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

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Mutations in the *katG* locus of catalase peroxidase in *Mycobacterium tuberculosis* (MTB) account for major isoniazid (INH) resistance. In the South China region, a collection of 906 respiratory specimens and 142 MTB isolates was used to evaluate the sensitivity and specificity of a PCR-RFLP method for the detection of INH resistance-associated mutations. Except for four catalase-negative MTB isolates, *katG* PCR for a 620-bp amplicon was successful for all purified MTB isolates. For respiratory specimens, diagnostic sensitivity and specificity of *katG* PCR was 85 and 100 %. Subsequent RFLP of the *katG* amplicons by *MspI* digestion identified that 51 % of INH-resistant MTB were associated with the Thr315 phenotype, and that codon 463 was a polymorphic site with no linkage to INH resistance. The Arg463 wild-type MTB isolates predominant in the Western world were replaced by isolates carrying Leu463 in the South China region. RFLP patterns of *katG* amplicons from respiratory specimens were identical to those of the corresponding MTB cultured colonies. This method has potential application for rapid diagnosis of INH resistance due to *katG* Ser315Thr mutation.

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INTRODUCTION

Tuberculosis remains a global threat to public health. The problem is further complicated by the emergence of multi-drug-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 Amplicor 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 Amplicor 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 *katG*904 (5'-AGCTCGTATGGCACCAGGAAC-3', forward primer, positions 904-923) and *katG*1523 (5'-TTGACCTCCCACCCGACTTG-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, 0.15 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× 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, CTG) were identified by RFLP using *MspI* digestion (restriction site C↓CGG).

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

Table 1. Comparative sensitivities and specificities of three PCR assays for detection of MTB

IS6110 PCR and Roche PCR were performed with undiluted extract. Values are number positive/total (% positive).

Isolates	IS6110 PCR	Roche PCR	katG PCR	
			Undiluted extract	3× concentrated extract
Respiratory specimens (n = 906)				
MTB culture-positive: (n = 233)				
Smear-positive (n = 187)	187/187 (100)	187/187 (100)	161/187 (86.1)	187/187 (100)
Smear-negative (n = 46)	27/46 (58.7)	13/46 (28.3)	9/46 (15.6)	11/46 (23.9)
MOTT culture-positive (n = 35)*	0/35 (0)	0/35 (0)	0/35 (0)	0/35 (0)
Culture-negative for <i>Mycobacterium</i> (n = 638)†	0/638 (0)	0/638 (0)	0/638 (0)	0/638 (0)
Purified MTB isolates (n = 142)				
Catalase-positive MTB (n = 138)	–	–	138/138 (100)	–
Catalase-negative MTB (n = 4)	–	–	0/4 (0)	–

*Nineteen *Mycobacterium avium*–*Mycobacterium intracellulare* complex isolates, eight *Mycobacterium chelonae*, four *Mycobacterium goodii*, two *Mycobacterium scrofulaceum* and two *Mycobacterium fortuitum* isolates.

†Including 28 isolates of *Streptococcus pneumoniae*, 13 isolates of *Haemophilus influenzae* and two isolates of *Pseudomonas aeruginosa*.

Fig. 1. Analytical sensitivity of IS6110 PCR (lanes A–F) and *katG* PCR (a–f) with serial dilutions of MTB Rv37 extracts. Lanes: A, a, 10^{-5} ; B, b, 10^{-6} ; C, c, 10^{-7} ; D, d, 10^{-8} ; E, e, 10^{-9} ; F, f, 10^{-10} ; M, molecular size marker ϕ X174.

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

Table 2. PCR-RFLP patterns of 620-bp *katG* fragments for 375 MTB isolates after *MspI* digestion

DNA fragments smaller than 30 bp are not shown.

Pattern	<i>MspI</i> RFLP DNA fragment (bp)						Amino acids	Phenotype	
	228	202	153	137	132	65		INH-susceptible (<i>n</i> = 273)	INH-resistant (<i>n</i> = 102)
A	+		+	+		+	Ser315+Arg463	38 (13.9 %)	7 (6.9 %)
B	+	+	+				Ser315+Leu463	235 (86.1 %)	43 (42.2 %)
C	+			+	+	+	Thr315+Arg463	0 (0 %)	13 (12.7 %)
D	+	+			+		Thr315+Leu463	0 (0 %)	39 (38.2 %)

Fig. 2. RFLP patterns of *katG* amplicons after *MspI* digestion. Lanes: 1, 3, 4, 7, 9 and 10, pattern B; 2, pattern A; 5, pattern C; 6 and 8, pattern D; M, molecular size marker ϕ X174. Details of RFLP patterns are given in Table 2.

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 *IS6110* or Roche PCR should be subjected to subsequent *katG* PCR. For *katG* PCR-positive samples, RFLP should be applied to identify the Ser315Thr substitution. For *katG* PCR-negative samples, routine mycobacterial culture should be monitored regularly so that MTB colonies identified can be used directly for *katG* PCR-RFLP. 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 *inhA* or *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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