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ORIGINAL ARTICLE

Correlating genetic mutations with isoniazid phenotypic levels of resistance in *Mycobacterium tuberculosis* isolates from patients with drug-resistant tuberculosis in a high burden setting

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Abstract

We analysed mutations in *katG, inhA* and *rpoB* genes, and isoniazid phenotypic resistance levels in *Mycobacterium tuberculosis* isolates from drug-resistant TB patients from São Paulo state, Brazil. Isolates resistant to the critical concentration of isoniazid in MGIT (0.1 µg/mL) were screened for mutations in *katG* 315 codon, *inhA* promoter region and *rpoB* RRDR by MTBDR*plus* assay and subjected to determination of isoniazid resistance levels by MGIT 960. Discordances were resolved by Sanger sequencing. Among the 203 isolates studied, 109 (54%) were isoniazid-monoresistant, 47 (23%) MDR, 29 (14%) polydrug-resistant, 12 (6%) pre-XDR and 6 (3%) XDR. MTBDR*plus* detected isoniazid mutations in 75% (153/203) of the isolates. Sequencing of the entire *katG* and *inhA* genes revealed mutations in 18/50 wild-type isolates by MTBDR*plus* (10 with novel mutations), resulting in a total of 32/203 (16%) isolates with no mutations detected. 81/83 (98%) isolates with *katG* 315 mutations alone had intermediate resistance. Of the 66 isolates with *inhA* C-15T mutation alone, 51 (77%) showed low-level, 14 (21%) intermediate and 1 (2%) high-level resistance. 5/6 (83%) isolates with mutations in both *katG* and *inhA* had high-level resistance. Inferred mutations corresponded to 22% (16/73) of all mutations found in *rpoB*. Mutations detected in *katG* regions other than codon 315 in this study might be potential new isoniazid resistance markers and could explain phenotypic resistance in some isolates without *katG* and *inhA* classic mutations. In our setting, 16% of isoniazid-resistant isolates, some with high-level resistance, presented no mutations either in *katG* or *inhA*.

Keywords Drug resistance · Gene sequencing · Isoniazid · Line probe assay · Multidrug-resistant tuberculosis · Rifampicin

Introduction

Drug resistance in *Mycobacterium tuberculosis* is due mainly to mutations in its genome. More than 95% of rifampicin (RIF) resistance conferring mutations occur in an 81-bp region (codons 507–533) of *rpoB* gene, known as the RIF resistance determining region (RRDR) [1]. As for isoniazid (INH) resistance, the most common associated mutations occur in codon 315 of *katG* and, in the promoter region of *inhA*, mostly at position − 15 [2]. Globally, 64%

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of phenotypic INH resistance is associated with *katG* 315 mutations, while mutations in *inhA* – 15 are observed in 19% of INH-resistant isolates [3, 4].

katG gene mutations have been associated with at least moderate- to high-level INH resistance, above the usual tested concentrations of 0.2 and 1 µg/mL in solid medium and 0.1 and 0.4 µg/mL in MGIT 960 liquid medium. *katG* S315T, the most frequent mutation found in this gene, is associated with minimum inhibitory concentrations (MICs) ranging from 2 to > 10 µg/mL in MGIT 960, while *inhA* promoter mutations have been associated with low-level resistance ($0.1 < \text{MIC} \leq 1$ µg/mL) in MGIT 960 [5].

Combinations of mutations in *katG* 315 and *inhA* promoter are known to confer high-level resistance (MIC > 10 µg/mL) [6]. However, previous studies have shown phenotypic heterogeneity of INH resistance-associated mutations, with a wide range of MICs observed among INH-resistant isolates [6–8].

A mutation leading to a decrease in drug susceptibility should not always exclude an anti-tuberculosis (TB) drug from a treatment regimen, since low-level resistance does not necessarily imply clinical resistance [5]. This is the case for INH, which can still be effective at elevated doses in patients infected by *M. tuberculosis* strains with low-level resistance, i.e. strains presenting specific *inhA* promoter mutations without *katG* mutations [4, 9]. On the other hand, isolates with *katG* 315 mutations only, particularly the high-confidence S315T and S315N, should be reported as high-level INH-resistant regardless of their MIC, so that INH is not used in the treatment [9]. Thus, patients harbouring isolates with mutations in *inhA* promoter only might be beneficiated by a treatment regimen with double dose of INH (600 mg) [10].

Here, we investigated genetic mutations in *rpoB, katG* and *inhA* genes of *M. tuberculosis* clinical isolates with different drug resistance profiles and associated *katG* and *inhA* mutations with INH phenotypic levels of resistance.

Methods

Setting and study population

The state of São Paulo is the most populous in Brazil, presenting a TB incidence of 40.8/100,000 in 2018 [11]. Adolfo Lutz Institute is the reference laboratory for São Paulo, receiving annually 8000 isolates of mycobacteria for species identification and drug susceptibility testing (DST). *M. tuberculosis* isolates from patients under risk of drug-resistant TB are subjected to DST by MGIT 960 (Becton Dickinson, MD, USA). These patients include smear-positive cases after 2 months on TB treatment, contacts of drug-resistant TB patients, retreatment cases, immunosuppressed persons, alcohol/illicit drugs abusers, healthcare workers, homeless individuals, indigenous, immigrants, inmates and inpatients.

Study design

This was a prospective study including all *M. tuberculosis* isolates referred to Adolfo Lutz Institute between January and December 2016, and resistant to 0.1 µg/mL of INH (along or not with other drugs) by MGIT 960 DST. Only one isolate per patient was included, except for patients with isolates showing different mutations along the study period.

