Rapid detection of *Mycobacterium tuberculosis* complex by real-time PCR in sputum samples and its use in the routine diagnosis in a reference laboratory

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Tuberculosis (TB) is an infectious disease of global distribution, constituting a serious public health problem in Brazil. São Paulo State, located in the south-east of Brazil, notified 16 580 new TB cases in 2013. The Instituto Adolfo Lutz is a public health reference laboratory for TB diagnosis for all the State. Considering that rapid and accurate diagnosis is essential for TB control, the aim of this study was to evaluate the use of an in-house real-time (RT)-PCR assay targeting the *mpt64* gene in the routine diagnosis of TB, and to compare this technique with smear microscopy and culture. From August 2012 to October 2013, 715 sputum samples from 657 patients were included in the study. Smear microscopy, culture, phenotypic and PRA-*hsp65* identification of mycobacteria, and *mpt64* RT-PCR were performed. With respect to confirmed TB cases (*n*=62/657; 9.4 %), smear microscopy had a sensitivity of 82.3 %. Culture and RT-PCR showed the same sensitivity, i.e. 90.3 %. Specificity was 99.7, 99.4 and 98.6 % for smear microscopy, culture and RT-PCR, respectively. *mpt64* RT-PCR showed high sensitivity and specificity for the detection of *Mycobacterium tuberculosis* complex in sputum samples. This technique can be deployed in laboratories that do not have a rapid test for TB available, enabling the performance of TB diagnosis in up to 5 h.

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INTRODUCTION

There were >9 million new cases of tuberculosis (TB) worldwide in 2013 (WHO, 2014). In Brazil, one of the 22 countries responsible for 80 % of all TB cases in the world, >71 100 new cases were notified in 2013 (Ministério da Saúde, 2014). Every year, 4000–5000 people die of TB in this setting (CVE, 2012).

São Paulo State is located in the south-east of Brazil and was responsible for 23 % of all new TB cases notified in 2013 (CVE, 2014). The Instituto Adolfo Lutz (IAL) is a public health reference laboratory for TB diagnosis and has units located in 13 cities of São Paulo State.

The most commonly used techniques for TB diagnosis in Brazil are smear microscopy and culture; however, the first lacks in sensitivity, whilst the second can take several weeks to yield results (de Waard & Robledo, 2007). To overcome these issues, many molecular diagnostic tools have been applied in TB diagnosis in the last decade, especially real-time (RT)-PCR, giving rapid and accurate results (Espy et al., 2006).

The use of RT-PCR for TB diagnosis has increased, as it has proven to be highly sensitive and specific for detection of the *Mycobacterium tuberculosis* complex (MTBC) directly in clinical samples or from culture isolates (Garcia-Quintanilla et al., 2002; Miller et al., 2002; Broccolo et al., 2003; Cleary...
et al., 2003; Armand et al., 2011; Lira et al., 2013). Many regions of the mycobacterial genome have been used in molecular diagnosis of TB, such as IS6110, 16SrRNA, hsp65, rpoB, sdaA, devR and mpt64 (Manjunath et al., 1991; Lachnik et al., 2002; Kim et al., 2004; Chakravorty et al., 2005; Negi et al., 2007; Hwang et al., 2013; Nimesh et al., 2013). mpt64 is found as a single copy in the genome of MTBC species (Lee et al., 1994), and has been used for both pulmonary and extrapulmonary TB diagnosis (Tan et al., 1995; Dar et al., 1998; Martins et al., 2000; Bhanu et al., 2005; Therese et al., 2005; Takahashi & Nakayama, 2006; Dil-Afreze et al., 2008; Kusum et al., 2012; Sethi et al., 2012).

In 2014, the Ministry of Health implemented GeneXpert MTB/RIF in some municipalities in Brazil, according to the number of new cases notified. This technology has been used in place of smear microscopy for the detection of new TB cases (Ministério da Saúde, 2013). However, not all laboratories in São Paulo State received this system and the ones that did not still have to use traditional techniques.

Considering the high prevalence of TB in São Paulo, the use of RT-PCR would allow a rapid diagnosis, breaking the chain of transmission and, therefore, helping to control the spread of the disease. Given the above, the aim of this study was to evaluate the performance of an in-house RT-PCR assay to detect MTBC directly in sputum samples from patients with pulmonary TB in the south-east of Brazil. In order to assess the use of RT-PCR in the routine diagnosis of TB, this technique was applied in a way that the workflow and diagnostic methodologies were maintained as recommended by the Ministry of Health.

