

## Detection of Isoniazid-Resistant *Mycobacterium tuberculosis* Strains by a Multiplex Allele-Specific PCR Assay Targeting *katG* Codon 315 Variation

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**We describe a simple multiplex allele-specific (MAS)-PCR assay to detect mutations in the second base of the *katG* gene codon 315, including AGC→ACC and ACA (Ser→Thr) substitutions that confer resistance to isoniazid (INH) in *Mycobacterium tuberculosis* clinical isolates. The 315 ACC allele is found in the majority of *Inh<sup>r</sup>* strains worldwide, especially in areas with a high incidence of tuberculosis. The 315 ACA allele is characteristic of the New York City multidrug-resistant (MDR) strain W and its progenies in the United States. The mutations in *katG315* are revealed depending on the presence or absence of an indicative fragment amplified from the wild-type allele of this codon. Initially optimized on the purified DNA samples, the assay was then tested on crude cell lysates and auramine-stained sputum slide preparations with the same reproducibility and interpretability of profiles generated by agarose gel electrophoresis. The MAS-PCR assay can be used for the detection of resistance to INH in clinical laboratories in regions with a high prevalence of MDR *M. tuberculosis* strains.**

Presently, tuberculosis (TB) reemergence and spread are of worldwide concern. The situation is aggravated by the increasing circulation of multidrug-resistant (MDR) *Mycobacterium tuberculosis* strains that are defined as resistant to at least rifampin (RIF) and isoniazid (INH), which comprise the backbone of antitubercular chemotherapy. While RIF resistance is essentially mediated by the *rpoB* hot spot region mutations (18), INH resistance is apparently controlled by a more complex genetic system that involves several genes, namely, *katG*, *inhA*, *kasA*, and *ahpC* (16, 17). Finally, extensive studies have demonstrated that INH resistance is most frequently associated with mutations in *katG*. The INH used for therapy is a prodrug and necessitates catalytic activation to be converted into an active form. Catalase-peroxidase, the product of the *katG* gene, was shown to accomplish this function, and the mutations in this gene mediate INH resistance (4, 23). Early studies showed that the complete deletion of the gene is rare, since peroxidase activity in KatG is necessary to detoxify host antibacterial radicals (4, 8, 19; T. C. Victor, G. S. Pretorius, J. V. Felix, A. M. Jordaan, P. D. van Helden, and K. D. Eisenach, Letter, Antimicrob. Agents Chemother. **40**:1572, 1996). For this reason, the predominant way of acquiring resistance via KatG alterations is through selection of those particular mutations that decrease catalase activity but still maintain the peroxidase activity of the enzyme at a certain level in viable *Inh<sup>r</sup>* organisms. Such mutations were found in up to 90% of *Inh<sup>r</sup>* strains, and one particular substitution in the

codon 315, AGC→ACC (Ser→Thr), was reported as the most frequent, apparently providing the optimal balance between the decreased catalase and sufficiently high peroxidase activities of KatG (16). This mutation was reported to be associated with intermediate or high levels of resistance to INH (1 to 10  $\mu\text{g/ml}$  [11, 16, 23]), though it has recently been suggested that the *katG315* alteration alone may not always be clinically significant (1). The prevalence of the *katG315* (AGC→ACC) mutation coding for the Ser→Thr substitution among MDR *M. tuberculosis* strains in the world, though generally high, varies in regions with low and high TB incidences, ranging from rare occurrences in Scotland (7) and Finland (11) to 26 to 30% in Singapore (10) and Madrid (15) to 52 to 64% in South Africa (9; Victor et al., letter) to 79% in Peru (6). In a previous study (13), the *katG* S315T mutation in *M. tuberculosis* isolates recovered from epidemiologically unlinked patients in northwestern Russia from 1996 to 2001 was screened by a PCR-restriction fragment length polymorphism (RFLP) assay. This mutation was found in 93.6% of 204 *Inh<sup>r</sup>* strains.

Particular methods described so far for the detection of *katG315* changes include rather elaborate DNA sequencing (6, 9, 11), cleavage fragment length polymorphism (3), single-strand conformation polymorphism (19), and molecular beacon (15) analyses where mutations in different *katG* codons were screened, along with PCR-RFLP (13; Victor et al., letter) and dot blot hybridization (21, 24) strategies based on PCR, followed by either restriction endonuclease analysis or DNA hybridization, respectively. The aim of the present study was to develop a simple and rapid assay for detecting INH resistance in *M. tuberculosis* clinical isolates based exclusively on *katG* PCR and agarose gel electrophoresis without any supplementary sequence analysis.

