Clinical data and molecular analysis of *Mycobacterium tuberculosis* isolates from drug-resistant tuberculosis patients in Goiás, Brazil

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Drug resistance is one of the major concerns regarding tuberculosis (TB) infection worldwide because it hampers control of the disease. Understanding the underlying mechanisms responsible for drug resistance development is of the highest importance. To investigate clinical data from drug-resistant TB patients at the Tropical Diseases Hospital, Goiás (GO), Brazil and to evaluate the molecular basis of rifampin (R) and isoniazid (H) resistance in Mycobacterium tuberculosis. *Drug susceptibility testing was performed on 124 isolates from 100 patients and 24 isolates displayed resistance to R and/or H. Molecular analysis of drug resistance was performed by partial sequencing of the* rpoB *and* katG *genes and analysis of the inhA promoter region. Similarity analysis of isolates was performed by 15 loci mycobacterial interspersed repetitive unit-variable number tandem repeat (MIRU-VNTR) typing. The molecular basis of drug resistance among the 24 isolates from 16 patients was confirmed in 18 isolates. Different susceptibility profiles among the isolates from the same individual were observed in five patients; using MIRU-VNTR, we have shown that those isolates were not genetically identical, with differences in one to three loci within the 15 analysed loci. Drug-resistant TB in GO is caused by* M. tuberculosis *strains with mutations in previously described sites of known genes and some patients harbour a mixed phenotype infection as a consequence of a single infective event; however, further and broader investigations are needed to support our findings.*

Key words: MDR-TB - MIRU-VNTR - *katG* - *rpoB*

Tuberculosis (TB) is a major world health problem with an estimated 9.4 million incidents of TB cases occurring globally in 2009 (equivalent to 137 cases per 100,000 inhabitants). Of the 9.4 million cases, an estimated 1.0-1.2 million (11-13%) were human immunodeficiency virus (HIV)-positive. Although the American continent is responsible for only 3% of the total TB burden, Brazil continues to be one of the 22 countries worldwide that contributes to the TB burden and accounts for 81% of all estimated cases (WHO 2010).

One of the most important challenges faced by TB control programs is the emergence and dissemination of drug-resistant TB worldwide, especially multidrug-resistant TB (MDR-TB) and extensively drug-resistant TB (XDR-TB). MDR-TB is caused by *Mycobacterium tuberculosis* that is resistant to at least rifampin (R) and isoniazid (H), and XDR-TB is defined as *M. tuberculosis* that is resistant to R, H, a fluoroquinolone and at least one of three injectable second-line drugs (i.e., capreomycin, kanamycin and amikacin) (Raviglione & Smith 2007). Drug resistance generally emerges due to the long period of treatment necessary for TB that favours poor therapy adherence, especially when therapy is without clinical supervision (Araújo-Filho et al. 2008, WHO 2008, Dye 2009, Santos et al. 2010). Of the utmost importance is the fact that MDR-TB and XDR-TB necessitate longer periods of treatment, usually requiring a hospital facility with biosafety concerns, in addition to the high mortality rate of XDR-TB (Curry 2008, WHO 2008).

Traditional drug resistance analysis is based on culture methods, which take a long time to produce results, demanding both labour and biosafety concerns. However, molecular approaches that rapidly detect mutations in specific genes have shown a good correlation with the results of culture susceptibility testing (ST), but these methods are not accessible in developing regions (Palomino 2009, Boehme et al. 2010). Thus, it is crucial to detect resistance to R because it is a good predictive marker of MDR-TB (Martin & Portaels 2007, Curry 2008). Early discovery of resistance allows for immediate adoption of the appropriate treatment regimens and, consequently, higher rates of treatment success.

The molecular characterisation of *M. tuberculosis* strains by DNA fingerprinting methods during the course of infection has revealed different genetic profiles in isolates from closely related infected individuals and within the same host. Those observations could be explained by mixed infections or microevolution of the infecting pathogens (Braden et al. 2001, Shamputa et al. 2006).

To better understand the microbiological aspects of drug-resistant TB infections, isolates from patients with TB resistant to R and/or H were evaluated. The clinical

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and the phenotypic resistance profiles were evaluated with emphasis on the analysis of mutations in the *rpoB*, catalase gene (*katG*) and *inhA* genes. Additionally, we investigated the genetic relationship among strains with different phenotypes from the same TB patient.

