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Diagnostic performance of the RT-qPCR method targeting 85B mRNA in the diagnosis of pulmonary *Mycobacterium tuberculosis* infection

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ABSTRACT

Background: Several nucleic acid amplification techniques (IS6110, 16S rRNA, and 85B mRNA) were developed for the rapid, direct detection of *Mycobacterium tuberculosis*. We aimed to assess the diagnostic performance of 85B mRNA-based RT-qPCR by comparing with the real-time PCR COBAS TaqMan MTB Kit while using the BACTEC MGIT 960 method as the gold standard.
Methods: 60 patients with confirmed pulmonary TB and 60 individuals without TB were included as the study and control groups, respectively. Sputum specimens were cultured using LJ and BACTEC MGIT 960 systems. Extracted DNA was used for COBAS PCR in a CONAS TaqMan 48 analyzer. 85B mRNA detection was performed by RT-qPCR.
Results: The sensitivity, specificity, positive predictive value, negative predictive value, and accuracy of COBAS TaqMan MTB Test were detected as 93.3%, 83.3%, 84.8%, 92.6%, and 88.3%, respectively. The same diagnostic parameters of RT-qPCR were: 98.3%, 95.0%, 95.2%, 98.3%, and 96.7%, respectively. According to the binary logistic regression analysis, RT-qPCR (OR: 19,924, $p < 0.001$) was identified as the more optimal test.
Conclusion: RT-qPCR targeting the 85B gene of *M. tuberculosis* seems to be a more useful and rapid technique than DNA-based methods for detecting live *M. tuberculosis* bacilli from sputum specimens.

Introduction

According to the latest 2015 World Health Organization (WHO) Global Tuberculosis Report, 10.4 million new tuberculosis (TB) cases were estimated worldwide and 480,000 of them were multidrug-resistant TB (MDR-TB). The death toll of TB rose to 1.4 million in 2015, making TB one of the top ten causes of death worldwide in the same year [1]. The WHO aims to reduce the global incidence of TB to less than 100 cases per million by 2035, but this goal seems impossible unless an effective TB vaccine is discovered [2]. People with latent *Mycobacterium tuberculosis* (LTBI) have the highest risk for progression to active TB; infection control measures that include preventive chemotherapy are recommended for these patients. Taken together, an effective approach toward managing the TB epidemic will include faster diagnosis of TB compared to the current diagnostic methods [3].

The most widely used conventional method for TB diagnosis is direct microscopy combined with culture; however, the sensitivity of microscopy is only around 50–60% [4]. To quicken the TB diagnosis, nucleic acid amplification tests for mycobacterial DNA/rRNA have been developed [5]. *M. tuberculosis* (MTB) cultures grow very slowly and conventional culture methods require at least one to two months for sufficient detection in clinical specimens, much longer than for other common bacterial infections. For this reason, most of the new diagnostic test strategies are focused on shorten-

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ing the detection time [6]. Several methods based on nucleic acid amplification techniques (e.g., IS6110, 65 kDa heat shock protein, 16S rRNA, 85B mRNA) were developed for the rapid direct detection of *M. tuberculosis* in clinical specimens [7–11]; however, these PCR methods cannot differentiate between viable and nonviable forms of TB [12].

Except for TB culture, conventional diagnostic methods cannot distinguish active *M. tuberculosis* specifically and the popular BACTEC MGIT (Mycobacteria Growth Indicator Tube) system takes a long time to detect TB bacilli. However, the antigen 85 complex is secreted in large quantities from growing mycobacteria [13]. The antigen 85 complex is composed of three 30–32-kDa proteins that are located in the extracellular space and secreted during the growth of *M. tuberculosis* in vitro through an energy-dependent process [14]. For the purpose of this study, we decided to use bacterial mRNA as an indicator of cell viability because its average half-life is three minutes [15].

mRNA degrades more rapidly than rRNA or DNA and, therefore, it seemed suitable for enabling a faster diagnosis compared to amplification tests directed at rRNA or DNA [16]. For a target, we selected mRNA coding for the 85B protein, one of three homologous proteins that constitute the antigen 85 complex of mycobacteria, which is present in all mycobacteria [17]. This complex has species-specific and shared epitopes [18]; therefore, it is both universally applicable while still permitting species detection using different primers.

In this study, we aimed to assess the diagnostic performance of 85B mRNA-based reverse-transcriptase quantitative polymerase chain reaction (RT-qPCR) by comparing the RT-qPCR results with those of the real-time PCR COBAS TaqMan MTB kit, using the BACTEC MGIT 960 method as the gold standard.