Routine drug susceptibility testing

DST for first-line drugs was performed on MGIT 960 using SIRE kit (Becton Dickinson, MD, USA). Critical concentrations tested were 1 µg/mL, 0.1 µg/mL, 1 µg/mL and 5 µg/mL of streptomycin, INH, RIF and ethambutol, respectively. The isolates were also tested against second-line drugs. Critical concentrations were 1 µg/mL of amikacin, 2.5 µg/mL of capreomycin and kanamycin, 2 µg/mL of ofloxacin and 1 µg/mL of levofloxacin [12–14]. Isolates were classified as INH-monoresistant when they presented INH resistance only; as polydrug-resistant when they were resistant to INH and other first-line drug(s), except RIF; as MDR when they presented resistance at least to INH and RIF; as pre-extensively drug-resistant (pre-XDR) when they were MDR and resistant to at least one of injectable second-line drugs or fluoroquinolones; and as extensively drug-resistant (XDR) when they were MDR and resistant to both injectables and fluoroquinolones. On January 2021, the WHO updated the definitions of both pre-XDR- and XDR-TB, which classify pre-XDR-TB as the disease caused by an MDR or RIF-resistant strain which is also resistant to a fluoroquinolone, and XDR-TB as TB caused by an MDR or RIF-resistant strain which is also resistant to a fluoroquinolone and bedaquiline and/or linezolid (https://www.who.int/news/item/27-01-2021-who-announces-updated-definitions-of-extensively-drug-resistant-tuberculosis). Here, we applied the previous definitions of pre-XDR- and XDR-TB, because this study was performed before these changes.

Determination of INH resistance levels

The INH-resistant isolates at 0.1 µg/mL were tested by MGIT 960 against 1 µg/mL, 3 µg/mL and 10 µg/mL of INH [6, 13, 15]. The tests were incubated into MGIT instrument according to Rüsch-Gerdes et al. (2006) [16]. Isolates with no growth at 1 µg/mL were considered low-level-resistant ($0.1 < \text{MIC} \leq 1$ µg/mL), while the ones growing at 1 µg/mL or at both 1 µg/mL and 3 µg/mL, but not at 10 µg/mL ($1 < \text{MIC} \leq 3$ µg/mL or $3 < \text{MIC} \leq 10$ µg/mL) were considered having intermediate resistance, and the ones growing

at all concentrations were considered high-level-resistant (MIC>10 µg/mL) [6, 15]. Despite the clinical breakpoint of 0.4 µg/mL of INH recommended by the Clinical & Laboratory Standards Institute (CLSI) for definition of low-level resistance, existing data on correlation of *inhA* promoter mutations and INH MICs are not enough to support this clinical breakpoint, according to the WHO [9]. Therefore, based on previous studies on MGIT 960 liquid medium, we decided to adopt the concentration of 1 µg/mL as a breakpoint for low-level INH resistance [6, 7, 15].

DNA extraction

DNA of the isolates was extracted by thermal lysis. One milliliter of the culture in MGIT was incubated at 95 °C for 20 min and frozen at −20 °C twice [17]. The lysates were kept at −20 °C until MTBDR*plus* and Sanger sequencing were performed.

Detection of mutations

All the isolates were subjected to GenoType MTBDR*plus* v. 2.0 (Hain Lifescience, Nehren, Germany) for detection of mutations in *katG* 315 codon, in *inhA* promoter region, and in *rpoB* RRDR, as described elsewhere [18, 19]. Sanger sequencing was performed for isolates with discordant results between DST and MTBDR*plus*, such as those with inferred mutations (wild type and mutant probes absent) and those which were phenotypically resistant to INH but presented no mutations by MTBDR*plus*. The *inhA* promoter was amplified and sequenced with primers *inhA*-1 and *inhA*-2 (positions −168 to 80 in reference to start codon) [20]. The whole *inhA* and *katG* genes were sequenced with primers *inhA*3 and *inhA*4 (positions −13 to 379), *inhA*3F and *inhA*5R (positions 381 to 840), and forward and reverse primers *katG*-P4, *katG*-P5, *katG*-P6, *katG*-P7 and *katG*-P8 (positions −135 to 2202 of *katG* plus 431 nucleotides after the end of the gene) [21]. Primers *rpoB*-1 and *rpoB*-2 were used to amplify and sequence a 350-bp fragment of *rpoB* encompassing the RRDR [22]. Each PCR reaction included 12.5 µL of PrimeSTAR Max DNA Polymerase (Takara Bio, Shiga, Japan), 5 pmol of primers for *inhA* promoter and *katG*, 10 pmol of primers for *inhA* coding region and *rpoB*, 2 µL of DNA template and PCR grade water for a final volume of 25 µL. Amplification comprised 30 cycles of 98 °C for 10 s, 55 °C for 5 s and 72 °C for 20 s. Amplicons were purified with ExoSAP-it (Affymetrix, CA, USA) and sequenced with ABI 3130×L Genetic Analyzer and the BigDye Terminator version 3.1 Kit (Applied Biosystems, CA, USA). Sequences were analysed using BioEdit v7.2.5 software and the web-based MUBII-TB-DB and BLAST tools [19].

Nucleotide sequence accession numbers

The sequences with novel mutations found in *katG* and *inhA* were deposited in GenBank (http://www.ncbi.nlm.nih.gov/) under the following accession numbers: MW809517-MW809528 and MW815499.

Data analysis

Fisher's exact test was used for comparisons between proportions. The significance threshold was set at 0.05. Statistical analyses were performed using the web-based OpenEpi program [23].