PATIENTS, MATERIALS AND METHODS

Patient enrolment and clinical data collection - This work is a descriptive study using a convenience sample size. During the period of September 2005-December 2007, 410 cultures were identified as *M. tuberculosis* based on traditional biochemical tests at Central Laboratory of Public Health of Goiás (LACEN-GO). ST was performed in 124 *M. tuberculosis* isolates from 100 patients following a physician's request and 24 of the isolates demonstrated resistance to at least one of the two drugs, H and R; these isolates were recovered from 16 patients. The patients were recruited and agreed to participate by signing an informed consent form. Clinical evaluations of the enrolled patients were obtained from the clinical charts at the Dr Anuar Auad Tropical Diseases Hospital, a reference centre for the treatment of infectious diseases. The patients' outcome was followed up until December of 2010 (Table I).

M. tuberculosis isolation, ST and DNA extraction - *M. tuberculosis* isolates were obtained from Lowenstein Jensen cultures of sputum, lymph node secretion and/or cerebrospinal fluid samples. ST for R, H, streptomycin (S), ethionamide (Et), and ethambutol (E) were performed according to Canetti et al. (1969). Isolated strains were stored in Sauton media with 10% glycerol at -80ºC.

Chromosomal DNA was extracted according to van Embden et al. (1993). DNA extractions were analysed by 0.8% agarose gel electrophoresis stained with 0.5 µg/mL of ethidium bromide, visualised on a Gel Doc System (Bio-Rad) and stored at -20ºC.

Partial amplification and sequencing of the rpoB and katG genes - Chromosomal DNA, obtained as described, was further diluted 10 times and 2 µL of each diluted DNA sample was used for the polymerase chain reaction (PCR) reactions. Partial amplification of the RNA polymerase gene *B* (*rpoB*) was performed using the pair of primers described by Siddiqi et al. (2002): *rpo*B1 (forward) 5'GGGAGCGGATGACCACCC3' and *rpo*B2 (reverse) 5'GCGGTACGACGGCGTTTCGATGAAC3'. Similarly, *katG* was partially amplified using the pair of primers described by (Marttila et al. 1996): *kat*G1 (forward) 5'GAAACAGCGGCGCTGATCGT3' and *kat*G2 (reverse) 5'GTTGTCCCATTTCGTCGGGG3'. The PCR reactions contained 0.1 ηmol of each primer for the *rpoB* or *katG* genes, 0.2 µM of dNTPs and one unit of Taq DNA polymerase (Invitrogen) in a final volume of 30 µL. Amplification was performed in a thermocycler (MJ-Biocyler) programmed with the following conditions: an initial denaturation step of 95ºC for 3 min followed by 35 cycles of denaturation at 92ºC for 40 sec, annealing at 56ºC for 40 sec, and extension at 72ºC for 1 min with a final extension cycle of 72ºC for 7 min. As a positive control, H37Rv *M. tuberculosis* (ATCC 27294) DNA was included. Five microlitres of the PCR products were analysed by 1.5% agarose gel electrophoresis stained with 0.5 µg/mL of ethidium bromide and visualised on a Gel Doc System (Bio-Rad). The expected sizes of the PCR products for the *katG* and *rpoB* genes were 209 and 332 base pairs (bp), respectively. The remaining PCR products were precipitated with isopropanol, resuspended in MiliQ grade water and sequenced using each primer from the PCR reactions. Sequencing reactions were performed with the BigDye Terminator Kit (Applied Biosystems) according to the manufacturer instructions and run on the ABI3130 Genetic Analyzer (Applied Biosystems). The Sequencing Analysis software package (version 3.3, Applied Biosystems) was used to analyse the data quality. Sequences were compared to the H37Rv strain sequences (Gene Bank accessions BX842574 and X68081) using ClustalX (version 2.0) and BioEdit (version 7.0) software for mutation identification. Visualised mutations were double-checked using DNA STAR version 5.1 software.

Analysis of mutations in the promoter of the inhA gene by real-time PCR (RT-PCR) - The samples were analysed for mutations at the ribosome-binding site in the promoter of the *mabA-inhA* operon by RT-PCR. The primer pair mabAF (5'CGAAGTGTGCTGAGTCACACCG3') and inhAR-mut (5'AGTCACCCCGACAACCTATTA3'), described by Herrera-León et al. (2005), was used to amplify a 146-bp fragment of the mutated region, while the primer pair mabAF and inhARwt (5'AGTCACCCCGACAACCTATCG3') was used in a separate RT-PCR to amplify a 146-bp fragment of the wild-type region. SYBR Green Supermix (Bio-Rad) was used for RT-PCR. RT-PCR was performed in an IQ5 thermocycler (Bio-Rad) at 94ºC for 1 min, 65ºC for 1 min and 72ºC for 1 min for 35 cycles. After amplification was complete, a final melting curve was recorded while the reaction mixture was slowly heated from 62-95ºC. Mutations were identified when amplification was detected with the mutant primer, but not with the wild-type primer.

