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Prevalence and dissemination of the Ser315Thr substitution within the KatG enzyme in isoniazid-resistant strains of *Mycobacterium tuberculosis* isolated in Uruguay

Gustavo Varela,[1] Sabina González,[1] Pilar Gadea,[1] Cecilia Coitinho,[2] Inés Mota,[1] Gladys González,[1] Fernando Goñi,[3,4] Carlos Rivas[2] and Felipe Schelotto[1]

Correspondence
Gustavo Varela
gvarela@higiene.edu.uy

[1]Department of Bacteriology and Virology, Institute of Hygiene, Universidad de la República, Av. Alfredo Navarro 3051, Montevideo, Uruguay

[2]Laboratory Department, Honorary Anti-Tuberculosis Commission, 18 de Julio 2175, Montevideo, Uruguay

[3]Department of Immunology, School of Chemistry, Universidad de la República, Av. Alfredo Navarro 3051, Montevideo, Uruguay

[4]Department of Neurology, New York University School of Medicine, New York, USA

The aim of this study was to determine the prevalence of Ser315Thr substitution in isoniazid (INH)-resistant strains of *Mycobacterium tuberculosis* in Uruguay. The *katG* gene of 62 INH-resistant strains was analysed by an RFLP-PCR assay. PCR products were digested with *Msp*I to detect Ser315Thr and Arg463Leu substitutions. A total of 16 of the 62 (26 %) INH-resistant strains analysed had a Ser315Thr substitution. Only one INH-resistant strain had an Arg463Leu substitution and two strains had a deletion in *katG*. Of the 16 strains with Ser315Thr, 15 showed different profiles using a double-repetitive-element PCR assay, demonstrating that there was no local dissemination of any particular strain. These findings are in agreement with published data from regions where the prevalence of tuberculosis (TB) is intermediate and may be due in part to the success of the local TB control programme.

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INTRODUCTION

The use of isoniazid (INH), rifampicin (RIF) and pyrazinamide constitutes the backbone of the directly observed therapy short-course, or DOTS, treatment for tuberculosis (TB). INH, a first-line antituberculosis drug, is very efficient at killing bacilli in the active metabolic state; however, the increase in INH-resistant strains has reduced the efficacy of this drug as a disease control agent in certain populations (WHO, 2000, 2004).

INH is a pro-drug activated by the bifunctional bacterial catalase–peroxidase enzyme encoded by the *katG* gene (Bardou *et al.*, 1998; Wallace & Griffith, 2005). It produces free radicals that in turn are toxic to different bacterial molecules (Sacchettini & Blanchard, 1996).

The mechanisms associated with INH resistance in *Mycobacterium tuberculosis* include mutations in several genes. These changes have been identified mainly in the *katG*, *inhA*, *kasA* and *ndh* genes. In addition, mutations in the *furA*, *iniA*, *iniB* and *iniC* genes have been found in INH-resistant strains (Piatek *et al.*, 2000; Ramaswamy & Musser, 1998; Ramaswamy *et al.*, 2003).

The prevalence of mutations in *katG* varies among different regions of the world, with rates ranging from 7 % in Finland to 94 % in North-West Russia (Marttila *et al.*, 1996; Mokrousov *et al.*, 2002). Approximately 70–90 % of the mutations in the *katG* gene are located in codon 315, with the most frequent substitution being serine to threonine (AGC→ACC) (Hazbón *et al.*, 2006; Herrera-León *et al.*, 2005; Leung *et al.*, 2003; Silva *et al.*, 2003).

The aim of this work was to determine the prevalence of the Ser315Thr substitution in INH-resistant strains of *M. tuberculosis* in Uruguay, and to determine whether it was associated with other epidemiological and phenotypic characteristics that could influence dissemination.

Abbreviations: DRE, double repetitive element; INH, isoniazid; RIF, rifampicin; TB, tuberculosis.

