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Note

A real-time PCR assay for detection of isoniazid resistance in *Mycobacterium tuberculosis* clinical isolates

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Abstract

A real-time PCR genotypic assay was developed for the detection of isoniazid (INH) resistance in *Mycobacterium tuberculosis*. The assay detects mutations C(−15)T and, possibly, G(−24)T in the regulatory region of the *inhA* gene and proved as sensitive and specific as nucleotide sequencing in all the clinical isolates tested. Our assays mapped the mutations efficiently in 10 out of 35 resistant isolates, thereby covering 29% of all resistant strains.

Keywords: Real-time PCR assay; Isoniazid resistance; *Mycobacterium tuberculosis*

Isoniazid (INH) is a critical component of the first-line multidrugs therapy of tuberculosis (TB). Mutations in several genes and genomic regions of *Mycobacterium tuberculosis* are involved in the occurrence of resistance to INH (Piatek et al., 2000; Slayden and Barry, 2000). The *katG* gene is the most commonly targeted, with the majority of mutations occurring at codon 315 in 30–90% of INH-resistant strains depending on geographical areas (Haas et al., 1997; Kiepiela et al., 2000; Mokrousov et al., 2002; Piatek et al., 2000). Another frequent target is the regulatory region of the *inhA* gene, where mutations have been reported in up to 32% of INH-resistant isolates (Kiepiela et al., 2000; Lee et al., 1999; Morris et al., 1995; Telenti et al., 1997). Mutations in other genomic regions, such as the promoter of *ahpC* gene and in the *kasA* gene, have been reported in 12–24% and 10–14% of INH-resistant strains, respectively (Kiepiela et al., 2000; Lee et al., 1999; Mdluli et al., 1998; Telenti et al., 1997). Genotypic assays that detect mutations within such regions are predictive of clinical drug resistance and have the potential to provide rapid detection of resistance in mycobacterial isolates. During the past few years, in fact, several genotypic assays have been developed for detection of the mutations responsible for INH resistance, particularly those at codon 315 of the *katG* gene (Telenti et al., 1997; Nachamkin et al., 1997; Garcia de Viedma et al., 2002; Piatek et al., 2000; Torres et al., 2000; Mokrousov et al., 2002). With regard to the regulatory

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region of *inhA* gene, mutations are frequently located 15 nucleotides upstream of the contiguous *mabA* gene that constitutes, together with *inhA*, a two-gene operon; the mutation consists in a C → T transition, and is usually indicated as C(−15)T (Ramaswamy and Musser, 1998) or C(209)T (Telenti et al., 1997); other mutations in this region occur less frequently, for example, mutation G(−24)T, T(−8)G/A (Kiepiela et al., 2000; Morris et al., 1995; Rouse et al., 1995; Slayden and Barry, 2000; Victor et al., 1999). In this paper we describe a simple and rapid real-time PCR-based genotypic assay designed to detect the mutations C(−15)T and G(−24)T. The assay was evaluated with a collection of 45 INH-resistant and 20 INH-susceptible clinical isolates of *M. tuberculosis* from 65 different patients; phenotypic drug resistance, determined by the radiometric BACTEC 460 TB system (Becton Dickinson, Towson, MD) (Siddiqi et al., 1981), was defined according to the proportional method as greater than 1% growth in the presence of 0.1 μg INH/ml. DNA was extracted by the Chelex 100 (Biorad) method from mycobacterial liquid cultures. Primers and hybridization probes for real-time PCR were designed by using the LightCycler Probe Design Software (Roche Applied Science, Germany). Forward (^{92}TGTGCTGAGTCACAC, melting temperature [T_m] 59.0 °C) and reverse (^{277}GACTGAACGGGATACGA, T_m 59.2 °C) primers amplify a 186-bp fragment of the regulatory region of the *inhA* gene (GeneBank accession no. U66801). Hybridization probes include a sensor probe (^{202}GGCGAGACGATAGGTTGTCG, T_m 64.1 °C), 5′ labeled with LC-Red 640 and 3′ phosphorylated, which hybridizes with the region containing the C(−15)T mutation site, and a 3′-fluorescein-labeled anchor probe (^{182}CCGATTTCGGCCCGGCC, T_m 68.0 °C), adjacent to the G(−24)T mutation site, which hybridizes two bases upstream from the former. A schematic representation of the *inhA* region including the C(−15)T and G(−24)T mutations, as well as the position of the hybridization probes, is given in Fig. 1. The PCR and hybridization reactions were carried out by the LightCycler instrument (Roche Applied Science) in glass capillaries in a volume of 20 μl containing 3 μl of template DNA, $MgCl_2$ 4 mM, primers 0.5 μM, probes 0.2 μM, and 2 μl of FastStart DNA Master Hybridization Probes (Roche Applied Science). PCR amplification comprised an initial denaturation step at 95 °C for 10 min, followed by 50 amplification cycles (with a temperature transition rate of 20 °C/s) of 95 °C for 10 s, 60 °C for 5 s, and 72 °C for 10 s, with monitoring of fluorescence during the annealing phase. Amplification was followed by a melting step consisting of 95 °C for 10 s, cooling to 45 °C for 10 s, and finally a slow rise in the temperature to 80 °C at a rate of 0.1 °C/s. Detection of mutated codons was obtained by the analysis of the melting curves derived from the continuous monitoring of probe hybridization status during slow heating, as the temperature at which a probe–target duplex melts, i.e. the melting temperature, gives qualitative information about the target sequence. The results of the real-time PCR assays were finally compared with the nucleotide sequences obtained with previously published primers (Telenti et al., 1997) by the ALFexpress DNA sequencer (Amersham Biosciences, UK).

