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Contribution of *kasA* Analysis to Detection of Isoniazid-Resistant *Mycobacterium tuberculosis* in Singapore

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Genotypic analysis of resistance to isoniazid (INH) in *Mycobacterium tuberculosis* is complex due to the various genes potentially involved. Mutations in ketoacyl acyl carrier protein synthase (encoded by *kasA*) were present in 16 of 160 (10%) INH-resistant isolates (R121K [*n* = 1], G269S [*n* = 3], G312S [*n* = 11], G387D [*n* = 1]). However, G312S was also present in 6 of 32 (19%) susceptible strains. *kasA* analysis contributed marginally to the performance of INH genotypic testing in Singapore. The significance of *kasA* polymorphisms in INH resistance should be carefully established.

Several genes and genomic regions of *Mycobacterium tuberculosis* participate in the development of resistance to isoniazid (INH), a frontline antituberculous drug. Mutations in the catalase-peroxidase gene (*katG*) diminish activation of INH, and structural or promoter mutations of enoyl acyl carrier protein reductase (encoded by *inhA*) modify the interaction of this drug target with INH. Mutations in the *oxyR-ahpC* intergenic region represent a surrogate marker for *katG* lesions (1, 3–9, 12–18).

Analysis of these regions does not, however, allow identification of all INH-resistant *M. tuberculosis* strains. The recent description of a novel target, ketoacyl acyl carrier protein synthase (encoded by *kasA*), involved in elongation of fatty acids intermediate in the biosynthetic pathway of mycolic acids, opened the possibility for identifying additional INH-resistant organisms (10). The aim of this study was to assess the contribution of *kasA* analysis to the investigation of INH resistance in a large collection of *M. tuberculosis* isolates from Singapore.

All drug-resistant isolates in Singapore are sent to the Central Tuberculosis Laboratory, Department of Pathology, Singapore General Hospital. Consecutive INH-resistant *M. tuberculosis* isolates collected from August 1994 to December 1996 (*n* = 160) and 32 susceptible controls were included in the study. Drug susceptibility testing was done by the BACTEC 460 radiometric method (Becton Dickinson, Towson, Md.), and the isoniazid concentration was 0.1 μg/ml.

Genotypic analysis by PCR amplification and sequencing targeted the codon 315 region of *katG* (codons 292 to 387) (5) and the promoter regions of *inhA* and *ahpC* (18). The entire *kasA* gene (GenBank accession no. Z70692) was investigated by amplifying three overlapping fragments with the oligonucleotide primers shown in Table 1.

Among INH-resistant strains, targeted analysis of *katG* identified mutations W300stop (*n* = 1), S302R (*n* = 1), S315T (*n* = 36), S315N (*n* = 5), and L336R (*n* = 2) and *katG* deletions in nine strains. To confirm that these deletions were not artifactual, PCR of the *katG* gene with primers to other regions of the gene was done (5). Mutation of *katG* at codon 315 was observed in 41 of 160 (26%) INH-resistant isolates.

Analysis of the *inhA* promoter identified the following nucleotide substitutions flanking the presumed ribosome binding site: −15 C→T (*n* = 43) and −8 T→A (*n* = 1) (numeration according to Ramaswamy and Musser [14]). A novel A→T substitution (*n* = 1) located 92 nucleotides 5′ of the ribosome binding site was also identified.

Analysis of the *oxyR-ahpC* intergenic region identified substitutions at positions −46 (G→A [*n* = 1]), −30 (C→T [*n* = 2]), −12 (C→T [*n* = 2]), and −6 (G→A [*n* = 1]) relative to the mRNA start site (14). Mutations in the 5′-terminal region of the *ahpC* gene product were observed at P2S (*n* = 1), associated with deletion of *katG*, and T5I (*n* = 1). Nucleotide substitutions in the defective *oxyR* gene were observed at nucleo-

TABLE 1. Oligonucleotide primer sequences used to amplify the *kasA* gene target[a]

Primer	Description	Sequence	Nucleotides
kasA1S	First fragment, sense	5′CGTTCAGGCGAGGCTTGAGG	30633–30652
kasA1AS	First fragment, antisense	5′CCGGTCTGGATCGACCTCCG	30983–30964
kasA2S	Second fragment, sense	5′GGACAGCTATGGGAGTCCGC	30936–30955
kasA2AS	Second fragment, antisense	5′ACCCAGCAATCGGGCCAACG	31463–31444
kasA3S	Third fragment, sense	5′GCACGCCAAAGCCCGTGGCG	31418–31437
kasA3AS	Third fragment, antisense	5′GGGCCTCGCGACCCGCGATG	31940–31921

[a] The *M. tuberculosis* sequence used to design the primers was obtained from GenBank (accession no. Z70692).

