

Characterization of *pncA* mutations of pyrazinamide-resistant *Mycobacterium tuberculosis* in Turkey

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SUMMARY

Mutations in the pyrazinamidase (PZase) gene (*pncA*) are considered the major mechanism of pyrazinamide (PZA) resistance in *Mycobacterium tuberculosis*. The aim of this study was designed to determine *pncA* mutations among ten PZA resistant and two PZA susceptible *M. tuberculosis* strains from Turkey and also to compare the PZase activity of them with the genotype. All isolates were identified by BACTEC NAP (P-nitro-alpha-acetylamino-beta-hydroxy-propionophenone) test and PCR-RFLP (Polymerase Chain Reaction- Restriction Fragment Length Polymorphism) method. Drug sensitivity tests were performed by BACTEC system. *PncA* mutations were detected by DNA sequence analysis. No mutation was detected in two PZA susceptible and three out of ten PZA resistant strains. While, two of the PZA resistant strains had mutations in the same region (Gly24Asp), two of the PZA resistant strains had mutations in different regions (Thr160Lys), (His51Pro). Three of the PZA resistant strains had frameshift as a 167 bp deletion at nucleotide position 102.

As a result, we detected two new mutations and a frameshift which may be responsible for PZA resistance in this study different from the other studies which previously 51st codon mutation was reported.

KEY WORDS: *Mycobacterium tuberculosis*, Drug resistance, Pyrazinamide, *pncA* mutations

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INTRODUCTION

Tuberculosis still remains one of the leading causes of morbidity and mortality worldwide. The first-line drugs used to treat tuberculosis include rifampin, isoniazide, pyrazinamide (PZA) and either ethambutol or streptomycin (Morlock *et al.*, 2000). During the 1990s, multidrug-resistant tuberculosis (MDR-TB), defined as resistance to at least isoniazid and rifampin, emerged as a threat to TB control in the worldwide (Brown *et al.*, 2000). In October 2006, the World Health Organization (WHO) approved extensively drug-

resistant tuberculosis (XDR-TB) in strains of MDR-TB which are also resistant to the second-line drugs such as fluoroquinolone and also resistant to at least one of the three following injectable drugs: capreomycin, kanamycin, and amikacin (WHO, 2006).

Although PZA is one of the most important first-line drugs for the treatment of *Mycobacterium tuberculosis* infection, the increasing frequency of PZA resistant strains also limits its effectiveness. PZA is a prodrug which is converted to bactericidal pyrazinoic acid (POA) by pyrazinamidase (PZase) produced by *M. tuberculosis* (Morlock *et al.*, 2000). Functioning exclusively in the acid environment (ph: 5.0-5.5) at the site of bacterial infection, PZA can kill semi-dormant and probably intracellular *M. tuberculosis* (Lee *et al.*, 2001).

In 1996, *pncA* gene in *M. tuberculosis* encoding PZase, was characterized (Scorpio *et al.*, 1996). The gene consists of 561 bp encoding a 20 kDa

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enzyme with 186 amino acids. DNA sequencing studies of the *pncA* gene from PZA-resistant and PZA-susceptible

M. tuberculosis strains have established a strong association between mutations in this gene and PZA resistance (Morlock *et al.*, 2000).

This study was designed to determine *pncA* mutations among PZA-resistant and susceptible *M. tuberculosis* isolates from Turkey. The resistance patterns of these isolates to other first-line drugs (isoniazid, rifampin, streptomycin and ethambutol) and some of the second line drugs (capreomycin, ethionamide, kanamycin, amikacin, ofloxacin, rifabutin) were also investigated. The aim of this study is to compare the PZase activity of *M. tuberculosis* strains with the genotype of the same strains and improve our understanding of molecular basis of PZA resistance in Turkey.

