Detection of non-tuberculous mycobacteria (NTMs) in lung samples using 16S rRNA

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BACKGROUND Non-tuberculous mycobacteria (NTMs) cause diseases known as mycobacteriosis and are an important cause of morbidity and mortality. The diagnosis of pulmonary disease caused by NTM is hampered by its clinical similarity with tuberculosis (TB) and by the lack of an accurate and rapid laboratory diagnosis.

OBJECTIVES Detect DNA from NTMs directly from lung samples using real-time polymerase chain reaction (qPCR) for amplification of 16S rRNA. Additionally, DNA sequencing (hsp65 and rpoB genes) was used to identify the species of MNTs.

METHODS A total of 68 sputum samples (54 with suspected NTMs and 14 with TB) from patients treated at a referral hospital were used.

FINDINGS Of these, 27/54 (50%) were qPCR positive for NTMs and 14/14 TB patients (controls) were qPCR negative with an almost perfect concordance (Kappa of 0.93) with the Mycobacterium spp. culture. Sequencing confirmed the presence of NTM in all positive samples. The most common species was Mycobacterium gordonae (33%), followed by Mycobacterium abscessus (26%), Mycobacterium fortuitum (22%), Mycobacterium avium (15%) and Mycobacterium peregrinum (4%).

MAIN CONCLUSIONS The qPCR technique for detecting NTMs targeting 16S rRNA has the potential to detect NTMs and rapidly differentiate from Mycobacterium tuberculosis. However, it is necessary to identify the species to help in the differential diagnosis between disease and contamination, and to guide the choice of the therapeutic scheme.

Key words: non-tuberculous mycobacteria (NTMs) - DNA - real-time PCR (qPCR)
The molecular techniques for identifying the species of NTMs that are being used are mainly based on the polymerase chain reaction (PCR), such as GenoType® Mycobacterium CM and PRA-hsp65, which have the advantage of quickly obtaining results, however, the high cost and/or complexity of the process limit its use in the routine.12

This study was developed to detect DNA from NTMs by real-time PCR (qPCR) in lung samples, with the aim of differentiating from the M. tuberculosis Complex and, additionally, to identify the species through sequencing for comparison of results.

SUBJECTS AND METHODS

Clinical isolates (culture) and lung samples of NTMs - All samples used in the study (68 sputum samples) were from patients at the Phthiology Outpatient Clinic of Hospital Sanatório Partenon (HSP), a state reference (Rio Grande do Sul, Brazil) for cases of NTMs, treated in the period from September 2018 to December 2019.

Clinical suspicion, as well as diagnosis, followed the criteria established in international guidelines.9 The study was approved by the Ethics Committee on Health Research of the Escola de Saúde Pública (CEPS-ESP) in accordance with the Resolution 466/2012, under the number CAAE 96556418.4.0000.5312 of September 24, 2018.

The smear slides, culture and GeneXpert/RIF tests were performed in the diagnostic routine of the HSP, as recommended.11,13 Patients with an insufficient sample (less than 500 µL) for all tests were excluded from the study, in addition to those being monitored for TB control and responding to treatment. The NTMs strains used as controls in this study came from the Institute of Biological Sciences of Universidade Federal do Rio Grande (Rio Grande, RS, Brazil).

DNA extraction - The extraction of DNA directly from culture samples and lung samples was performed by the method known as “sonication”.14 The concentration of DNA extracted from the cultures was determined by spectrophotometry using the Eppendorf BioSpectrometer® basic equipment (Hamburg, Germany).

The study was divided into three stages: standardisation of the qPCR technique with control strains; detection of NTM DNAs in lung samples using the standardised technique and sequencing of the positive samples.

qPCR standardisation from clinical isolates - A qPCR (TaqMan® hydrolysis linear probes) methodology was standardised for amplification of the 16S rRNA region15 in the 7500 equipment (Applied Biosystems). For this, 11 DNAs from NTMs extracted from culture and confirmed by sequencing were used, as described in item C (Table I).

Primer efficiency was evaluated from a serial dilution using known amounts (100 ng) of M. avium DNA. The detection limit was defined using known concentrations of DNA from cultured M. avium and serially diluted, in ultrapure water, in factor 10 (16 ng to 0.016 fg) (Figure).

