Fast test for assessing the susceptibility of *Mycobacterium tuberculosis* to isoniazid and rifampin by real-time PCR

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Mycobacterium tuberculosis *is the bacterium that causes tuberculosis (TB), a leading cause of death from infectious disease worldwide. Rapid diagnosis of resistant strains is important for the control of TB. Real-time polymerase chain reaction (RT-PCR) assays may detect all of the mutations that occur in the* M. tuberculosis *81-bp core region of the* rpoB *gene, which is responsible for resistance to rifampin (RIF) and codon 315 of the* katG *gene and the* inhA *ribosomal binding site, which are responsible for isoniazid (INH). The goal of this study was to assess the performance of RT-PCR compared to traditional culture-based methods for determining the drug susceptibility of* M. tuberculosis. *BACTEC™ MGIT™ 960 was used as the gold standard method for phenotypic drug susceptibility testing. Susceptibilities to INH and RIF were also determined by genotyping of* katG, inhA *and* rpoB *genes. RT-PCR based on molecular beacons probes was used to detect specific point mutations associated with resistance. The sensitivities of RT-PCR in detecting INH resistance using* katG *and* inhA *targets individually were 55% and 25%, respectively and 73% when combined. The sensitivity of the RT-PCR assay in detecting RIF resistance was 99%. The median time to complete the RT-PCR assay was three-four hours. The specificities for tests were both 100%. Our results confirm that RT-PCR can detect INH and RIF resistance in less than four hours with high sensitivity.*

Key words: *Mycobacterium tuberculosis* - RT-PCR - drug resistance - INH - RIF

Mycobacterium tuberculosis is the bacterium that causes tuberculosis (TB), a leading cause of death from infectious disease worldwide. In 2008, approximately two million deaths and nine million new cases of TB were reported and 5% of these were resistant to anti-TB drugs (WHO 2009a, b). In Brazil, TB was the fourth leading cause of death from infectious disease in 2008 and the first for acquired immune deficiency syndrome patients (MS/SVS 2010), with an incidence of 48 per 100,000 inhabitants (SESSP/SVE 2006, MS/SVS 2009). From the 11,900 isolates processed in our laboratory during the last three years (2007-2009), 15% exhibited resistance to at least one drug and 5.5% were multidrug-resistant (MDR).

Rifampin (RIF) resistance is an excellent marker for MDR TB. Approximately 95% of all RIF-resistant strains contain mutations localised in an 81-bp core region of the *rpoB* gene (Telenti et al. 1993, Riska et al. 2000) and all mutations that occur in that region result in RIF resistance (Telenti et al. 1993, El-Hajj et al. 2001). In contrast to RIF, 75% of isoniazid (INH)-resistant strains have mutations either in codon 315 of the *katG* gene or in the *inhA* ribosomal binding site accompanied by mutations in the *ahpC* gene and/or the *oxyR* intergenic region (Piatek et al. 2000, Rossetti et al. 2002). In Brazil, similar percentages of mutations in the *rpoB* and *katG* genes have been described (Valim et al. 2000, Rossetti et al. 2002).

Rapid diagnosis of resistant strains is important for the control of TB. The isolation and subsequent susceptibility testing of *M. tuberculosis* remain the gold standard, but this process takes many weeks to complete (Wada et al. 2004, MS/SVS/DVE 2008). The real-time polymerase chain reaction (RT-PCR*)* assay is faster and may detect all mutations that occur in the *M. tuberculosis rpoB* 81-bp core region, the *katG* 315 codon and the *inhA* ribosomal binding site (Piatek et al. 2000, El-Hajj et al. 2001, Marín et al. 2004, Ruiz et al. 2004, Wada et al. 2004, Yesilkaya et al. 2006, Boehme et al. 2010). In this study, we investigated an RT-PCR assay based on molecular beacon probes to determine susceptibility and/or resistance of *M. tuberculosis* isolates to INH and RIF in comparison with the phenotypic gold standard method that, in our study, was considered to be the automatised BACTEC MGIT 960 susceptibility assay (Becton Dickinson. Available from: finddiagnostics.org/export/sites/default/resource-entre/find_documentation/pdfs/ mgit_manual_nov_2007.pdf). The goal was to assess whether RT-PCR is able to produce sensitive, specific and faster results than the traditional method for routine laboratory drug-susceptibility testing.

