Mycobacteriology

# Direct detection of isoniazid-resistant *Mycobacterium tuberculosis* in respiratory specimens by multiplex allele-specific polymerase chain reaction

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## Abstract

This study evaluated the feasibility of using 2 multiplex allele-specific polymerase chain reaction (MAS-PCR) assays targeting 2 mutations (codon 315 of the *katG* gene and the 15th nucleotide preceding the *mabA-inhA* operon) to directly detect isoniazid (INH)-resistant *Mycobacterium tuberculosis* in cultured isolates and respiratory specimens. A total of 203 *M. tuberculosis* isolates and 487 respiratory specimens were investigated. The MAS-PCR assays successfully amplified all *M. tuberculosis* isolates and acid-fast bacilli smear-positive specimens while only 49.2% of the smear-negative specimens exhibited positive MAS-PCR results. The MAS-PCR assays identified 83.4% and 79.2% of the resistant strains in the culture isolates and respiratory specimens, respectively. All the inferred genotypes were in complete accordance with subsequent DNA sequence analyses. This study suggested the application of our improved MAS-PCR protocols to provide the rapid identification of INH-resistant *M. tuberculosis* directly in respiratory specimens. The technical simplicity, short turnaround time, and low cost of this molecular strategy should facilitate routine diagnostic services in developing areas with a high prevalence of drug-resistant tuberculosis.
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## 1. Introduction

Tuberculosis is a major cause of infectious morbidity and mortality with 9.2 million new cases and 1.7 million deaths worldwide each year (World Health Organization, 2008). Although isoniazid (INH) remains the central chemotherapeutic and prophylactic drug used for tuberculosis treatment, recent increases in INH-resistant *M. tuberculosis* have imperiled the future utility of the drug. Previous studies indicated that the median rate of primary INH resistance is 7.3% (range from 1.5% to 32%) and that

the rates of acquired resistance is between 5.3% and 70% globally (World Health Organization, 1997). The overall rate of resistance to INH is 4.8% in Hong Kong (Department of Health, 2004).

INH resistance is likely to be mediated by several molecular mechanisms, only a subset of which have been completely elucidated. The predominant mechanism is associated with mutations in *katG*, particularly in codon 315. Our previous study indicated that approximately 50% of INH-resistant *M. tuberculosis* strains in Hong Kong were found to harbor mutations in *katG*315 (Leung et al., 2003). One particular base substitution at codon 315, Ser→Thr (AGC→ACC), was found to be the most frequently mutated allele. This mutation appears to reduce the efficiency of INH

activation without abolishing catalase–peroxidase activity, which detoxifies antibacterial radicals (Kapetanaki et al., 2005). About 8–30% of isoniazid-resistant *M. tuberculosis* isolates were shown to carry a mutation located at the 15th nucleotide preceding the *mabA*-inhA operon in our previous report (Leung et al., 2006). This mutation causes overproduction of the INH drug target and results in INH resistance via a titration mechanism (Ramaswamy and Musser, 1998). INH resistance was also shown to be mediated by mutations within the structural region of *inhA*, although the prevalence of this occurrence is relatively low, ranging from 0.5% to 5% (Hazbon et al., 2006, Zhang et al., 2005). Other genes, such as *kasA*, *ndh*, and *ahpC*, have also been shown to be associated with INH resistance. Those mutations, however, are extremely rare and usually coexist with other hotspot mutations in *KatG* and *inhA-mabA*, limiting their utility for rapid diagnosis of INH resistance (Hazbon et al., 2006, Lee et al., 2001, Mdluli et al., 1998, Ramaswamy et al., 2003, Sreevatsan et al., 1997).

PCR-based molecular techniques have become popular for the rapid identification of *M. tuberculosis*. Our previous studies reported the effectiveness of the application of one-tube nested *IS6110*-PCR to diagnose *M. tuberculosis* in both purified isolates and clinical specimens (Yam et al., 1998, Yuen et al., 1997). This study extends the approach to the genotypic prediction of INH resistance in *M. tuberculosis* by using multiplex allele-specific polymerase chain reaction (MAS-PCR).