Results of the real-time PCR assays are summarized in Table 1. In particular, all the 20 *M. tuberculosis* isolates phenotypically susceptible to INH showed derivative melting curves that corresponded to the INH-susceptible control strain *M. tuberculosis* H37Rv; the probe's T_m for these isolates was 62.6 ± 0.6 °C (mean ± standard deviation). Nucleotide sequencing confirmed the wild-type genotype for all the isolates. Of the 45 INH-resistant strains, 9

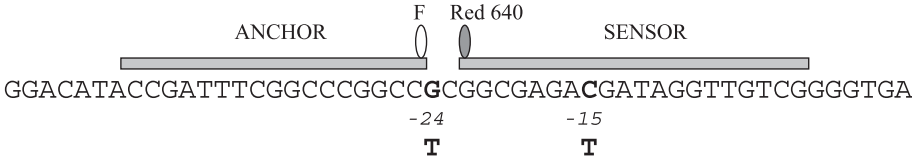

Fig. 1. Schematic representation of the regulatory region of the *inhA* gene that includes the mutations for resistance to INH in *M. tuberculosis*. The hybridization probes used in the real-time PCR assay are indicated by the gray boxes above the corresponding complementary sequence. The mutations studied and the corresponding nucleotide substitutions are indicated in bold. The fluorescein (F) and the Red 640 labels are indicated.

Table 1
Characteristic melting temperatures for *inhA* mutation detection probe

No. of isolates	INH Phenotypic test[a]	Nucleotide sequence	Real-time PCR ($T_m \pm$ S.D.)
21[b]	susceptible	wild type	62.6 ± 0.6
9	resistant	C(−15)T	58.2 ± 0.2
1	resistant	G(−24)T	61.0 ± 0.2[c]
35	resistant	wild type	62.7 ± 0.5

[a] Determined by radiometric BACTEC system.
[b] *M. tuberculosis* H37Rv is included.
[c] Data derived from four determinations.

showed melting curves with peak T_m of 58.2 ± 0.2 °C, while 1 isolate yielded a melting curve with peak T_m of 61 ± 0.2 °C; nucleotide sequencing showed mutation C(−15)T for all the former isolates and mutation G(−24)T for the latter. The other 35 isolates phenotypically resistant to INH yielded basically the same T_m (62.7 ± 0.5 °C) as INH-susceptible isolates, thus indicating a wild-type genotype for the studied region. Indeed, nucleotide sequencing confirmed the absence of mutations in the entire regulatory region of the *inhA* gene. The melting curves of representative isolates with a wild-type genotype, and C(−15)T and G(−24)T mutations are given in Fig. 2.