SUBJECTS, MATERIALS AND METHODS

M. tuberculosis isolates - We selected the first 988 isolates of *M. tuberculosis* received in our laboratory between October 30 2008-March 13 2009 for phenotypic drug-susceptibility testing from TB patients living in greater São Paulo, Brazil. *M. tuberculosis* lysates were prepared by transferring colonies from a Löwenstein-Jensen slant into a 2 mL screw-cap tube containing

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1 mL of H_2O. The suspension was boiled for 20 min and frozen at -70ºC overnight. The thawed suspension was than centrifuged for 10 min at 10,000 *g* and the supernatant was diluted 1/10 in H_2O. The lysate suspensions were kept at -20ºC until used.

Automatised BD Bactec™ MGIT™ 960 test - We used the BACTEC™ MGIT™ 960 system (Becton, Dickson and Company, Maryland, USA) as the gold standard method for phenotypic drug susceptibility testing, following the manufacturer's instructions. The INH and RIF-susceptible *M. tuberculosis* strain H37Rv (ATCC 27294) was used as a wild type control for susceptibility testing, for RT-PCR assays and for *rpoB*, *katG* and *inhA* DNA sequencing analysis.

PCR amplification and DNA sequencing - The amplification conditions and nucleotide sequences of the primers used to amplify the 555-bp segment of the *katG* gene containing codons 313-318 (GenBank access X68081 - *katG* 315), a 248-bp segment of the *inhA* gene (GenBank access Z79701) and a 189-bp segment of the 81-bp core of the *rpoB* gene (GenBank access L27989) were previously described (Telenti et al. 1997, Piatek et al. 1998, 2000, El-Hajj et al. 2001, van Doorn et al. 2003, Wada et al. 2004) (Table I). DNA amplification was verified on 2% agarose gels. The amplified PCR products were purified using a PureLink™ PCR Purification Kit (Invitrogen Corporation, Carlsbad, CA, USA). DNA sequencing was performed using the Big Dye Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) with an ABI Prism 3130xl DNA Sequencer (Applied Biosystems). The selection of *M. tuberculosis* isolates for DNA amplification and sequencing was based on the phenotypic susceptibility testing results for INH and RIF. All *M. tuberculosis* isolates that were phenotypically resistant to INH (120) or RIF (74) were submitted to gene sequencing for *katG* and *inhA* or *rpoB*, respectively (Supplementary data). For comparison purposes, additional numbers of *M. tuberculosis* INH and RIF-sensitive isolates were also submitted for *katG* and *inhA* and *rpoB* gene sequencing, approximately 10% of the 868 (n = 90) and 914 (n = 95), respectively (Supplementary data).

RT- PCR - All 988 *M. tuberculosis* isolates were tested for *katG*, *inhA* and *rpoB* using single-target RT-PCR assays. The sensitivity and specificity of the RT-PCR assays were calculated using the phenotypic resistance test results as the gold standard. To test for INH resistance, two sets of primers and probes were used to hybridise to two different amplicon targets (Table I). One molecular beacon probe was complementary to codons 313-318 of the *katG* gene and the other was complementary to the *inhA* ribosomal binding site. To test for RIF resistance, one set of primers and five probes (SW143, SW89, SW111, SW112 and SW182) were used to hybridise to a single *rpoB* amplicon such that their probe sequences spanned the entire core region, with overlapping sequences of one to three nucleotides (Table I). The RT-PCR probes were molecular beacons designated to hybridise to targets that did not contain mutations associated with antibiotic resistance. Drug resistance was indicated by the absence of a characteristic increasing fluorescent signal during PCR amplification. All assays were performed in duplicate and in sealed wells on a 96-well microtitre plate (Roche Diagnostics, Indianapolis, Ind). Reactions were performed in a Roche LightCycler 480 II (Roche Diagnostics, Indianapolis, Ind). Each reaction mixture contained 5 µL of lysed suspension diluted 1/10 in water, 2 µL of each primer, 2 µL of probe and 12.5 µL of 2X TaqMan® Universal master mix (Applied Biosystems). PCR-certified water (Roche Diagnostics, Indianapolis, Ind) was added to bring the reaction volume to 25 µL. PCR mixtures were first incubated for 2 min at 50ºC and 10 min at 95ºC and then for 45 cycles of 30 s at 95ºC, annealing at 1 min at 50ºC (55ºC for the *inhA* reaction) and 30 s at 60ºC.

