Research Paper

Validation of a real-time PCR assay for the molecular identification of *Mycobacterium tuberculosis*

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Abstract

Mycobacterium tuberculosis is the major cause of tuberculosis in humans. This bacillus gained prominence with the occurrence of HIV, presenting itself as an important opportunistic infection associated with acquired immunodeficiency syndrome (AIDS). The current study aimed to develop a real-time PCR using Eva Green technology for molecular identification of *M. tuberculosis* isolates. The primers were designed to Rv1510 gene. Ninety nine samples of *M. tuberculosis* and sixty samples of *M. bovis* were tested and no sample of the bovine bacillus was detected by the qPCR. Statistical tests showed no difference between the qPCR and biochemical tests used to identify the *Mycobacterium tuberculosis*. The correlation between tests was perfect with Kappa index of 1.0 ($p < 0.001$, CI = 0.84 - 1.0). The diagnostic sensitivity and specificity were 100% (CI = 95.94% - 100%) and 100% (CI = 93.98% - 100%). This qPCR was developed with the goal of diagnosing the bacillus *M. tuberculosis* in samples of bacterial suspension. TB reference laboratories (health and agriculture sectors), public health programs and epidemiological studies probably may benefit from such method.

Key words: *Mycobacterium tuberculosis*, diagnosis, real time PCR.

Introduction

Tuberculosis (TB) is one of the oldest and most serious known diseases. Studies using DNA isolated from cattle bones found in North America, which were dated to 17,000 BC, showed the presence of *Mycobacterium tuberculosis* (Rothschild *et al.*, 2001). During the Industrial Revolution, numerous cases of tuberculosis emerged due to the agglomeration of workers (Costa, 1985). In the first half of the twentieth century, the disease terrorized Europe, and a diagnosis meant a death sentence for 50% of infected individuals, mostly children and youth (Daniel *et al.*, 1984).

There was hope that tuberculosis would be eradicated by 2000 in developed countries, However, with the AIDS pandemic, pulmonary tuberculosis has become the third most common opportunistic infection in HIV-positive patients (Job, 1998). The occurrence of TB co-infection with human immunodeficiency virus (HIV) caused a change in the epidemiological trends of TB, which now presented as an important opportunistic pathogen associated with acquired immunodeficiency syndrome (AIDS) (Carvalho, 2006). According to estimates from the World Health Organization (WHO), one third of the world population is infected with *Mycobacterium tuberculosis*; every year, 8 million people become ill and 2,9 million people die as a result of TB infection (WHO, 2013). Of the 8 million cases registered annually, 95% occur in developing countries (Brasil, 2002). In the past, pulmonary tuberculosis was used as an indicator of peripheral development.

While mycobacterium culture is the gold standard for confirmation of a TB diagnosis, the process requires more than three weeks for a conclusive result and is not species-level specific. The major problems in mycobacteria

identification by phenotypic methods are the variety of biochemical tests required and the time needed for completion of the tests (Telenti *et al.*, 1993). These tests can be laborious, time consuming, inaccurate and non-reproducible, can generate ambiguous results and require adequate facilities with a high biosecurity level (Huard *et al.*, 2003).

Molecular biology techniques are widely applicable to laboratory diagnoses of infectious diseases caused by viruses (Fonseca Jr. *et al.*, 2010), bacteria (Cortez *et al.*, 2006), parasites or fungi (Sotiriadou *et al.*, 2013). DNA fragment amplification opened excellent prospects for the detection of infectious agents. PCR has several advantages compared to traditional diagnosis methods. PCR is highly sensitive, specific and rapid. To produce a positive diagnosis, fewer bacterial cells are required, and the bacteria do not need to be viable, which means that inadequately preserved samples can be used (Abrahão, 1999, Narayanan, 2004). Real-time PCR (qPCR) has further advantages, including precision, reproducibility, accuracy, quality control processes and reduced contamination. In addition, qPCR eliminates the need for electrophoresis after the cycling reaction. Furthermore, this technique reduces the analysis time to three or four days, which is important in laborious and lengthy microbiological diagnoses, such as those required for TB.

Faster diagnoses of human TB is of significant clinical importance. There are commercial methods available to detect *M. tuberculosis*, but these techniques rely on multiple probes or detect *Mycobacterium tuberculosis* complex (MTC) but not the specific species (Rossau *et al.*, 1997). Thus, the aim of this study was to develop a real-time PCR assay using bacterial suspensions and Eva Green® to identify *M. tuberculosis* as an alternative to phenotypic tests.