METHODS

Study design. Sputum samples that were routinely sent to the IAL unit located in Santo André City (Santo André IAL) for smear and culture were included in the study. These samples were prospectively collected from patients with suspected pulmonary TB that attended at healthcare units of four cities in the southern region of São Paulo, i.e. Diadema, Mauá, Rio Grande da Serra and Santo André, between August 2012 and October 2013. Sample size (N) was calculated using the formula: 

\[ N = \frac{Z^2 \cdot p(1-p)}{D^2}, \]

where \(Z\) is the anticipated prevalence of TB amongst new cases in the study setting (\(P=8\%\)), \(D\) is the precision (95% confidence interval) and \(Z\) is the value from the normal distribution for a 95% confidence (\(Z=1.96\)) (Bonita et al., 2006), which gave us a sample size of 707. A total of 715 sputum samples with a minimum volume of 4 ml were collected from 657 patients that had no previous history of TB and were analysed. Clinical data, including radiologic and laboratory findings, were also collected from each patient using TB-WEB, an information system from the surveillance department of São Paulo in which all TB cases are notified and followed.

TB case definition. TB cases were considered as patients that had one or more smear-positive results and/or a culture-positive result for MTBC, or patients with smear- and culture-negative results, but notified at TB-WEB.

Smear microscopy and culture. Ziehl–Neelsen staining was performed directly in unprocessed sputum at the healthcare unit laboratories, according to the recommendations of the Ministry of Health (Ministério da Saúde, 2008). Samples were then sent to Santo Andre IAL, where they were equally split in two aliquots: one for culturing and the other for nucleic acid extraction. For culturing, samples were decontaminated by the modified Petroff method for inoculation in MGIT tubes (Becton Dickinson) or by the swab method for inoculation in Ogawa–Kudoh slant (Pedro et al., 2011). MGIT tubes were incubated for 6 weeks and Ogawa–Kudoh cultures were incubated for 8 weeks. Every positive culture was subjected to Ziehl–Neelsen staining to confirm the presence of acid-fast bacilli (AFB) and cord formation, and to exclude contamination. Mycobacterial isolates were identified by conventional phenotypic methods and by primers for 65S (Chimara et al., 2008). Unprocessed sputum aliquots for nucleic acid extraction were stored at −20 °C and sent to São Paulo IAL.

Nucleic acid extraction. At São Paulo IAL, sputum samples were processed with 2% N-acetyl-L-cysteine-sodium hydroxide (NAC-NaOH), followed by centrifugation at 3000 g for 15 min (Ministério da Saúde, 2008). Sediments were resuspended in 1 ml ultrapure water and boiled at 100°C for 30 min for mycobacterial inactivation. An aliquot of 25 μl lysozyme at a concentration of 0.2 mg ml⁻¹ was added to 200 μl processed samples, which were incubated at 37°C for 30 min. Total nucleic acids were then extracted and purified with a Genomic DNA from Tissue kit (Macherey-Nagel), according to the manufacturer's instructions. A vial containing 200 μl ultrapure water was used as a negative control. Purified nucleic acids were eluted in 200 μl elution buffer and stored at −20 °C until nucleic acid amplification.

RT-PCR for mpt64. The mpt64 gene (GenBank accession number NC_000962) primer and probe sequences are described in Table 1 (Takahashi & Nakayama, 2006). The human rnaseP gene (GenBank accession number NM0006413) was amplified separately as a control for the presence of PCR inhibitors and to monitor the efficiency of nucleic acid extraction (Table 1). Amplified products were detected by the use of TaqMan probes labelled at the 5’ position with FAM and at