All reactions for RIF testing were initially performed as a single-target RT-PCR assay with one set of primers and one FAM-labelled probe per reaction tube for all 988 isolates. After all probes had been individually tested, all phenotypically RIF-resistant and the same ~10% additional RIF-sensitive isolates mentioned above were re-evaluated by dual-target RT-PCR assay. We prepared two sets of dual-target RT-PCR assays: (i) the HEX-labelled probe SW182 plus the FAM-labelled probe SW112 and (ii) the HEX-labelled probe SW89 plus the FAM-labelled probe SW111. Each combination was used in a different RT-PCR assay. Probe SW143 was not used for dual-target assays because we did not identify any mutation in that region based on a single-target RT-PCR assay or by DNA sequencing.

C_T values from 18-39 were considered positive, meaning that no mutation was present on the gene fragment being tested, therefore indicating susceptibility. C_T values equal to zero or equal to or greater than 40 were considered negative, indicating the presence of a drug resistance mutation. C_T values between 1-17 were not found among the tested isolates.

Ethics - This study was approved by the ethical review board of the São Paulo University (CONEP/265/08).

RESULTS

Phenotypic determination of resistance - Of the 988 isolates studied, 120 (12%) and 74 (7%) were phenotypically INH or RIF-resistant, respectively, and 65 (7%) were resistant to both drugs. Sixty-five (88%) of the 74 RIF-resistant isolates were also resistant to INH. The overall sensitivity to both drugs determined by the phenotypic test was 87% (859/988) [95% confidence interval (CI): 85, 89].

Mutation frequencies - We characterised mutations in the *katG*, *inhA* and *rpoB* gene *loci*. Among INH-resistant isolates, mutations of the *katG* gene (55%; 66/120) were more frequent than in the *inhA* gene (25%; 30/120); only eight (7%; 8/120) presented mutations on both genes. No mutations were identified among the 90 INH-sensitive isolates submitted to DNA sequencing.

katG - Among INH-resistant isolates, all mutations were at codon 315. At this codon, the substitution from AGC to ACC, leading to the amino acid change serine (S) to threonine (T), was observed in 59 (91%) isolates. In addition, single-nucleotide polymorphisms from AGC (S) to AAC (N), AGA (R), CGC (R) and ACG (T) were seen in two (3%), two (3%), one (1.5%) and one (1.5%) isolates, respectively (Table II).

TABLE I

Primers and probes for gene amplification, sequencing and real-time polymerase chain reaction (RT-PCR)