Materials and Methods

qPCR

The oligonucleotides designed in this study were based on the complete genome sequence of *M. tuberculosis* (Cole *et al.*, 1998). First, the sequences were subjected to BLAST® (http://blast.ncbi.nlm.nih.gov/Blast.cgi) for verification of genomic differences (Altschul *et al.*, 1997). Then, the selected target sequences available on GenBank (NCBI, http://www.ncbi.nlm.nih.gov/) were aligned in BioEdit (16). After checking the ideal regions, namely, those present only on *M. tuberculosis*, primers were designed in the Primer3Plus program (Untergasser *et al.*, 2007) and analyzed using the Oligo Analyser 3.1 program (IDT, USA) for verification of secondary structures. *In silico* analytical specificity of the primers was tested with the PrimerBlast program (NCBI, http://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi?LINK_LOC=BlastHome) (Altschul *et al.*, 1990).

The region chosen for the specific detection of *M. tuberculosis* DNA was the gene Rv1510. Primers Mtub.115.F: 5'TTCGATATTCGCGGTGTTTT3' (318446-318465, referring to BX842576.1) and Mtub.115.R: 5'CGCAACTATTTGGGTGGAG3' (318542-318560) amplify a 115 bp segment of the gene. The ideal qPCR reactions contained 10 pmol of each primer (IDT, USA), 1.5 U Hot Start GoTaq Polymerase (Promega, USA), 20% Colorless GoTaq Hot Start Buffer 5x, 1.5 mM $MgCl_2$, 1 µL of 20x Eva Green (Biotium, USA), 2 µL Rox 10x (Biotium, USA), 10 mM dNTPs and 2 µL of DNA to a final volume of 20 µL. All tests were performed on a Rotorgene 3000 (Qiagen, Germany) with the following program: denaturation at 95 °C for 3 min followed by 35 cycles at 95 °C for 10 s, 60 °C for 15 s and 72 °C for 20 s with fluorescence readings. The melting curve was performed with the following parameters: 45 s at 72 °C and 3 s per degree up to 99 °C. DNA extraction was evaluated by qPCR targeting ribosomal DNA. Primers M.sp.rDNA.133.F (5'GTCCTTGGAGACGTTCCTCA3') and M.sp.rDNA.133.R (5'CACGTCGATCACCACG TAGA3') were used with the same reagent concentrations and cycling conditions that were used for Rv1510 detection.

Samples

Standard *M. tuberculosis* strains H37Rv CRNC 23 and H37Ra CRNC 25 were used as positive controls, and *M. bovis* AN5 Mexico CRNC 36, *M. bovis* AN5 CRNC 02, *M. bovis* AN5 CRNC 01 and *M. bovis* BCG CRNC standard strains were used as negative controls for all tests in this study.

Phenotype-based speciation methods

All positive AFB cultures were subjected to additional phenotypic speciation methods, including biochemical tests such as catalase at room temperature and at 68 °C, niacin, nitrate, pyrazinamidase, and urease. Drug susceptibility testing (DST) was used to distinguish mycobacterial strains (Job, 1998).

Molecular-based speciation methods

Two loops of each mycobacteria replica that grew on Lowenstein Jensen or Stonebrink media were transferred into a microcentrifuge tube containing 400 µL of 1X TE buffer and were separately subjected to heat to kill and lyse the cells. The inactivated isolates were stored at 2 to 8 °C until use in the PCR reactions.

A rapid differentiation of 99 *M. tuberculosis* isolates and 60 *M. bovis* isolates was performed by molecular methods to confirm the real situation of the study samples. *M. tuberculosis* samples were previously isolated and kindly provided by Embrapa Gado de Leite. *M. bovis* isolates were collected in Lanagro/MG slaughterhouses from tissues with lesions characteristic of bovine tuberculosis and tested in qPCR previously validated by our group (Sales et al., 2014a, Sales et al., 2014b). The mycobacterial DNA was

subjected to amplification of the *pncA* gene (Scorpio and Zhang, 1996) and detection of the *pncA* polymorphism by cleavage of the amplicon with *Eco*065I, as previously described (Barouni *et al.*, 2004) for the genetic identification of *M. bovis* or *M. tuberculosis*. This analysis was carried out at the Laboratory of Molecular Biology Applied to Mycobacteria, Oswaldo Cruz Institute, Fiocruz.

Validation criteria

The parameters for the validation methodology were performed according to the Manual of Diagnostic Tests and Vaccines for Terrestrial Animals (OIE, 2010).

Reaction efficiency

The reaction efficiency was tested by examining 10-fold dilutions in 1X TE buffer of DNA extracted from the positive controls. Each dilution was tested in duplicate and the concentration estimated on a Nanovue® (GE Healthcare, USA) spectrophotometer. A number of standard curves were generated to determine the optimal primer concentration.

