Real-time polymerase chain reaction as an alternative method for diagnosis of multidrug-resistant tuberculosis: can it stand alone in this concern?

Gamal Rabie Agamy[a,b], Safaa Moktar Wafy[b], Asmaa Omar Ahmed[c], Lamees Mohamed Bakar[b]

Background Delays in diagnosing multidrug-resistant tuberculosis (MDR-TB) are responsible for higher tuberculosis morbidity and mortality and its subsequent transmission. Molecular assays such as real-time PCR (RT-PCR) used to identify drug resistance in *Mycobacterium tuberculosis* are more rapid than standard drug susceptibility testing.

Objectives The aim of this study was to evaluate the diagnostic performance of the Anyplex MTB/MDR RT-PCR assay in detecting MDR-TB strains.

Patients and methods Sputum samples were collected from 29 patients with symptoms and radiological findings suggestive of active pulmonary tuberculosis, with at least one of three sputum smear samples showing acid-fast bacilli and/ or sputum culture isolates positive for *M. tuberculosis.* The results obtained by RT-PCR were compared with those obtained by the *Mycobacterium* growth indicator tube SIRE method.

Results *M. tuberculosis* was confirmed in 29 specimens. Only six cases determined as MDR-TB were obtained by *Mycobacterium* growth indicator tube SIRE. For detection of rifampicin-resistant and isoniazid-resistant strains, the RT-PCR assay yielded a sensitivity of 62.5 and 66.66% and specificity of 80 and 95%, respectively. The overall sensitivity of that assay was 64.2% and specificity was 88.88%.

Conclusion RT-PCR is an easy and reliable assay for rapid detection of MDR-TB in clinical specimens. However, RT-PCR should be followed by a culture method to increase the overall sensitivity of that assay.

[a]ERS National Delegate of Egypt, [b]Chest Diseases and Tuberculosis Department, [c]Microbiology and Clinical Pathology Department, Faculty of Medicine, Assuit University, Assuit, Egypt

Correspondence to Dr. Lamees Mohamed Bakar, PhD, Fellow at Chest Diseases and Tuberculosis Department, Faculty of Medicine, Assuit University Hospital, Assuit, 71526, Egypt. Fax: +20882333327;
e-mail: lameesbakar@yahoo.com

Introduction

In recent years, the emergence of drug-resistant strains, defined as resistance to at least isoniazid (INH) and rifampicin (RIF), has been an important problem and is threatening the control of tuberculosis (TB) [1]. Therefore, the rapid detection of drug resistance is essential to begin effective therapies and protect the community from TB.

Drug resistance in *Mycobacterium tuberculosis* is due to mutations in genes or promoters of genes activating the drug or encoding the drug targets, which are detectable in the majority of drug-resistant isolates [2]. Ninety-five percent of mutations associated with RIF resistance occur mainly in an 81-bp RIF-resistance-determining region of the *rpoB* gene [3]. Mutations associated with INH resistance occur mainly in the *katG* gene (codon 315), the *inhA* gene and regulatory region, and the *ahpC* regulatory region [4].

Several molecular methods have been previously described for drug susceptibility testing (DST) of *M. tuberculosis*, including real-time PCR (RT-PCR), for simultaneous detection of *M. tuberculosis* and its drug susceptibility [5].

A key advantage of genotypic drug susceptibility assays over phenotypic assays is the shorter time required for the assay, with genotypic assays requiring just hours to complete in contrast to phenotypic tests that can take weeks. Because of the need to grow the organism, phenotypic methods require weeks of culture, during which the patient may be treated with the wrong antibiotics, resulting in poorer treatment outcome or the transmission of resistant strains [6].

This study aimed to evaluate the diagnostic efficacy of RT-PCR in the detection of RIF-resistant and INH-resistant *M. tuberculosis* strains. The drug susceptibility obtained with the *Mycobacterium* growth indicator tube (MGIT) SIRE served as the gold standard for comparison with that obtained with the Anyplex II MTB/multidrug-resistant (MDR) RT-PCR assay.

Patients and methods
M. tuberculosis isolates were obtained from 29 patients attending the outpatient clinic of the Department of Chest in 1 year, selected from those presenting with symptoms and radiological findings suggestive of active pulmonary TB, with at least one of three sputum smear samples showing acid-fast bacilli and/or sputum culture isolates positive for *M. tuberculosis.*

Specimen collection
Three early morning sputum specimens on 3 consecutive days were collected from each patient [7]. The specimens were transported to the TB lab. of the Microbiological Unit in the Department of Clinical Pathology, Faculty of Medicine, Assiut University, and were screened by direct Ziehl–Neelsen smear for acid-fast bacilli, and further processing of the specimens was performed.

