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Genotypic and Phenotypic Characteristics of Tunisian Isoniazid-Resistant *Mycobacterium tuberculosis* Strains

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Forty three isoniazid (INH)-resistant *Mycobacterium tuberculosis* isolates were characterized on the basis of the most common INH associated mutations, *katG*315 and *mabA* -15C→T, and phenotypic properties (i.e. MIC of INH, resistance associated pattern, and catalase activity). Typing for resistance mutations was performed by Multiplex Allele-Specific PCR and sequencing reaction. Mutations at either codon were detected in 67.5% of isolates: *katG*315 in 37.2, *mabA* -15C→T in 27.9 and both of them in 2.4%, respectively. *katG* sequencing showed a G insertion at codon 325 detected in 2 strains and leading to amino acid change T326D which has not been previously reported. Distribution of each mutation, among the investigated strains, showed that *katG* S315T was associated with multiple-drug profile, high-level INH resistance and loss or decreased catalase activity; whereas the *mabA* -15C→T was more prevalent in mono-INH resistant isolates, but it was not only associated with a low-level INH resistance. It seems that determination of catalase activity aids in the detection of isolates for which MICs are high and could, in conjunction with molecular methods, provide rapid detection of most clinical INH-resistant strains.

***Keywords*:** *M. tuberculosis*, isoniazid resistance, new *katG*326 mutation, *katG*315 and *mabA* -15C→T mutations, phenotypic properties

Although tuberculosis (TB) is a preventable and treatable infectious disease, it still causes 8 million new TB infections per year with 250,000 to 400,000 multidrug resistance (MDR) cases (WHO, 2008). Isoniazid (INH), a synthetic bactericidal agent, was first reported to be an effective anti-TB drug in 1952, displaying particular potency against *Mycobacterium tuberculosis* and *M. bovis* (Banerjee *et al*., 1994). Mutants resistant to INH have emerged since then, and today such mutants account for as many as 5% of the clinical *M. tuberculosis* isolates in Tunisia (Slim *et al*., 2004).

The molecular genetic basis of isoniazid (INH) resistance is still not well understood. Actually, it's thought that this resistance may arise through distinct mutations frequencies in the *katG*, *inhA* genes (regulatory and structural regions), *oxyR-aphC* intergenic region, and *kasA* (Silva *et al*., 2003). Although diverse array mutations are uniquely represented among INH resistant strains, amino acid substitutions located at position 315 in *katG* gene are the most abundant, and in some geographic areas, this mutation was associated with MDR (Dobner *et al*., 1997; Marttila *et al*., 1998; Mokrousov *et al*., 2002a). It was also reported, that most INH-resistant strains carrying *katG*315 mutation showed a significant decrease in catalase activity associated with high-INH level MICs (van Soolingen *et al*., 2000; Ramaswamy *et al*., 2003). Otherwise, several studies showed that approximately 25% of clinical INH-resistant *M. tuberculosis* isolates contains mutations within the promoter or structural regions of the *inhA* locus (Rouse *et al*., 1995; Musser *et al*., 1996). Nucleotide substitution in region flanking a presumed ribosomal binding site (RBS) located in the upstream region of the *mabA-inhA* operon is thought to increase the target levels, thereby causing resistance by a drug titration mechanism (Ramaswamy and Musser, 1998). The point mutation -15C→T *mabA-inhA* promoter region was the most frequently involved in INH-resistance (Madison *et al*., 2004).

The aims of our study were to: i) determine the prevalence of the *katG*315 and *mabA* -15C→T mutations in Tunisian INH-resistant *M. tuberculosis* clinical strains and, ii) to evaluate the association of these mutations with INH resistance level, MDR, patient's status, and catalytic activity.

Materials and Methods

Bacterial strains

During January 2005 to July 2008, 495 *M. tuberculosis* strains isolated in Rabta university hospital (located in Tunis City, Tunisia) were investigated for INH resistance. 7 MDR *M. tuberculosis* strains isolated from a Hedi Chaker hospital located in Sfax region (south of Tunisia) were also included in our study. These strains were selected according to their resistance profile and patient's status (previously treated cases). A pan-sensitive and two INH-resistant isolates (low- and high-INH resistances) of *M. tuberculosis* were also included as references.