Limit of Detection (LOD) and Analytical Specificity

The determination of the LOD was performed by the 10-fold dilution of the standard strain. The dilutions were tested in triplicate, and the LOD was determined as the dilution at which all replicates were positive. The LOD was confirmed by repeating the qPCR twenty-one times.

The following strains were used in analytical specificity tests: 60 *M. bovis* isolates, *M. fortuitum* CRNC 10, *M. kansasii* CRNC 48 and CRNC 18, *M. gordonae* CRNC 16, *M. avium* D4 CRNC 05, *M. avium paratuberculosis* CRNC 26, *M. avium* 1500 CRNC 15 and 2045 CRNC 14, *M. intracellulare* CRNC 17, *M. marinum* CRNC 19, *M. scrofulaceum* CRNC 49, *M. scrofulaceum* CRNC 20, *M. szulgai* CRNC 21, *M. triviale* CRNC 22, *M. fortuitum peregrinum* CRNC 11, *M. phei* CRNC 12, *Rhodococcus equi* CRC 09/01, *Corynebacterium pseudotuberculosis* CRC 09/02 and *Nocardia asteroides* CRC 10/01.

Repeatability

The repeatability was estimated using seven samples of *M. tuberculosis* and three samples of *M. bovis*. We extracted DNA from seven samples on three different days and subjected the extracted nucleic acid to qPCR. To assess possible changes in the test, a second analyst performed the technique using the same criteria and the same samples.

Robustness

We analyzed the assay robustness with potential issues that may occur, such as an improperly calibrated pipette or thermocycler, to evaluate how these problems would impact the diagnosis of human tuberculosis. Five *M. tuberculosis* samples and five *M. bovis* samples were used in a qPCR assay with a 1 °C increased annealing tempera-

ture and 10% less enzyme, $MgCl_2$ and primers. A second qPCR was performed with a 1 °C decreased annealing temperature and 10% more enzyme, $MgCl_2$ and primers.

Statistical analysis

We used the McNemar test to compare paired proportions, the kappa test to determine the measure of agreement between two tests for each sample and the sensitivity and specificity analysis (Kraemer, 1992).

For the diagnostic sensitivity and diagnostic specificity analyses, 99 *M. tuberculosis* isolates from Brazil were used. Each sample was simultaneously tested by phenotypic and qPCR methods. The qPCR method was compared with the phenotype-based speciation method as a gold standard.

Results

qPCR

The average melting temperature for positive samples in the qPCR for *M. tuberculosis* was 94.11 °C with a standard deviation of 1.31 °C. The qPCR was able to detect up to 9.88 x 103 copies/µL in a standard curve with an R^2 of 99% and with 78% efficiency (Table 1). The average melting temperature for positive samples in the qPCR for ribosomal DNA was 87.2 °C.

Repeatability and robustness

The qPCR had similar results when performed on different days and by a different analyst. The average melting temperature was 94.72 °C with a standard deviation of 0.26 °C. No amplification was detected when *M. bovis* DNA was used. Variations in cycle thresholds (Ct) and melting temperatures detected in the robustness tests are described in table 2 and Figure 1.

Statistical analyses

The McNemar test showed no significant difference between the qPCR (p = 0.3173) and microbiological isolation methods, and the Kappa test showed perfect agreement (0.98) between the two tests.

Sensitivity and specificity

The qPCR detected only DNA extracted from *M. tuberculosis*. All other nucleic acid from other *Mycobacte*-

Table 1 - Mean values σ of Ct and standard deviations of qPCR.

Copies/µL	Concentration (ng/µL)	Ct	σ
9.88×10^6	47.5	20.56	0.64
9.88×10^5	4.7	24.03	0.24
9.88×10^4	0.47	28.95	0.48
9.88×10^3	0.047	32.64	0.41
9.88×10^2	0.0047	—	—

Table 2 - Variation of melting temperature and cycle threshold in robustness tests for qPCR.