Results

Phenotypic resistance to first- and second-line drugs

There were 203 isolates resistant to INH at 0.1 µg/mL by MGIT 960, in the study period. Most of the isolates were obtained from sputum ($n=194$, 95%), followed by bronchoalveolar lavage ($n=4$, 2%), tracheal aspirate ($n=2$, 1%), pleural fluid ($n=2$, 1%) and bone tissue ($n=1$, 1%). These isolates belonged to 200 patients, as 3 patients had 2 isolates presenting different mutations (2 polydrug-resistant isolates from one patient, and 2 MDR isolates from the other two patients). Among the 203 isolates, 109 (54%) were INH-monoresistant, 47 (23%) MDR, 29 (14%) polydrug-resistant, 12 (6%) pre-XDR (all of them resistant to fluoroquinolones) and 6 (3%) XDR.

INH resistance levels

Among the 203 isolates tested, 75 (37%) were INH low-level-resistant ($0.1 < \text{MIC} \leq 1$ µg/mL), 107 (53%) showed intermediate resistance [34 (17%) with $1 < \text{MIC} \leq 3$ µg/mL, and 73 (36%) with $3 < \text{MIC} \leq 10$ µg/mL], and 21 (10%) were high-level-resistant (MIC > 10 µg/mL). Most of the 75 isolates with low-level resistance were INH-monoresistant (88%, $n=66$) (Fig. 1). Interestingly, INH-monoresistant isolates accounted for 33% ($n=7$) of high-level-resistant isolates (Fig. 1).

Mutations detected by MTBDR*plus*

Regarding INH-associated mutations, of the 203 isolates tested, 82 (40%) had mutations only in *katG* 315: 79 (39%) with S315T1 (AGC > ACC), 2 (1%) with S315T2 (AGC > ACA) and 1 with an inferred mutation. Sixty-six isolates (33%) showed mutations only in *inhA*, all with C-15T. Mutations in both genes were detected in 5 (3%) isolates:

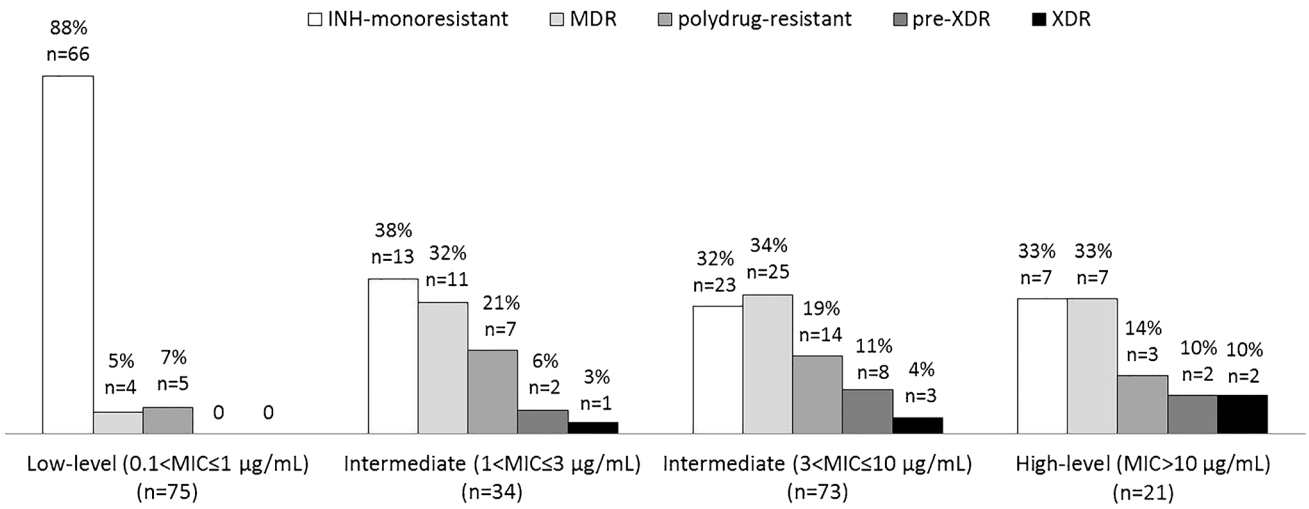

Fig. 1 Isoniazid resistance levels of *Mycobacterium tuberculosis* isolates with different drug-resistance profiles