Assay validation was performed in triplicate with six mixtures of different concentrations, starting with primers and probes at 10 ng (oligonucleotide sequences are based on the study by Kim et al.15). Analysing the amplification profile from the curve formed and based on the level of detectable fluorescence (threshold). The smallest amount of primers and probe with the best performance was considered as the standard for the reaction.16

Detection of NTMs by qPCR in a lung sample - The qPCR for amplification of 16S rRNA from NTMs was performed as standardised, using DNAs extracted directly from sputum samples, by sonication, as described above. The positive control (PC) was a DNA from M. avium and the negative control (NC) was ultrapure water. After carrying out the assay using 16S rRNA as a target, the extracted DNAs were amplified by PCR using primers that amplify regions of the IS6110 to identify the presence of M. tuberculosis complex DNA or mixed infections.17 The reactions used M. tuberculosis DNA from the reference strain H37Rv as PC and ultrapure water as NC.18

The analysis of the amplification curve followed the predefined parameters (threshold and baseline). An evaluation of the methodology was performed by the amplification of DNAs from previously genotyped mycobacteria.

Molecular identification of NTMs species by sequencing - Molecular identification of NTMs in qPCR-positive samples was performed by sequencing the rpoB and hsp65 genes after PCR amplification with primers described by Telenti and coworkers.19 PCR for the rpoB gene was performed with an initial denaturation step at 94°C (5 min), proceeding with 40 cycles of denaturation at 94°C (90 s), hybridisation at 65°C (2 min), extension 72°C (3 min) and a final extension cycle at 72°C (10 min). The PCR for the hsp65 gene follows the model described above, changing to 45 cycles of denaturation at 94°C (1 min), hybridisation at 60°C (1 min) and 72°C extension (1 min). The reactions were performed on the Applied Biosystems® Veriti® 96-Well ThermalCycler StepOne equipment. As controls, DNAs from NTMs previously identified by culture and confirmed in the sequencing were used (Table I) and as NC, ultrapure water. After checking the DNA amplifications on an agarose gel, the PCR amplicons were subjected to purification by polyethylene glycol (PEG) described by Rosenthal et al.20 Cycling with the Big Dye® Kit version 3.1 (Applied Biosystems) was performed according to the manufacturer’s instructions.21

The PCR products were labeled with 5 pmol of the TB11 primer (5’-ACCAACGATGGTGTTGTCAT-3’, for the hsp65 gene) or with 5 pmol of the MycoF primer

Graph of the analysis of the detection limit and evaluation of the technical efficiency (This figure has not been previously published in any journal).
The test reproducibility was confirmed by the DNA amplification pattern of the 11 NTMs used (Table I).

Detection of NTM DNA in lung samples - The 68 lung samples analysed consisted of: 54 from patients with suspected mycobacteriosis (NTM) and 14 from patients with TB (tested for acid-fast bacilli - AFB, M. tuberculosis culture and GeneXpert/RIF positive) used as negative controls to assess specificity.

In 28/54 (52%), the culture was positive for Mycobacterium (NTMs) that do not belong to the M. tuberculosis complex. Of these, 27/28 (96.4%) were positive in the qPCR test for NTMs with CT ranging from 12-17 (Table II). All 26 samples negative for NTMs culture and the 14 samples that were from patients with tuberculosis did not have qPCR amplification for NTM (sensitivity and specificity were 100% and 96%, respectively).

All 27 samples positive for the presence of NTM DNA in the qPCR were sequenced. The identified NTM species were M. gordonae in 9/27 (33%), M. abscessus in 7/27 (26%), M. fortuitum in 6/27 (22%), M. avium in 4/27 (15%) and M. peregrinum in 1/27 (4%) of the samples.

PCR results were also compared with smear slides tests. Eleven (40.7%) samples that were negative by sputum smear microscopy were positive by qPCR. All were positive in culture for NTM. Only one sample, with positive smear slide, did not show qPCR amplification.

The performance of qPCR, performed from DNA from lung samples, compared to culture, showed an almost perfect agreement (Kappa = 0.93), and with the AFB test the agreement was substantial (Kappa = 0.62).

**DISCUSSION**

Molecular methods for identifying mycobacterial species are more accurate and faster when compared to conventional methods.  