Species	Sample	Robustness 1*		Robustness 2	
		Ct	Melting	Ct	Melting
M. tuberculosis	1	12.95	94.4	19.44	94.1
	2	18.31	94.4	29.44	94.5
	3	16.30	94.5	26.73	94.4
	4	17.37	93.56	24.88	93.63
	5	19.36	94.43	31.76	93.6
M. bovis	1	—-	—-	—-	—-
	2	—-	—-	—-	—-
	3	—-	—-	—-	—-
	4	—-	—-	—-	—-
	5	—-	—-	—-	—-

*Robustness 1: increase in 1 °C in annealing temperature and 10% less enzyme, $MgCl_2$ and primers; Robustness 2: decrease by 1 °C temperature annealing and 10% more enzyme, $MgCl_2$ and primers.
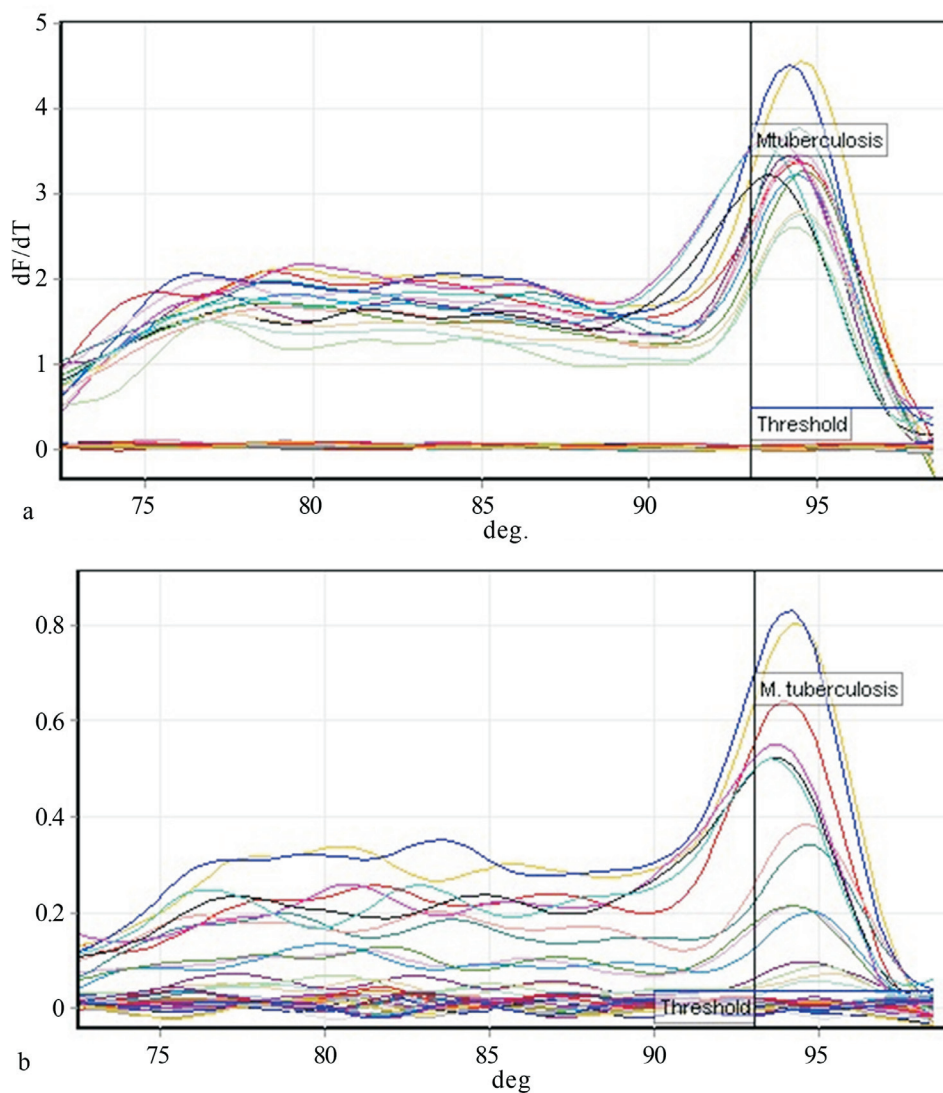

Figure 1 - Denaturation curve (dF/dT *vs.* temperature) of positive and negative samples used in robustness 1 (a) and robustness 2 (b) for qPCR-Mtub-115.

rium and another bacterial species tested in this work were negative in all of the performed tests. The diagnostic sensitivity and specificity were 100% (CI = 95.94% - 100%) and 100% (CI = 93.98% - 100%), respectively.

Discussion

Phenotypic tests used to identify *Mycobacterium* isolates are time consuming and unreliable. The use of molecular tests to correctly identify microorganisms is fast, reliable and sensitive. The qPCR developed in this study did not amplify DNA from any of the *Mycobacterium* spp. other than the *M. tuberculosis* samples used in the analytical specificity tests; positive results were only realized for the *M. tuberculosis* strains used as positive controls.

