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Mutations in *katG*, *inhA*, and *ahpC* Genes of Brazilian Isoniazid-Resistant Isolates of *Mycobacterium tuberculosis*

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The presence of mutations in specific regions of the *katG*, *inhA*, and *ahpC* genes was analyzed with 69 *Mycobacterium tuberculosis* isoniazid-resistant isolates from three Brazilian states. Point mutations in codon 315 of the *katG* gene were observed in 87.1, 60.9, and 60% of the isolates from Rio Grande do Sul, Rio de Janeiro, and São Paulo, respectively. Mutations in the *inhA* gene were identified only in one isolate from RJ State, and the *ahpC* promoter region revealed mutations in distinct positions in 12.9, 21.7, and 6.7% of the isolates from RS, RJ and SP, respectively.

Tuberculosis still represents a serious health problem in several parts of the world. The disease is endemic in Brazil, where about 80,000 new tuberculosis cases were reported in 2000 (27).

Several studies have evaluated genes and genomic regions of *Mycobacterium tuberculosis* involved in the development of resistance to isoniazid (INH), such as *katG*, encoding catalase-peroxidase, which transforms INH into its active form, *inhA*, encoding a putative mycolic acid synthesis enzyme involved in cell wall formation, and *ahpC*, encoding alkyl hydroxiperoxidase, which acts as a component of the antioxidant reductase (3, 18, 28). More recently, resistant strains have been reported to contain mutations in the *kasA* (ketoacyl acyl carrier protein synthase) and *ndh* (NADH dehydrogenase) genes (10, 11).

Worldwide INH resistance is more frequently associated with mutations in the *katG* gene. However, no data from Latin America have been reported so far. The present study analyzed a total of 69 Brazilian INH-resistant *M. tuberculosis* isolates by single-strand conformation polymorphism (SSCP), DNA sequencing, and restriction fragment length polymorphism (RFLP) in order to identify possible mutations in the *katG*, *inhA*, and *oxyR-ahpC* genes.

INH-resistant *M. tuberculosis* isolates were randomly selected from samples collected in the period of 1996 to 1999. The isolates and respective data of susceptibility tests were kindly provided by Laboratório Central do Rio Grande do Sul (RS) ($n = 31$), Centro de Referência Professor Hélio Fraga (RJ) ($n = 23$), and Instituto Adolfo Lutz of São Paulo (SP) ($n = 15$). Isolates were cultivated on Ogawa medium (9), and the tests to analyze their sensitivity to INH, rifampin (RIF), ethambutol (EMB), ethionamide (ETH), pyrazinamide (PZA), and streptomycin (STR) were performed according to the proportional method (4) on Löwenstein-Jensen medium. *M. tuberculosis* H37Rv and the laboratory standard strain 1575 were used as INH-sensitive and -resistant controls, respectively (Table 1). The MIC was defined as the lowest concentration of INH showing complete inhibition of bacterial growth (22).

Genomic DNA was extracted from *M. tuberculosis* grown in Löwenstein-Jensen medium using cetyl-trimethyl ammonium bromide according to the method of van Soolingen et al. (25). The *katG*, *inhA*, and *oxyR-ahpC* genes were amplified using primers (Table 2) designed with the aid of the Primer 3 program. Amplifications were carried out in a thermocycler Mini-Cycler–Hot Bonnet PTC–150 (MJ Research) as follows: 94°C for 2 min, 55°C for 1 min, and 72°C for 2 min for 30 cycles. Amplification products were analyzed by electrophoresis in 1.5% agarose gels, purified, and used for sequencing.

Nonradioactive SSCP analyses of the *katG*, *inhA*, and *ahpC* genes were performed to identify genetic alterations or possible point mutations (5). Sequencing was conducted in an Applied Biosystems ABI model 373 A automated DNA sequencer, using the same primers used for the PCR-SSCP analysis an the *Taq* DyeDeoxy terminator cycle sequencing kit (Applied Biosystems Inc.). Mutations in codon 315 of the *katG* gene were observed in the majority of the *M. tuberculosis* isolates. Those isolates with no detectable point mutation by this method were further investigated by sequencing of the complete *katG* gene.

RFLP analysis was performed according to standard procedures (23). After hybridization with the probe, the results were detected by an enhanced chemiluminescence direct nucleic acid detection system (ECL Direct System; Amersham) according to the manufacturer's recommendations.