MATERIALS AND METHODS

Identification of *Mycobacterium tuberculosis* complex

Twelve clinical isolates of *M. tuberculosis* were provided from different cities of Turkey (Table 1). These isolates obtained from different patients were grown in BACTEC 7H12B media (Becton Dickinson Diagnostic Instrument System, USA). All isolates were identified by BACTEC NAP (P-nitro-alpha-acetylamino-beta-hydroxy-propionophenone) test (Becton Dickinson Diagnostic Instrument System, USA) and PCR-RFLP (Polymerase Chain Reaction- Restriction Fragment Length Polymorphism) method. The PCR-RFLP method was based on the amplification of a 439-bp segment of the 65-kDa heat shock protein (*hsp65*) gene, and the restriction enzyme analysis of PCR products was performed by using *BstEII* and *HaeIII*. RFLP patterns were compared by using the PCR-RFLP algorithm (from <http://www.hospvd.ch:8005>).

PZase activity testing

PZase activity testing of *M. tuberculosis* complex strains was applied by a modified method of that described by Wayne (Wayne, 1974). Briefly, two tubes of Dubos agar (Difco, Oxford, UK) containing 100 mg/ml PZA were surface - inoculated with a loopful of a mature culture of *M. tuberculosis*. All tubes were incubated for 4 and 7 days, respec-

tively. One millilitre of 1% ferrous ammonium sulphate (Sigma) was added to the each tube, and they were reincubated at 4°C for 4 h. A pink band at the interface indicates a positive result for PZA hydrolysis. The results were compared with two *M. tuberculosis* reference strain, ATCC 13950 and ATCC 12478 as positive and negative controls for the PZase production, respectively.

Antimicrobial susceptibility test

Drug sensitivity tests were performed by BACTEC radiometric system (Becton Dickinson Diagnostic Instrument System, USA) with the following antimicrobial agents: isoniazid, 0.1 µg/ml; ethambutol, 2.5 µg/ml; rifampin, 2.0 µg/ml; streptomycin, 2.0 µg/ml; capreomycin, 1.25 µg/ml; ethionamide, 1.25 µg/ml; kanamycin, 5.0 µg/ml; amikacin 1.0 µg/ml, ofloxacin, 2.0 µg/ml and rifabutin 0.5 µg/ml (Pfyffer *et al.*, 1999).

Preparation of DNA for PCR

Fresh colonies were collected from LJ medium, mixed with 200 µl distilled water until a suspension was obtained. Suspensions containing acid-fast bacteria were heated at 80°C for 60 minutes and then mycobacterial DNAs were isolated from the sample using the Nucleospin tissue isolation kit (Macherey-Nagel GmbH & Co KG Germany).

PCR

A total of 5.0 µl of the DNA extract was added to a reaction tube containing 45 µl of the PCR mixture [50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1.5 mM MgCl₂, 200 µM (each) deoxynucleoside triphosphate, 0.5 µM (each) primers *pncA*-11 (5'-GCTTGCGGCGAGCGCTCCA-3') and *pncA*-8 (5'-GGTTGGGTGGCCCGCCGCTCAG- 3'), and 1.25 U of *Taq* polymerase (Promega)] (Morlock *et al.*, 2000). The amplification was carried out using SANYO MIR D 40 thermal cycler. The reaction tube was first heated for 10 min at 95°C and then subjected to 35 cycles of amplification (1 min at 95°C, 1 min at 55°C, 2 min at 72°C), followed by 10 min extension at 72°C. The presence of amplified product was confirmed by agarose gel electrophoresis.

Sequencing

Firstly, the product obtained after PCR was purified with Microcon YM-100 (Millipore) spin column and sequenced with Big Dye terminator v3.0

TABLE 1 - Characteristics of PZA-resistant and PZA-susceptible clinical strains of *M. tuberculosis*.