Sputum processing
The specimens were decontaminated and liquefied by 2% *N*-acetyl-l-cysteine NaOH according to the manufacturer's instructions [8]. Finally, after the last centrifugation step, the processed samples were used for Ziehl–Neelsen smear preparation (concentration method), as inoculum for culture on MGIT and for DNA extraction.

Culture on *Mycobacterium* growth indicator tube medium
Inoculation of 200 μl of processed specimens on MIGT should be carried out according to the manufacturer's instructions and kept at 37°C. The tubes are to be read daily, starting on the third day of inoculation, for 2 weeks. The MGIT are visually located on an UV transilluminator and compared with positive and negative control tubes. The positive control should show a high amount of fluorescence (very bright orange color at the bottom of the tube and an orange reflection on the meniscus). The negative control should have very little or no fluorescence. Positive tubes should be stained for acid-fast bacilli, and subculture for DST should be carried out with MGIT SIRE tubes.

Drug susceptibility testing
Mycobacterium growth indicator tube SIRE Kit
We used the instructions provided by the manufacturer for sensitivity testing. The modified critical concentrations of the provided drugs (SIRE, Becton Dickinson Microbiology System of antibiotic susceptibility by MGIT SIRE Kit; Becton Dickinson Microbiology System, Cockeysville, Maryland, USA) were adopted: INH, 0.1 μg/ml; RIF, 1 μg/ml [9].

Anyplex II MTB/MDR detection assay
Sputum specimens were examined by the Anyplex II MTB/MDR detection assay for the simultaneous detection of *M. tuberculosis* and its resistance to first-line anti-TB drugs (INH and RIF) using the CFX96 RT-PCR System (Bio-Rad). It covers six mutations causing INH resistance in the *katG* gene and *inh A* promoter region, and 15 mutations causing RIF resistance in the *rpoB* gene.

DNA extraction
A part of the processed samples was subjected to DNA extraction using the Epicenter kit according to the manufacturer's instructions with the DNA extraction solution included in the Anyplex II MTB/MDR detection kit from Seegene System (Seegene, Anyplex RT-PCR, Taewon) [10].

Real-time polymerase chain reaction
Each sample was tested in two separate reactions (MTB/NTB and MTB/MDR) according to the manufacturer? s instructions. One reaction was used for the amplification and detection of MTB in sputum, and the second was used for detection of multidrug-resistant tuberculosis (MDR-TB) of the MTB-positive sample.

The wild-type control was designed to exhibit the same result pattern with a drug-susceptible *M. tuberculosis* sample, and the drug-resistant result of unknown samples was analyzed on the basis of the result of the wild-type control.

Statistical analysis
The results of the antimicrobial susceptibility test and RT-PCR assay were analyzed using the computer software SPSS (statistical package for the social science, version 10.999; SPSS Inc., Chicago, Illinois, USA).

Results
The study was carried out on sputum samples from 29 patients (17 male and 12 female) with an age range of 19–75 years.

RT-PCR carries the advantages of simultaneous detection of *M. tuberculosis* (Table 1) and its susceptibility, with availability of the results within 1 working day in comparison with 12–14 days when MGIT SIRE is used (Table 2).

MGIT SIRE revealed that 20% (6 out of 29 cases) of the isolated TB bacilli were MDR-TB bacilli.

Table 1 Mean time required for isolation of *Mycobacterium tuberculosis*

Sample results	MGIT TB isolation (days)	RT-PCR (h)
Positive	10 (3–14)	4
Negative	17 (11–21)	4

MGIT, *Mycobacterium* growth indicator tube; RT-PCR, real-time PCR; TB, tuberculosis.

Table 2 Mean time required for drug susceptibility testing of *Mycobacterium tuberculosis*

Sample results	MGIT SIRE TB susceptibility testing (days)	RT-PCR (h)
Susceptible strains	10 (3–14)	24
Resistant strains	17 (11–21)	24

MGIT, *Mycobacterium* growth indicator tube; RT-PCR, real-time PCR; TB, tuberculosis.

Table 3 shows results for detection of MDR (resistance to RIF and/or INH) in 29 cases with Anyplex II MTB/MDR detection assay compared with MGIT SIRE susceptibility results.

Out of 29 specimens that were tested with MGIT SIRE and RT-PCR susceptibility tests, nine (31%) specimens were determined resistant to INH with RT-PCR, and eight of these specimens were in agreement with RT-PCR when tested with the MGIT SIRE. Only one specimen was determined genotypically resistant and phenotypically susceptible (Table 3).

As regards RIF susceptibility results, five (17.2%) out of 29 specimens were determined resistant to RIF with RT-PCR and MGIT SIRE. Only one specimen was determined genotypically susceptible and phenotypically resistant (Table 3).