The culturing of mycobacterial isolates was performed on Löwenstein-Jensen (LJ) medium (BioRad, France) and all *M. tuberculosis* strains were biochemically characterized and confirmed by the AccuProbe

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method (Gen-Probe Inc., USA).

Drug susceptibility testing

Drug susceptibility for anti-TB drugs tested in routine in our laboratory was carried out on LJ medium according to the standard procedure (Canetti *et al*., 1969). The critical concentrations of rifampicin (RMP), isoniazid (INH), ethambutol (EMB), Streptomycin (SM), Ciprofloxacin (CIP), ethionamide (ETH), and pyrazinamide (PZA) were 40, 0.2, 1, 10, 10, 100, and 20 μg/ml, respectively. Resistance to INH was defined as ≥1% growth on INH-containing medium compared to the rate of growth on control medium.

To determine INH MICs, the agar proportion method was performed on LJ medium containing varying concentrations of INH (0.1, 0.2, 1, and 10 μg/ml). The critical concentration for high-level INH resistance was defined as ≥1 μg/ml on LJ medium (Ramaswamy and Musser, 1998).

Catalase activity assay

Evaluation of catalyse activity was performed on all tested and reference strains following a standard protocol (Kent and Kubica, 1985). Briefly, a suspension of the isolate was prepared from freshly growing 21 days LJ agar *M. tuberculosis* culture with 500 μl of phosphate solution M 5 (pH 7), and then incubated at 37°C for 30 min. 500 μl of 1:1 mixture of Tween 80 (Sigma, Tunisia) and hydrogen peroxide (Sigma) was added, and the mixture was incubated for 5 min at room temperature before measuring (in mm) the height of the bubbles. For convenience, we have classified the catalase activity as types I, II, and III according to values obtained with reference strains tested (24 mm for pan-susceptible strain and 6 to 18 mm for high- or low level INH-resistant isolates, respectively). Type I corresponds to strains with loss of catalase activity (0 mm); strains having a catalase activity ranged between 1 to 6 mm were defined as type II and, type III represents strains in which catalase activity varied between 7 to 18 mm.

DNA extraction

It was performed as previously described by Buck *et al.* (1992) with some modifications. Briefly, 2 or 3 colonies of *M. tuberculosis* were suspended in 300 μl of distilled water. After heat killing at 95°C for 30 min, a sonication was held at room temperature for 25 min, and the suspension was centrifuged at 14,000×g for 5 min. Supernatant was kept at -20°C until used.

Typing of resistance mutation *katG*315

Multiplex allele-specific (MAS) PCR targeting *katG*315 mutations was carried out using the assay developed and performed by Mokrousov *et al.* (2002b), with some modifications at the concentrations of used primers. katg0F and katg4R (Invitrogen, Tunisia) were used at 30 pmol, and katg5R primer at 40 pmol (Invitrogen). The amplified products (Perkin-Elmer 9700, Applied Biosystems, France) were electrophoresis (Mini-Sub® Cell 12 cm, Bio-Rad, Tunisia) in 2% agarose gel (Bio-Rad) in 1× Tris-Borate-EDTA buffer (Bio-Rad), and visualized under UV light.

Typing of resistance mutation *mabA* -15C→T

The detection of *mabA* -15C→T mutation was performed by using allele-specific primer, inhAPF2, of which the 3′ end is positioned to pair with the C base at position -15 in *mabA-inhA* promoter region as described by Yang *et al.* (2005).

The PCR reaction mixture performed on 25 μl included: 1 μM of each inhAPF2 and inhAP15 primers (Invitrogen), 5 μl of 5× reaction buffer, 2 mM $MgCl_2$, 200 μM concentrations of each deoxynucleoside triphosphate, 1 U of recombinant *Taq* DNA polymerase, and 1 μl of DNA template. The thermocycling (Perkin-Elmer 9700, Applied Biosystems) parameters used the following conditions: an initial denaturing at 96°C for 3 min, 23 cycles of 95°C for 50 sec, 64°C for 1 min, and 72°C for 1 min, and a final extension at 72°C for 7 min. The PCR products were examined for binding presence by 2% agarose gel (Bio-Rad) electrophoresis (Mini-Sub® Cell 12 cm, BioRad) in 1× Tris-Borate-EDTA buffer (Bio-Rad), and visualized under UV light. A sensitive and resistant INH *M. tuberculosis* strains was included respectively as positive and negative PCR reaction control.