Gene target	Primers and probes	Name	Sequence (5'-3')	Tm (ºC)	Final concentration (µM)	Reference
katG	Amplification	katGf1	GCAGATGGGGCTGATCTACG	60	0.4	Wada et al. (2004)
		katGr1	AACTCGTCGGCCAATTCCTC			
	Sequencing	katGf2	GGGCTTGGGCTGGAAGAG	50	0.16	van Doorn et al. (2003)
		katGr2	ACAGGATCTCGAGGAAACTGTTGT			
	RT-PCR primers	katGf3	CGTCGGCGGTCACACTTTCGGTAAGA	50	0.6	Piatek et al. (2000)
		katGr3	TTGTCCCATTTCGTCGGGGTGTTCGT		0.6	
	RT-PCR probe	katGpb	FAM-CCGAGGCACCAGCGGCATCGACCTCGG-BHQ1		0.3	
inhA	Amplification and sequencing	TB92	CCTCGCTGCCCAGAAAGGGA	58	0.4 and 0.16	Talenti et al. (1997)
		TB93	ATCCCCCGGTTTCCTCCGGT			
	RT-PCR primers	inhAf	GTGGACATACCGATTTCG	55	0.3	Piatek et al. (2000)
		inhAr	CTCCGGTAACCAGGACTGAACGGG		0.3	
	RT-PCR probe	inhAPb	FAM-CGAGGCCGACAACCTATCGTCTCCCTCG-BHQ1		0.1	
rpoB	Amplification and sequencing	rpoBf1	GGAGGCGATCACACCGCAGACGTT	60	0.4 and 0.16	El Hajj et al. (2001)
		rpoBr1	ACCTCCAGCCCGGCACGCTCACGT			
	RT-PCR primers	rpoBf2	GGCCGGTGGTCGCCGCG	50	0.3	Piatek et al. (1998)
		rpoBr2	ACGTGACAGACCGCCGGGC		0.3	
	RT-PCR probes	SW143	FAM-CGAGCTCAGCTGGCTGGTGCGCTCG		0.3	
		SW89	FAM or HEX-GCTACGGAGCCAATTCATGGACCAGACGTAGC		0.3	
		SW111	FAM-CCACGCCGACAGCGGGTTGTTCGTGG		0.3	
		SW112	FAM-CCACGCTTGTGGGTCAACCCCCGTGG		0.3	
		SW182	FAM or HEX-CCTGGGCCGACTGTCGGCGCTGCCAGG		0.3	

Tm: temperature of melting.

inhA - Among INH-resistant isolates, mutations at the ribosomal binding site at the -15 or -16 positions were found with substitutions from CGT-CAT (87%) and CTC-ATC (13%) (Table II).

rpoB - Among RIF-resistant (74) and RIF-sensitive isolates (95), mutations at the 81-bp core fragment region of the *rpoB* gene were investigated; 75 resistant and 94 sensitive isolates were identified by sequencing (Supplementary data). The most frequently mutated codon was 531 (67%): TCG-TTG (64%; 48/74) and TCG-TGG (3%; 2/75), followed by codon 526 (Table II) (23%; 17/75), codon 516 (GAC-GTC) (8%; 6/75) and codons 522 (TCG-TTG) and 533 (CTG-CCG) (1%; 1/75 each). Codon 526 (23%; 17/75) was polymorphic, with five different substitutions from CAC: GAC (47%; 8/17), TAC (35%; 6/17), TGC, CTC and TGC (6%; 1/17 each) (Table II). All mutations detected by DNA sequencing were located in four of the five different probe complementary regions of the 81-bp core region of the *rpoB* gene (SW89, SW111, SW112 and SW182). No mutations were detected in the SW143 probe complementary region.

Single-target RT-PCR assay - INH - Of the 988 isolates analysed, 91% (900/988) were sensitive and 9% (88/988) were resistant as determined by RT-PCR (Supplementary data). The overall sensitivities of the assay in detecting INH resistance using *katG* and *inhA* targets individually were 55% (66/120) and 25% (30/120), respectively (95% CI: 46-64 and 18-34, respectively). When the results of both targets were combined, the overall sensitivity was 73% (88/120) (95% CI: 64-81). The specificities for the tests were both 100% (95% CI: 97-100). All of the mutations identified by sequencing the *katG* and *inhA* gene probe complementary regions were detected by RT-PCR assays. Therefore, the specificity and sensitivity in detecting the point mutations in both genes were 100%. No discrepant results were obtained between duplicates.