Materials and methods

Study area and groups

This is a two-centre case-control study case-control study that took place between January 2016 and August 2016 involving the following participants:

1. Medical Microbiology Department of Cerrahpasa Medical Faculty, Istanbul University
2. Clinics and Microbiology Laboratory of Yedikule Chest Disease Education and Research Hospital, Istanbul

Initially, 112 clinical sputum samples were obtained with highly suspicious clinical/radiological/histopathological evidence of pulmonary TB from patients who were referred to the outpatient clinics of Yedikule Chest Disease Education and Research Hospital (94 samples) and Cerrahpasa Medical Faculty (18 samples). Total RNA extractions from decontaminated samples were stored at −70 °C until analysis. Fifty-two specimens were not used due to contamination, no growth in culture, receiving anti-tuberculosis treatment, etc. Finally, 60 patients with confirmed pulmonary TB, as determined through both the Lowenstein-Jensen (LJ) and BACTEC MGIT 960 systems, (BD Diagnostics, Sparks, MD) were included in this study. The control group was comprised of 60 individuals with no evidence or complaints of pulmonary TB; they applied to the clinics with various other conditions (e.g., cough, dyspnea, etc.) Their sputum specimens were also cultured using both the LJ and BACTEC MGIT 960 systems. The sex distribution and mean age among the patient and control groups, respectively, were 36/24 and 38.97 years (range 21–76 years) and 33/27 and 45.91 years (range 19–86 years). Microscopy and culture studies were applied both in Yedikule Chest Disease Education and Research Hospital (94 samples) and Cerrahpasa Medical Faculty (18 samples). After collection of sample and formation of study groups, total RNA isolation, DNA isolation, COBAS Taqman and 85B mRNA reactions were made at medical microbiology laboratory of Cerrahpaşa Medical Faculty.

Table 1
85B mRNA oligonucleotide primers and probe information.

Name	Sequence	Ref
85B mRNA forward	5-TCAGGGGATGGGGCCTAG-3	[19]
85B mRNA reverse	5-GCTTGGGGATCTGCTGCGTA-3	[19]
85B mRNA probe	5-FAM-TCGAGTGACCCGGCATGGGAGCGT-Tamra-3	[20]

Abbreviations: References (Ref.) [19,20].

The inclusion criteria for the study group were as follows: confirmed pulmonary TB cases with clinical/radiological, histopathological and microbiological evidence of pulmonary TB and not receiving anti-tuberculosis treatment before the onset of the study. All participants signed a written informed consent form approved by the Clinical Research Ethics Board of Istanbul University, Cerrahpasa Faculty of Medicine (No: 83045809/604.01, Date: 08.10.2015). The same Institutional Ethics Board also approved this study.

Processing of specimens

Analysis was performed at the Microbiology Laboratory of Yedikule Chest Disease Education and Research Hospital. All samples were processed for microscopy of smear examination and cultured [both in the BACTEC MGIT 960 and LJ systems]. The mycobacterial isolates obtained in culture were subjected to limited biochemical testing for species characterization using the BACTEC NAP TB Differentiation Test Kit, (Becton Dickinson, Sparks, MD, USA).

Molecular methods

MTB DNA detection with COBAS TaqMan MTB kit

The *Mycobacterium* genome contains a highly conserved region of approximately 1500 nucleotides encoding the gene for 16S rRNA. The Cobas TaqMan MTB Test uses *Mycobacterium* genus specific primers to define a sequence within this region. *M. tuberculosis* DNA was extracted manually from decontaminated patient samples using the AMPLICOR Respiratory Specimen Preparation Kit (Roche Diagnostics GmBH, Mannheim, Germany) according to the manufacturer's instructions. After DNA isolation, a commercial COBAS TaqMan 48 MTB Kit (Roche Diagnostics GmBH, Mannheim, Germany) was used immediately in the COBAS TaqMan 48 Analyzer (Roche Diagnostics GmBH, Mannheim, Germany). 50 μL of DNA extract were used in each PCR reaction. The results were automatically analyzed using the manufacturer's software.

85B mRNA detection with RT-qPCR

85B mRNA from *M. tuberculosis* was extracted from decontaminated samples using TRIzol (Invitrogen, Carlsbad, CA, USA) according to manufacturer's instructions including a modification described previously [19]. RNA concentration and purity ratios (OD260/280, OD260/230) were measured using the NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA). Purified RNA was stored at −70 °C until analysis. The primers and probe used were specific to the 85B sequence and amplify a 130 bp region (GenBank Accession number: X62398) [19,20] (Table 1).

RT-PCR was performed on a LightCycler 480 II Real-Time PCR System (Roche Diagnostics GmBH, Mannheim, Germany) using the 2X OneStep qRT-PCR Mastermix Kit (PrimerDesign Ltd. Southampton, UK) according to manufacturer's instruction.

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