METHODS

***M. tuberculosis* isolates.** In Uruguay, the Mycobacteriology Laboratory of the Honorary Anti-Tuberculosis Commission is the

national reference centre for mycobacteria, and receives at least 95 % of the specimens detected in the country as a whole. In this study, we analysed all *M. tuberculosis* cultures resistant to INH (62 strains) isolated between 1999 and 2005 at this laboratory from the respiratory tracts of 69 patients. We also studied 14 INH-susceptible strains. Ten of these isolates corresponded to five patients who presented with a susceptible strain before treatment and an INH-resistant strain during the course of standard treatment with RIF, INH and pyrazinamide. These cases were defined as treatment failure. Another four isolates were recovered from two patients defined as having a relapse of TB. Clinically and epidemiologically relevant information from each patient including human immunodeficiency virus status and demographic data were obtained from the National Tuberculosis Register of Uruguay.

Catalase activity assay. A semi-quantitative catalase test was carried out on all strains according to a standard procedure (Vincent *et al.*, 2003). *M. tuberculosis* H37Rv was included as a positive control.

Drug-susceptibility testing. INH resistance was determined using the proportion method in Löwenstein–Jensen medium containing 0.2 or 1 μg INH ml^{-1} (Canetti *et al.*, 1963).

Bacterial DNA isolation. For DNA extraction, a loopful of each bacterial growth on Löwenstein–Jensen medium was suspended in 1 ml distilled water, boiled for 10 min in a water bath, frozen at −80 °C overnight, thawed and centrifuged at 12 000 r.p.m. in a microfuge for 10 min. The supernatants were used as DNA templates for amplification reactions.

RFLP-PCR for *katG*. The reaction protocol was carried out as described by Leung *et al.* (2003). The primers used were katG-F 904 (5′-AGCTCGTATGGCACCGGAAC-3′) and katG-R 1523 (5′-TTGACCTCCCACCCGACTTG-3′). Amplification was performed in 25 μl PCR mixture containing 0.2 μM each primer, 2 mM $MgCl_2$, 1.5 U *Taq* DNA polymerase (Invitrogen) and 200 μM dNTPs. The amplification steps were as follows: initial denaturation for 4 min at 95 °C; 45 cycles of 1 min at 94 °C, 60 °C for 1 min and 72 °C for 1 min; and a final extension for 10 min at 72 °C. The 620 bp fragment of the *katG* gene was digested at 37 °C for 4 h with *Msp*I restriction endonuclease (New England Biolabs), followed by electrophoresis in a 6 % polyacrylamide gel. The pattern of bands was visualized under UV light using ethidium bromide. This procedure was applied to all strains.

Double-repetitive-element (DRE) PCR. This assay was performed as described by Friedman *et al.* (1995) and Varela *et al.* (2005) on all RFLP-PCR pattern C strains, on the ten isolates recovered from patients with treatment failure and on the four strains isolated from two patients with a relapse of TB. The primers used were Ris1 (5′-GGCTGAGGTCTCAGATCAG-3′) and Ris2 (5′-ACCCCATCCTTTCCAAGAAC-3′), and Pntb1 (5′-CCGTTGCCGTACAGCTG-3′) and Pntb2 (5′-CCTAGCCGAACCCTTTG-3′). The amplification products were examined by 2 % (w/v) agarose gel electrophoresis. A GeneAmp PCR system 2700 thermal cycler (Applied Biosystems) was used for all amplification reactions.

RESULTS AND DISCUSSION

This is believed to be the first study in Uruguay of the association of INH resistance with the AGC→ACC substitution at codon 315 of the *katG* gene. A total of 16 of the 62 (26 %) INH-resistant *M. tuberculosis* strains showed pattern C by RFLP-PCR (Fig. 1, lane 3), which corresponds to the Ser315Thr substitution. Similar results have been obtained in Singapore where the prevalence of

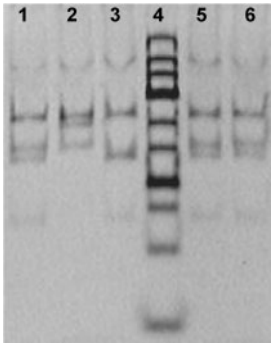

Fig. 1. RFLP-PCR patterns of *katG* amplicons after *Msp*I digestion. Lane: 1, 5 and 6, strains IH9, IH17 and IH65, respectively, with pattern A; 2, strain IH8 with pattern B; 3, strain IH43 with pattern C; 4, 25 bp DNA ladder (low range GeneRuler; Fermentas).