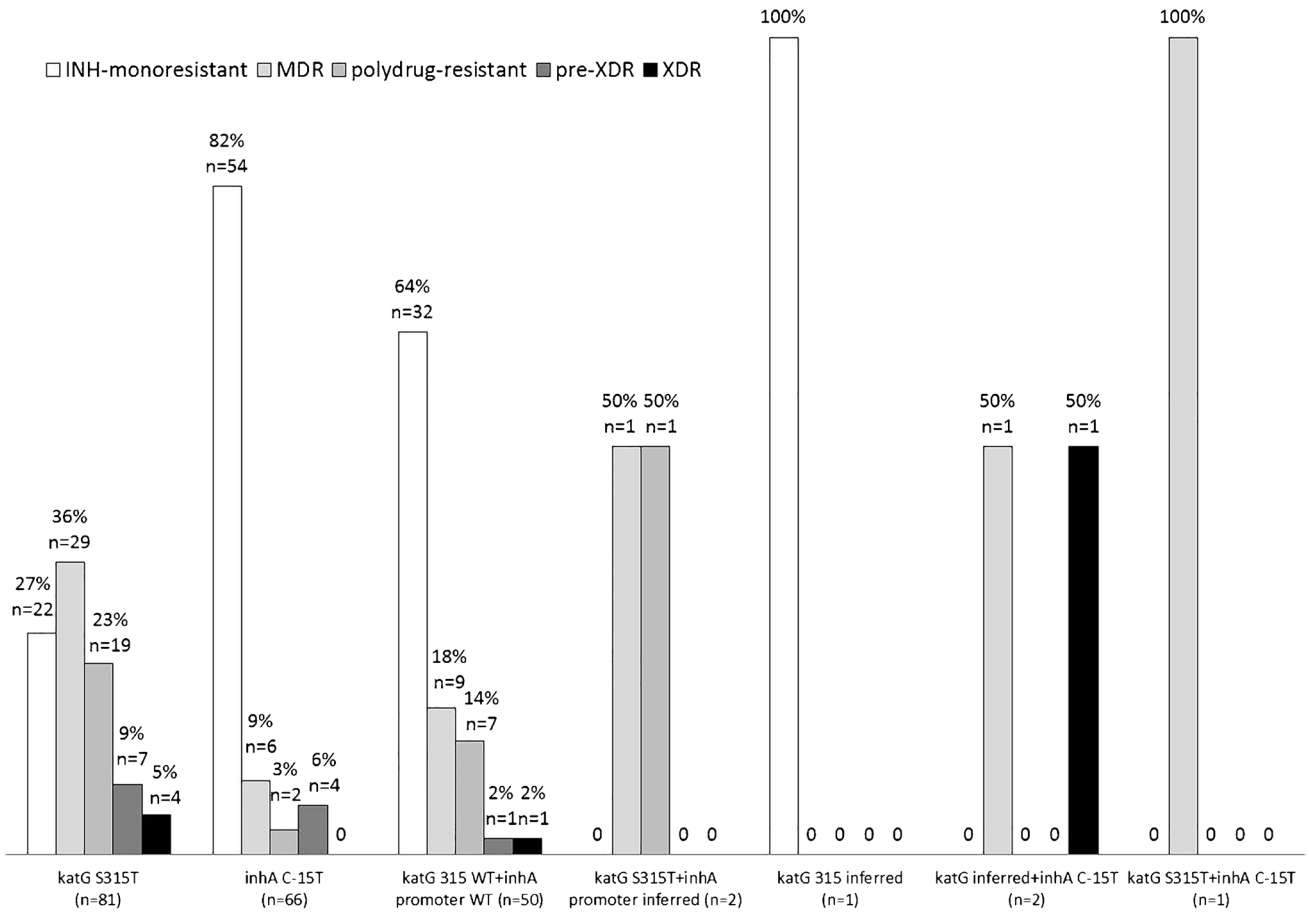

Fig. 2 Mutations in *katG* and *inhA* genes detected by MTBDR*plus* in *Mycobacterium tuberculosis* isolates with different drug-resistance profiles

katG S315T1 + *inhA* inferred ($n = 2$), *katG* inferred + *inhA* C-15T ($n = 2$) and *katG* S315T1 + *inhA* C-15T ($n = 1$) (Fig. 2). A considerable proportion of INH-resistant isolates (25%, $n = 50$) presented no mutations in any of these targets.

Figure 2 shows the distribution of *katG* 315 and *inhA* promoter mutations according to the phenotypic resistance of the isolates. While the *katG* S315T mutation alone was found mainly among INH-monoresistant ($n = 22$, 27%), MDR ($n = 29$, 36%) and polydrug-resistant ($n = 19$, 24%) isolates, the majority of the isolates ($n = 54$, 82%) with only *inhA* C-15T mutation were INH-monoresistant. Most of the isolates showing no mutations either in *katG* 315 and *inhA* promoter were INH-monoresistant ($n = 32$, 64%); however, 18% ($n = 9$) and 14% ($n = 7$) of the isolates without *katG* 315 and *inhA* promoter mutations were MDR and polydrug-resistant, respectively. Absence of these mutations was also detected in 1 pre-XDR and 1 XDR isolates.

As for RIF-associated mutations, most of the isolates (64%, $n = 130/203$) did not present any mutation in *rpoB* (all of them were phenotypically RIF-susceptible, 105 INH-monoresistant and 25 polydrug-resistant). Among the 73 isolates with *rpoB* mutations, the most prevalent was S531L mutation, which occurred alone in 51 (70%) isolates: 37 MDR, 11 pre-XDR and 3 XDR. Four (6%) isolates presented D516V mutation (2 MDR and 2 XDR), and 1 (1%) pre-XDR isolate had H526Y mutation. One (1%) MDR isolate presented both S531L and D516V mutations. *rpoB* inferred mutations occurred in 16 (22%) isolates, of which 4 were INH-monoresistant, 4 polydrug-resistant, 7 MDR and 1 pre-XDR.

Mutations detected by Sanger sequencing

Fifty-five isolates were subjected to *katG* and *inhA* sequencing (Table 1). Among the 50 isolates with no mutations detected either in *katG* 315 and *inhA* promoter by MTBDR*plus,* 18 (36%) presented mutations by sequencing, while 32 (64%) continued without mutations. Point mutations were the most prevalent in *katG* and occurred in isolates with different levels of resistance to INH. Four isolates presented heteroresistance (defined as the coexistence of susceptible and resistant organisms to a drug in the same *M. tuberculosis* population): 1 with *katG* S315R mutation (which was not detected by MTBDR*plus*) and 3 with mutations in other codons. The 3 isolates with deletions presented high levels of resistance: A122G – 3 < MIC ≤ 10 µg/mL, R463L + A478R – MIC > 10 µg/mL and K590_None – MIC > 10 µg/mL. The latter isolate presented also a silent mutation in *inhA* (G76G) (Table 1).