katG sequencing

DNA sequencing of the 435 bp *katG* region was performed by using katg0F and katg4R primers with an ABI PRISM Dye terminator cycle sequencing ready reaction kit (Applied Biosystems) and the reactions were analysed on an ABI PRISM 310. The BLAST 2 SEQUENCES computer program was used for DNA sequence comparisons (http://www.ncbi.nlm.nih.gov/BLAST).

Statistical analysis

Categorical data were analyzed by the chi-square test. A *P* value of <0.05 was considered statistically significant.

Results

Among the 495 isolates collected in Rabta hospital during the study period, 36 (7.2%) showed INH resistance: 12 were mono-INH resistant, 6 were MDR (according to the definition of the World Health Organisation as resistance against at least to INH and RMP), 14 were resistant to INH and SM, and 4 were resistant to INH and ETH.

In our study, 7 MDR strains (isolated from Sfax area) have been included and then a total of 43 investigated INH-resistant strains were classified in two distinct populations: 12 mono-INHr and 31 strains resistant to INH and one other drug (multiple-drug resistant).

The presence of *katG*315 and/or *mabA* -15C→T mutations was observed in 67.5% (29/43) of clinical *M. tuberculosis* INH-

Table 1. Frequency of mutations among mono-INHr and multiple-drug resistant *M. tuberculosis* isolates

Studied population	Mutations				
	*katG*315 (%)	*katG* T326D (%)	*mabA* -15C→T (%)	*katG*315+*mabA* -15C→T (%)	No mutations (%)
Mono-INHr (N=12)	3(25)	-	5(41.7)	-	4(33.3)
MDR (N=13)	7(53.8)	2(15.4)	1(7.7)	1(7.7)	2(15.4)
INH + SM (N=14)	6(42.9)	-	3(21.4)	-	5(35.7)
INH + ETH (N=4)	-	-	3(75)	-	1(25)

Table 2. Distribution of mutations according to patient's status

Mutations	New cases (N=27)	Treated patients (N=16)
*katG*315	9 (33.3%)	7 (43.8%)
katG T326D	-	2 (12.5%)
mabA -15C→T	9 (33.3%)	3 (18.7%)
*katG*315+*mabA* -15C→T	-	1 (6.3%)
No mutations	9 (33.3%)	3 (18.7%)

resistant strains. Prevalence of each studied mutation, according to resistance profile, was summarized in Table 1. *katG*315 and *mabA* -15C→T mutations were detected respectively, in 16 (37.2%) and 12 (27.9%) cases of the total resistant strains. Simultaneous mutations at both loci were detected in 1 MDR isolate (Table 1).

Our sequencing results showed that all *katG*315 mutations detected in 17 isolates (16 isolates which carried only the *katG*315 and 1 isolate with the *mabA* -15C→T mutation) were a Ser315Thr substitution (A*G*C→A*C*C). Moreover, for the remaining 26 strains, no *katG*315 mutation was detected by sequencing, confirming thus the MAS-PCR results, but a G insertion at codon 325 leading to amino acid change in codon 326 (T326D), and which has not been previously reported, was detected in 2 MDR strains. Besides, no mutations were observed in 27.9% (12/43) of isolates (Table 1).

Our results showed that the distribution of *katG*315/*mabA* -15C→T mutations was different between mono-INHr and multiple-drug resistant strains. Among all strains which have only the *katG*315 mutation, 18.8% (3/16) were mono-INH resistant and 81.3% (13/16) were resistant to INH and one other drug (Table 1). However, in strains with the *mabA* -15C→T mutation, 41.7 (5/12) were mono-INH resistant and 58.3% (7/12) were multiple-drug resistant population (Table 1). Moreover, the *mabA* -15C→T mutation is thought to presumably cause overexpression of *inhA*, the target of INH and ETH, thus resulting in co-resistance to these both anti-tuberculosis drugs (Lee *et al*., 2000). Our results showed that 25% (3/12) of strains carrying the *mabA* -15C→T mutation were INH and ETH resistant (Table 1). In fact, 3 of 4 strains (75%) which were INH–ETH resistant had the *mabA* -15C→T mutation.