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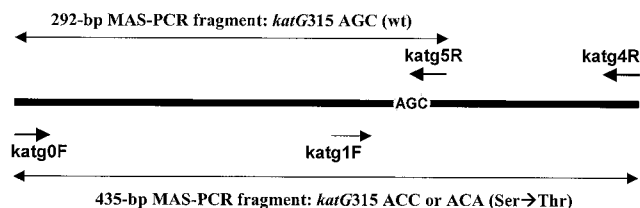


FIG. 1. Schematic view of the *katG* gene fragment targeted by MAS-PCR assay using three primers (katg0F, katg5R, and katg4R [boldface arrows]). Short arrows indicate primers; long double-headed arrows depict the indicated PCR fragments. The *katG* codon 315 AGC is in the shaded box. The katg1F and katg4R primers are used in the PCR-RFLP assay.

#### MATERIALS AND METHODS

**Bacterial strains and susceptibility testing.** The 80 *M. tuberculosis* isolates were recovered from 80 unrelated adult (15 to 63 years) patients with newly diagnosed pulmonary TB. They originated from St. Petersburg and three neighboring provinces in northwestern Russia (Leningrad, Novgorod, and Pskov) and were admitted to the hospitals of the St. Petersburg Institute of Phthisiopulmonology and City Anti-Tuberculosis Dispensary of St. Petersburg between 1999 and 2001.

Species identification of the isolates was based on standard microbiological tests: colony morphology, acid-fast staining, and biochemical tests (27, 28). Löwenstein-Jensen medium was used for the cultivation of isolates, and susceptibility testing was performed by the absolute concentration method as recommended by the Russian Ministry of Health (order no. 558 of 28 June 1978) and has been described previously (25). A microbial suspension containing  $5 \times 10^8$  organisms/ml was prepared according to the McFarland turbidity standards and was diluted 1:10; then, 0.2 ml of the dilution was added to Löwenstein-Jensen medium with or without a drug. The culture tubes were incubated at 37°C, and growth was monitored after 3 weeks of incubation and assessed as previously described (28). An isolate was considered resistant if bacterial growth occurred at a concentration of 1 µg of INH per ml, 20 µg of RIF per ml, 5 µg of streptomycin (STR) per ml, 2 µg of ethambutol (EMB) per ml, and 100 µg of pyrazinamide (PZA [pH 5.6]) per ml. The method of absolute concentration was previously shown in our laboratory to give results concordant with those generated by the proportion method in a comparative study with the National Mycobacterial Reference Laboratory in Turku, Finland (25).

**DNA preparation.** DNA used for the PCR analysis was either extracted from cultured cells and purified as described by van Embden et al. (20) or obtained by resuspending a mycobacterial colony in 200 µl of 1× TE buffer (10 mM Tris-HCl and 1 mM EDTA [pH 7.4], with or without Chelex 100 [5%, wt/vol; Sigma]), followed by boiling for 20 min. After centrifugation, the DNA-containing supernatant (cell lysate) was harvested and stored at 4°C until used. The DNA of the strain M22 (W) with *katG315* ACA sequence was kindly provided by Peter Small and Alexander Pym (Stanford University Medical Center).

The DNA samples from auramine-stained sputum smears (cell counts graded as 1+, 2+, or 3+ [27]) were obtained by scraping the material from the glass slide to a sterile Eppendorf tube and then resuspending it in a total volume of 75 µl of extraction buffer (15% Chelex 100 [Sigma], 0.5% Nonidet P-40, [Sigma], 0.5% Tween 20), followed by incubation for 20 min in a boiling water bath. After centrifugation, the removed supernatant was used for PCR.

Control for contamination during microbiological and genetic experiments was performed as recommended in references 28 and 5, respectively. In particular, a negative control sputum slide was processed along with the test slides. A control of possible contamination with previously amplified amplicons was performed by including a negative control sample (distilled water) in each PCR run; no contamination was detected.

**PCR-RFLP analysis.** A primer pair, katg1F (5'-AGTCGTATGGCACCGG AAC) and katg4R (5'-AACGGGTCCGGGATGGTG) (Fig. 1), was used to amplify a *katG315* fragment (*katG* positions 904 to 1103 in strain H37Rv [GenBank accession number X68081, positions 2882 to 3081]) as described previously (13). The amplified fragment was digested with *HapII* (Amersham Pharmacia Biotech); the obtained restriction fragments were electrophoresed in 1.5% standard agarose gels (Quantum Bioprobe) and visualized under UV light. The *katG* codon 315 missense mutation AGC-Ser→ACC-Thr created an additional *HapII* site (C↑CGG) and could be detected by cleavage with this restriction endonuclease (or its isoschizomer, like *MspI*). As a result, the size of the largest digest