Mycobacterial interspersed repetitive unit-variable number tandem repeat (MIRU-VNTR) typing - Amplification of 15 loci (580, 2996, 802, 960, 1644, 3192, 424, 577, 2165, 2401, 3690, 4156, 2163b, 1955 and 4052) was performed according to (Supply et al. 2006). The PCR products were analysed on a 2% agarose gel stained with ethidium bromide and visualised on a Gel Doc System (Bio-Rad). Each gel contained 100-bp ladder standards (Invitrogen) and the number of bp in the target VNTR loci was estimated using Quantity One (Bio-Rad) software. The number of repetitions of various MIRU-VNTR loci of each strain were determined and regarded as an allele profile. DNA from *M. tuberculosis* H37Rv (ATCC 27294) was included as a positive control for each amplification reaction. A phylogenetic tree was constructed by entering the allele profiles into the MIRU-VNTR plus database (Allix-Béguec et al. 2008) using the unweighted pair group method with an arithmetic mean algorithm.

Ethics - The Ethical Committee of Federal University of Goiás approved this study under the protocol UFG-006/2005.

TABLE I

Clinical characteristics and phenotypical drug resistance profile of the drug-resistant tuberculosis (TB) patients in the state of Goiás, Brazil

Patient number	Gender (age in years)	Status HIV	Clinical form	Drug resistance	Interval between TB diagnosis and drug resistance	Last treatment regimen[a]	Outcome (as of December 2010)
1	F (39)	Neg	Non cavitary bilateral lung	R, H, Z	1 year	Amk, Ofx, E, Trd, Amx/Clv	Clinical and microbiological cure in 2008 after 18 months of treatment.
2	M (47)	Neg	Cavitary unilateral lung	R, H	1 year	S, Ofx, E, Z, Trd	Under treatment.
3	M (45)	Pos	Non cavitary bilateral lung	H	3 months	R, H, E, Z	Clinical cure in 2008 after nine months of treatment.
4	M (39)	Neg	Cavitary bilateral lung	R, H, E	7 years	Amk, Ofx, Z, Et, Trd	Clinical and microbiological failure in 2009.
5	F (60)	NA	Non cavitary unilateral lung	H	3 years	S, E, Et, Z	Clinical cure in 2009 after 12 months of treatment.
6	M (42)	Pos	Disseminated (non cavitary bilateral lung disease/lymph node)	R	1 year	S, E, H, Z	Death in 2009.
7	M (38)	Neg	Cavitary unilateral lung disease	R, H	1 year	S, E, Et, Z	Clinical cure in 2007 after 12 months of treatment.
8	F (40)	Neg	Cavitary bilateral lung	R, H, S	2 years	Ofx, Et, Clr, Z, Trd	Death in 2008.
9	M (58)	Neg	Non cavitary bilateral lung	H	10 years	R, H, E, Z	Clinical and microbiological cure in 2007 after nine months of treatment
10	M (35)	Neg	Non cavitary unilateral lung	R, H	3 years	Amk, Ofx, Z, Et, Trd	Loss of follow-up in 2007.
11	M (35)	NA	Pulmonary without specification	R, H	NA	NA	Transferred out.
12	M (43)	Neg	Disseminated (cavitary unilateral lung/ meningoencephalitis)	R, Et	4 years	NA	Transferred out.
13	F (37)	Neg	Cavitary unilateral lung	R, H	11 years	S, E, Et, Z	Loss of follow-up in 2009.
14	M (24)	Neg	Cavitary bilateral lung	R, H	2 years	Amk, Lfx, E, Z, Trd	Under treatment.
15	M (62)	Neg	Cavitary bilateral lung	R,H, E ,Et	2 years	Amk, Ofx, Clr, Z, Trd	Death in 2007.
16	F (33)	Neg	Non cavitary lung	R, H, Z, S	6 years	Amk, Ofx, E, Et, Trd	Clinical and microbiological cure in 2009 after 18 months of treatment.

a: most of the patients had more than two previous treatments; Amk: amikacin; Amx/Clv: amoxacilin/clavulanate; Clr: clarithromycin; E: ethambutol; Et: ethionamide; F: female; H: isoniazid; HIV: human immunodeficiency virus; Lfx: levofloxacin; M: male; NA: not available; Neg: negative; Ofx: ofloxacin; Pos: positive; R: rifampin; S: streptomycin; Trd: terizidone; Z: pyrazinamide.