Distribution of mutations among MDR strains showed that the *katG*315 was detected in 53.8% (7/13) of cases and the *mabA* -15C→T in 7.7% (1/13). Moreover, simultaneous presence of these mutations was detected in 7.7% (1/13) of MDR strains.

Besides and according to our results, the prevalence of *katG*315 and *mabA* -15C→T mutations varied significantly between MDR and mono-INH resistant strains (P=4.26).

Patient medical records showed that 27 strains were isolated from new cases and 16 were from previously treated cases. Mutations were detected in 70.4 (18/27) and 75% (13/16) of new and treated cases, respectively (Table 2).

There was no significant difference in frequency of mutations between the groups of isolates recovered from new and previously treated cases (P=0.28).

Distribution of each mutation according to patient's status was summarized in Table 2. Our results showed that in 16 strains recovered from treated cases, 81.3% (13/16) carried a *katG* or *mabA* mutation; while in new cases we detected the presence of mutation in 66.6% (18/27) of isolated.

MICs INH testing results showed that 30 (69.8%) of isolates had a high level of INH-resistance (MICs=1 or 10 μg/ml) and 13 (30.2%) were resistant at 0.2 μg/ml. All 16 INH-resistant isolates with a *katG*315 mutation, had a MIC of 1 or 10 μg/ml. However, it's interesting to note that 50% of isolates with *mabA* -15C→T mutation had a MIC of 1 or 10 μg/ml; the remaining 50% had MIC equal to 0.2 μg/ml. In strains with novel mutation *katG* T326D, the MIC was of 1 μg/ml.

The MIC was of 10 μg/ml in the isolate in which simultaneous mutations were detected and for strains with no mutations, they were observed in each MIC category with a largest frequency in strains with a low INH level resistance (0.2 μg/ml).

The catalase activity tested for all INH-resistant isolates was ranged from 0 to 18 mm, and our results showed that, 3 strains had the type I characterized by the loss of catalase activity, 24 strains had the type II distinguished by a catalase activity comprised between 1 to 6 mm and 16 isolates showed the type III in which values varied from 7 to 18 mm. *katG*315 mutation was more prevalent in type II (68.8%) but the *mabA* -15C→T was frequently observed in type III (58.3%). The distribution of catalase activity according to INH MICs and *katG*315, *mabA* -15C→T mutations was summarized in Table 3. There was a significant difference in frequency of *katG*315/*mabA* -15C→T mutations between the groups of strains with type I and II catalase activity and, type III group (P=6.61). The new mutation was only present in type II.

Discussion

The emergence of *M. tuberculosis* multidrug-resistant strains had resulted in fatal outbreaks in many countries. The development of INH resistance is a common first step in the evolution to MDR (van Rie *et al*., 2001), so it would be beneficial to detect rapidly the most of clinical INH-resistant strains.

In Tunisia, compilation data of 2006 WHO report showed that the global incidence of TB infection was about 25 per 100,000 inhabitants. MDR was found in 2.7% of new TB cases, and in 36% of previously treated TB cases (WHO, 2008). The global INH resistance was about 5.2% in 2006 (Slim *et*

Table 3. Catalase activity, MIC of INH and *katG*315 and/or *mabA* -15C→T, *katG* T326D mutations in INH-resistant investigated strains

Type of catalase activity	INH MICs (1 or 10 μg/ml)	INH MICS (0.2 μg/ml)	*katG*315	*mabA* -15C→T	*katG*315+*mabA* -15C→T	*katG* T326D
Type I: 0 mm (N=3)	3	-	3	-	-	-
Type II: 1 to 6 mm (N=24)	24	-	11	5	1	2
Type III: 7 to 18 mm (N=16)	3	13	2	7	-	-

al., 2004).