Sequencing of the 2 isolates with *katG* S315T and *inhA* promoter inferred mutation revealed had *inhA* G-17 T mutation, and both showed high-level INH resistance. Of the 3 isolates with *katG* inferred mutations, 2 had S315N mutation (one presented heteroresistance and the other presented also the *katG* A506T mutation), and the other had S315G mutation alone (Table 1).

rpoB RRDR sequencing was performed for the 16 isolates with inferred mutations by MTBDR*plus* (Table 2). The most frequently mutated codon was 526, with 6 isolates presenting mutations in this target, 5 of them phenotypically RIF-susceptible (2 with H526L, 2 with H526N and 1 with H526S).

INH resistance levels and mutations

Considering the results from MTBDR*plus* and sequencing together, we assessed the association between INH resistance levels and mutations in *katG* and *inhA* for the 203 isolates (Table 3) (Fig. 3). Overall, 99 (49%) isolates presented mutations in *katG* only, of which 83 (84%), occurred in 315. All these 83 isolates showed intermediate- to high-level INH resistance. Among the 16 isolates with mutations in *katG* codons other than the 315, only 4 had low-level resistance. Of the 66 (33%) isolates with *inhA* C-15T mutation only, 51 (77%) showed low-level resistance, 14 (21%) had intermediate resistance and 1 (2%) showed high-level resistance. Among the 6 isolates with mutations in both *katG* and *inhA*, 5 (83%) were high-level INH-resistant, while 1 (17%) was intermediate. Even after sequencing of entire *katG* and *inhA*, no mutations were detected in 32/203 (16%) isolates, of which 20 were low-level-resistant, 6 intermediate and 6 high-level INH-resistant.

Discussion

We investigated mutations related to INH and RIF resistance in 203 M. *tuberculosis* clinical isolates with different drug resistance profiles and associated mutations found in *katG* and *inhA* with INH phenotypic levels of resistance. MTBDR*plus* kit was able to identify INH resistance-associated mutations in 75% (153/203) of the isolates. Still, 25% (50/203) of the isolates phenotypically resistant to INH would be diagnosed by the test as not having mutations, which means that resistance was not detected. Analysing the entire *katG* and *inhA* genes by sequencing enhanced the molecular diagnosis of INH resistance, as mutations in other codons of *katG* were found in 17/50 isolates (plus one S315R mutant), resulting in a total of 32/203 (16%) isolates without mutations in these targets.

We observed that low-level INH resistance was detected more frequently in INH-monoresistant isolates (66/109, 61%) than in MDR (4/47, 9%, $p < 0.0001$) and polydrug-resistant (5/29, 17%, $p = 0.0002$) isolates. Considering MTBDR*plus* results only, frequencies of *katG* S315T mutation in MDR (29/47, 62%, $p < 0.0001$), and polydrug-resistant (19/29, 66%, $p < 0.0001$) isolates were significantly higher

Table 1 Mutations in *katG* and *inhA* genes detected by Sanger sequencing in *Mycobacterium tuberculosis* isolates phenotypically resistant to isoniazid

Isolates with *katG* 315 wild type + *inhA* promoter wild type by MTBDR*plus* (*n* = 50)					
Whole *katG* sequencing	**Whole *inhA* sequencing**	**No. isolates**	**Phenotypic resistance profile**	**MIC INH (µg/mL)**	**INH resistance level**
Y98C (TAC > TGC)	Wild type	1	MDR	> 1 ≤ 3	Intermediate
A106V (GCG > GTG)	Wild type	1	INH-monoresistant	> 0.1 ≤ 1	Low
A122G (delCGGCGGCGC)	Wild type	1	INH-monoresistant	> 3 ≤ 10	Intermediate
P232T (CCG > ACG)*	Wild type	1	INH-monoresistant	> 10	High
G299C (GGC > TGC) + wild type	Wild type	1	INH-monoresistant	> 10	High
W300R (TGG > CGG) + C549G (TGT > GGT)	Wild type	1	INH-monoresistant	> 1 ≤ 3	Intermediate
S315R (AGC > AGG) + wild type	Wild type	1	INH-monoresistant	> 10	High
D381N (GAC > AAC)* + L611L (CTC > CTT)	Wild type	1	MDR	> 10	High
W438R (TGG > CGG)* + wild type	Wild type	1	pre-XDR	> 10	High
Q439R (CAG > CGG)	Wild type	1	MDR	> 10	High
R463L (CGG > CTG) + **A478R (1430delG)***	G76G (GGC > GGT)	1	XDR	> 10	High
I497T (ATC > ACC)*	Wild type	1	INH-monoresistant	> 3 ≤ 10	Intermediate
K590_None (1769delA)*	Wild type	1	Polydrug-resistant	> 10	High
N660D (AAC > GAC)* + wild type	Wild type	1	MDR	> 0.1 ≤ 1	Low
W668Y (TGG > TAC)*	Wild type	1	INH-monoresistant	> 0.1 ≤ 1	Low
G680R (GGC > CGC)*	Wild type	1	INH-monoresistant	> 1 ≤ 3	Intermediate
A726T (GCT > ACT)	Wild type	1	Polydrug-resistant	> 0.1 ≤ 1	Low
W728G (TGG > GGG)*	Wild type	1	MDR	> 10	High
Wild type	Wild type	32	-	-	-
Isolates with *katG* S315T + *inhA* promoter inferred by MTBDR*plus* (*n* = 2)					
Whole *katG* sequencing	***inhA* promoter sequencing**	**No. isolates**	**Phenotypic resistance profile**	**MIC INH (µg/mL)**	**INH resistance level**
Not performed	G-17T	1	MDR	> 10	High
Not performed	G-17T	1	Polydrug-resistant	> 10	High
Isolate with *katG* 315 inferred + *inhA* promoter wild type by MTBDR*plus* (*n* = 1)					
***katG* 315 sequencing**	**Whole *inhA* sequencing**	**No. isolates**	**Phenotypic resistance profile**	**MIC INH (µg/mL)**	**INH resistance level**
S315N (AGC > AAC) + wild type	Wild type	1	INH-monoresistant	> 3 ≤ 10	Intermediate
Isolates with *katG* 315 inferred + *inhA* C-15T by MTBDR*plus* (*n* = 2)					
***katG* sequencing**	**Whole *inhA* sequencing**	**No. isolates**	**Phenotypic resistance profile**	**MIC INH (µg/mL)**	**INH resistance level**
S315G (AGC-GGC)[a]	Not performed	1	MDR	> 1 ≤ 3	Intermediate
S315N (AGC > AAC) + A506T (GCC > ACC)[b]	Not performed	1	XDR	> 10	High