Strains	Cities of patients	PZA	INH	RIF	EMB	SM	AK	OFX	RFB	ETM	CAP	KAN	Nucleotide Changes	Amino Acid Changes
2T	Antep	R	R	R	S	S	S	S	R	S	S	S	G 71 A	G 24→D
3T	Antep	R	R	R	S	S	S	S	R	S	S	S	G 71 A	G 24→D
4T	Izmir	R	R	R	S	R	S	S	R	S	S	S	No mutation	No mutation
5T	Izmir	R	R	R	R	S	S	S	R	S	S	S	C 479A	T 160→K
6T	Izmir	R	R	R	S	R	S	S	R	S	S	S	No mutation	No mutation
7T	Ankara	R	R	R	R	R	S	S	R	R	S	S	102, deletion (167-bp)	Frameshift
8T	Istanbul	R	R	R	R	R	S	R	S	R	S	S	No mutation	No mutation
10T	Istanbul	R	R	R	R	S	S	S	R	S	S	S	102, deletion (167-bp)	Frameshift
11T	Istanbul	R	R	R	S	R	S	S	R	S	S	S	102, deletion (167-bp)	Frameshift
12T	Edirne	R	R	R	R	R	S	S	R	R	S	S	A152 C	H 51→P
13T	Istanbul	S	R	R	S	S	S	S	S	S	S	S	No mutation	No mutation
14T	Istanbul	S	R	R	S	S	S	S	S	S	S	S	No mutation	No mutation

PZA: Pyrazinamide INH: Isoniazid RIF: Rifampin EMB: Ethambutol SM: Streptomycin. AK: Amikacin OFX: Ofloxacin RFB: Rifabutin ETM: Ethionamide. CAP: Capreomycin KAN: Kanamycin R: Resistant S: Susceptible.

cycle sequencing kit (ABI PRISM). Next, free dye-terminators were removed with DyeEx 2.0 (Qiagen) kit. Finally, the purified PCR products were sequenced in an ABI PRISM 310 genetic analyzer (Applied Biosystems, Inc., Foster City, Calif.). The sequence of each isolate was compared with that of the PZA-sensitive wild-type sequence (GenBank accession number U59967).

RESULTS

All isolates were identified as *M. tuberculosis* complex by PCR-RFLP analysis of *hsp65* and

BACTEC NAP test. All susceptible *M. tuberculosis* isolates produced PZase, whereas all resistant isolates were PZase negative. The susceptibility test results of twelve strains to PZA and other antituberculous drugs were shown in Table 1. These isolates were detected as the causative agents of MDR-TB but not XDR-TB. Among them, two and ten of the strains were detected as PZA susceptible and PZA resistant, respectively.

Three resistant isolates and all of the susceptible isolates had wild-type *pncA*. A total of four different mutations were detected and these mutations were dispersed along the *pncA* gene. *PncA* mutations were observed at the following amino

acid residues: Gly24Asp, His51Pro, Thr160Lys. A 167 bp deletion at nucleotide position 102 were detected in three isolates (Table 1).

DISCUSSION

Drug resistance may cause a serious negative impact on both patient outcome and control of transmission, so it is critical to detect accurately any drug resistance as soon as possible (Morlock *et al.*, 2000). Reproducible susceptibility testing is essential for effective management of tuberculosis (Davies *et al.*, 2000). Conventional PZA susceptibility testing is labour intensive and the result vary among laboratories.

In this study, two and ten of the strains were found PZA susceptible and resistant by BACTEC method. While all of the PZAase positive strains were sensitive to PZA respectively negative ones resistant to PZA. Thus, agreement between the methods of BACTEC 460 and PZAase testing was 100%. These data were consistent with those of previously published reports (Sekiguchi *et al.*, 2007). However Miller *et al.* (Miller *et al.*, 1995), compared the two methods in 428 *M. tuberculosis* complex strain and they detected, 98.2% and 100% agreement in PZA susceptible and resistant isolates, respectively.

The results of PZase testing method should be only interpreted in one direction - a negative result indicating PZA resistance - whereas care should be taken in interpreting positive test results (Mestdagh *et al.*, 1999).

