Detection of katG Ser315Thr substitution in respiratory specimens from patients with isoniazid-resistant Mycobacterium tuberculosis using PCR-RFLP

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INTRODUCTION

Tuberculosis remains a global threat to public health. The problem is further complicated by the emergence of multidrug-resistant tuberculosis as a consequence of the widespread use and incautious administration of antibiotics (WHO, 2000). Rapid diagnosis and appropriate chemotherapy become the first priorities in controlling growing epidemics. Nowadays, isoniazid (INH), ethambutol, rifampicin, pyrazinamide and streptomycin are important components of first-line anti-tubercular regimens. INH has a much more complex resistance mechanism than the other four compounds. Several studies have revealed different putative molecular targets of INH, including catalase peroxidase (katG) (van Soolingen et al., 2000), enoyl-acyl reductase (inhA) (Basso et al., 1998), alkyl-hydroperoxide reductase (ahpC) (Sherman et al., 1996), β-ketoacyl-acyl carrier protein synthase (kasA) (Mdluli et al., 1998) and NADH dehydrogenase (ndh) (Lee et al., 2001). However, a predominant mutation in the katG locus, Ser315Thr, accounts for more than 50 % of INH-resistance phenotypes, according to earlier reports (Musser et al., 1996; van Soolingen et al., 2000). Mycobacterial strains lacking the entire katG gene would also exhibit an INH-resistant phenotype (Heym et al., 1995).

In this study, we developed a simple PCR-RFLP method for direct detection of katG Ser315Thr-associated INH-resistant Mycobacterium tuberculosis (MTB) in clinical isolates and respiratory specimens. A total of 906 respiratory specimens and 142 MTB isolates were used to evaluate the specificity and sensitivity of this assay. Results were compared with commercial and in-house PCR assays for MTB, anti-mycobacterial susceptibility testing and DNA sequencing.

Abbreviations: AFB, acid-fast bacilli; INH, isoniazid; MOTT, Mycobacterium sp. other than tuberculosis; MTB, Mycobacterium tuberculosis.
METHODS

Specimens and isolates. Between December 1999 and February 2002, 906 respiratory specimens (828 expectorated sputum and 78 broncho-alveolar lavage samples) collected from patients suffering from chest symptoms and/or chest radiographic infiltrates of undetermined origin included 675 patients from Queen Mary Hospital and 231 out-patients of Polyclinics of the Department of Health in Hong Kong. An additional 142 clinical isolates of MTB were collected from three major cities in the Pearl River delta of the South China region of China: Hong Kong (96 isolates), Macau (27 isolates) and Guangzhou (19 isolates). Specimens were processed for direct smear followed by concentration for acid-fast bacilli (AFB) culture as described previously (Nolte & Metchock, 1995). After concentration, digested sediments were divided equally for AFB culture and subsequent PCR assays. Cultures positive for AFB were identified using the AccuProbe hybridization assay (Gen-Probe) and conventional biochemical tests. Catalase test for MTB was performed according to the method of Nolte & Metchock (1995). Antimicrobial susceptibility testing was performed using the proportional method (NCCLS, 2000).

DNA extraction for PCR. For the digested sediments, the Roche Cobas Amplipcr extraction protocol was adopted as described previously (Yam et al., 1998; Yuen et al., 1997). A final volume of 200 μl DNA extract was used for subsequent PCRs (undiluted extract). For samples requiring further DNA concentration and purification, 100 μl DNA extract was further purified using mini-columns (gel extraction kit; Qiagen) according to the manufacturer’s instructions and 30 μl purified DNA was eluted (3× concentrated extract). For purified culture of MTB on Lowenstein–Jensen medium, a uniform bacterial suspension (McFarland standard no. 1) was made in 0.1 M Tris/HCl, pH 7.5. For the MTB standard strain Rv37, the bacterial suspension was further diluted from $10^{-3}$ to $10^{-10}$ for colony counting using Middlebrook agar plates. All bacterial suspensions were subsequently heated at 100 °C for 30 min, frozen at −80 °C, thawed at 80 °C and centrifuged at 15 000 g for 10 min; the supernatant was used directly for subsequent PCR assays. For the MTB standard strain Rv37, serial dilutions of heated extract were used for determination of analytical sensitivity of the IS6110 and katG PCR assays.

