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 the enoyl 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 drug target with INH. Mutations in the 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 mycocic 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 S′-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 targeta

<table>
<thead>
<tr>
<th>Primer</th>
<th>Description</th>
<th>Sequence</th>
<th>Nucleotides</th>
</tr>
</thead>
<tbody>
<tr>
<td>kasA1S</td>
<td>First fragment, sense</td>
<td>5′CGTTCAAGGGAGGACCTGAGG</td>
<td>30633–30652</td>
</tr>
<tr>
<td>kasA1AS</td>
<td>First fragment, antisense</td>
<td>5′CCGTCCTGGAGCCTCCCG</td>
<td>30983–30964</td>
</tr>
<tr>
<td>kasA2S</td>
<td>Second fragment, sense</td>
<td>5′GGACAGCTATGTTGACCTCGC</td>
<td>30936–30955</td>
</tr>
<tr>
<td>kasA2AS</td>
<td>Second fragment, antisense</td>
<td>5′ACCCAGCAATGGGACCCACG</td>
<td>31463–31444</td>
</tr>
<tr>
<td>kasA3S</td>
<td>Third fragment, sense</td>
<td>5′GGCCATTCCGAGCCACCACG</td>
<td>31418–31437</td>
</tr>
<tr>
<td>kasA3AS</td>
<td>Third fragment, antisense</td>
<td>5′GGGCTTCCGAGCCACCACG</td>
<td>31940–31921</td>
</tr>
</tbody>
</table>

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

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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 = 1]), 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).

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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**REFERENCES**


tution at codon 463 of the katG gene in isoniazid-resistant Mycobacterium tuberculo-
bacterium tuberculosis complex organisms recovered from diseased hu-