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Rapid Detection of *Pnca* Mutations in Pyrazinamide-Resistant *Mycobacterium tuberculosis Clinical isolates*

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Abstract: Pyrazinamide (PZA), an analog of nicotinamide, is a prodrug for tuberculosis which requires conversion to the bactericidal compound pyrazinoic acid by bacterial pyrazinamidase activity. Mutations leading to a loss of pyrazinamidase activity cause PZA resistance in *Mycobacterium tuberculosis*. Thus, the detection of pyrazinamidase activity makes the discrimination of PZA-resistant tuberculosis possible. However, the detection of the pyrazinamidase activity of *M. tuberculosis* isolates needs a large amount of bacilli and is therefore time consuming. In this study PCR mediated automated DNA sequencing was used to check for prevalence of Pyrazinamide (PZA) resistance among treatment failure cases of pulmonary tuberculosis. A total of 50 clinical isolates from treatment failures cases were isolated by culture and were analyzed for mutation in *pncA gene* by automated DNA sequencing. 39 clinical strains showed mutation in *pncA gene* out of 50 clinical isolates of *Mycobacterium tuberculosis.* The identified mutations were dispersed along the *pncA* gene, but some degrees of clustering of mutations were found at the following regions: Gly132, Lys96, Lys72, Pro69 (7.7%) Asp12 (10.3%) and Leu85 (15.4%). We found a significant proportion of resistance to PZA among treatment failure cases. If we are to prevent an epidemic of drug-resistant tuberculosis we must take steps to ensure that all treatment failure patients are diagnosed early and effectively treated. This will reduce treatment cost and potentially limit the spread of PZA drug-resistance.

Key words: PZA resistance · Mycobacterium tuberculosis · Mutation · PCR · DNA sequencing

INTRODUCTION

Tuberculosis (TB) caused by members of *Mycobacterium tuberculosis* complex is one of the common human diseases, causing 3,000,000 deaths per year worldwide [1]. While the disease is associated with economic impoverishment, TB is on the rise in many industrialized nations. The spread of TB is due to immigration, the emergence of drug-resistant strains [2] and the AIDS epidemics. The increasing number of drug-resistant *M.tuberculosis* strains has made the rapid identification of susceptibility clinically important because those patients suffering from these strains require specialized antibiotic treatment.

PZA, an analog of nicotinamide, is not active against *M. tuberculosis* under normal culture conditions [3], but it is active in acid medium (pH 5.5) [4] and in host macrophages [5]. The mode of action of PZA is not understood. It is thought that the bacterial enzyme pyrazinamidase (PZase) is required to convert PZA to pyrazinoic acid (POA), which is toxic to *M. tuberculosis* [6], but the target of PZA or POA is unknown. Resistance to PZA develops readily and in a fashion analogous to INH resistance [7], PZA-resistant *M. tuberculosis* strains lose both PZase and nicotinamidase activities[8]. These two enzyme activities are due to a single enzyme that acts on both nicotinamide and PZA [6]. Loss of PZase correlates with resistance to PZA and negative PZase tests for clinical isolates of *M. tuberculosis* are indicative of PZA resistance [4, 5, 9]. PZA is one of the most important drugs for anti-TB short course chemotherapy. PZA is converted to pyrazinoic acid by mediation of pyrazinamidase in *M. tuberculosis* and it inhibits fatty acid synthesis. Reduced pyrazinamidase activity

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correlates well with resistance to PZA. The mutations that confer PZA resistance have been investigated on the pyrazinamidase gene (*pncA*) by Scorpio and Zhang [10]. Thus, finding mutations on the *pncA* gene makes the rapid detection of PZA resistant *M. tuberculosis* possible. In the present study, we analyzed 50 PZA-resistant clinical isolates of *M. tuberculosis* for potential mutations in the *pncA* gene to gain further insight into the genetic basis of PZA resistance and to address the correlations between PZA resistance, PZase activity and *pncA* mutations. Thirty nine of the 50 clinical isolates were found to contain *pncA* mutations, as revealed by DNA sequence analysis. The *pncA* mutations could be rapidly identified by the PCR-single-strand conformation polymorphism (SSCP) technique.

MATERIALS AND METHODS

Bacterial Samples: A total of 50 clinical strains of *Mycobacterium tuberculosis* were isolated from sputum samples of treatment failure (CAT II) cases hospitalized at State TB Training and Demonstration Centre (Intermediate Reference Laboratory) Government Hospital for Chest Diseases, Pondicherry, during the period from Jan 2006 to Nov 2008. Among the 50 sputum samples collected, 32 samples were from patients of Pondicherry state and 18 samples were from the other states of India. All specimens were processed immediately and aliquots of the decontaminated specimens were kept at -20°C.

Sputum Processing for AFB Culture: Sputum in excess of 5 ml was discarded into the disinfectant bath. To each volume of remaining sputum, 2 volumes of 4% NaOH was added taking care to avoid contact between the specimen bottle rim and the NaOH flask. (If less than 5 ml of sputum is received, 2 times that volume of 4% NaOH was added.) The caps were screwed firmly on, ensuring at the same time that the bottle tops are not broken or chipped. The bottles were shaken by hand for 1 minute. Then the bottles were placed in a rack on the shaking machine and were left to shake gently for 20 minutes. The specimens were removed from the shaker. The sputum bottles were centrifuged for 15 minutes at 4000rpm make sure that the centrifuge buckets were counter balanced. After the bottles were removed from the centrifuge, the supernatant was carefully poured off into the disinfectant bath and the rim of each bottle was wiped with sterile filter paper. The bottles were filled with 20 ml of sterile distilled water, shaken by hand to mix the deposit and were centrifuged for 15 minutes at 4000rpm.The supernatant was poured off as before and again the neck of the bottle was wiped with sterile filter paper. Finally the sediment was inoculated with a 5 mm diameter loop onto the pre-sterilized and numbered Lowenstein-Jensen's slopes. The inoculated media was placed in the 37°c incubator [11, 12].

