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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.

Table 2
Baseline characteristics of all study participants.

Characteristics	Study group (*n*: 60)	Control group (*n*: 60)	Total (*n*: 120)
Age (Years)			
Median (min–max)	38.97 (21–76)	45.92 (19–86)	42.44 (19–86)
Sex			
Male	36 (60%)	33 (55%)	69 (57.5%)
Female	24 (40%)	27 (45%)	51 (42.5%)
Geographic origin			
From the city of Istanbul	52 (86.7%)	50 (834%)	102 (85%)
From Turkey (outside of Istanbul)	5 (8.3%)	3 (5%)	8 (6.67%)
Foreign nationality	3 (5%)	7 (11.6%)	10 (8.33%)

Table 3
Positivity ratios of different tests in pulmonary samples for study group.

Methods	Positive %	Negative %
LJ	60 (100)	–
MGIT 960	60 (100)	–
ZN	47 (78.3)	13 (21.7)
cobas Taqman MTB test	56 (93.3)	4 (6.7)
85B mRNA-based RT-qPCR method	59 (98.3)	1 (1.7)

The qPCR reaction was performed in a total volume of 20 μL consisting of 5 μL of total RNA (40 ng/μL), 10 μL of 2X qPCR Mastermix Kit (PrimerDesign Ltd. Southampton, UK), 1 μL of primer/probe mix (FAM reporter), and 4 μL of nuclease free water. PCR conditions were as follows: reverse transcription at 55 °C for 10 min and enzyme activation at 95 °C for 2 min followed by 50 cycles of denaturation for 10 s at 95 °C and annealing for 60 s at 60 °C. LightCycler 480 software (version 1.5) automatically analyzed the results.

Statistical methods

The diagnostic methods were compared using Fisher's exact test or chi-square test. The results for sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) were obtained using the BACTEC MGIT 960 and LJ systems as a gold standard. Student's *t*-test was used to compare mean ages and sex. Binary logistic regression was used to analyze the variables. All statistical analyses were performed using SPSS, version 20 (IBM Corporation, Somers, NY, USA) and MedCalc, version 13 (MedCalc, Mariakerke, Belgium) statistical software packages.

Results

The baseline characteristics of the study group (60 patients with confirmed pulmonary TB) and control group are shown in Table 2.

Positivity ratios of different methods in pulmonary samples for study group were shown in Table 3.

Results of the COBAS TaqMan MTB Test and 85B mRNA-based RT-qPCR method in relation to gold standard method (MGIT 960 method) are detailed in Table 4.

Sensitivity, specificity, PPV, NPV, and accuracy of COBAS TaqMan MTB Test were determined to be as 93.3% (95% CI: 83.8–98.15%), 83.3% (95% CI: 71.48–91.71%), 84.8%, 92.6% and 88.3%, respectively. The same diagnostic parameters of 85B mRNA-based RT-qPCR method were as follows: 98.3% (95% CI: 91.06–99.96%), 95.0% (95% CI: 86.08–98.96%), 95.2%, 98.3% and 96.7%, respectively (Table 5).

Table 5
Diagnostic performance parameters of COBAS TaqMan MTB DNA Test and 85B mRNA-based RT-PCR method.

	COBAS TaqMan MTB DNA	85B mRNA-based RT-PCR
Sensitivity	93.33	98.33
Specificity	83.33	95.00
PPV	84.85	95.16
NPV	92.59	98.28
Efficiency	88.33	96.67

Abbreviations: PPV; positive predictive value, NPV; negative predictive value.

The results of the binary logistic regression analysis of the 85B RNA-based RT-qPCR method and the COBAS TaqMan MTB Test are shown in Table 4. The 85B mRNA-based RT-PCR method (OR: 19,924, $p < 0.001$) was identified as a successful test for pulmonary TB diagnosis when BACTEC MGIT 960 was considered as a dependent variable (Table 6).

Discussion

We determined the sensitivity and specificity of the 85B mRNA-based RT-qPCR method for the diagnosis of pulmonary TB as 98.3% and 95.0%, respectively. Our diagnostic sensitivity values were higher than those of Jou et al. [16], Therese et al. [21] and Negi et al. [22] and similar to the results of Saraswat et al. [23] and Montenegro et al. [24]. However, our specificity results were lower than those of Jou et al. [16] and Montenegro et al. [24]. Our sensitivity and specificity results were 93.3% and 83.3%, respectively, for the COBAS TaqMan MTB Test and lower than the 85B mRNA-based RT-qPCR method. We found that the 85B mRNA-based RT-qPCR method is more useful for the diagnosis of pulmonary TB. In contrast, Negi et al. [22] reported the sensitivity and specificity of PCR targeting IS6110 were 83% and 94.59%, respectively, while the same values for 85B were 70.4% and 89.18%. However, the ability to differentiate between live and dead bacilli makes the 85B mRNA-based RT-qPCR method more useful than DNA-based PCR methods.