The purpose of this study was to detect NTMs directly from lung samples from patients, using a qPCR with specific primers (16S rRNA) only for NTMs, thus being able to rapidly separate these from the M. tuberculosis complex. The 16S rRNA gene contains highly conserved and variable regions, being universally used to identify mycobacteria. In our study, these primers amplified DNA from several NTMs with an efficiency of 98% (90-100%), with a test detection limit of 160 pg for amplification of 100% of triplicates. Similar analytical sensitivity analyses were used in the study by Peixoto and his coworkers.

The standardised qPCR technique showed an almost perfect agreement with the culture (Kappa 0.93), which was confirmed by sequencing. Only one sample was positive for culture, but negative for qPCR and sequencing. In all the others, it was possible to detect DNA from NTMs. This negative sample had culture and positive AFB testing. In the hypothesis that it really is an NTM, it is believed that, possibly, this lack of amplification could be related to PCR inhibitors present in the sample, which were not totally removed in the DNA extraction process, or loss of DNA during extraction, since the targets (rpoB and hsp65) used in the sequencing also did not amplify.
Agreement with AFB testing was substantial (Kappa = 0.62), that is, 11 AFB negative samples were detected by PCR. Greater detection by qPCR was already expected, due to the need for a high bacterial load (around 10,000 bacilli per mL of sample) for sputum smear positivity, unlike qPCR, where low DNA concentrations of NTMs could already be amplified.

When testing the 14 samples from TB positive patients, no amplification was detected, which may allow the use of qPCR to differentiate between TB and NTM. The presence of TB/NTM coinfection has been reported, but it was not detected in this study.

These results suggest that the technique may be promising for detecting NTMs directly from lung samples, ruling out a diagnosis of TB and indicating the possibility of NTM infection/disease in the patient. The target used in this study (16S rRNA), together with the hsp65 gene, has been described for the identification and differentiation of NTMs from the M. tuberculosis complex.

Sequencing was used to identify species detected as NTMs. This is described by many authors as the molecular gold standard for the identification of mycobacteria, due to its high discriminatory capacity, allowing the characterisation of bacterial species. The hsp65 and rpoB genes have been described for this purpose. The most frequently identified NTM species in this study was M. gordonae (33%), being a common mycobacterial species. M. gordonae is considered to be a low virulence strain that is also very associated with laboratory contamination; hence the importance of other...
er factors to make the differential diagnosis of disease caused by NTM, thus reinforcing the need for careful procedures in order to differentiate contamination from infection,\textsuperscript{(3,8,40)} since \textit{M. gordonae} hardly causes disease. \textsuperscript{(37)} The results of the study by Shin et al.\textsuperscript{(38)} suggested that the clinical sensitivity of a test may be strains dependent.

\textit{Mycobacterium abscessus} was the second (26%) most frequently identified bacteria, followed by \textit{M. fortuitum} (22%) and \textit{M. avium} (15%). These potentially pathogenic NTM species are among the most frequent related to lung diseases, both in southern Brazil and in other regions of the country.\textsuperscript{(3,8,40)}

The limitation found in the present study was the small number of samples analysed. Despite this, the results showed a possibility for the rapid identification of NTM, excluding a diagnosis of TB, especially in patients with symptoms and suspicion of TB, facilitating the beginning of early treatment and contributing to reduce the transmission of this disease.

In conclusion - The results suggest that the application of the qPCR technique to detect NTMs using 16S rRNA as a target has the potential to detect NTMs and differentiate them, quickly and efficiently, from \textit{M. tuberculosis}. However, it is still necessary to identify the species to assist in the differential diagnosis between disease and contamination, guiding the choice of the therapeutic scheme.

ACKNOWLEDGEMENTS

To the Postgraduate Program in Cellular and Molecular Biology Applied to Health - Lutheran University of Brazil (ULBRA), Institute of Biological Sciences of Universidade Federal do Rio Grande (Rio Grande, RS, Brazil) and Centre for Scientific and Technological Development (CDCT-RS).

AUTHORS’ CONTRIBUTION

FCLM, GLB and CC contributed to developing methodology, experiments, and scientific writing; KS and TSS contributed to the experiments; GU, CFD and RBB contributed to the scientific writing and clinical analysis of patients; PEAS contributed with a bank of strains for standardising techniques and scientific writing; MLRR coordinated the execution of the study and the orientation and correction of the scientific writing. The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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