<table>
<thead>
<tr>
<th>Gene target</th>
<th>Primers and probes</th>
<th>Sequence (5’→3’)</th>
<th>Final concentration (nM)</th>
<th>Tm (°C)</th>
<th>Amplicon length (bp)</th>
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<tbody>
<tr>
<td>mpt64</td>
<td>Forward primer</td>
<td>GTGAACTGAGCAAGCAGACCG</td>
<td>300</td>
<td>60</td>
<td>76</td>
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<tr>
<td></td>
<td>Reverse primer</td>
<td>GTTCTGATAATTCACCGCTCC</td>
<td>300</td>
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<tr>
<td></td>
<td>TagMan Probe*</td>
<td>FAM-TATGGAAGAGCTGAGCAGCG-BHQ1</td>
<td>100</td>
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<tr>
<td>rnaseP</td>
<td>Forward primer</td>
<td>AGATTGGACCCTGAGGCGCC</td>
<td>300</td>
<td>60</td>
<td>65</td>
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<tr>
<td></td>
<td>Reverse primer</td>
<td>GACGCGCTGTTCGCACAGT</td>
<td>300</td>
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<tr>
<td></td>
<td>TagMan Probe*</td>
<td>FAM-TTCTGAGCGGCGCCAATCTGGCG-BHQ1</td>
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*Probe 5’-end-labelled with FAM (6-carboxylfluorescein) and 3’-end-labelled with BHQ1 (Black Hole Quencher 1).
the 3’ position with BHQ1 (Table 1). RT-PCR was performed with a Roche LightCycler 480 II system (Roche Diagnostics). A total reaction volume of 25 µl was used for all samples and contained 5 µl purified DNA, 12.5 µl 2 × TaqMan Universal Master Mix (Applied Biosystems), 2 µl each primer (forward and reverse) at 300 nM, 2 µl probe at 100 nM and 1.5 µl PCR-grade water (Roche Diagnostics). Cycling conditions were one cycle at 50 °C for 2 min and one cycle at 95 °C for 10 min followed by a two-step PCR (45 cycles of 15 s at 95 °C and 1 min at 60 °C). All samples were tested in duplicate, and each reaction included two wells for positive control (M. tuberculosis reference strain H37Rv, ATCC 27294), two wells for negative control of DNA extraction and purification (ultrapure water) and four controls without DNA (two for the Master Mix preparation area and two for the DNA addition area). These controls were used to rule out the possibility of amplification failure or cross-contamination. The results were analysed by LightCycler 480 II SW version 1.5.0 SP3 software (Roche Diagnostics). The total time for amplification, detection and analysis using this protocol was ~110 min for 30 samples. Fluorescence measurements were made in every cycle. The threshold cycle (Ct) value is the cycle at which there is a significant increase in fluorescence and this value is associated with an exponential growth of PCR product during the log-linear phase.

Lower limit of detection (LLD). The LLD for mpt64 was calculated in duplicate using extracted and purified H37Rv DNA. DNA was diluted in ultrapure water to a concentration of 10 ng µl⁻¹. From this concentration, DNA was diluted in serial 10-fold dilutions until 10⁻⁸ or 0.1 fg µl⁻¹.

Interpretation of results. In this study, cut-off for positive and negative results was defined based on Ct values. Samples with Ct ≤ 36 were considered positive for mpt64. There were no inconclusive samples as all samples that had Ct > 36 were smear- and culture-negative, and these patients were not notified at TB-WEB. For rnaseP, all samples with Ct ≤ 42 were considered positive and there were also no inconclusive results. Samples with Ct = 0 or > 36 for mpt64 and Ct = 0 or > 42 for rnaseP were considered negative.

This study was approved by the IAL ethics board (protocol number 79574/2012).

RESULTS

There were 62 (9.4%) confirmed new TB cases, either bacteriologically or by patient notification at TB-WEB, amongst the 657 patients with suspected TB. Each of the 62 TB cases had only one sample included in the study. Of the 715 samples analysed, 53 (7.4%) were smear-positive; 60 (8.4%) were culture-positive for mycobacteria, 610 (85.3%) were culture-negative and 45 (6.3%) cultures were contaminated. Fifty-six (93.3%) of the 60 positive cultures were identified as MTBC, one (1.7%) as Mycobacterium intracellulare and two (3.3%) as belonging to the group of achromogen rapid growers.

The LLD for the assay was determined to be 2 pg for the mpt64 gene, with a minimum Ct of 19 and a maximum of 36. The reaction had an efficiency of 94.6% with a slope of −3.4. For accurate results, reactions should have an efficiency as close to 100% as possible, which is equivalent to a slope of −3.32 and means that the RT-PCR product is doubling at each cycle.