*Novel mutations

[a]Only *katG* 315 codon sequenced

[b]Whole *katG* gene sequenced

than in INH-monoresistant isolates (22/109, 20%). The *inhA* C-15T mutation was also more frequent in INH-monoresistant isolates (54/109, 50%) than in MDR (6/47, 13%, $p < 0.0001$) and polydrug-resistant (2/29, 7%, $p < 0.0001$) isolates. Marttila et al. (1998) and Hazbón et al. (2006) also found association between S315T mutation and MDR isolates [24, 25].

Mutations in *katG* and *inhA* are the most frequently encountered in INH-resistant isolates [26]. Among the 171 isolates with any *katG* and/or *inhA* mutation detected

Table 2 Mutations in *rpoB* detected by Sanger sequencing in *Mycobacterium tuberculosis* isolates presenting inferred mutations in *rpoB* by MTBDR*plus*

Isolates with *rpoB* inferred mutations by MTBDR*plus* (n = 16)				
rpoB RRDR sequencing	*katG*	*inhA*	No. isolates	Phenotypic resistance
F505L (TTA > TTC) + H526N (CAC > AAC)	S315T	C-15T	1	MDR
T508A (ACC > GCC)	S315T	Promoter wild type	1	INH-monoresistant
Q513E (CAA > GAA)	S315T	Promoter wild type	1	Pre-XDR
D516F (GAC > TTC)	K590_None	Whole gene wild type	1	Polydrug-resistant
D516I (GAC > ATC)	S315T	Promoter wild type	1	MDR
Del518 (delAAC)	D381N + L611L	Whole gene wild type	1	MDR
	Q439R	Whole gene wild type	1	MDR
S522L (TCG > TTG)	S315T	Promoter wild type	1	MDR
H526L (CAC > CTC)	S315T	Promoter wild type	1	INH-monoresistant
	S315T	G-17T	1	MDR
	S315T	G-17T	1	Polydrug-resistant
H526N (CAC > AAC)	W300R + C549G	Whole gene wild type	1	INH-monoresistant
	Whole gene wild type	Whole gene wild type	1	INH-monoresistant
H526S (CAC > TCC)	S315T	Promoter wild type	1	Polydrug-resistant
S531W (TCG > TGG)	S315T	Promoter wild type	1	MDR
L533P (CTG > CCG)	S315T	Promoter wild type	1	Polydrug-resistant

by MTBDR*plus* and sequencing in our study, 99 (58%) had mutations in *katG* alone, while 66 (39%) had only the C-15T *inhA* mutation, and six (3%) had mutations in both genes.

Table 3 Association between mutations in *katG* and *inhA* genes and levels of resistance to isoniazid in *Mycobacterium tuberculosis* clinical isolates

Mutation	INH resistance level								
	Total	Low (0.1 < MIC ≤ 1.0 µg/mL)		Intermediate (1.0 < MIC ≤ 3.0 µg/mL)		Intermediate (3.0 < MIC ≤ 10.0 µg/mL)		High (MIC > 10 µg/mL)	
	n	*n*	%	*n*	%	*n*	%	*n*	%
katG									
S315T	81	0	0	18	22	62	77	1	1
S315 other[a]	2	0	0	0	0	1	50	1	50
Other single mutations[b]	14	4	29	2	14	2	14	6	43
Other double mutations[c]	2	0	0	1	50	0	0	1	50
Total	**99**	**4**	**4**	**21**	**21**	**65**	**67**	**9**	**9**
inhA									
C-15T	**66**	**51**	**77**	**9**	**14**	**5**	**8**	**1**	**1**
katG + *inhA*									
S315T + C-15T	1	0	0	0	0	0	0	1	100
S315T + G-17T	2	0	0	0	0	0	0	2	100
S315 other[a] + C-15T	2	0	0	1	50	0	0	1[d]	50
R463L/A478R + G76G	1	0	0	0	0	0	0	1	100
Total	**6**	**0**	**0**	**1**	**17**	**0**	**0**	**5**	**83**
katG wild type + *inhA* wild type	**32**	**20**	**63**	**3**	**9**	**3**	**9**	**6**	**19**

