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

Sueli Lemes de Ávila Alves\(^1,2\), Fabiana Sarcinelli Metzker\(^1\), João Alves de Araújo-Filho\(^1,3\), Ana Paula Junqueira-Kipnis\(^1\), André Kipnis\(^1/+\)

\(^1\)Instituto de Patologia Tropical e Saúde Pública, Universidade Federal de Goiás, Rua 235/1ª Av. s/n, 74605-050 Goiânia, GO, Brasil
\(^2\)Laboratório Central de Saúde Pública Dr. Giovanni Cysneiros, Goiânia, GO, Brasil
\(^3\)Hospital de Doenças Tropicais Dr Anuar Auad, Goiânia, GO, Brasil

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 (Branden 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...
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 partial amplification 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): \(rpoB\)I (forward) 5’GGGAGCGGATGACCACCC3’ and \(rpoB\)II (reverse) 5’GCGGTACGACGCCGCTTTCTAGAAC3’. Similarly, \(katG\) was partially amplified using the pair of primers described by Martilla et al. (1996): \(katG\)I (forward) 5’GAAACAGCGCGTGATCTG3’ and \(katG\)II (reverse) 5’GGTGTCACCATTTCGTCGGG3’. The PCR reactions contained 0.1 µmol 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’CGAAGTGTGCTGATCCACCCG3’) 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’AGTCACCCCGACAACTATCG3’) 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>
<thead>
<tr>
<th>Patient number</th>
<th>Gender</th>
<th>Status</th>
<th>Clinical form</th>
<th>Drug resistance</th>
<th>Interval between TB diagnosis and drug resistance</th>
<th>Last treatment regimen&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Outcome (as of December 2010)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>F</td>
<td>NA</td>
<td>Non cavitary unilateral lung</td>
<td>H</td>
<td>3 years</td>
<td>S, E, Et, Z</td>
<td>Clinical cure in 2009 after 12 months of treatment.</td>
</tr>
<tr>
<td>9</td>
<td>M</td>
<td>Neg</td>
<td>Non cavitary bilateral lung</td>
<td>H</td>
<td>10 years</td>
<td>R, H, E, Z</td>
<td>Clinical and microbiological cure in 2007 after nine months of treatment</td>
</tr>
<tr>
<td>11</td>
<td>M</td>
<td>NA</td>
<td>Pulmonary without specification</td>
<td>R, H</td>
<td>NA</td>
<td>NA</td>
<td>Transferred out.</td>
</tr>
<tr>
<td>12</td>
<td>M</td>
<td>Neg</td>
<td>Disseminated (cavitary unilateral lung/ meningoencephalitis)</td>
<td>R, Et</td>
<td>4 years</td>
<td>NA</td>
<td>Transferred out.</td>
</tr>
</tbody>
</table>

<sup>a</sup>: most of the patients had more than two previous treatments; Amk: amikacin; Amx/Clv: amoxicilin/clavulanate; Clr: clarithromycin; E: ethambutol; Et: ethionamide; F: female; H: isoniazid; HIV: human immunodeficiency virus; Lfx: levofoxacin; 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 nine (56.3%) patients. A bilateral lung commitment was confirmed contact with an MDR-TB index case. (data not shown), although one of the patients had a contact with MDR-TB and a short interval for resistance (Santos et al. 2010). In this study, two patients presented 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 (Drobeniuk 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).
Potential risk factors for MDR-TB development are the presence of bilateral and extensive pulmonary cavi -
ties (Barroso et al. 2003, Kaplan et al. 2003); our data
corroborates with these reports because a predominance
of bilateral and cavitary pulmonary disease was ob-
served 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 \textit{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 ob-
served in the different \textit{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 present-
ed mono-resistance to H and the second isolate obtained
eight months later acquired an additional resistance to R,
confirmed by mutation in codon 456 of the \textit{rpoB} gene.

The evolution of drug resistance patterns observed in
the \textit{M. tuberculosis} isolates from patients 4 and 8 was
unexpected, as it went from an MDR to a mono-resis-
tance 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 iso-
lates 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 dif-
ferences) than the isolates from patient 4, even though
those isolates were obtained only one month apart. Two
hypotheses could be formulated to explain these chang-
es: first, a mutation could reverts the susceptibility of
\textit{M. tuberculosis} to H or, second, the patients could harbour
mixed bacterial populations, for example, MDR and
mono-resistant strains that could have been artificially

\begin{table}[h]
\centering
\begin{tabular}{llcccc}
\hline
Patient & Collection date & SS & ST & \textit{katG} & \textit{inhA} & \textit{rpoB} \\
\hline
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 & 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 & 315 ser → thr & ND & ND \\
6A & 3 June 2007 & LN & R & 315 ser → thr & ND & 451 his → tyr \\
6B & 19 June 2007 & LN & R & 315 ser → thr & 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 \\
\hline
\end{tabular}
\caption{Drug susceptibility phenotype of the isolated strains and molecular analysis of mutations in \textit{katG}, \textit{rpoB} and \textit{inhA} genes of \textit{Mycobacterium tuberculosis}.}
\end{table}
missed during laboratory ST due to culture manipulations (Matthys et al. 2009, Richardson et al. 2009). Mutation reversion has been recently reported for MDR \( M. \) \( \text{tuberculosis} \) (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 \( M. \) \( \text{tuberculosis} \) 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 \( M. \) \( \text{tuberculosis} \) isolates from the same individual. We demonstrated that \( M. \) \( \text{tuberculosis} \) 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 \( \text{ST} \) 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 \( M. \) \( \text{tuberculosis} \) strains with mutations in the \( \text{rpoB} \) and \( \text{katG} \) genes and in the promoter region of the \( \text{inhA} \) gene. In addition, some patients harbour a mixed phenotype/genotype of \( M. \) \( \text{tuberculosis} \) 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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