The sensitivity and specificity of the RT-PCR susceptibility test for detecting INH resistance using the MGIT SIRE test as reference were 62.5 and 81%, respectively.

The sensitivity and specificity of the RT-PCR susceptibility test for detecting RIF resistance using the MGIT SIRE test as reference were 66.66 and 95%, respectively.

Overall, this assay showed a sensitivity of 64.2% and specificity of 88.88% for the detection of MDR strains.

Discussion

Globally, in 2015, 3.5% of new and 20.5% of previously treated TB cases were estimated to have had MDR-TB

Table 3 Comparison between real-time polymerase chain reaction and *Mycobacterium* growth indicator tube SIRE drug susceptibility results

Antimicrobial agents	Number of isolates with drug susceptibility test [*N* (%)]	
	RT-PCR	MGIT 960
INH		
Resistant strains	9 (31)	8 (27.5)
Susceptible strains	20 (68.9)	21 (72.4)
RIF		
Resistant strains	5 (17.2)	6 (20.6)
Susceptible strains	24 (82.7)	23 (79.3)

INH, isoniazid; MGIT, *Mycobacterium* growth indicator tube; RIF, rifampicin; RT-PCR, real-time PCR.

[11]. However, patients are often not expeditiously diagnosed, resulting in the delay of appropriate treatment as well as poorer treatment outcomes for patients and the propagation and spread of MDR-TB [6].

Conventional methods for DST of MDR-TB require between 2 and 5 weeks. Implementation of easy and rapid methods for DST is thus of paramount importance to limit the spread of drug-resistant TB [12].

The Anyplex II MTB/MDR RT-PCR assay has been proposed as an alternative approach to detect drug-resistant strains, as the results obtained with the conventional susceptibility methods of *M. tuberculosis* come too late to influence a timely decision on patient management [13].

The use of MGIT SIRE for MDR-TB identification gave results of 20, 20.68, and 27.58% for overall MDR, RIF resistance, and INH resistance, respectively. Similar to our results, El-Sayed Zaki and Hassanin [14] reported that the rate of MDR-TB was 20%, whereas the rates of resistance to RIF and INH in Egypt were 20 and 20%, respectively. A resistance rate of 14.8% for MDR-TB, 16.3% for RIF, and 20.7% for INH were reported by Abdullal [15].

In the current study, 29 specimens were tested with both methods, the MGIT SIRE and Anyplex MTB/MDR assay, for detection of RIF and INH resistance. Overall, we reported six specimens that were determined as MDR strains with MGIT SIRE. The Anyplex II MTB/MDR RT-PCR assay showed that four specimens out of the six MDR strains were compatible with the MGIT SIRE results, whereas two specimens out of the six MDR strains were determined as false-negative results (susceptible by RT-PCR, resistant by MGIT SIRE), therefore

becoming inconsistent with MGIT SIRE results. One specimen was determined with Anyplex II MTB/MDR RT-PCR assay as an MDR strain that would have been interpreted as a susceptible strain with MGIT SIRE (false-positive results). The discrepancies between the molecular and phenotypic methods reported in this study have been reported by others previously, wherein phenotypically resistant strains (detected by MGIT SIRE) were detected as susceptible strains by molecular methods (RT-PCR) [16–18].

The false-negative results (two specimens) could be explained by the fact that phenotypic assays can detect resistance mediated by other mechanisms, as well as unknown mechanisms that are not yet understood. False-positive results could be explained by the presence of mixed populations of resistant and susceptible *M. tuberculosis* bacilli in the initial sputum specimen in which the mutant genes are recognized by the molecular assay and therefore can be considered as masking or dominating the susceptible genes.

RT-PCR susceptibility results showed 62.5% sensitivity for detection of INH resistance and 66.66% for detection of RIF resistance, respectively. Overall, this assay showed a sensitivity of 64.2% and a specificity of 88.88% for the detection of MDR strains.

Goncalves *et al.* [13] reported that the sensitivity of the RT-PCR assay in detecting INH resistance was 55% and that for detecting RIF resistance was 99%. The specificity of both tests was 100%. Another study by ElFeky and ElShimy [11] reported that the sensitivity and specificity for detection of INH resistance were 83.3 and 100%, respectively.

The differences in results in the different studies could be explained by the geographic and genetic variation in the distribution of drug-resistant strains of *M. tuberculosis*; different methods used to perform the phenotypic susceptibility testing, difference in the year of *M. tuberculosis* isolation, and the use of different sizes of sample collections might partially explain the present findings.