RIF - Of the 988 isolates analysed, 92.4% (913/988) were sensitive and 7.6% (75/988) were resistant as determined by RT-PCR (Supplementary data). The overall sensitivity of the RT-PCR assay in detecting RIF resistance using all five different probes was 99% (73/74) (95% CI: 93, 100) when compared to the phenotypic assay. One of the RIF phenotypically resistant isolates was RIF-sensitive by RT-PCR assay. The nucleotide sequence analysis of this isolate revealed that a mutation at codon 522 (TCG-TTG), covered by probe SW111, was not detected by RT-PCR assay. We found a discrepancy between the phenotypic and genotypic results (RT-PCR and DNA sequencing) for RIF resistance for two *M. tuberculosis* isolates: one at codon 516 (GAC-TAC) and the other at codon 526 (CAC-CTC), using probes SW89 and SW112, respectively. No discrepant results were obtained between duplicates.

Dual-target RT-PCR assay - The combination of two probes for each of the dual-target RT-PCR assays for RIF was based on the prevalence of mutations at the 81-bp core region of the *rpoB* gene. We excluded probe SW143 from both combinations because there were no mutations present on the DNA region complementary to probe SW143. All 74 phenotypically RIF-resistant isolates and 95 (10%) of phenotypically RIF-sensitive isolates were used to evaluate the two dual-target RT-PCR assays (SW182 + SW112 and SW89 + SW111). There was 100% correlation between the results of single and dual-target RT-PCR assays. No discrepant results were obtained between duplicates.

TABLE II

Gene mutation found associated with anti-tuberculosis drug resistance in São Paulo isolates

Gene	Isolates with mutation	DNA position		Base changed to	Isolates (n)	Change (%)	Codon (%)
		Number	Bases				
rpoB	75	516	GAC	GTC	5	7	8
				TAC	1	1	-
		522	TCG	TTG	1	1	1
		526	CAC	CGC	1	1	23
				TGC	1	1	-
				CTC	1	1	-
				GAC	8	12	-
				TAC	6	8	-
		531	TCG	TTG	48	64	67
				TGG	2	3	-
		533	CTG	CCG	1	1	1
katG	65	315	AGC	ACC	59	91	100
				AAC	2	3	-
				AGA	2	3	-
				CGC	1	1.5	-
				ACG	1	1.5	-
inhA	30	-15	G	A	26	87	100
		-16	C	A	4	13	-

DISCUSSION

Because of the slow growth of *M. tuberculosis,* long delays in the diagnosis of drug-resistant TB occur when conventional culture-based susceptibility assays are used (the time required is, on average, 2-4 weeks). More rapid approaches that use genetic analysis for the detection of mutations associated with drug resistance have been described (Telenti et al. 1993, Kapur et al. 1994, Torres et al. 2000, 2003, El-Hajj et al. 2001, Garcia de Viedma et al. 2002, Garcia de Viedma 2003, van Doorn et al. 2003, Marín et al. 2004, Wada et al. 2004, Yesilkaya et al. 2006, Boehme et al. 2010) and the RT-PCR assay has been proposed as an alternative approach to detect drug-resistant organisms (Kapur et al. 1994, Piatek et al. 2000, Garcia de Viedma et al. 2002, Marín et al. 2004, Ruiz et al. 2004, Kocagoz et al. 2005, Boehme et al. 2010). In this study, we assessed reported RT-PCR assays to predict the drug resistance of *M. tuberculosis* isolates.

The RT-PCR results and DNA sequencing analysis obtained in this study were consistent for all *katG* and *inhA* mutations, except for one *rpoB* mutation. In this isolate, probe SW111 was not able to detect a mutation at codon 522 (TCG-TTG). We are unable to explain this discrepancy despite numerous repetitions of MGIT 960, DNA sequencing and RT-PCR assays. The mutation missed by probe SW111 is close to the 3' end of its sequence, only three nucleotides before the molecular beacon hairpin arm. It is possible that the binding capacity of probe SW111 is affected when mismatches are too close to that area. We also identified discrepancy between phenotypic and genotypic results of the RIF resistance of two *M. tuberculosis* isolates. DNA sequencing predicted that RIF resistance of these two isolates was consistent after three rounds of DNA sequencing and RT-PCR testing, but they were phenotypically RIF-sensitive, even after repetitions. According to our data, RT-PCR and MGIT failed to detect 1% (1/74) and 2% (2/95) of RIF-resistant isolates, respectively.

