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Contribution of *dfrA* and *inhA* Mutations to the Detection of Isoniazid-Resistant *Mycobacterium tuberculosis* Isolates▽

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Screening of 127 isoniazid (INH)-resistant *Mycobacterium tuberculosis* isolates from Singapore for mutations within the *dfrA* and *inhA* genes revealed mutations in 0 and 5 (3.9%) isolates respectively, implying that mutations in *dfrA* do not contribute to the detection of INH-resistant *M. tuberculosis* and that mutations within *inhA* are rare. Thirty-seven (29%) of the 127 isolates had no mutations in any of the genes implicated in INH resistance (*katG*, *kasA*, and *ndh*; *inhA* and *ahpC* promoters), suggesting that there are new INH targets yet to be discovered.

Mycobacterium tuberculosis remains a major health concern worldwide, with approximately 2 billion people currently infected worldwide and 9.2 million new cases in 2006 (13, 27). The emergence of multidrug-resistant tuberculosis has increased global efforts to understand the molecular mechanisms of drug resistance in *M. tuberculosis*.

Isoniazid (INH) for *M. tuberculosis* chemotherapy has always been used as first-line treatment (25) since it was found to exhibit powerful bactericidal activity against the disease by effectively causing loss of acid fastness in *M. tuberculosis* through inhibition of mycolic acid synthesis (9). However, its widespread use, justified by its high specificity, low cost, and considerably low toxicity, has seen treatment failures due to increasing resistance to the drug.

Clinical resistance to INH is widely known to be caused by mutations within the *katG* and *inhA* genes and the promoter region of *inhA* (2, 13, 18, 19, 25, 29). Other genes, such as *kasA* and *ndh*, have been implicated in INH resistance, but mutations in these genes are rare and have been observed in INH-susceptible isolates and/or in association with *katG* mutations (8, 11, 12, 14, 15, 18, 25, 28). The *ahpC* gene is involved in the cellular regulation of oxidative stress (18, 22). Mutations within the *oxyR-ahpC* intergenic region that result in increased expression of alkyl hydroperoxidase are considered to compensate for peroxide sensitivity due to loss of KatG function found in clinical resistant strains (10, 22, 28). Nevertheless, screening of INH-resistant clinical isolates of *M. tuberculosis* for mutations within *kasA*, *ndh*, and *ahpC* is often performed.

We have previously extensively characterized INH-resistant isolates for mutations within all of these regions; however, 57 (36%) of 160 INH-resistant isolates from Singapore had no detectable alterations (11, 12). This finding suggests that mutations in other genes that confer resistance to INH may exist.

Recently, dihydrofolate reductase, encoded by the *dfrA* gene, was proposed as a new target for INH (1) as it has been reported that NADP-bound INH had been shown to inhibit dihydrofolate reductase, an enzyme essential for nucleic acid synthesis. Also, the importance of mutations within the structural region of enoyl reductase (InhA), encoded by the *inhA* gene, have been reported where a single point mutation allele

TABLE 1. Oligonucleotide primers used for the amplification of the entire *dfrA*[a] and *inhA*[b] genes

Gene	Primer sequence	Annealing temp (°C)	Amplicon size (bp)	Nucleotide positions
dfrA (F)	GAC GAA GCG ATG AGG AGA AG	55	632	300,058–300,072
dfrA (R)	TCG TTG TGA AGA ACT ACG ATC C			300,699–300,678
inhA (F)1	CTA CAT CGA CAC CGA TAT GAC	55	700	290,948–290,968
inhA (R)1	GAC CGT CAT CCA GTT GTA G			291,647–291,629
inhA (F)2	GCA TCA ACC CGT TCT TCG AC	55	677	291,469–291,488
inhA (R)2	TAA TGC CAT TGA TCG GTG ATA C			292,145–292,124

[a] The *M. tuberculosis* sequence used to design the primers was obtained from GenBank (accession no. BX842580.1).
[b] The *M. tuberculosis* sequence used to design the primers was obtained from GenBank (accession no. BX842576.1).