PCR assays for MTB 16S rRNA and IS6110. The Cobas Amplipcr MTB test (Roche) uses genus Mycobacterium-specific biotinylated primers to amplify a sequence of 584 bp within a 1500-bp region encoding the 16S rRNA of MTB (Yuen et al., 1997). An internal control was incorporated in each reaction to monitor PCR inhibitors. A manual one-tube nested PCR for IS6110 was performed as described previously (Chan et al., 1996; Yam et al., 1998; Yuen et al., 1997).

PCR-RFLP for katG. Each PCR contained 10 μl DNA extract. Primers katG904 (5’-AGCTCGGATGCGACCCGGAA-3’, forward primer, positions 904–923) and katG1523 (5’-TTGACCTCCACCCGCACTTG-3’, reverse primer, positions 1523–1502) (Uhl et al., 1996) were used to amplify a 620-bp fragment of katG. Each 100 μl reaction contained 10 mM Tris/HCl (pH 8.3), 50 mM KCl, 2 mM MgCl₂, 0.1-15 mM dNTPs, 0.2 μM each primer and 2 U AmpliTaq Gold polymerase (Perkin Elmer). To activate the Taq polymerase, the mixture was first incubated at 94 °C for 12 min. Subsequent temperature cycling for 45 cycles started at 94 °C for 1 min, 60 °C for 1 min and 72 °C for 1 min. A 10 μl aliquot of the PCR product was electrophoresed for 1 h through 2 % agarose gel in 1 X TBE. For samples positive for the 620-bp fragment of katG, a 5 μl aliquot of the PCR product was digested using 2 U MspI restriction endonuclease (Amersham Pharmacia Biotech) in a 10 μl reaction at 37 °C for 4 h, followed by electrophoresis in a 6 % polyacrylamide gel. In this study, the INH-resistance mutation in katG codon 315 (Ser, AGC→Thr, ACC) and polymorphic variation in codon 463 (Arg, CGG or Leu, CTT) were identified by RFLP using MspI digestion (restriction site TC/CCGG).

DNA sequencing of 620-bp katG amplicons. To verify the point mutations detected by PCR-RFLP, 620-bp katG amplicons of 20 randomly selected MTB isolates (10 INH-susceptible and 10 INH-resistant) were sequenced using BigDye technology and an ABI 377 Genetic Analyzer (Applied Biosystems).

RESULTS AND DISCUSSION

Among 906 respiratory specimens shown in Table 1, 233 were culture-positive for MTB and 35 were culture-positive for ‘Mycobacterium sp. other than tuberculosis’ (MOTT), while the remaining 638 were culture-negative for Mycobacterium. Of 187 samples that were AFB smear-positive and culture-positive for MTB, 161 (86 %) were positive for katG PCR, whereas IS6110 PCR and Roche PCR provided 100 % sensitivity for direct detection of MTB in these specimens. This result is concordant with an experiment on analytical sensitivity that showed that IS6110 PCR was 10 times more sensitive than the katG PCR (Fig. 1). End-point detection of MTB for IS6110 PCR and katG PCR was found to be 1-5 and 15 c.f.u., respectively. Using a simple column-elution concentrator, all 187 AFB smear-positive specimens and 11/46 (24 %) AFB smear-negative specimens were positive for katG PCR. A total of 233 specimens were subsequently confirmed to be culture-positive for MTB, all of which were found to be catalase-positive. A specificity of 100 % was exhibited for the three PCR assays on 906 respiratory specimens with diagnostic sensitivity of 92 % for IS6110 PCR, 86 % for Roche PCR and 85 % for katG PCR. The katG PCR is highly specific for MTB, in that none of the specimens with MOTT or those that were culture-positive for other respiratory bacterial pathogens showed a positive result. By Roche PCR, 4.8 % specimens overall were detected to contain PCR inhibitors (data not shown).