Of the 715 samples analysed, 650 (90.9%) were negative and 65 (9.1%) were positive for the mpt64 gene by RT-PCR. rnaseP amplification occurred in all 715 samples, meaning that there were no PCR inhibitors in the samples that were negative for mpt64.

Table 2 shows the sensitivity, specificity, positive predictive value, negative predictive value and accuracy of smear microscopy, culture and RT-PCR according to confirmed TB cases. Smear microscopy detected 51 of the 62 TB cases (sensitivity of 82.3%), whilst culture and RT-PCR detected 56 of these cases, showing the same sensitivity of 90.3%. Specificity values were 99.7, 99.4 and 98.6% for

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<th>Table 2. Results of smear microscopy, culture and RT-PCR according to confirmed TB cases</th>
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<td>Method</td>
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<tr>
<td>Smear microscopy</td>
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<td>mpt64 RT-PCR</td>
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†The final diagnosis of TB was based on the results of smear microscopy, culture, clinical and radiological findings.
‡One sample had an isolate identified as M. intracellulare and was negative for mpt64.
§All were non-TB mycobacteria isolates: one M. intracellulare, one M. kansasii and two achromogen rapid growers, and did not show amplification for mpt64.
¶Two samples were confirmed for TB by RT-PCR.
*43 samples were contaminated in culture.
smear microscopy, culture and RT-PCR, respectively. All samples with non-TB mycobacteria (NTM) isolation were negative for the mpt64 gene, showing that this target was specific for the MTBC.

Of the 51 TB cases detected by smear microscopy, 47 were confirmed by culture and 49 were positive for mpt64 (92.2 and 96.1% sensitivity, respectively). Of the 11 smear-negative TB cases, nine were confirmed by culture and seven were positive for mpt64 by RT-PCR (81.8 and 63.6% sensitivity, respectively).

Two TB cases were detected only by RT-PCR – one was smear- and culture-negative, and the other was smear-negative and contaminated in culture. One of these patients was initiated in treatment only 4 months after the sputum collection date.

**DISCUSSION**

Traditional techniques for the bacteriological diagnosis of TB are based on visualization of AFB in clinical specimens by microscopy and growing these micro-organisms in culture for later identification. In Brazil, sputum smear microscopy is the most commonly used technique for the diagnosis of pulmonary TB, because it is simple, rapid and inexpensive, despite its low sensitivity (60–70%) (WHO, 2002). Culture, which is the gold standard, but can take several weeks to give results, is performed in the following cases: repeated smear-negative results, positive smear at the second month of treatment, suspicion of extrapulmonary TB, suspicion of mycobacteriosis, contacts with multidrug-resistant TB, patients with a history of previous treatment, immunosuppressed patients, immigrants, indigenous patients and populations with a higher risk of TB infection (prisoners, homeless, inpatients) (Ministério da Saúde, 2011).

In this study, culture and RT-PCR detected a higher number of confirmed TB cases in comparison with microscopy, as was also found by Sethi et al. (2012) and Nimesh et al. (2013). Moreover, RT-PCR showed high specificity, as found by Broccolo et al. (2003) and Armand et al. (2011). The high sensitivity of microscopy we encountered could be due to the fact that only sputum samples of at least 4 ml were included in our study, favouring the detection of AFB. Also, the high specificity of this technique can be explained by the fact that only one of the four samples with isolation of NTM was smear-positive.

It is well documented that molecular methods show good sensitivity in smear-positive samples, but are less sensitive in paucibacillary samples (Greco et al., 2006; Armand et al., 2011). We found the sensitivity of RT-PCR in smear-positive and -negative samples to be 96.1 and 63.6%, respectively. Armand et al. (2011) reported similar findings with GeneXpert MTB/RIF and an in-house RT-PCR for IS6110.

False-negative results in molecular tests can be explained by a low load of mycobacteria, the presence of polymerase inhibitors and the uneven distribution of bacilli in paucibacillary samples (Reischl et al., 1998). There were six false-negative samples by RT-PCR in our study, of which four were smear-negative, two had one to nine AFB and only one was negative in culture. It is possible that mycobacterial DNA was lost during sputum aliquoting in Santo Andre IAL or pre-treatment for DNA extraction.