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TABLE 2. *M. tuberculosis* isolates with mutations in the *inhA* gene

Isolate	Mutation	Amino acid substitution	No. (%) of isolates	Mutation(s) at:				
				katG	*ahpC*	*inhA* promoter	*kasA*	*ndh*
I59	GGA→GGC	G3G	1 (0.8)					
I82	ATC→ACC	I194T	1 (0.8)				T6T (ACC→ACT), H307H (CAC→CAT)	
IR27	GGA→GGC	G3G	1 (0.8)		−6 (G→A)[a]	−15 (C→T)		
IRS7	ATC→ACC	I21T	1 (0.8)		27 (G→T)[b]	−15 (C→T)		
MDR2	ATC→ACC	I21T	1 (0.8)			−15 (C→T)		

[a] Position relative to the mRNA start site.
[b] Nucleotide substitution within the defective *oxyR* gene.

(S94A) transferred by using specialized linkage transduction was able to confer clinically relevant levels of resistance and inhibit mycolic acid synthesis (18, 26). Most studies investigating mutations in the *inhA* gene analyzed the promoter region of *inhA* and not the region within the gene (6–8, 16, 17). Thus, we aimed to screen for mutations within both the *inhA* and *dfrA* genes of INH-resistant *M. tuberculosis* isolates to determine whether mutations within these genes contribute to INH resistance.

A total of 127 INH-resistant clinical isolates and 15 INH-sensitive isolates from Singapore were included in this study in order to gain molecular insight into INH resistance. DNA extracted from the isolates was analyzed by amplifying two overlapping fragments for the entire *inhA* gene and one fragment for the entire *dfrA* gene (Table 1). Amplification of the genes through PCR was done by using specific oligonucleotide primers for the respective gene fragments (Table 1). PCR products were purified (NucleoSpin Extract Column II; Macherey-Nagel) and directly sequenced with the BigDye Terminator sequencing kit V3.1 (Applied Biosystems), followed by analysis on the 3130XL Genetic Analyzer (Applied Biosystems). Confirmation of mutations was done by reamplification and resequencing. The nucleotide sequences obtained were aligned against the reference sequences of the respective genes of *M. tuberculosis* reference strain H37Rv by using the DNASTAR SeqMan II software (Lasergene).

Screening of the entire *inhA* gene revealed mutations in five (3.9%) of 127 INH-resistant isolates (synonymous mutation GGA→GGC at nucleotide position 9 or G3G [$n = 2$], I21T [$n = 2$], or I194T [$n = 1$]). Of the five isolates with mutations, four also had mutations in the *ahpC* promoter, the *inhA* promoter, and the *kasA* gene, which were detected in our previous study (Table 2) (11). Such an occurrence might suggest that some of the observed substitutions are not unique mutations that actually contribute to INH resistance. Also, the low mutational frequency within the *inhA* gene among INH-resistant isolates suggests this (11). Furthermore, a previous study reported that a previously known mutation, at position 491 of the *rrs* gene, was in fact a polymorphism within the F11 family and not actually involved in streptomycin resistance (24). Hence, mutations found, especially rarely occurring ones, should not be hastily perceived to confer resistance.

Screening of the entire *dfrA* gene did not reveal any mutations in any of the 127 INH-resistant isolates, suggesting that *dfrA* mutations may not contribute to INH resistance and that screening for *dfrA* mutations in INH-resistant isolates is unnecessary.

To determine if the mutations detected within the *inhA* gene are mutations and not polymorphisms, we screened 15 INH-susceptible isolates and found no mutations in *inhA*. These isolates also did not have mutations in the *dfrA* gene.

Mutations at both I21T (3, 5, 18) and I194T (5, 13) were previously reported to confer resistance to INH. Ile21 and Ile194 were shown to be 2 of the 10 most important amino acid residues making conserved hydrogen bonds with NADH cofactor in the wild-type InhA protein, which encourages INH sensitivity (21). Amino acid replacements will result in lowered NADH affinity and therefore confer resistance to INH since InhA inactivation is dependent on the presence of bound NADH (4, 13, 20, 23, 28).

Of the 127 INH-resistant samples we have screened, there remain 37 samples (29%) with no mutations in any of the genes reported to be involved in INH resistance. This possibly implies that there are new INH targets yet to be discovered. Our findings suggest that mutations in *dfrA* do not contribute to the detection of INH-resistant *M. tuberculosis* and that mutations within the *inhA* gene are rare.

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