Molecular Characterization of Isoniazid Resistance in *Mycobacterium tuberculosis*: Identification of a Novel Mutation in *inhA*


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Tuberculosis (TB) remains a public health issue in many parts of the world (20). The situation is further complicated by the emergence of multidrug-resistant TB (18). Multidrug-resistant TB is recognized as infection with *Mycobacterium tuberculosis* resistant to at least isoniazid (INH) and rifampin. Mutations in dispersed gene loci including *katG* (catalase-peroxidase), the promoter region of *ahpC* (alkyl hydroperoxidase), *inhA* (enoyl-acyl reductase), *kasA* (β-ketoacyl ACP synthase), *mabA* (3-ketoacyl reductase), and *ndh* (NADH dehydrogenase) have been found to be associated with INH resistance (2, 4, 7, 10–11, 22). Our previous study using PCR-RFLP to detect the *KatG* amino acid substitution Ser315Thr successfully identified 51% of INHR *M. tuberculosis* strains among 375 clinical isolates from the South China region (9). In the present study, a multiplex allele-specific PCR (MAS-PCR) was used to detect INH-negative strains in the South China region between 1999 and 2002 were tested for susceptibility to the antimycobacterial agent INH by using a Michaelis-Menten equation and plotted in Lineweaver-Burk coordinates. Among 375 clinical isolates, 371 were successfully amplified by *mabA* and *katG* MAS-PCRs (12) for rapid diagnosis of INHR *M. tuberculosis* was evaluated. INHR isolates negative for *katG* 315 alterations were subsequently subjected to DNA sequencing of various gene loci associated with INH resistance. A novel mutation in *inhA* was characterized to elucidate non-*katG*-related resistance mechanisms.

Three hundred seventy-five *M. tuberculosis* isolates collected from patients suffering from tuberculosis in Hong Kong and the South China region between 1999 and 2002 were tested for susceptibility to the antitubercular agent INH by using 7H10 medium containing INH at 0.2 or 1.0 μg/ml (9, 13). Mycobacterial DNA was extracted as described previously (19).

The *KatG* MAS-PCR protocol was essentially adopted from the work of Mokrousov et al. (12). A mutation at codon 315 would yield an amplicon of 435 bp (Fig. 1a), and wild-type *katG* would yield a smaller amplicon of 293 bp. For wild-type strains, two fragments of 451 bp and 119 bp were amplified, while a single 451-bp fragment was amplified for mutants (Fig. 1b). Performance of *mabA* MAS-PCR was verified by DNA sequencing of 100 randomly selected isolates (50 susceptible and 50 resistant) using an ABI3700 genetic sequencer (Applied Biosystems) as described previously (21). Sequencing primers used for different gene loci are listed in Table 1.

Both wild-type and Ile194Thr InhA proteins were expressed for kinetic analysis using the pET-15b expression vector (Novagen, Madison, Wis.) and *Escherichia coli* BL21(DE3) as the host. All kinetic reactions were carried out in 30 mM piperazine-N,N'-bis(2-ethanesulfonic acid) (PIPES) buffer, pH 6.8, using 2-trans-hexadecenyl coenzyme A as a substrate, at 25°C (3, 14). Following NADH oxidation at *A*₅₅₀ steady-state *Kₘ* values for NADH were determined with variable concentrations of NADH at fixed saturating concentrations of the substrate. The experiment was repeated with three separate preparations of purified recombinant proteins. Data were fitted to a Michaelis-Menten equation and plotted in Lineweaver-Burk reciprocal form with GraphPad Prism v4.0 software to generate estimates of *Kₘ* and *V*ₘₐₓ values.

Among 375 clinical isolates, 371 were successfully amplified by *katG* and *mabA* MAS-PCR assays. Four INHR isolates previously identified as catalase negative (9) were positive only by *mabA* MAS-PCR. Fifty-two of the 102 resistant isolates exhibited *katG* codon 315 alteration (Fig. 2a). The remaining 50 resistant isolates, as well as the 273 susceptible isolates, showed no mutation in *katG* codon 315. The findings completely agree with our previous antimycobacterial susceptibility testing, PCR-RFLP, and DNA sequencing results (9). With *mabA* MAS-PCR, 32 of 102 resistant isolates were identified as having a mutation in *mabA* bp –15 (Fig. 2b). The remaining 70 resistant isolates and the 273 susceptible isolates exhibited no mutation in the corresponding region. The results also agree with DNA sequencing results of 100 randomly selected isolates (50 resistant and 50 susceptible). Further analysis revealed that 79 (77.5%) resistant isolates carried one or both of the mutations with 100% specificity. The remaining 22.5% may harbor mutations in other regions of *katG* and *inhA* associated with

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point mutations in eight catalase-positive and INH\textsuperscript{R} isolates, among which seven strains exhibited point mutations upstream of \textit{ahpC}, with the DNA sequence of the coding region unaltered. Previous studies showed that mutations in promoter regions of \textit{ahpC} in INH-resistant \textit{M. tuberculosis} could overexpress alkyl hydroperoxidase to combat oxidative damage. Such overexpression does not directly relate to the initiation of INH resistance (1, 5, 8).