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TABLE 2. Genetic characterizations of 160 INH-resistant isolates and 32 INH-susceptible isolates from Singapore

Phenotype (*n*)[a]	No. (%) with indicated genotypes	Genotype[b]			
		katG	*inhA*	*ahpC-oxyR*	*kasA*
INH-R (160)	36 (23)	Mut	—[c]	—	—
	6 (4)	Del	—	—	—
	31 (19)	—	Mut	—	—
	4 (3)	—	—	Mut	—
	3 (2)	—	—	—	Mut
	2 (1)	Mut/Del	Mut	—	—
	4 (3)	Mut/Del	—	Mut	—
	5 (3)	Mut/Del	—	—	Mut
	4 (3)	—	Mut	Mut	—
	7 (4)	—	Mut	—	Mut
	1 (0.5)	Mut	Mut	—	Mut
	57 (36)	—	—	—	—
INH-S (32)	25 (78)	—	—	—	—
	1 (3)	—	—	Mut	—
	6 (19)	—	—	—	Mut

[a] INH-R, INH resistant; INH-S, INH susceptible.
[b] Mut, mutation; Del, deletion.
[c] —, no detectable mutation.

tides 18 (G→A [*n* = 2]), 27 (G→T [*n* = 1]), and 28 (C→A [*n* = 1]). All *oxyR* mutations were observed in the presence of other mutations established to be associated with resistance, e.g., *katG* S315T or an *inhA* promoter mutation. While an association of *ahpC* coding region mutations with INH resistance remains plausible, the functional role of *oxyR* mutations remains doubtful.

Overall, the targeted strategy identified *katG* mutations in 54 of 160 strains (34%), *inhA* mutations in 45 strains (28%), and *oxyR-ahpC* mutations in 12 strains (7.5%) (Table 2). Twenty-three of 160 INH-resistant strains (14%) carried more than one mutation. No alterations were identified in susceptible strains, with the exception of one isolate having a point mutation in the defective *oxyR* gene (nucleotide 18).

Analysis of *kasA* identified a number of polymorphic sites both in resistant and in susceptible isolates (Tables 2 and 3). Sixteen resistant isolates presented mutations (R121K [*n* = 1], G269S [*n* = 3], G312S [*n* = 11], G387D [*n* = 1]); however, most (13 of 16) presented mutations associated with resistance in other genes. A particular polymorphism, G312S, was also present in 6 of 32 (19%) susceptible strains.

The present study raises two relevant points for discussion of the implementation of genotypic strategies for detection of drug resistance in *M. tuberculosis*. First, it demonstrates that targeted approaches that limit the number of genetic regions analyzed may not be universally applicable. The strategy implemented in Singapore (analysis of the codon 315 region and the promoter regions of *inhA* and *oxyR-ahpC*) detected mutations in 100 of 160 (62.5%) resistant strains, while it proved successful in Spain (detection of 87% of resistant strains). Additional mutations could be present in *katG* regions not included in the analysis or in the structural *inhA* gene or could correspond to unidentified mechanisms of resistance (2, 11, 14).

Geographical differences in the frequencies of specific mutations are also apparent in analysis of data from other studies: the *katG* gene was mutated at codon 315 in 64% of INH-resistant strains from South Africa and central and western Africa (4) but in only 26% of Singaporean isolates; mutations in the regulatory region of the *inhA* gene have been reported in 6.5 to 21.6% of INH-resistant isolates (7, 12–15); and *oxyR-ahpC* intergenic region substitutions have been reported in 24.2 to 32.9% of INH-resistant isolates (7, 17). Interestingly, investigation of the same set of isolates for *rpoB* mutations associated with rifampin resistance demonstrated the same prevalence and distribution of specific mutations as are present in other geographical regions (data not shown).

TABLE 3. Genetic polymorphisms of the *kasA* gene in INH-resistant and -susceptible clinical isolates of *M. tuberculosis*

Phenotype (*n*)	Codon	Amino acid change	Mutation	No. (%) of isolates with indicated mutation
INH resistant (160)	121[a]	Arg→Lys	AGG→AAG	1 (0.6)
	269	Gly→Ser	GGT→AGT	3 (2)
	312[b]	Gly→Ser	GGC→AGC	11 (7)
	387[a]	Gly→Asp	GGC→GAC	1 (0.6)
		None	None	144 (90)
INH susceptible (32)	312	Gly→Ser	GGC→AGC	6 (19)
		None	None	26 (81)

[a] Novel mutation.
[b] Polymorphism also present in susceptible isolates.

In the case of INH, discrepant results between studies likely reflect different geographical prevalences of specific genotypes. Certainly, the possibility of a limited number of epidemic strains contributing to these differences needs to be assessed. These geographical differences in the prevalences of specific polymorphisms were underscored by our previous report on the *katG* R463L substitution in Singaporean isolates, where this substitution constitutes a frequent natural polymorphism unrelated to INH resistance (8). Therefore, information regarding the frequencies and types of mutations or deletions which have been documented in one country or geographical region may not be applicable elsewhere.

Due to the limited performance of the chosen targeted approach to INH resistance, we investigated the contribution of *kasA* analysis to the overall performance of targeted genotypic detection of INH resistance. Mdluli et al. (10) identified *kasA* polymorphisms in 4 of 28 (14.3%) INH-resistant isolates (codons 66, 269, 312, and 413) but not among 43 INH-susceptible strains. While *kasA* polymorphisms (codons 121, 269, 312, and 387) were identified in 10% of INH resistant isolates in the present study, the most frequent substitution (G312S) was also shown to be a frequent polymorphism (19%) among susceptible strains. In this study, mutation of *kasA* did not represent a frequent event associated with INH resistance, and analysis of this target contributed minimally to the diagnostic strategy.

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