Among the 69 isolates analyzed, 45 had MICs of >16 μg/ml, 20 had MICs of 16 μg/ml and 4 had MICs of ≤8 μg/ml. The

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TABLE 1. Resistance patterns of *M. tuberculosis* isolates from Brazil used in this study

Isolate(s)[a]	Resistance[b]
RS01, RS03, RS05, RS07, RS10, RS13, RS17, RS18, RS21, RS22, RS23, RS25, RS29, RS30, RS31	INH
RJ02, RJ10, RJ13, RJ14, RJ15, RJ16, RJ17, RJ18, RJ19, RJ20, RJ21, RJ22, RJ23; SP02, SP04, SP05, SP06, SP07, SP08, SP09, SP11, SP12, SP14, SP15; RS06, RS08, RS12, RS14, RS28	INH, RIF
RS27	INH, STR
RS11	INH, PZA
RJ04, RJ05	INH, RIF, EMB
RJ08, RS04, SP01, SP10	INH, RIF, PZA
SP13, RS09, RS24, RS26	INH, RIF, STR
RS16	INH, RIF, PZA, EMB
RS02, RS15	INH, RIF, STR, EMB
RJ03, RJ07, SP03, RS19, RS20	INH, RIF, PZA, STR
RJ01	INH, RIF, PZA, ETH, EMB
RJ06, RJ09, RJ11, RJ12	INH, RIF, PZA, STR, EMB, ETH

[a] Letters in isolate names denote state of origin.

mutation in codon 315 of the *katG* gene was associated with a relatively high level of drug resistance (≥16 μg/ml) (data not shown). The results of our study indicate that the MIC for most isolates (59.4%) exhibited high levels of INH resistance, which in previous studies have been more frequently associated with the absence of catalase-peroxidase (2, 28).

DNA sequencing analysis of 69 INH-resistant isolates from three different Brazilian states showed that most of the detected mutations corresponded to single-nucleotide mutations previously described (2, 14, 24). Codon 315 of the *katG* gene was the most affected by point mutations, with frequencies of 87.1, 60.9, and 60% in isolates from RS, RJ, and SP, respectively (Table 3). As previously reported, frequencies of specific mutations may vary according to geographical region (1, 6, 7, 12, 13, 16). The *katG* mutation S315T was more prevalent among *M. tuberculosis* isolates from RS. Moreover, the results of RFLP analysis (see below) indicate that the isolates are not related.

Although isolates from RJ and SP showed similarity in the frequency of mutations in the *kat*G gene, differences were observed in the frequency of specific amino acid alterations in codon 315. Changes S315T (56.5%) and S315I (4.3%) were detected in RJ isolates. In isolates from SP the observed changes were S315T (46.7%) and S315N (13.3%). Isolates from RS presented the mutation S315T (80.6%) and a less prevalent one, S315N (6.4%). No mutation in codon 315 was observed in four, nine, and six isolates from RS, RJ, and SP, respectively. The *katG* gene was completely sequenced in these isolates, and nucleotide substitutions were observed in four isolates from RJ and one isolate from RS, leading, respectively, to the following alterations: Q439P, M126I, R484H, L101P, and T275P. Insertions were observed in one isolate (position Cins1297) from RS and in one (position Gins7) from SP. A nonsense mutation (W90stop) was identified in one isolate from RJ. Five out of nine isolates from RJ, two out of four isolates from RS, and five out of six isolates from SP presented no mutations in this gene.

A 206-bp fragment of the *inhA* gene corresponding to part of the coding region (codons 62 to 131) was analyzed. The presence of mutations in the *inhA* gene was identified in one RJ isolate containing the mutation S94A. No mutations in this gene were observed in isolates from RS and SP (Table 3). Our results are in agreement with previous studies showing a low frequency of mutations in the coding region of the *inhA* gene (3, 15).

The *oxyR-ahpC* promoter region (359 bp) revealed mutations in distinct positions in RJ and RS isolates with frequencies of 21.7 and 12.9%, respectively, and in 6.7% of the SP isolates (Table 3). Furthermore, we observed association with mutations in *katG* and *ahpC* genes in 3.2% of RS isolates and 13.0% of RJ isolates. In others reports, mutations in the *ahpC* promoter region were found in approximately 13 to 18% of INH-resistant isolates, and in general they are associated with *katG* mutations (8, 17, 19, 21).

The number of mutations varied among isolates from different states. Our findings showed that the RJ isolates were more likely to contain mutations in all analyzed genes, including simultaneous mutations in the *katG* and *ahpC* genes. Differences in the frequency of mutations in another gene, *rpoB*, were also observed in *M. tuberculosis* isolates from the same Brazilian states (26).