TB is intermediate and the Ser315Thr substitution represents 26 % of the INH-resistant isolates (Lee *et al.*, 1999). In contrast, in North-West Russia where the prevalence of TB is 278.2 per 100 000 of the population, Mokrousov *et al.* (2002) found a prevalence of the Ser315Thr substitution close to 95 %, which strongly suggests a dissemination of particular strains.

We analysed at least 95 % of the INH-resistant strains present in Uruguay during the period 1999–2005 and could not establish any epidemiological link between 15 of the 16 patients infected with pattern C strains. In addition, 15 of the 16 strains with RFLP-PCR pattern C had different profiles by DRE-PCR, suggesting that there is no predominant pattern C/INH-resistant strain linked to dissemination (Fig. 2a).

It has been postulated that Ser315Thr substitution confers intermediate- to high-level resistance to INH by binding of KatG to INH without suppressing the catalase activity (Abate *et al.*, 2001; Cardoso *et al.*, 2004; Gagneux *et al.*, 2006; Zhang *et al.*, 2005). We found that 13 of the 16 strains (81 %) with pattern C grew in the presence of 1 μg INH ml^{-1}.

van Soolingen *et al.* (2000) postulated that strains that already have the Ser315Thr substitution have more possibility of acquiring mutations in other genes. In this respect, we found that 8 of the 16 strains (50 %) with the Ser315Thr substitution showed resistance to other drugs such as RIF and streptomycin, whereas only 5 out of 33 INH-resistant strains (15 %) with pattern A by RFLP-PCR (which corresponds to serine in position 315 and arginine in position 463) (Fig. 1, lanes 1, 5 and 6) showed other phenotypes associated with resistance.

A total of 12 of the 16 strains with Ser315Thr substitution were recovered from patients defined as new TB cases according to World Health Organization recommendations (WHO, 2002). Two of these strains showed the same band profile by DRE-PCR assay (Fig. 2b) and were isolated from

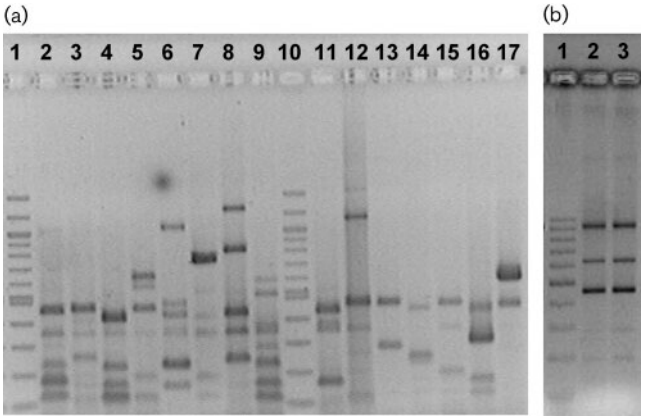

Fig. 2. (a) DRE-PCR of INH-resistant strains of *M. tuberculosis* with pattern C recovered from patients without epidemiological links. Lane: 1 and 10, 100 bp DNA ladder (New England BioLabs); 2, *M. tuberculosis* H37Rv; 3, strain IH11; 4, strain IH18; 5, strain IH24; 6, strain IH27; 7, strain IH31; 8, strain IH36; 9, strain IH41; 11, strain IH43; 12, strain IH45; 13, strain IH52; 14, strain IH59; 15, strain IH63; 16, strain IH72; 17, strain IH74. (b) DRE-PCR from INH-resistant strains of *M. tuberculosis* with pattern C recovered from two brothers living together. Lane: 1, 100 bp DNA ladder (GeneRuler; Fermentas); 2, strain IH33 and 3, strain IH38.

two brothers who lived together, which suggests a common origin of infection or transmission between them. These results are in agreement with previous reports that showed that INH-resistant strains of *M. tuberculosis* with a Ser315Thr substitution are capable of producing secondary cases of TB (Gagneux *et al.*, 2006). The remaining four strains with a Ser315Thr substitution were obtained from patients who had previously received antituberculosis treatment.

As reported in other regions (Bakonyte *et al.*, 2003; Haas *et al.*, 1997; Marttila *et al.*, 1996), the presence in Uruguay of a *katG* gene deletion was low, being found in only 2 (3 %) of the 62 INH-resistant strains. These strains grew in the presence of INH at a concentration of 1 μg ml^{-1} and showed no catalase activity.