In one of these studies, Jou et al. [16] developed a single-tube, nested, reverse-transcription PCR method for the detection of viable *M. tuberculosis* by targeting bacterial 85B mRNA wherein a positive signal indicates the presence of a recently viable organism. They developed specific primers and their detection limit was 40 colony-forming units (CFU) in smear-negative sputum samples

Table 4
Results of two diagnostic tests in relation to the MGIT 960 method.

Cobas TaqMan MTB Test	MGIT 960 Method		85B mRNA-based RT-qPCR method	MGIT 960 Method	
	Positive (*n*)	Negative (*n*)		Positive (*n*)	Negative (*n*)
Positive (60)	56	10	Positive (60)	59	3
Negative (60)	4	50	Negative (60)	1	57
Total (120)	60	60	Total (120)	60	60

Table 6
Binary logistic regression for two methods in the study and control groups.

Variables	B	SE	Wald	*p*	OR	95% CI for OR	
						Lower	Upper
Method 1	1.204	0.720	2.794	0.095	3.332	0.812	13.669
Method 2	2.992	0.670	19.968	<0.001	19.924	5.364	74.012
Constant	2.473	1.715	2.080	0.149	11.858		

Abbreviations: Method 1; COBAS TaqMan MTB test; Method 2: 85B mRNA-based RT-qPCR method; B – beta regression coefficient; SE – standard error; Sig – significant; OR – odds ratio.
CI_{95} – confidence interval.

and 12 CFU in clinical sputum specimens. Their test sensitivity was 83%, which was greater than the sensitivity of TB culture (75%). The specificity of their PCR assay was 100% and was not inhibited by the addition of isoniazid. In another study, Saraswat et al., [23] used a modified molecular method, single test reverse-transcription (STN RT-PCR), which works through the amplification of a 216 bp region of the mRNA encoding the 85B antigen for the diagnosis of active genital TB. The results of their modified STN RT-PCR method were the same as the MGIT method (17/60, positive). They concluded that their STN RT-PCR method can be used for the early diagnosis of active genital TB from clinical endometrial tissue specimens. Negi et al. [22] compared the PCR amplification of *M. tuberculosis*-specific DNA sequences (IS6110, 65 kDa, 38 kDa) and mRNA coding for the 85B protein in pulmonary and extra-pulmonary samples for TB. They showed that each PCR test had a higher positivity than the BACTEC culture ($p < 0.05$) and no significant difference was detected between their four PCR protocols ($p > 0.05$). The primer specific for amplifying the 123 bp IS6110 fragment had the highest positivity (83%) and the sensitivity of the 85B mRNA assay was 70.4%. In pulmonary samples, the sensitivity of the 85B protein assay was also lower (67.6%). The specificity of the mRNA coding for the 85B protein was 89.18%.

Montenegro et al. [24] recently assayed the performance of an 85B mRNA RT-qPCR method for the diagnosis of pulmonary TB and its treatment. Fifty-six patients with signs of pulmonary TB were evaluated by culture and RT-qPCR in a 30-day period of anti-tuberculosis treatment. We focused on the diagnostic sensitivity of 85B mRNA-based RT-qPCR method over the follow-up results. While 51 sputum samples from 56 patients with a confirmed pulmonary TB were positive by culture, 52 (93%) were positive by RT-qPCR. In the study of Therese et al. [21], 301 sputum samples from TB patients were studied using the STN RT-PCR method with 100% specificity and 69.1% sensitivity. All of their 82 patients with negative cultures and positive RT-PCR were eventually diagnosed with TB. Maksymowicz-Mazur et al. [25] also reported the high specificity and sensitivity of quantitative analysis of 85B mRNA in *M. tuberculosis* cultures isolated from TB patients. On the other hand, in a study targeting IS6110 DNA, Yuen et al. [26] reported that 70% of 41 patients with TB were positive by PCR four weeks after the onset of therapy, but only 32% of the cultures were positive; this study shows the disadvantages of DNA-based molecular diagnosis because the results of the culture and the IS6110 DNA analysis do not match. DNA-based methods can also detect dead bacilli, while mRNA-detecting methods only show live bacilli and will give negative results after successful anti-tuberculosis treatment. In another study, Eisenach et al. [27] reported DNA positivity 500 days after the onset of therapy.