The MIC of INH for the last strain was $>1.0$ $\mu$g/ml, and there was a point mutation at bp 581 of \textit{inhA} (GenBank accession no. AF06077) causing the amino acid substitution Ile194Thr (ATC $\rightarrow$ ACC). Compared with wild-type InhA of H37Rv (ATCC 27294), purified protein with Ile194Thr showed a 5-fold increase in $K_m$ without a significant increase (1.3-fold only) in $V_{\text{max}}$ (Table 2 and Fig. 3). The high $K_m$ suggested that under cellular concentrations of NADH, Ile194Thr affects the binding of NADH to the enzyme and the decrease of reaction. Unless a very high concentration of NADH is available, which is unlikely, since the cellular concentration of NADH is less than 10 $\mu$M (14), the reaction rate cannot be raised to normal wild-type levels. This finding is also consistent with previous X-ray crystallography data (http://au.expasy.org) showing that isoleucine 194 lies within the binding cleft of the enzyme and in close proximity with the oxygen atom of NADH (16). It is likely that isoleucine 194 participates in hydrogen bonding with the docked NADH. Recently, molecular dynamics simulations also showed Ile194 as 1 of the 10 most important amino acid residues making conserved H bonds with NADH cofactor in wild-type InhA protein (17). It is quite

\begin{table}[h]
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\begin{tabular}{llc}
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Gene locus (size [bp]) & Primer, sequence & Position in reference to start codon (bp) \\
\hline
\textit{katG} (2,223) & katG-1F, 5'CCTCGGGGTATCATCCATCGTG3' & $\sim$57 to $\sim$39 \\
& katG-1R, 5'AAAGCGGCGCGGACGGCTG3' & 396 to 378 \\
& katG-2F, 5'GGGATGCTGAGGCGGGCCG3' & 373 to 391 \\
& katG-2R, 5'ACGTTCGGAATGTGCA3' & 754 to 736 \\
& katG-3F, 5'CGCCGCGGTCGACATTG3' & 729 to 746 \\
& katG-3R, 5'GACCGTCATCCAGTTGTAG3' & 1104 to 1096 \\
& katG-4F, 5'CTGCAGGCGGCGGGCGGCG3' & 1110 to 1119 \\
& katG-4R, 5'GTTCGGCGGGCCAGGGCGC3' & 1500 to 1480 \\
& katG-5F, 5'CGCCAACGGTGGTCGCATC3' & 1473 to 1491 \\
& katG-5R, 5'GGCGACGTGAAACGTCG3' & 2220 to 2202 \\
\textit{inhA} (810) & inha-1F, 5'GCAATGGTGATATGGCCACTGAC3' & $\sim$38 to $\sim$13 \\
& inha-2F, 5'CGGCTGACCGAGGGGATCG3' & 250 to 268 \\
& inha-3F, 5'CCACATCTCGGAGGATTCG3' & 381 to 399 \\
& inha-4R, 5'GACCGTCATCCAGTTGAG3' & 510 to 492 \\
& inha-5R, 5'TTCCGCTGCGCCGACAGACG3' & 860 to 840 \\
\textit{kasA} (1,251) & kasA-1F, 5'GGGCGGACGTGTTTGCGG3' & $\sim$24 to $\sim$3 \\
& kasA-2F, 5'GTGGAGAGGCTACGACGATG3' & 365 to 391 \\
& kasA-3F, 5'GCCAAGCCTGCTGGCCGATTGTC3' & 775 to 798 \\
& kasA-4R, 5'CGACCCCCGGATGTTGCTTTC3' & 1270 to 1250 \\
\textit{mabA} (744) & mabA-1F, 5'GTTAAGGCTGCTGCGCGAATC3' & $\sim$59 to $\sim$41 \\
& mabA-2R, 5'CTGATCCTGGCGCGATG3' & 384 to 368 \\
& mabA-3R, 5'GATTCCGCTAACCAGAATC3' & 807 to 789 \\
\textit{ndh} (1,392) & ndh-1F, 5'CACGTGAGTCTCGGCGGCTGAC3' & $\sim$40 to $\sim$18 \\
& ndh-2F, 5'GTGGAGAGGCTACGACGATG3' & 295 to 315 \\
& ndh-3F, 5'TGGACAGGCTGCGGCGC3' & 462 to 480 \\
& ndh-4F, 5'CAAGGCGGCGGCTGCGGCG3' & 952 to 973 \\
& ndh-5R, 5'GGACATGCGCTGACGATG3' & 1154 to 1135 \\
& ndh-6R, 5'AGCTCTCGCGAATGTCGAC3' & 1441 to 1418 \\
\textit{oxyR-ahpC} (107) & oxyR-ahpC-F, 5'CCGCGCAGCGCGACCTCGG3' & 398 to 417 (oxyR) \\
& oxyR-ahpC-R, 5'ATTATCGCCAATGGTACG3' & 28 to 9 (\textit{ahpC}) \\
\hline
\end{tabular}
\caption{Primers used for DNA sequencing}
\end{table}
likely that substitution of the isoleucine alkyl chain with a hydroxyl group of threonine disrupts the hydrogen bond pattern around NADH and reduces the affinity of NADH to InhA. Subsequently, a larger proportion of the cellular InhA molecules would be left in the non-NADH-bound form as a result of the lowered affinity. According to Rozwarski et al. (16), InhA in its NADH-bound form is more susceptible to the attack of activated INH than in its free molecule form. A lowered affinity of NADH therefore protects most of the InhA molecules from INH. Alternatively, if Ile194Thr InhA has a decreased affinity with NADH, its affinity with NADH-isonicotinic adduct will also be reduced. According to Rawat et al. (15), activated INH can bind with free NADH, forming an adduct molecule to block the enzymatic reaction of InhA even if InhA is in its non-NADH-bound form. Lowered affinity with NADH-isonicotinic adduct promotes the release of the adduct from the enzyme and allows normal substrate catalysis to proceed. Either scenario could result in INH resistance in this mutant with a wild-type katG sequence.

This study evaluated a MAS-PCR protocol suitable for rapid diagnosis of INH-resistant M. tuberculosis. The enzyme kinetics study of an Ile194Thr mutant opens a path to better understanding of the molecular basis of non-katG-related INH resistance mechanisms.

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REFERENCES