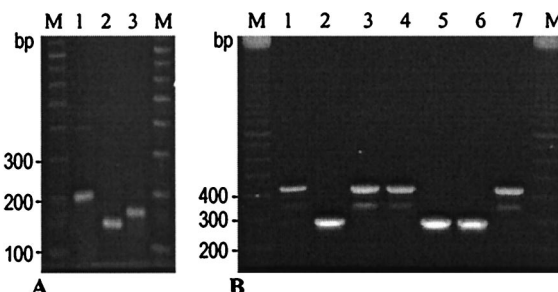


FIG. 2. *katG* 315 PCR-RFLP (A) and MAS-PCR (B) profiles of the *M. tuberculosis* strains. (A) Lanes: 1, 200-bp PCR fragment used for *HapII*-RFLP analysis; 2, strain with *katG* 315 AGC→ACC mutation; 3, strain with *katG315* wild-type allele. (B) Lanes: 1, 3, and 4, strains with the *katG315* AGC→ACC mutation; 2, 5, and 6, strains with the *katG315* wild-type allele; 7, strain with the AGC→ACA mutation (strain W); M, 100-bp DNA ladder (Amersham Pharmacia Biotech).

was 132 bp for the mutated (315 AGC→ACC) allele, the INH-resistant strain, and 153 bp for the *katG315* wild-type or differently mutated alleles (Fig. 2A, lanes 2 and 3, respectively). The shorter 10- to 21-bp digests ran outside of the gel and were not considered.

**MAS-PCR assay.** The multiplex allele-specific (MAS)-PCR assay was previously developed for *embB306* mutational analysis (12). In the present study, we applied this strategy to distinguish between the wild-type and mutated alleles of *katG315* (Fig. 1). The assay used three primers. The inner reverse primer katg5R was positioned so that its 3' end paired with the second base (G) of the codon 315 wild-type allele (AGC) (Fig. 1). Consequently, in the absence of mutation in this position in *katG315*, a 292-bp fragment was amplified by the outer forward primer katg0F and the inner reverse primer katg5R (Fig. 2B, lanes 2, 5, and 6). If a mutation (e.g., AGC→ACC) occurred, this resulted in a mismatch at the 3' end of this wild-type inner primer and, under the appropriate stringent PCR conditions, in the absence of the 292-bp PCR product. Two outer primers, katg0F and katg4R, flanked the entire *katG315* region under investigation (Fig. 1). In the case of the *katG315* wild-type allele, amplification of this 435-bp fragment was prevented under selected reaction conditions due to the concurrent action of the katg5R inner primer. The 435-bp fragment was amplified in only the strains with *katG315* mutations (Fig. 2B, lanes 1, 3, 4, and 7) and was therefore indicative of the INH resistance phenotype.

The following primers were used for a single tube PCR targeting a portion of the *katG* gene (*katG* positions 669 to 1103 in strain H37Rv [accession number X68081, positions 2647 to 3081]): two outer primers, forward primer katg0F (5'-GCAGATGGGGCTGATCTACG) and reverse primer katg4R, and one inner reverse primer, katg5R (5'-ATACGACCTCGATGCCG). A purified DNA sample (0.1 µl) or 10 µl of lysate or sputum slide preparation was added to a PCR mixture (final volume of 30 µl) that contained 30 pmol each of katg0F and katg5R, 40 pmol of katg4R, 1.5 mM MgCl<sub>2</sub>, 1 U of recombinant *Taq* DNA polymerase (MBI Fermentas), and 200 µM concentrations of each deoxynucleoside triphosphate. The reaction was performed in a PTC-100 thermal controller (MJ Research, Inc.) under the following conditions: initial denaturation at 96°C for 3 min; 5 cycles at 95°C for 1 min, 64°C for 1 min, and 72°C for 30 s; 5 cycles at 95°C for 1 min, 62°C for 40 s, and 72°C for 30 s; 20 cycles (25 cycles for sputum slide or lysate probes) at 94°C for 1 min, 60°C for 40 s, and 72°C for 30 s; and final elongation at 72°C for 3 min. The amplified fragments were electrophoresed in 1.5% agarose gels and visualized under UV light.

**Sequencing of the *katG* fragment.** The 500-bp *katG* fragment was amplified with the KG1 (5'-AATCGATGGGCTTCAAGACG) and KG2 (5'-CTCGTAG CCGTACAGGATCTCG) primers. The PCR (35 cycles) was performed as follows: 94°C for 1 min, 56°C for 1 min, and 72°C for 1 min. PCR products were analyzed in 2% agarose gels, purified with a Qiagen purification kit (Qiagen), and sequenced with the [<sup>32</sup>P]-end-labeled KG2 primer as described in reference 26.

#### RESULTS AND DISCUSSION

The *M. tuberculosis* clinical strains studied included 15 Inh<sup>s</sup> and 65 Inh<sup>r</sup> isolates from new TB cases in the northwestern region of the Russian Federation from 1999 to 2001 (13). The

Inh<sup>s</sup> strains were pan-susceptible. The Inh<sup>r</sup> strains were also resistant to RIF and STR ( $n = 57$ ); to RIF, STR, and EMB ( $n = 2$ ); to RIF, STR, and PZA ( $n = 4$ ); and to RIF, STR, EMB, and PZA ( $n = 2$ ).