If we consider that any mutation on the 81-bp core region of the *rpoB* gene confers RIF resistance, these two cases suggest that sometimes RT-PCR may be more accurate than the phenotypic assay. Similar discrepancies have been described, such as when a mutation at codon 533 was compatible with a clinically RIF-resistant case, but was overlooked by the BACTEC method (Riska et al. 2000).

All 75 (100%) RIF-resistant isolates obtained by genetic sequencing in this study had mutations in the *rpoB* gene. This contrasts with previous data showing that RIF-resistant isolates in Brazil exhibited *rpoB* mutation frequencies varying from 80-89% (Valim et al. 2000, de Miranda et al. 2001, Clemente et al. 2008). Previous data on *katG* reported that mutations occur in approximately 60-85% of INH-resistant isolates (Rossetti et al. 2002, Cardoso et al. 2004, Hofling et al. 2005, Clemente et al. 2008) but, in our study, mutation of the *katG* gene was seen in 55% (65/118) of the INH-resistant isolates. The reasons for these discrepancies are not clear, but may be related to different methods used to perform the phenotypic susceptibility testing, differences in year and geographical site of *M. tuberculosis* isolation and the use of different sizes of sample collections.

RT-PCR cannot completely replace culture-based susceptibility tests because conventional testing can detect resistance due both to genetic mechanisms that are not yet understood and to a larger number of drugs. There are a number of limitations to the widespread use of PCR-based techniques; resistance to anti-TB drugs may involve changes in multiple genes and at multiple possible locations within a gene. However, the fact that the majority of the INH and RIF resistance of *M. tuberculosis* isolates are due to point mutations on the *katG*, *inhA* and *rpoB* genes, respectively, molecular approaches such as RT-PCR are promising.

Our results confirm that RIF-resistant *M. tuberculosis* can be detected in less than 4 h using a very specific and sensitive assay. Moreover, the assay is a powerful tool because it is simple to perform and readily automatable for high-throughput screening and the results enable faster diagnosis and immediate decision-making to determine appropriate drug therapy.

The rapid detection of *M. tuberculosis* resistance continues to be an important public health matter. Commercial assays, such as GeneXpert (Cepheid), which detects *M. tuberculosis* and resistance to RIF, demonstrated good performance with clinical specimens (Helb et al. 2010, Lawn & Nicol 2011, Zeka et al. 2011). In our study, the use of RT-PCR to determine *M. tuberculosis* resistance to RIF was also very promising and its application in clinical samples will be explored in future studies.

The cost of in-house molecular designs could be equivalent to, or even less expensive than, the costs of phenotypic antibiograms if the new liquid-culture media are used. However, cost, speed and professional expertise are not the only factors to consider. The molecular analysis of resistance provides additional information and essential data that are not available if only phenotypic antibiograms are obtained. Genotypic analysis also specifies the resistance mutation involved, which helps to identify strains with high and/or broad levels of resistance (van Soolingen et al. 2000, Garcia de Viedma 2003). The optimal approach for drug susceptibility testing of MTB will likely involve both methodologies.

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Susceptibility and resistence to isoniazid (INH), rifampin (RIF) or both in 988 isolates obtained by phenotypic and molecular methods [real-time polymerase chain reaction (RT-PCR)]

Methods	Susceptible (S) or resistant (R)	INH	RIF	INH + RIF
		n (%)	n (%)	n (%)
Phenotypict	S	868 (88)	914 (93)	859 (87)
	R	120 (12)	74 (7)	65 (7)
RT-PCR	S	900 (91)	913 (92.4)	878 (89)
	R	88 (9)	75 (7.6)	110 (11)