RESULTS

During the period of September 2005-December 2007, 410 cultures were identified as *M. tuberculosis*-positive based on traditional biochemical tests performed at LACEN-GO. ST was performed on 124 *M. tuberculosis* isolates from 100 TB patients following a physician's request; 24 (19.35%) of the isolates obtained from 16 patients presented with resistance to at least one of the two drugs, H and R.

The individuals with *M. tuberculosis* isolates resistant to H and/or R were evaluated and their clinical characteristics are presented in Table I. Eleven patients were male (68.7%) and the mean age was 42.3 years old (range 24-62 years old). Among the comorbidities presented by the patients, HIV and leprosy were present in 12.5% and 6.3% of the cases, respectively.

The diagnosis of drug-resistant TB occurred at three-132 months (average of 42.9 months) after the TB diagnosis. In addition to R and H resistance, phenotypic resistance to E, S and Et was also detected (Table I). Due to the small number of individuals investigated, no predisposing factor was statistically associated with the development of monodrug and/or multidrug resistance (data not shown), although one of the patients had a confirmed contact with an MDR-TB index case.

Lung cavity was the main characteristic lesion present in nine (56.3%) patients. A bilateral lung commitment was observed in eight patients. Disseminated TB was present in two patients; one was HIV-positive and the other was infected with *Mycobacterium leprae* with chronic use of prednisone and thalidomide. None of the HIV-positive patients presented with cavitary lung disease.

Among the studied patients, only six were clinically cured, three of which also had negative cultures for acid-fast bacilli. The clinical follow-ups of four of the patients were interrupted due to their transfer to another care centre or to treatment abandonment; consequently, the final outcome for these patients remains unknown. Three deaths occurred. Three patients remained under treatment until the end of this study, one of which had acid-fast bacilli-positive sputum.

During the course of the treatment, some patients received at least four drugs with certain or almost certain effectiveness based on drug susceptibility tests and individual tolerability (Table I). The others patients received the standard regimens for re-treatment/failure or monoresistant TB according to the current guidelines of the Brazilian Ministry of Health (MS/SVS 2010).

Ten patients (62.5%) had MDR *M. tuberculosis* isolates, while monoresistance to H was observed in only five patients (31.3%) and one (6.3%) had an isolate with monoresistance to R. Five patients (31.3%) had more than one culture sample obtained at different time points during the course of the disease; these patients had samples taken at different times due to the failure of the sputum to become negative during chemotherapy (Table II). Interestingly, the ST profile of the different isolates from the same patient varied significantly. For example, in patient 4 and 8, their first isolated cultures had an MDR phenotype, while posterior isolates presented with monoresistance to R. Inversely, and more understandable, the first isolate from patient 13 was resistant to H and the second culture that was isolated eight months later presented with an MDR phenotype.

The differences observed in ST among the different isolates from the same patient prompted us to investigate the molecular basis of drug resistance. As shown in Table II, all but one of the 17 *M. tuberculosis* isolates resistant to R had a mutation in previously described regions of the *rpoB* gene. Similarly, most *M. tuberculosis* isolates (11 out of 17) resistant to H presented with mutations in the analysed regions of either the *katG* or *inhA* genes.

To further investigate the genetic relatedness of the isolates, we performed a similarity analysis based on MIRU-VNTR typing (Figure). The isolates from the different TB patients were not genetically related. The isolates obtained from the same patient at different periods of time presented a unique, but closely associated, MIRU-VNTR type. The isolates from the same patient showed allele differences in one-three different loci. Only patient 4 presented with two isolates (2 out of 4) with an identical MIRU-VNTR type.

DISCUSSION

In the present study, we demonstrated that the molecular basis of the R and H resistance of *M. tuberculosis* isolated from the studied patients was attributed to known mutations in the *rpoB* and *katG* genes and in the promoter region of the *inhA* gene. The isolates from the same patient had different drug susceptibility profiles; genetic analyses of those isolates using MIRU-VNTR revealed that they were not genetically identical, with one-three loci differences.