Concordances of 89.8, 98.5, and 86.9% between SSCP and

TABLE 2. Primers used for amplification of DNA segments from *katG*, *inhA*, and *oxyR-ahpC* genes[a]

Gene	Primer sequence	Primer name	Size of product (bp)	Primer coordinates
*kat*G	5′-CATGAACGACGTCGAAACAG-3′	katG 1	232	2153887–2156109
	5′-CGAGGAAACTGTTGTCCCAT-3′	katG 2		
*ahp*C	5′-GCCTGGGTGTTCGTCACTGGT-3′	ahpC 1	359	2726191–2726778
	5′-CGCAACGTCGACTGGCTCATA-3′	ahpC 2		
*inh*A	5′-GAACTCGACGTGCAAAAC-3′	inhA 1	206	1674200–1675009
	5′-CATCGAAGCATACGAATA-3′	inhA 2		

[a] The primer coordinates refer to the positions in the *M. tuberculosis* H37Rv genome (GenBank accession number AL123456).

TABLE 3. Mutations identified in 69 INH-resistant isolates of *M. tuberculosis* from Brazil

No. of isolates (origin)	No. of mutated isolates, specific mutation(s)			
	katG	*inhA*	*ahpC*	*katG/ahpC*
31 (RS)	25, S315T; 2, S315N	—[a]	1, G(−6) A; 1, G(−9) A; 1, C(−30)T; 1, C(−39)T	1, S315T/G(−6)A
23 (RJ)	13, S315T; 1, S315I	1, S94A	1, G(−6) A; 1, C(−15)T; 1, C(−30)T; 1, T(−40)C; 1, F10I	1, S315T/G(−6)A; 1, S315I/T(−40)C; 1, S315T/F10I
15 (SP)	7, S315T; 2, S315N	—	1, C(−39)T	—

[a] —, no mutation.

DNA sequencing were observed for the *katG*, *inhA*, and *ahpC* genes, respectively. There was no agreement between the results obtained by the drug susceptibility tests and the SSCP results for 16 isolates. Eight out of sixteen isolates, defined as INH resistant by the proportion method ("gold standard"), showed an INH-sensitive pattern by SSCP. The DNA sequencing analysis confirmed mutations in the *katG* gene (codon 315) of one of these isolates, in the *ahpC* gene of six isolates, and in the *inhA* gene of one isolate, indicating the likely occurrence of SSCP false-negative results. Eight out of sixteen isolates, defined as INH resistant by the proportion method, showed an INH resistant pattern by SSCP, but the DNA sequencing analysis did not confirm the presence of mutations. It is possible that these isolates contain mutations either in other regions of the analyzed genes or in other genes not evaluated in the study. Similar to the results described by Telenti et al. (21), part of the discrepancies observed here could be due to an abnormal SSCP profile. As expected (7, 14), DNA sequencing was the most accurate method for detection of mutations associated with drug resistance.

IS*6110* RFLP analyses of *M. tuberculosis* isolates with mutations in the *katG* gene showed that all isolates with alteration in codon 315 exhibited different DNA fingerprinting whatever the source of the isolate. Heterogeneity in *IS*6110 RFLP patterns was previously described (12). Hence, further analyses of isolates from other Brazilian regions are necessary to evaluate and confirm the lack of clonality observed with the *M. tuberculosis* isolates.

The presence of mutations in each of the three studied genes was used as a criterium to evaluate the possible contribution of each mutated gene for the identification of INH-resistant strains. Analyzing only the mutation in the *katG* gene, it was possible to detect mutation in 87.1, 60.9, and 60% of isolates from RS, RJ, and SP, respectively. When mutations in the *ahpC* gene were analyzed in association with *katG*, these percentages increased to 96.7, 69.6, and 66.7%, respectively. Among isolates from RJ, the inclusion of mutations in the *inhA* gene in the analysis increased the number of identified resistant isolates to 74%. The RS and SP isolates do not contain mutations in this sequenced region. These data support the evidence that these genes are implicated in most of the INH resistance described for different isolates (6).

The results presented in this work suggest that there are differences in the isolates from RJ when compared to isolates from SP and RS, such as the presence of mutations in all genes studied. This fact might be explained by differences in the chemotherapeutic regimen and in treatment compliance rates among the states. In RJ, for instance, treatment failure due to noncompliance is at a higher level than in RS and SP (20).

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