The qPCR tests described here do not detect three species of the MTC: *M. canettii*, *M. africanum* 1 and *M. africanum* 2. However, these results do not invalidate the use of this qPCR assay outside of Africa. *M. africanum* and *M. canettii* cause human tuberculosis but are restricted to Africa or cases closely related to that continent. Medical treatment for infections caused by these species is the same as for *M. tuberculosis* (Frothingham *et al.*, 1999, van Soolingen *et al.*, 1997). There are a few cases related to *M. africanum* in countries closely related to West Africa. Spain, for example, has more than 7500 cases of tuberculosis per year but registered only 57 cases caused by *M. africanum* between 2000 and 2010 (Isea-Peña *et al.*, 2012). We focused on the differentiation of *M. tuberculosis* and *M. bovis*. TB due to *Mycobacterium bovis*, the causative agent of bovine tuberculosis, is clinically indistinguishable from human TB due to *M. tuberculosis* and is an important cause of infection in developing countries (Silva *et al.*, 2013). This discrimination is possible only by the use of specific culture media and subsequent characterization by phenotypic and molecular speciation methods. The treatment for *M. bovis* infection differs from *M. tuberculosis* infection due to the natural resistance to pyrazinamide (Ruggiero *et al.*, 2007).

The efficiency of the qPCR was 78%, indicating the presence of inhibitors in the reaction, likely primer dimers or the formation of secondary structures in the amplicon. In fact, the primers have several repetitions of nucleotides, which increase the possibility of nonspecific reactions. The amplified region is rich in guanine and cytosine (G+C = 64%), which may hinder both the dissociation of amplicons and the formation of new amplicons due to the secondary structures (Raso *et al.*, 2011). Thus, this qPCR is not recommended for quantification of samples; however, the number of samples used in this work show that it is a reliable method to correctly identify *M. tuberculosis*.

Repeatability and reproducibility tests are required to establish a routine technique in the laboratory as stated in ISO 17025 and Principles and methods of validation of diagnostic assays for infectious diseases (OIE, 2010). This step is critical in the validation process and demonstrates that the method can be easily duplicated by another analyst and does not suffer interference from continuous repetition. The robustness tests showed that the diagnosis may be influenced by several factors together, such as a reduction in reagent amount and an increase in annealing temperature. The increase in reagent amount and the decrease in annealing temperature improved the performance of the qPCR. These changes did not amplify *M. bovis* used in the robustness tests, but it could reduce specificity, so we chose not to use it in our diagnostics.

This qPCR was developed with the goal of diagnosing the bacillus M. *tuberculosis* in bacterial suspension samples. Although not recommended for quantification, validation with a high number of positive and negative samples shows that it provides repeatability, reproducibility and diagnostic accuracy. It is important to validate the PCR with many samples. There are reports in literature that show unspecific results for detection of *M. tuberculosis* even when using regions considered safe for genetic characterization in MTC like RD9 and RD12 (Ueyama *et al.*, 2014). The method validated in this work could be used as an alternative to phenotypic tests in TB diagnosis, as it is a fast, reliable technique that uses a less expensive chemistry in real time PCR. It should also be taken into account that *M. tuberculosis* is the most prevalent agent in human TB. In addition to the fact that a large number of samples and tests (repeatability, reproducibility and robustness) have been used to validate these techniques, the qPCR developed in this work offers advantages over other PCR tests, which rely only on single nucleotide polymorphisms (Goh *et al.*, 2006), require electrophoresis in agarose gels (Shah *et al.*, 2002) or were tested on only few samples (Bakshi *et al.*, 2005). Teo *et al.* (2013) and Lee *et al.* (2010) demonstrated the importance of a PCR to correct identify *M. bovis* BCG infection, but used a low number of samples and a multiplex PCR based on electrophoresis in agarose gels. Reddington *et al.* (2011) used a multiplex real time PCR based on hydrolysis probe, buts as this kind of PCR is more expensive, it is of limited use in developing countries where tuberculosis is more prevalent. The qPCR validated in this work has advantages over these cited PCR tests, as it does not require agarose gel electrophoresis to visualize the results, it uses one of the least expensive qPCR fluorophores, and it is able to correctly identify *M. tuberculosis* in less than three hours after bacterial isolation. The results indicate that the qPCR reported here can be used in clinical laboratories that are implementing a quality system aimed at improving their ability to consistently produce valid results.

Bacterial suspensions suspected of containing *M. tuberculosis* could be subjected to this qPCR. If no amplification occurs, then the suspension may contain mycobacteria other than *M. tuberculosis*. In these cases, the same bacterial suspensions could be subjected to other phenotypic or molecular speciation methods to identify the correct spe-

cies. Finally, TB reference laboratories (health and agriculture sectors), public health programs and epidemiological studies will likely benefit from this method.

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