Evaluating the clinical profile of the enrolled TB patients, as shown elsewhere (Drobeniewski et al. 2002, Barroso et al. 2003, Araújo-Filho et al. 2008, WHO 2008), it was observed that the long period of time between the first diagnosis of TB and the resistance diagnosis is probably the result of poor clinical practices, such as not routinely performing culture and susceptibility tests. It is also important to point out that the previous TB treatment was not supervised because at the time the patient enrolled, direct observed treatment was not adopted. Only recently, there was a change in the Brazilian guidelines recommending culture and susceptibility tests in all re-treatment cases, in cases with confirmed contact with MDR-TB patients, in cases where sputum remains positive at the second month of treatment and in cases with treatment failure (MS/SVS 2010).

We have previously shown that in Goiás (GO) there is an unexpected high rate of resistance among naïve treated patients (13.6%), suggestive of primary drug resistance (Santos et al. 2010). In this study, two patients presented clinical evidence for primary drug resistance (i.e., contact with MDR-TB and a short interval for resistance development). However, the long period for resistance development/detection observed in the present study also suggests the possibility that the majority of drug-resistant cases are secondary cases in which the drug resistance emerged after inadequate previous treatments (Martin & Portaels 2007, Araújo-Filho et al. 2008, Curry 2008).

TABLE II

Drug susceptibility phenotype of the isolated strains and molecular analysis of mutations in *kat*G, *rpo*B and *inhA* genes of *Mycobacterium tuberculosis*

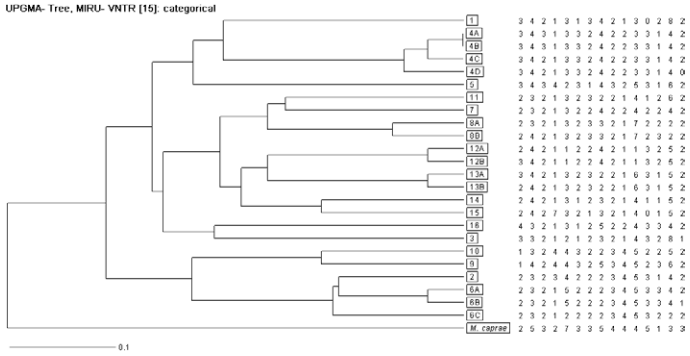
Patient[a]	Collection date	SS	ST	*katG*[b]	*inhA*[c]	*rpoB*[b]
1	29 December 2006	Sp	H + R	315 ser → thr	ND	456 ser → leu
2	2 January 2006	Sp	H + R	315 ser → thr	ND	456 ser → leu
3	23 January 2007	Sp	H	315 ser → thr 297 gly → gly	ND	ND
4A	30 August 2005	Sp	H + R	NM	NM	456 ser → leu
4B	10 March 2006	Sp	H + R	NM	NM	456 ser → leu
4C	17 November 2006	Sp	R	ND	ND	456 ser → leu
4D	12 April 2007	Sp	R	ND	ND	456 ser → leu
5	2 April 2007	Sp	H	297gly → gly	ND	ND
6A	3 June 2007	LN	R	ND	ND	451 his → tyr
6B	19 June 2007	LN	R	ND	ND	451 his → tyr
6C	13 December 2007	Sp	R	ND	ND	451 his → tyr
7	13 July 2006	Sp	H + R	315 ser → thr	ND	447 ser → leu
8A	31 July 2006	Sp	H + R	NM	NM	456 ser → leu
8B	31 August 2006	Sp	R	ND	ND	456 ser → leu
9	23 August 2006	Sp	H	NM	NM	ND
10	28 October 2007	Sp	H + R	NM	NM	NM
11	28 September 2006	Sp	H + R	NM	NM	456 ser → leu
12A	6 October 2006	CSF	H	NM	Yes	ND
12B	23 November 2006	Sp	ND	NM	Yes	456 ser → leu
13A	10 February 2006	Sp	H	315 ser → asn	ND	ND
13B	4 October 2006	Sp	H + R	315 ser → asn	ND	456 ser → leu
14	17 November 2006	Sp	H + R	NM	Yes	456 ser → leu
15	28 November 2006	Sp	H + R	NM	Yes	456 ser → leu
16	8 December 2006	Sp	H	315 ser →asn	ND	ND

a: each number represents one patient and letters corresponds to different isolates from the same patient; *b*: mutations found by partial gene sequencing; *c*: mutation detected by real-time polymerase chain reaction; CSF: cerebrospinal fluid; H: isoniazide; LN: lymph node; NM: no mutations; ND: not done; R: rifampin; Sp: sputum; SS: sample source; ST: susceptibility testing.