First, the sample under investigation was assessed by the *katG315* PCR-RFLP assay (13). None of the 15 Inh<sup>s</sup> strains had the *katG315* AGC→ACC mutation. Since other, rarely described mutations in codon 315 confer INH resistance (11), we assumed that these Inh<sup>s</sup> strains had a wild-type allele of codon 315 (AGC). The *katG315* AGC→ACC alteration was observed in all 65 Inh<sup>r</sup> strains. Sequence analysis of six randomly selected strains confirmed the PCR-RFLP assay results: the *katG315* mutation (AGC→ACC) was found in three Inh<sup>r</sup> strains, and the *katG315* wild-type allele (AGC) was identified in three Inh<sup>s</sup> strains.

The MAS-PCR assay was evaluated to detect *katG* codon 315 alteration in the strains studied. All 15 Inh<sup>s</sup> strains produced a single MAS-PCR 292-bp band (Fig. 2B, lanes 2, 5, and 6), as expected, implying no mutation in the *katG315* second base. A single MAS-PCR 435-bp fragment was amplified in all the 65 Inh<sup>r</sup> strains harboring the AGC→ACC mutation (Fig. 2B, lanes 1, 3, and 4). The generation of an either 292- or 435-bp fragment provided a PCR quality confirmation so as to rule out eventual false-negative results due to lack of amplification. In general, the MAS-PCR results on the *katG315* variation were concordant with those generated by PCR-RFLP analysis for all the strains tested. A mutation in the *katG315* second base, one other than AGC→ACC, should have also resulted in the amplification of a single 435-bp fragment, though such mutations are rare (8, 16) and were not found in our isolates.

The high prevalence of the *katG315* ACC mutant allele among Inh<sup>r</sup> *M. tuberculosis* clinical isolates in northwestern Russia (93.6% for all isolates), especially among the strains of the Beijing family (100% for the Beijing isolates from new TB cases from 1996 to 2001), was previously shown (13). The strains of this family are known to be highly prevalent in East Asia and in the countries of the former Soviet Union, and they have also been reported in other geographical regions, including North America (2, 14, 22). The notorious New York City strain W, which caused several MDR-TB outbreaks in the United States in the nineties, also belongs to the Beijing family (2). However, unlike other members of this family, one of the specific features of the W (New York City) strain and its progenies is that their INH resistance is associated with a rare mutation in the *katG* codon 315, AGC→ACA (Ser→Thr) (16). This double mutation does not create a *HapII* site (CCGG), and PCR-RFLP analysis cannot distinguish it from the wild-type allele. In contrast, the MAS-PCR assay that we used to detect the AGC→ACC mutation allowed us to also identify the AGC→ACA mutation. In such a case, a mismatch at the 3' end of the inner wild-type primer *katG5R* (Fig. 1) is even more dramatic, as it involves two bases of codon 315. MAS-PCR was performed on one available W strain that harbored the *katG315* ACA allele. This analysis unambiguously identified the presence of this mutation and hence the INH resistance phenotype of the W strain (Fig. 2B, lane 7).

The MAS-PCR assay was initially performed on the purified DNA preparations with which the PCR conditions and concentrations were optimized. The assay was then tested on a

selection of 22 DNA samples from crude cell lysates and sputum slide preparations, all from the same patients (seven strains, Inh<sup>s</sup>, *katG315* AGC; 15 strains, Inh<sup>r</sup>, *katG315* ACC). The MAS-PCR profiles obtained from smear preparations and purified DNA from cultured cells were equally unambiguous in interpretation and concordant for each of the 22 patients examined (data not shown).

A limited number of gene codons has recently been suggested to reliably predict resistance to the major drugs in the majority of *M. tuberculosis* strains in areas with high TB incidence (21). However, for RIF, a minimum of three codons of *rpoB* should be analyzed and neither wild-type nor mutant alleles can be detected by any restriction endonuclease. On the other hand, the so noticeably prevalent *katG315* AGC→ACC or ACA mutations (6, 9, 13, 15; Victor et al., letter) present markers of choice for INH resistance detection. The *katG315* MAS-PCR assay can therefore be suggested as a simple and rapid tool for detecting with high probability the INH resistance in *M. tuberculosis* clinical strains. It is also applicable for direct detection in stained sputum smear preparations, which should facilitate the adequate and timely choice of antituberculosis therapy. The assay is easy to perform and to interpret; it is based on a single tube PCR and minigel electrophoresis without any further extension and can be implemented into the routine practices of clinical laboratories in areas with a high prevalence of MDR *M. tuberculosis* strains. However, preliminary evaluation of the *katG315* mutations' distribution is a prerequisite for each particular setting prior to large-scale implementation.

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