[a]Mutation in *katG* 315 other than the S315T

[b]Single *katG* mutations in a codon other than the 315

[c]Double *katG* mutations outside the 315 codon

[d]Isolate with *katG* S315N + A506T double mutations

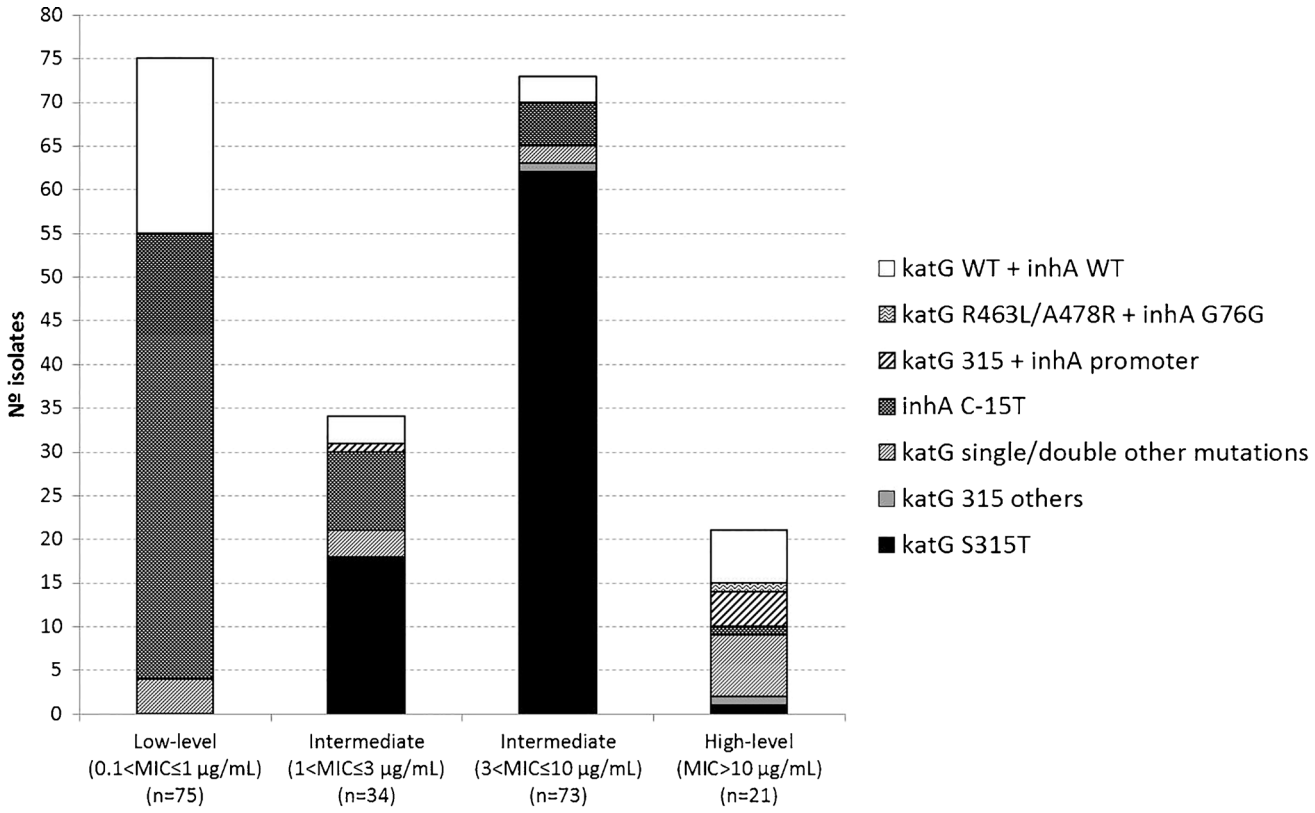

Fig. 3 Isoniazid resistance levels and mutations in *katG* and *inhA* genes detected by MTBDR*plus* and Sanger sequencing in *Mycobacterium tuberculosis* isolates

In this study, mutations in *katG* 315 were associated with an intermediate level of INH resistance, presented by 81/83 (98%) isolates with mutations in this region only. Regarding mutations in *inhA* promoter alone, 51/66 (77%) isolates with C-15T substitution showed low-level resistance to INH. Cambau et al. (2015) found 93% of isolates presenting *katG* S315T mutation with intermediate-level resistance ($3 \leq \text{MIC} \leq 10$ µg/mL) and 83% of isolates with *inhA* promoter mutations having low-level resistance ($0.1 < \text{MIC} \leq 1$ µg/mL) [15]. Lempens et al. (2018) found that 83% of isolates with *katG* S315T mutation had intermediate resistance ($3.2 \leq \text{MIC} \leq 12.8$ µg/mL), while isolates with *inhA* C-15T showed low-level ($0.8 \leq \text{MIC} \leq 1.6$ µg/mL) and intermediate resistance [8]. In our study, 14 (21%) and 1 (2%) of the 66 isolates with *inhA* C-15T mutation alone had respectively intermediate- and high-level resistance.

Concurrent mutations in *katG* and *inhA* have been associated with high-level INH resistance. In our study, 5/6 (83%) isolates with mutations in both genes presented INH MIC > 10 µg/mL, while only 1 isolate had intermediate resistance. Similar results were found by Lempens et al. (2018), showing that molecular tests such as MTBDR*plus* are accurate in identifying isolates with high-level INH resistance which cannot be overcome by increasing INH dose in the treatment [8].