In this study, two PZA-susceptible *M. tuberculosis* strain failed to show any mutations of the *pncA* gene like in many studies (Barco *et al.*, 2006, Brown *et al.*, 2000, Lee *et al.*, 2001, Lemaitre *et al.*, 1999, Tracevska *et al.*, 2004). We detected that three PZA resistant isolates did not have any *pncA* mutations in concordance with previously reported studies (Lemaitre *et al.*, 1999, Morlock *et al.* 2000, Park *et al.*, 2001). Mutation of the *pncA* gene was suggested as the major mechanism of PZA resistance but resistant strains containing the wild-type gene have also been described, suggesting additional resistance mechanisms exist besides a lack of PZase activity (Lee *et al.*, 2001, Mestdagh *et al.*, 1999). In some previously reported studies, a few of PZA resistant strains were detected PZase positive without *pncA* mutations

(Barco *et al.*, 2006, Denkin *et al.*, 2005, Portugal *et al.*, 2004, Rodrigues *et al.*, 2005). These findings support the hypothesis that other mechanisms may be involved in PZA resistance, possibly alteration in PZA uptake, increased POA active efflux or mutations leading to the modification or amplification of an unknown POA target. Cumulative reports indicate that between 72 and 97% of PZA-resistant isolates may harbour *pncA* mutations and the frequencies of these mutations were varied among studies (Huang *et al.*, 2003, McCammon *et al.*, 2005, Morlock *et al.*, 2000, Portugal *et al.*, 2004, Tracevska *et al.*, 2004). In this study, the frequency of *pncA* mutation appeared to be 70% in PZA-resistant *M. tuberculosis* although the number of studied samples were less. It has been recommended that initial screening by DNA-based methods followed by conventional culture-based methods for any mutation negative isolates; might be appropriate (Lee *et al.*, 2001). We also suggest this recommendation. Theoretically, the same mutations of the *pncA* gene would be rarely present in unrelated isolates because mutations are distributed throughout the *pncA* gene, and no major hot spots have been observed (McCammon *et al.*, 2005).

His51Pro (A → C) mutation detected in one of our PZA-resistant *M. tuberculosis* complex strain has also been reported from Thailand, Portugal, Japan and United States of America studies (Hirano *et al.*, 1998, Portugal *et al.*, 2004, Suzuki *et al.*, 2002). A total of seven papers were collected for analysis of the mutations in the *pncA* gene from Korea, Singapore, Japan, Peru, Turkey, Canada, Bangladesh, Azerbaijan and Scotland. The most frequent mutation of this gene is A → C occurring in 24 out of 114 strains (21%) (O'Sullivan *et al.*, 2005).

Some authors have also mentioned a certain degree of conservation of *pncA* mutations at amino acid residues 3-17, 61-76, and 132-142 of the PZAase protein (Lemaitre *et al.*, 1999, Barco *et al.*, 2006).

Two of the mutations (Gly 24 Asp), (Thr 160 Lys) that we described in this report have not been detected yet to our best knowledge. A 167 bp deletion at nucleotide position 102 detected in three PZA resistant *M. tuberculosis* complex strains have not been reported in literature yet. Deletions at 80 nucleotide in length at position 151, 234 nucleotide in length at position 56 from Korea, 68

nucleotide in length at position 419 from France and 68 nucleotide in length at position 195 from Azerbaijan have been reported (Lee *et al.*, 2001, Park *et al.*, 2001, Lemaitre *et al.*, 1999, Mestdagh *et al.*, 1999). There appears to be a higher tendency toward nucleotide deletion and insertion in the *pncA* gene compared to mutations found in *rpoB* gene related to rifampin-resistance (Lee *et al.*, 2001). Five PZA susceptible and, five PZA resistant Turkish strains studied in British laboratories were investigated and various mutations were detected in resistant ones. These various mutations (H57P, S59P, H82D + S65S, R148S, deletion 80-118) were different from the mutations that we found here (Brown *et al.*, 2000).

It takes 1-6 weeks or one week to identify the activity of PZase by conventional test methods and PZase method, respectively. but detection of mutation in *pncA* gene needs only two days by DNA sequence analysis. In this study, we described the high diversity of the *pncA* gene in strains of PZA-resistant *M. tuberculosis* from Turkey.

Furthermore, our study reveals that three new mutations in the *pncA* gene of *M. tuberculosis* may possibly be involved in resistance to PZA.

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