In conclusion, RT-PCR is rapid, specific, and a technically affordable molecular technique for detection of MDR-TB in clinical specimens. The optimal approach for DST of *M. tuberculosis* will likely involve both conventional methods (culture) and a molecular method (RT-PCR) to identify resistant strains missed by RT-PCR assay when used alone. This approach should facilitate the adequate management of MDR-TB and limit the extent and severity of MDR-TB transmission and infection.

Conflicts of interest
There are no conflicts of interest.

References

1 Espinal MA. The global situation of MDR-TB. *Tuberculosis (Edinb)* 2003; **83**:44–51.

2 Soini H, Musser JM. Molecular diagnosis of mycobacteria. *Clin Chem* 2001; **47**:809–814.

3 Lee AS, Lim IH, Tang LL, Wong SY. High frequency of mutations in the rpoB gene in rifampin-resistant clinical isolates of *Mycobacterium tuberculosis* from Singapore. *J Clin Microbiol* 2005; **43**:2026–2027.

4 Hazbon MH, Brimacombe M, Bobadilla del Valle M, Cavatore M, Guerrero MI, Varma-Basil M, et al. Population genetics study of isoniazid resistance mutations and evolution of multidrug-resistant *Mycobacterium tuberculosis*. *Antimicrob Agents Chemother* 2006; **50**:2640–2649.

5 Bergval IL, Vijzelaar RN, Dalla Costa ER, Schuitema AR, Oskam L, Kritski AL, et al. Development of multiplex assay for rapid characterization of *Mycobacterium tuberculosis*. *J Clin Microbiol* 2008; **46**:689–699.

6 Ong DC, Yam WC, Siu GK, Lee AS. Rapid detection of rifampicin- and isoniazid-resistant *Mycobacterium tuberculosis* by high-resolution melting analysis. *J Clin Microbiol* 2010; **48**:1047–1054.

7 Lagrange PH, Thangaraj SK, Dayal R, Despande A, Ganguly NK, Girardi E, et al. A toolbox for tuberculosis diagnosis: an Indian multicentric study (2006-2008): microbiological results. *PloS one* 2012; **7**:e43739.

8 Kent PT, Kubica GP. *Public Health mycobacteriology: A guide for the level III laboratory*. Georgia: US Departement of Health and Human Services, CDC Atlanta; 1985.

9 Barenfanger J. Making your lab safe against multi-drug resistant *Mycobacterium tuberculosis*. *Clin Microbiol Newsl* 1993; **15**:76–80.

10 Greer CE, Wheeler CM, Manos MM. Sample preparation and PCR amplification from paraffin-embedded tissues. *PCR Methods Appl* 1994; **3**:S113–S122.

11 ElFeky DS, ElShimy A. Evaluation of the diagnostic performance of Anyplex II MTB/MDR/XDR detection assay for Detection and antimicrobial susceptibility testing of *Mycobacterium tuberculosis* from sputum specimens. *Int J Curr Microbiol App Sci* 2015; **4**:657–669.

12 Ramirez MV, Cowart KC, Campbell PJ, Morlock GP, Sikes D, Winchell JM, et al. Rapid detection of multidrug-resistant *Mycobacterium tuberculosis* by use of real-time PCR and high-resolution melt analysis. *J Clin Microbiol* 2010; **48**:4003–4009.

13 Goncalves MG, Fukasawa LO, Oliveira RS, Salgado MM, Harrison LH, Shutt KA, et al. Fast test for assessing the susceptibility of *Mycobacterium tuberculosis* to isoniazid and rifampin by real-time PCR. *Mem Inst Oswaldo Cruz* 2012; **107**:903–908.

14 El-Sayed Zaki M, Hassanin N. DNA sequence analysis of rpoB Gene Mutations in rifampicin resistant *Mycobacterium tuberculosis*. *J Rapid Meth Aut Microbiol* 2006; **14**:237–248.

15 Abdullal AK. *Direct and rapid detection of multidrug resistant Mycobacterium tuberculosis (MDR-MT) in sputum samples*. London, UK: World Congress of Infectious Diseases 10–12 August 2015.

16 Chan RC, Hui M, Chan EW, Au TK, Chin ML, Yip CK, et al. Genetic and phenotypic characterization of drug-resistant *Mycobacterium tuberculosis* isolates in Hong Kong. *J Antimicrob Chemother* 2007; **59**:866–873.

17 Nimri L, Samara H, Batchoun R. Detection of mutations assaciated with multidrug resistant *Mycobacterium tuberculosis* clinical isolates. *FEMS Immunol Med Microbiol* 2011; **62**:321–327.

18 Plinke C, Cox HS, Kalon S, Doshetov D, Rusch-Gerdes S, Niemann S. Tuberculosis ethambutol resistance: concordance between phenotypic and genotypic test results. *Tuberculosis (Edinb)* 2009; **89**:448–452.