Taken altogether, our results show that the real-time PCR assay described here is 100% concordant with the more laborious nucleotide sequencing method. The assay allows to detect the mutation C(−15)T with maximal sensitivity and specificity. A similar assay, which employs a different set of sensor/anchor hybridization probes that allows detection of C(−15)T mutation, has recently been published by Torres et al. (2003). Our assay also proved able to detect the mutation G(−24)T, adjacent to the anchor probe. However, as this mutation was harbored by only 1 of our 45 INH-resistant isolates, the result needs to be validated on a suitable number of isolates.

In conclusion, our LightCycler real-time PCR assay, covering 29% of all resistant isolates, appears to be excellent for rapid and reliable prediction of C(−15)T- and, possibly, G(−24)T-based phenotypical resistance to INH of *M. tuberculosis* isolates. The assay, used in combination with other available real-time PCR-based assays for *katG* gene mutations (Garcia de Viedma et al., 2002; Torres et al., 2000), might provide a rapid and accurate tool to identify most of the known mutations conferring resistance to INH in clinical isolates, as well as directly in clinical samples, which would minimize the time needed for initiation of a specific anti-TB chemotherapy.

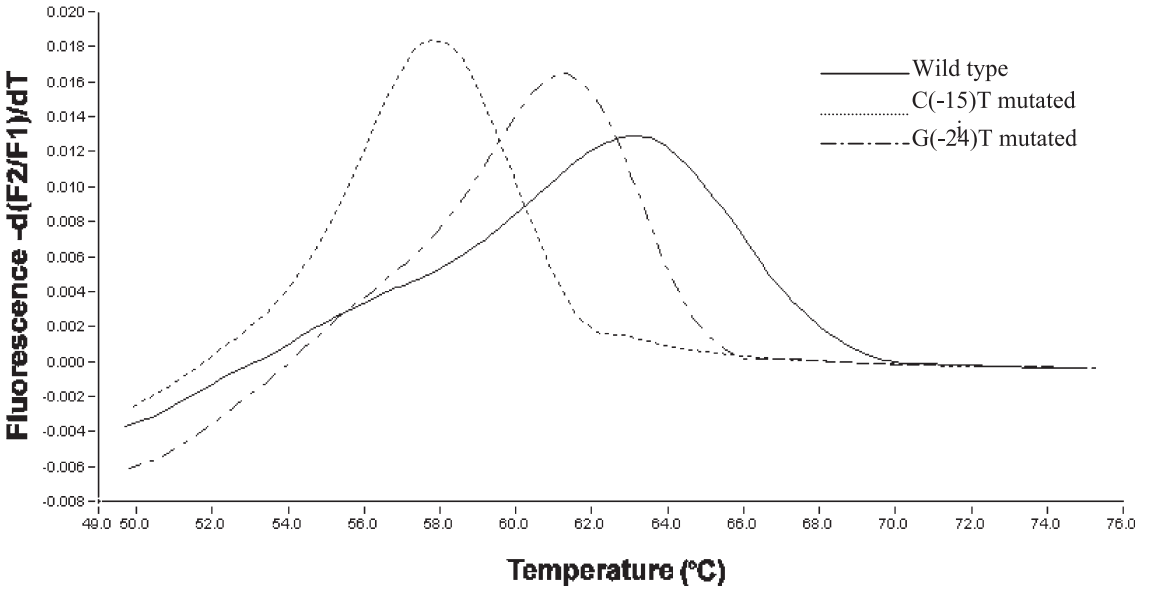

Fig. 2. Representative experimental melting patterns for wild type, C(−15)T and G(−24)T isolates, as measured in fluorimetric channel F2 by LightCycler.

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