The katG amplicons were further analysed by RFLP using MspI digestion (Table 2) and four distinct RFLP patterns were generated (Fig. 2). All RFLP patterns of katG amplicons from the 198 respiratory specimens were identical to those of the corresponding MTB isolates, indicating that katG PCR provides reliable detection in clinical specimens. Four catalase-negative MTB isolates were katG PCR-negative and confirmed INH-resistant by susceptibility testing. These isolates confer INH resistance by partial or complete deletion of katG, accounting for the negative katG PCR result (Heym et al., 1995). Among the 375 MTB isolates (233 isolates from respiratory specimens and 142 isolates from purified culture), the proportional method identified 273 INH-susceptible and 102 INH-resistant MTB isolates. Of the 102 INH-resistant isolates (Hong Kong, 71 isolates; Macau, 19; Guangzhou, 12), 52 (51 %) isolates exhibited phenotype Thr315 (RFLP patterns C and D). The remaining 50 (49 %) resistant isolates showed phenotype Ser315 (RFLP patterns A and B). There was no documentation of an outbreak during the study period, and all RFLP patterns were distributed randomly among isolates from the three cities (data not shown). Thr315 is 100 % specific for INH resistance, since no susceptible isolates exhibited Thr315. Automated DNA sequencing of the 620-bp katG amplicons from 10 randomly
selected INH-susceptible isolates (four isolates of RFLP pattern A and six isolates of RFLP pattern B) and 10 INH-resistant isolates (two, one, three and four isolates, respectively, of RFLP patterns A–D) verified 100% sequence accuracy of the point mutations detected by PCR-RFLP. DNA sequencing also revealed no mutation other than Ser/Thr315 and Arg/Leu463 within the 620-bp katG amplicons for the 20 isolates. Arg463 and Leu463 were identified in 20% (20/102) and 80% (82/102), respectively, of the resistant isolates. Codon 463 is a polymorphic site that does not contribute to INH resistance (van Doorn et al., 2001). In this study, Leu463 was the predominant wild-type MTB isolate in the South China region, replacing the major Arg463 wild-type found in the Western world (Cockerill et al., 1995).

Previous findings showed rapid detection of INH resistance selected INH-susceptible isolates (four isolates of RFLP pattern A and six isolates of RFLP pattern B) and 10 INH-resistant isolates (two, one, three and four isolates, respectively, of RFLP patterns A–D) verified 100% sequence accuracy of the point mutations detected by PCR-RFLP. DNA sequencing also revealed no mutation other than Ser/Thr315 and Arg/Leu463 within the 620-bp katG amplicons for the 20 isolates. Arg463 and Leu463 were identified in 20% (20/102) and 80% (82/102), respectively, of the resistant isolates. Codon 463 is a polymorphic site that does not contribute to INH resistance (van Doorn et al., 2001). In this study, Leu463 was the predominant wild-type MTB isolate in the South China region, replacing the major Arg463 wild-type found in the Western world (Cockerill et al., 1995).
in purified MTB colonies using a real-time PCR assay (Garcia de Viedma et al., 2002) or allele-specific PCR assay (Mokrousov et al., 2002): this is the first report of direct detection of INH resistance-associated mutations of MTB in clinical specimens with highly specific results. Our protocol requires a simple laboratory set-up suitable for a diagnostic microbiology service and the turnaround time can be shortened from 8–10 weeks to 3 days. In routine practice, respiratory specimens positive for \( \text{MTB} \) can be used directly for PCR-katG. The proposed diagnostic algorithm would possibly shorten the turnaround time for identification of INH-resistant MTB associated with katG Ser315Thr substitution. However, this PCR-RFLP method only detected 51% of all INH-resistant MTB in this study. The remaining resistant isolates possibly acquired the INH-resistant phenotype by accumulation of novel mutations in katG or known mutations of other gene loci, such as \( \text{inhA} \) or \( \text{ahpC} \) (Basso et al., 1998; Lee et al., 2001; Mdluli et al., 1998; Sherman et al., 1996). Further understanding of INH-resistance mechanisms in MTB will facilitate the development of PCR-based protocols for rapid diagnosis of the pathogen in clinical specimens.

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