not able to identify 5.3% of clinical isolates belonging to MTBC, suggested using additional phenotypic or molecular testing for MPT64 negative isolates[16]. In this sense, our study points out that the use of PCR, targeting *IS6110*, would be an effective alternative to identify MTBC in isolates from TB patients but negative by the MPT64 assay. It is noteworthy that in-house PCR, despite requiring more time and more resources than the MPT64 assay, is a cheaper method than commercial molecular assays[4].

This study has one limitation, the presence of mutations in the mpt64 gene was not investigated in negative isolates by the *MPT64* assay. However, it was possible to propose a workflow for laboratories that carry out the diagnosis of TB through liquid culture systems, and an alternative for additional testing of positive cultures after performing the MPT64 assay. In addition, it is noteworthy that the methods used in the study can also be applied to growths obtained from a culture in solid media, which remain widely used.

A rapid and accurate diagnosis of TB is fundamental for disease control. Although the BACTEC MGIT liquid culture system allows time reduction for diagnosis and increases detection of TB cases, methods capable of confirming the MTBC in positive cultures should continue to be standardized, tested, and optimized to reach the ideal requirements of simplicity, speed, and effectiveness, to be implemented in different settings. The molecular and immunological methods evaluated in the present study have differences in the execution and detection of MTBC in cultures, but they are rapid tools for the laboratory diagnosis of TB.

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