Other studies also reported false-negative results by RT-PCR in paucibacillary samples (Miller et al., 2002; Cleary et al., 2003; Lira et al., 2013). These authors observed an inversely proportional relationship between the Ct values and the number of AFB detected in microscopy, as also encountered in our study (data not shown).

According to García-Quintanilla et al. (2002), PCR sensitivity loss may be due to the detection of a single-copy gene, as is the case of mpt64 (Lee et al., 1994). In this study, we tested the hypothesis of a possible mutation in the sites where the primers and probe hybridize. Thus, we performed RT-PCR from the isolates available from five of the false-negative samples (as one was culture-negative) and all of them were positive for mpt64, thus discarding this hypothesis.

The mpt64 RT-PCR assay detected the MTBC in two samples from patients whose conventional tests (smear and culture) were negative/contaminated, but were diagnosed with TB by clinical/radiological findings. This resulted in late notification of one of these patients, delaying initiation of treatment and increasing the probability of transmission.

Five of the nine samples that were false-positive by RT-PCR showed Ct values of 35–36, which is very close to the maximum Ct obtained in the LLD to mpt64 detection. Gomez et al. (2011) also reported positive results by PCR in sputum samples from patients without TB. They argued that these patients may be paucibacillary, or there may have been cross-contamination during sputum collection or laboratorial procedures. The samples analysed in this study were collected in many health facilities from four municipalities in São Paulo State and thus we cannot guarantee that all sputum collection steps were performed adequately with regard to avoiding cross-contamination. As for the laboratory procedures, work flow was unidirectional, and separate areas were used for culture, DNA extraction, RT-PCR reagent preparation and addition of the samples to the reaction plates.

None of the four samples with NTM isolation was positive for mpt64, showing the specificity of this target to MTBC detection, as reported by Takahashi & Nakayama (2006), Kusum et al. (2012), Sethi et al. (2012) and Nimesh et al. (2013). All of the patients infected with NTM were notified and treated for TB. One had a change in diagnosis after 6 months of treatment, another had already been treated previously and the remaining two were still being treated for TB. In these cases, RT-PCR results suggested an indirect diagnosis of NTM infection, ruling out TB. This indirect diagnosis is very useful in smear-positive cases with negative results in RT-PCR and clinical/radiological findings compatible with the disease.
The mean time for obtaining results with GeneXpert MTB/RIF is ~3 h per sample, but the limitation is that the equipment acquired by the Ministry of Health has only four modules, besides the high cost of the cartridges, which would hinder its use in developing countries such as Brazil. Considering a workload of 8 h, the laboratories receiving the four-module equipment will be able to analyse up to 16 samples per day.

The in-house mpt64 RT-PCR assay enabled the run of 30 samples per plate, in 110 min. Adding this to the time needed for pre-treatment, DNA extraction of the samples and preparation of the PCR plate, the results for 30 samples would be available in ~5 h. Molecular in-house techniques may have lower costs, as the laboratory can use pre-existing multiuse platforms, as is the case for São Paulo IAL, which performs routine diagnosis of several infectious diseases by RT-PCR.

The aim of our study was to evaluate the applicability of an in-house RT-PCR assay in the routine diagnosis of TB in a reference laboratory, without changes in the workflow and the techniques recommended by the Ministry of Health. The exclusion of sputum samples <4 ml is a limitation of our study, because these samples are the most commonly received samples by the laboratory. As culture and DNA extraction were performed in two different laboratories, we had to include samples with a larger volume, so that they could be split.

Moreover, the most commonly used methods for sputum treatment in routine diagnosis in Brazil are modified Petriflask and Kudoh swabs, which are not recommended for pre-treatment of samples for DNA extraction. This is another reason why we included samples with a larger volume.

The in-house RT-PCR technique evaluated in this study, which demonstrated the same sensitivity as the gold standard method of culture, can be implemented in the diagnostic routine of laboratories in countries with a high incidence of TB, such as Brazil, but that do not have a rapid test for TB diagnosis available. From the decontaminated sputum samples for smear and culture, an aliquot would be separated for RT-PCR testing. The results would be available on the same day of the samples' arrival in the laboratory, representing a relevant gain in time of diagnosis in comparison with culture. Thus, these laboratories could also rely on a tool for the rapid diagnosis of TB that would be used together with smear microscopy.

REFERENCES


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