In our study, isolates presenting mutations in *katG* regions other than the 315 codon ($n = 17$) showed varying levels of INH resistance. To the best of our knowledge, 10 of these isolates presented novel mutations (P232T, D381N, W438R, A478R, I497T, K590_None, N660D, W668Y, G680R and W728G), of which 8 showed high-level resistance. Previous studies also detected different degrees of INH resistance in isolates with mutations in *katG* other than in 315 [8, 27, 28]. The only isolate with a mutation in the coding region of *inhA* (G76G) had also two mutations in *katG*, and high-level resistance.

The great variability of mutations we encountered along *katG*, including isolates harbouring more than one mutation, might be explained by the fact that this gene is located in a highly variable region of the genome containing repetitive DNA sequences, which favours the instability of the region and the high frequency of mutations [29]. *katG* point mutations were more frequent than deletions in our study as previously reported elsewhere [27–29].

The WHO has just released a catalogue of > 17,000 M. *tuberculosis* mutations and their association with drug resistance [30]. The 10 novel *katG* mutations we found are not

present in this catalogue, which comprises isolates from 41 different countries. Except for the *katG* R463L mutation (which is considered a polymorphism and is not associated with drug resistance), the other mutations we found in *katG* regions outside the 315 codon that are present in the WHO catalogue have a very low frequency, so that their association with INH resistance is classified as uncertain. The scarcity of information on these *katG* mutations in the catalogue reinforces the importance of this work, as more data on association of INH MIC levels and infrequent mutations are needed.

The current CLSI clinical breakpoint to define INH low-level resistance (0.4 µg/mL) does not correspond to the upper end of the MIC distribution of *inhA* promoter mutants, hindering the adoption of such concentration to decide if the patient can be treated with high-level INH or not. According to Ghodousi et al. (2019) results on MIC distributions of *inhA* promoter mutants, a clinical breakpoint of 1 or 2 µg/mL would be more suitable than 0.4 µg/mL to phenotypically separate isolates between low- and high-level INH-resistant [31].

Our data show that either isolates with *katG* 315 mutations only or with *inhA* C-15T mutation alone have overlapping MICs that fall into the intermediate category. Similar results were found by Lempens et al. (2018), showing that *inhA* promoter mutations do not always indicate very low, and *katG* mutations do not necessarily cause very high levels of INH resistance levels [8]. As a consequence, the Global Laboratory Initiative recommends that, once a *katG* 315 mutation is detected by LPA, the result should be reported as high-level INH resistance, while an isolate with an *inhA* promoter mutation should be reported as "at least" low-level-resistant (http://www.stoptb.org/wg/gli/assets/documents/LPA_test_web_ready.pdf). Further research is necessary to elucidate whether high-dose INH contributes to successful outcomes in patients with low- or intermediate-level resistance, caused by a *katG* or *inhA* mutation alone.

All RIF-resistant isolates by MGIT 960 had *rpoB* mutations detected by MTBDR*plus*. Among the 73 isolates with *rpoB* mutations, 22% ($n = 16$) showed inferred mutations (50% of them were phenotypically susceptible to RIF at 1.0 µg/mL). Such borderline mutations (mutations which are detected as RIF-susceptible by phenotypic DST) are related to a higher risk of treatment failure or relapse when the standard first-line regimen with RIF is used in the treatment [32, 33]. If isolates presenting borderline mutations are misdiagnosed as RIF-susceptible and treatment with RIF is inadequately maintained, RIF resistance might spread in the community. Therefore, the WHO has just released a report in which the RIF critical concentration in MGIT decreased to 0.5 µg/mL, so that misclassification of these mutations can be reduced [9].

This study has limitations. We sequenced entire *katG* and *inhA* genes only for isolates with MTBDR*plus* wild-type results, so we might have missed mutations in other regions of these genes in isolates with *katG* and/or *inhA* mutations found by MTBDR*plus*. Moreover, we did not investigate other genes known to be related with INH resistance, such as those that regulate *katG* expression (*furA-katG* and *sigI* intergenic region), *ahpC, oxyR, kasA* and *ndh,* and genes involved in efflux pump mechanisms [26, 29]. Mutations in these genetic regions might explain INH resistance in isolates with wild-type results for both *katG* and *inhA* whole genes in our study. We also did not assess RIF resistance level determination for the inferred *rpoB* mutations.

Conclusions

We have shown that mutations in *katG* 315 are associated with intermediate- to high-level resistance to INH, while *inhA* C-15T is related to low- and also intermediate-level resistance in *M. tuberculosis* clinical isolates. We have described 10 novel mutations in different regions of *katG* in isolates with varying levels of resistance to INH. Such mutations might be potential new INH resistance markers and could explain INH resistance in isolates without classic mutations in *katG* 315 and *inhA* promoter region. In our setting, 16% of INH-resistant isolates, some with high-level resistance, did not show mutations in either *katG* or *inhA* genes.

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Author contribution JMWP and RSO conceived the study and designed the experiments; JMWP and FFM performed and analysed MTBDR*plus* tests; JMWP, APB and MCSR performed and analysed gene sequencing; JMWP collected laboratory data; JMWP and RSO analysed the data; JMWP wrote the draft; and JMWP, RSO, APB and LF reviewed and edited the draft. All authors read and approved the final manuscript and agreed to be responsible for all aspects of the work.

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Data availability The datasets generated and analysed during the current study are available from the corresponding author on reasonable request.

Code availability Not applicable.

Declarations

Ethics approval This study was approved by Adolfo Lutz Institute Ethics Committee (Plataforma Brasil no. 1.473.145).

Consent to participate Not applicable.

Consent for publication Not applicable.

Conflict of interest The authors declare no competing interests.

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