A single INH-resistant strain with pattern B corresponding to Ser315 and Leu463 was recovered from one immigrant of Chinese origin, who arrived in Uruguay 2 years previously (Fig. 1, lane 2). The low occurrence of this mutation agrees with a previous study conducted in the neighbouring country of Brazil (Cardoso *et al.*, 2004).

All strains recovered from five individual cases defined as treatment failure showed pattern A by RFLP-PCR. These results suggest that the Ser315Thr substitution is not frequently associated with this situation. Four out of five of these cultures showed sequences related to *katG* by PCR and had no catalase activity (Table 1). The strains from different individuals with treatment failure showed different band patterns by DRE-PCR assay, but in each individual patient the same band distribution was seen for the INH-susceptible and INH-resistant strains recovered before and after treatment (Fig. 3).

Two pairs of strains corresponding to relapses of TB after 1 and 2 years, respectively, showed pattern A in the INH-resistant isolates and displayed an identical band distribution by DRE-PCR assay (Fig. 1). A total of 15 (45 %) of the 33 remaining INH-resistant strains with pattern A were isolated from patients who had not received antituberculosis treatment and all INH-susceptible isolates analysed showed pattern A in RFLP-PCR (Fig. 1). Three strains showed particular and unique profiles not described before and apparently not involving Ser315Thr or Arg463Leu.

In summary, we found the following results. (i) In Uruguay the prevalence of the Ser315Thr substitution in KatG in INH-resistant *M. tuberculosis* strains was intermediate (26 %) and similar to figures reported in regions with a comparable prevalence of TB. The intermediate prevalence of Ser315Thr substitution precludes the use of RFLP-PCR as a rapid tool for the screening of INH resistance. (ii) Using DRE-PCR, we found no clonal dissemination of any

Table 1. INH-susceptible and INH-resistant *M. tuberculosis* strains isolated from patients with treatment failure in Uruguay

Patient no.	HIV status*	Time between isolation of the two strains (days)	Other drugs to which the second isolate was resistant	Height (mm) in catalase test		DRE-PCR pattern (both strains)	S315T/R463L substitutions (both strains)†
				INH-S strains	INH-R strains		
CLA 14	Negative	62	–	15	0	A/A	−/−
CLA 23	Negative	60	RIF	18	0	B/B	−/−
CLA 32	Negative	68	–	23	0	C/C	−/−
CLA 48	Negative	63	–	8	0	D/D	−/−
CLA 57	Negative	66	–	18	16	E/E	−/−

HIV, Human immunodeficiency virus; INH-R, INH resistant; INH-S, INH susceptible.
*Determined by enzyme immunoassay (AxSYM; Abbott Laboratories).
†−, absence of substitutions as determined by RFLP-PCR.

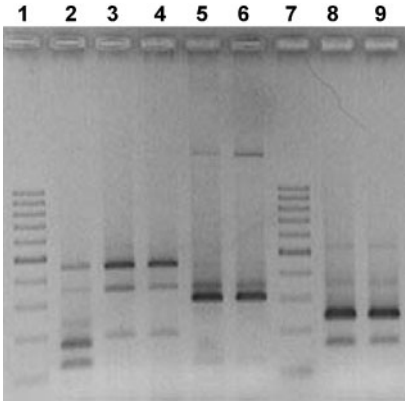

Fig. 3. DRE-PCR of strains of *M. tuberculosis* recovered from three of five patients with treatment failure. Lane: 1 and 7, 100 bp DNA ladder (GeneRuler; Fermentas); 2, *M. tuberculosis* H37Rv; 3 and 4, strains A and B isolated from patient CLA 14; 5 and 6, strains A and B isolated from patient CLA 23; 8 and 9, strains A and B isolated from patient CLA 32. Strains identified as A corresponded to INH-susceptible isolates recovered before treatment and strains identified as B to INH-resistant isolates recovered 2 months after starting the antituberculosis treatment.

particular INH-resistant strain of *M. tuberculosis* carrying the Ser315Thr substitution. (iii) Deletions in the *katG* gene in INH-resistant strains of *M. tuberculosis* are rare. (iv) More-refined techniques are needed to map the whole array of mutations/deletions leading or related to INH resistance and multidrug resistance. A comprehensive classification of *M. tuberculosis* strains will be critical in determining future antituberculosis therapies in a dynamic and efficient TB control programme.

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