Potential risk factors for MDR-TB development are the presence of bilateral and extensive pulmonary cavities (Barroso et al. 2003, Kaplan et al. 2003); our data corroborates with these reports because a predominance of bilateral and cavitary pulmonary disease was observed in the majority of the patients (Table I and data not shown). Thus far, it has not been shown if and how cavities induce resistance. Timm et al. (2003) have shown that the cavity imposes an environment pressure, such as oxygen and iron deprivation, that leads to an increase in the metabolic changes of *M. tuberculosis*. It is possible that these metabolic changes could be associated with higher rates of mutations (Timm et al. 2003).

The finding of diverse drug susceptibility profiles observed in the different *M. tuberculosis* isolates from the same patient at different time points could be explained by the development of resistance to additional drugs (Johnson et al. 2006, Bergval et al. 2009); this could be the case for patient 13, for whom the first isolate presented monoresistance to H and the second isolate obtained eight months later acquired an additional resistance to R, confirmed by mutation in codon 456 of the *rpoB* gene.

The evolution of drug resistance patterns observed in the *M. tuberculosis* isolates from patients 4 and 8 was unexpected, as it went from an MDR to a monoresistance phenotype; combining this observation with the genetic profile of the isolates from patients 4 and 8, it is interesting to point out that while the first two isolates from patient 4 were identical, the next two isolates had a single allele difference in loci 580 and 4156. The interval of time over the sample collection period (5-9 months) could have favoured this event. In contrast, the isolates from patient 8 were more divergent (2 loci differences) than the isolates from patient 4, even though those isolates were obtained only one month apart. Two hypotheses could be formulated to explain these changes: first, a mutation could reverts the susceptibility of *M. tuberculosis* to H or, second, the patients could harbour mixed bacterial populations, for example, MDR and monoresistant strains that could have been artificially



Dendogram of similarity analysis of the 24 isolates included in this study. Isolates from the same patient have the same number with different letters. The bar indicates genetic distance calculated by the unweighted pair group method with an arithmetic mean method. Isolate *M. caprae* mycobacterial interspersed repetitive unit-variable number tandem repeat (MIRU-VNTR) profile from MIRU-VNTR plus database was included as outlier strain. Allele numbers for each locus are presented in the following order of loci from left to right: 424, 577, 580, 802, 960, 1644, 1955, 2163b, 2165, 2401, 2996, 3192, 3690, 4052 and 4156.

missed during laboratory ST due to culture manipulations (Matthys et al. 2009, Richardson et al. 2009). Mutation reversion has been recently reported for MDR *M. tuberculosis* (Richardson et al. 2009) in a patient receiving chemotherapy without R and H; although we could not prove this was the case for our results, it is an unlikely explanation because the patients were receiving H therapy during the sampling period (data not shown).

TB infection with a heterogeneous *M. tuberculosis* population can be caused by a re-infection event or by an evolutionary genetic variation of a single infection event (Small et al. 1993, Andrews et al. 2008). To address this question, we performed MIRU-VNTR typing to analyse the genetic relatedness of the different *M. tuberculosis* isolates from the same individual. We demonstrated here that *M. tuberculosis* isolates from the same patient had closely related MIRU-VNTR types, which suggested that they originated from a common strain, supporting the theory of mutational changes during the course of infection and rejecting the possibility of re-infection. Additionally, it has been proposed that drug resistance imposes a fitness cost to bacteria (Gagneux et al. 2006); if this is the case, this finding could explain the persistence of the heterogeneous bacterial population in those individuals and, during sampling times, a particular strain with or without drug resistance would be preferentially selected.

The present paper had a small sample size and this fact could be the consequence of several factors, including a lack of awareness of the assistant physicians that ST should be prescribed. It is important to stress that all available drug-resistant isolates from GO during the period of 2005 and 2007 were evaluated.

We concluded that drug-resistant TB in GO is caused by *M. tuberculosis* strains with mutations in the *rpoB* and *katG* genes and in the promoter region of the *inhA* gene. In addition, some patients harbour a mixed phenotype/genotype of *M. tuberculosis* populations that probably originated from a single infective event and evolved during disease progression; however, further and broader investigations are needed to support our findings.

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