The use of MAS-PCR to detect *katG*315 and *mabA*-15 mutations in *M. tuberculosis* clinical isolates has been reported in our previous study (Leung et al., 2006). We have also utilized MAS-PCR to identify isoniazid resistance in a recent case of fluoroquinolone-resistant multidrug-resistant tuberculosis (Lau et al., 2009). However, the sensitivity and specificity of this molecular technique for detection of INH-resistant *M .tuberculosis* from clinical specimens have not been fully evaluated.

In this study we modified the described primers (Leung et al., 2006, Mokrousov et al., 2002) and reoptimized the PCR cycling condition to facilitate DNA amplification from clinical specimens. A DNA purification step was also implemented before the MAS-PCR assays to remove PCR inhibitors present in human samples.

A total of 203 *M. tuberculosis* isolates and 487 respiratory specimens were used to evaluate the specificity and sensitivity of the *katG*315 MAS-PCR and *mabA*-15 MAS-PCR assays. The performance was compared to PCR-sequencing assays and conventional antimycobacterial susceptibility testing.

## 2. Materials and methods

### 2.1. Isolates and respiratory specimens

This study consisted of 2 sets of samples for evaluation of the reoptimized MAS-PCR assays for detection of

INH-resistant *M. tuberculosis* in purified isolates and respiratory specimens.

#### 2.1.1. Set 1

Laboratory reference strain, H37Rv, and 203 nonduplicated clinical isolates of *M. tuberculosis* collected between 2003 and 2007, including 145 INH-resistant and 58 INH-susceptible *M. tuberculosis* strains, were used to validate the reoptimized MAS-PCR protocols in our setting.

#### 2.1.2. Set 2

Between 2007 and 2009, nonduplicated respiratory specimens from 487 in-patients with chest symptoms and/or chest radiographic infiltrates of undetermined origin were collected from 3 general hospitals in Hong Kong. All specimens were routinely examined by acid-fast bacilli (AFB) staining followed by digestion using sputasol-sodium hydroxide (Yuen et al., 1997). The digested sediments *were* divided equally *for AFB cultur*e and the *IS6110* PCR assay for *M. tuberculosis* complex as described in our previous studies (Chan et al., 1996, Yam et al., 1998, Yuen et al., 1997).

### 2.2. Antimycobacterial susceptibility test

Susceptibility test for INH, rifampin (RIF), streptomycin (STR), and ethambutol (EMB) was carried out using the agar proportional method (Clinical and Laboratory Standards Institute, 2003). In brief, the mycobacterial suspension was prepared and adjusted to the McFarland 1 standard. A 100-μL aliquot of 100- and 10 000-fold dilution samples was inoculated onto drug-containing and drug-free sectors of Middlebrook 7H10 quadrant plates. The following drug concentrations were used: INH (0.2 μg/mL), RIF (1 μg/mL), EMB (5 μg/mL), and STR (2 μg/mL). Plates were incubated and examined weekly for up to 3 weeks. Whenever the drug-free sector grew at least 50–150 colonies and the percentage of the colonies grown in the drug-containing sector was ≧1%, the result was interpreted as resistant.

### 2.3. DNA extraction

For both purified isolates and respiratory specimens, mycobacterial DNA was extracted using the Roche Cobas Amplicor extraction kit as described previously (Yam et al., 2004).

### 2.4. IS6110 PCR for M. tuberculosis identification

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). Unpurified DNA extract (10 μL) was used as the template for this assay.

### 2.5. Identification of non-tuberculosis mycobacteria

*M. avium* and *M. intracellulare* were identified by a previously described PCR protocol (Li et al., 1996). For non-*M. avium* complex MNT, the species were identified by