According to the results from the binary logistic regression analysis on the 85B RNA-based RT-qPCR method and COBAS TaqMan MTB Test, the 85B mRNA-based RT-PCR method (OR: 19,924, $p < 0.001$) was identified as a more optimal test for pulmonary TB diagnosis when BACTEC MGIT 960 results were used as a dependent variable.

In other studies, the 85B mRNA method has been used for assessing the treatment outcome for pulmonary or extra-pulmonary TB. The slow growth rate of *M. tuberculosis* in culture and the inability of DNA-based PCR assays to differentiate live or dead bacilli have directed researchers to the 85B mRNA method. Desjardin et al. [20] compared the *M. tuberculosis* 85B mRNA, 16S rRNA, and IS6110 DNA methods in terms of anti-tuberculosis treatment outcomes. Their study was the first to evaluate the usefulness of mRNA as potential surrogate markers for measuring the success of anti-tuberculosis treatment. In their study, which included 19 smear-positive pulmonary tuberculosis samples, they showed that the levels of 85B mRNA declined after the initiation of therapy. Therefore, they concluded that the rapid disappearance of *M. tuberculosis* mRNA from sputum suggests that it is a good indicator of microbial viability and a useful marker for the rapid assessment of response to chemotherapy. 16S rRNA decreased rapidly (ten-fold during the first week) but 85B mRNA was undetectable in the majority of their patients by day seven, except for the patient with treatment failure.

Interestingly, the 85B antigen is involved in cell wall biosynthesis and also serves a target of the immune response. Wilkinson et al. [28] reported that the level of 85B mRNA increased approximately 54 times, compared to only 14.6 times for 16S ribosomal rRNA, during the first 24 h of intracellular *M. tuberculosis* infection. Immunization with Ag85B DNA or purified Ag85B protein induced a strong antigen-specific CD4+ T cell and IFN-γ response and protected against TB. Vaccination with a related Ag85B DNA sequence may also induce immunopathology, as has been shown in guinea pigs; however, after the vaccination, the guinea pigs died as a result of lymphocytic granulomas [29]. Moreover, Wu et al. [30] applied Ag85B DNA intranasally in a murine model of asthma and they observed the inhibition of eosinophilic airway inflammation through the down-regulation of Th2 cytokines and up-regulation of Th1 cytokines. They suggested that Ag85B DNA may be used in the development of DNA vaccines, further confirming that Ag85B not only serves as a component for cell wall biosynthesis but also as a target of the immune response.

In conclusion, our results suggest that the performance of the mRNA-based RT-qPCR method is better for diagnostic purposes than the DNA-based COBAS TaqMan MTB Test. In conventional methods, Ziehl-Neelsen (ZN) smear microscopy, which has poor sensitivity and multiple repeats are required to diagnosis of TB. The other conventional mycobacterial culture method, although considered as the gold standard but is slow and usually takes 2–6 weeks time to yield a final result and requires proper infrastructure and technical expertise. Our positivity rates for ZN smear microscopy was very low (78.3%) when compared culture and molecular methods. Therefore, there is a need for a reliable, cost-effective detection method with high sensitivity and specificity. The need for biomarkers in TB is most important under two conditions that are crucial for new TB diagnostics: in active pulmonary TB disease, to monitor anti-tuberculosis treatment outcomes; and in latent *M. tubercu-*

losis infection, to indicate reactivation risk and predict treatment success. Using 85B mRNA as a diagnostic tool seems to be a good candidate for detecting active pulmonary TB disease and monitoring anti-tuberculosis treatment outcomes. RT-qPCR targeting the 85B gene of *M. tuberculosis* is a more useful and rapid technique than DNA-based methods for detecting live *M. tuberculosis* bacilli from sputum samples. Future investigations include comprehensive large-scale studies, extra-pulmonary specimens, and to evaluate its early diagnosis potential in active pulmonary TB.

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Competing interests

None declared.

Ethical approval

This study was approved by the Clinical Research Ethics Board of Istanbul University, Cerrahpasa Faculty of Medicine (No: 83045809/604.01, Date: 08.10.2015). All patients provided their informed consent to participate in the study.

Authors' contributions

All authors have approved of the final article. Conceived and designed the experiments: BK, SS, EC, MD. Performed the experiments: MD, NO, ST, GA, PY, HOD. Analyzed the data: OU, Contributed reagents/materials/analysis tools: HBT, TZ. Wrote the paper: BK SS.

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