This study showed that 37.2 and 27.9% of INH-resistant *M. tuberculosis* isolates have respectively the *katG*315 and *mabA* -15C→T mutations. This is in concordance with previous studies in which 12 to 75% of INH-resistant strains contained mutations either in codon 315 of the *katG* gene or in *inhA* ribosomal binding site (Rouse *et al*., 1995; Musser *et al*., 1996; Marttila *et al*., 1998).

A common *katG*315 missense mutation (A*G*C→A*C*C or A*CA*) with amino acid change of serine to threonine (Ser315 Thr) was described in various geographic areas (Haas *et al*., 1997; Abate *et al*., 2001), and about 10 to 25% of other mutations were located in different *katG* loci (Musser *et al*., 1996). Our sequencing results showed the presence of Ser315Thr in 17 isolates and allowed us to detect a novel mutation (*katG* T326D) in 2 MDR investigated strains. This area of *katG* gene was chosen for sequencing investigation because the most frequently INH-resistance mutations occurred between codon 249 to codon 350 (Haas *et al*., 1997; Lavender *et al*., 2005; Guo *et al*., 2006; Hazbóm *et al*., 2006). None of the isolates had an entire deletion of *katG*, evidence of its rare occurrence in clinical isolates.

This high prevalence of Ser315Thr mutation among INH-resistant *M. tuberculosis* isolates may be favored because it possibly decreases INH activation without abolishing catalase-peroxidase activity, a potential virulence factor (Pym *et al*., 2002). It was demonstrated that Ser315 mutations were associated with high-level INH resistance and their frequencies depend significantly on a drug resistance pattern (Piatek *et al*., 2000; Hillemann *et al*., 2005). However, several data reported that *mabA* -15C→T mutation was relative to low-level INH resistance (Guo *et al*., 2006).

In this study, we evaluated the possible correlation between *katG*315, *mabA* -15C→T, the new *katG*326 mutation and INH resistance level, MDR profile and catalytic activity.

Our results showed that *katG* Ser315Thr mutation was significantly more common in MDR isolates, but conversely, the *mabA* -15C→T was more frequent in mono-INHr isolates. All of 16 isolates with only *katG*315 mutation had a high-level INH resistance. However, among the 12 strains which carried only the *mabA* -15C→T mutation, 50% showed a high-level INH resistance. These findings suggest that this mutation can be present by itself and could be associated with a high-level INH resistance. Nevertheless, it would be useful to see for other *mabA* polymorphisms in these clinical strains, and probably seeking for the presence of mutations identified in different loci associated with INH-resistance.

Correlation between catalase activity and INH resistance has been known for a long time (Middlebrook, 1954). The loss of catalase activity is more consistent with a high-level of INH resistance (Zhang *et al*., 1992). This was also observed in our study: of the 30 strains with high INH MICs (1 or 10 μg/ml), 3 expressed no catalase activity and 24 had a reduced activity (1 to 6 mm). We have also noted that the loss of catalase activity was found in strains carried the *katG*315 mutation.

Despite the high percentage -72.1% of strains with *katG*315 and/or *mabA* -15C→T or *katG* T326D – which are resistant to INH, INH+SM, INH+ETH, or MDR, 27.9% do not have these mutations and are resistant to the previously mentioned groups. Therefore, further expansion must be made of the results of non-mutating strains as research for *inhA* mutations, point mutations in the *aphC-oxyR* intergenic region (Wilson and Collins, 1996; Piatek *et al*., 2000), or mutations in the *kasA* gene (Mdluli *et al*., 1998; Lee *et al*., 1999).

It's clear that in our clinical strains further investigation of implicated INH-resistance loci is needed, and it will be very attractive to include other strains isolated from distinct Tunisian geographic region, in order to evaluate the real prevalence of the investigated mutations in our country. Nevertheless, on basis of our results, using of INH in the treatment of tuberculosis patients must be prescribed with caution at Rabta University Hospital due to the high rate of *katG*315 mutation. We noted also, that Ser315Thr was most frequently observed in MDR cases whatever the geographic origin of strains (Tunis and Sfax) and we may consider that determination of catalase activity aids in the detection of isolates for which MICs are high and could, in conjunction with molecular methods, provide rapid detection of most clinical INH-resistant strains.

Nucleotide sequence accession number

The sequence with novel mutation found in this study has been deposited in GenBank under accession number FJ169893.

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