Mycobacterium DNA Extraction: One loopful of culture was taken in 100µl of sterile distilled water and was homogenized. The entire homogenized samples were treated with 50 µl of lysozyme (10mg/ml) at 37°C for overnight incubation. 70µl of 14% SDS and 6µl of Proteinase K (10 mg / ml) was added to precipitate the proteins and was incubated at 65°C for 15 minutes. 10 µl of 5M NaCl and 80µl of CTAB/NaCl were added to remove the polysaccharides and unwanted residues and was incubated at 65°C for 10 minutes. 800 µl of Phenol: Chloroform: Isoamylalcohol (25:24:1) mixture was added to remove the proteins from preparation of nucleic acid. The chloroform denatures the proteins while Isoamylalcohol reduces foaming during extraction and facilitates the separation of the aqueous and organic phase. Centrifuged at 10,000 rpm for 10 minutes. The supernatant was transferred to a fresh tube and 600µl of Isopropanol was added to precipitate the DNA and incubated overnight at -20°C.Centrifuged at 12000 rpm in 4°C for 10 minutes. The pellet was washed with 70% ethanol to remove any remaining solutes. The pellet was air-dried and was dissolved in 20µl of 1x TE buffer [13].

PCR Amplification: The isolated template DNA was amplified using IS6110 primer in an authorized thermal cycler (Eppendorf Gradient Cycler). This confirms the template DNA as *Mycobacterium tuberculosis*. The PCR reaction was set up as follows using the primer for *Mycobacterium IS6110* amplification F 5'GTGAGGGCATCGAGGTGG 3' (10pmol/µl) R 5'CGTAGGCGTCGGTCA CAAA 3' (10pmol/µl). The PCR cycling parameters were 94°C for 5 minutes; followed by 40 cycles of 94°C for 1 minute, 57°C for 1 minute and 72°C for 1 minute; and a final extension of 72°C for 10 minutes. The PCR was then kept at hold at 4°C for 15 minutes. The amplified PCR product was withdrawn from thermal cycler and run on a 2% Agarose gel in TAE buffer. The Ethidium bromide stained gels were observed in a UV Trans illuminator and photographed using a Geldoc.

PCR Amplification of Drug Target Gene: The isolated template DNA was amplified using *pncA* primers (P_1 5'GTC GGTCATGTTCGCGATCG and P_2

5'TCGGCCAGGT AGTCGCTGAT) in an authorized thermal cycler (Eppendorf Gradient Cycler). The PCR cycling parameters were 94 °C for 5 minutes; followed by 40 cycles of 94°C for 1 minute, 57°C for 1 minute and 72°C for 1 minute; and a final extension of 74°C for 10 minutes. The PCR was then kept at hold at 4°C for15 minutes. The amplified PCR product was withdrawn from thermal cycler and run on a 2% Agarose gel in TAE buffer. The Ethidium bromide stained gels were observed in a UV Trans illuminator and photographed using a Geldoc.

Agarose Gel Electrophoresis: The gel running tray was placed in a clean gel casting tray to form the gel uniformly and the comb was fixed at one end. 400mg of agarose (2%) powder was added to 20ml of 0.75x TAE and was boiled for few seconds to dissolve the agarose completely Less than 1µl of Ethidium bromide (0.5mg/ml) was added into the hand bearable heat 250 ml conical flask containing melted agarose gel and was poured into the gel running tray. 1µl of gel loading dye was transferred into a 5x5 cm size Para film. To it 5µl of polymerized DNA was added and was mixed thoroughly. The whole volume aliquot of amplified sample with gel loading dye was loaded into a well of 2% agarose gel in 0.75x TAE buffer and was subjected to electrophoresis for 30 minutes at 100 volts. The gel was observed under UV Transilluminator for specific DNA bands and was photographed. The DNA bands were identified according to the size by comparing with the molecular weight marker (100 bp DNA ladder) loaded in a separate lane.

Bioanalyser: DNA dye concentration and DNA gel matrix were allowed to equilibrate at room temperature. 25µl of dye concentration was added to DNA gel matrix.Vortexed and transferred to spin filter and Centrifuged at 2240g for 15 minutes. The gel dye was allowed to settle at room temperature for 30 minutes. A new DNA chip was placed on the chip priming station. 9µl of gel dye mix was pipetted into the well marked as G and the chip priming station was closed. The plunger was pressed down until it is held by the chip for 60 seconds. After 5 seconds the plunger was pulled back slowly to 1 ml position. The chip priming station was opened and 9µl of gel dye was pipetted into the well marked G and 1µl of ladder was added to the well labeled ladder. 5µl of marker was pipetted into all 12 sample wells and in ladder well. 1µl of sample was added into the well. The chip was placed in the Laser Induced Fluorescent instrument (LIF) and the results were interpreted.

PCR-SSCP Analysis: PCR products (5 ml containing about 0.5 to 1 mg of DNA) were denatured by boiling for 5 to 10 min in formamide dye (95% formamide, 10 mM sodium hydroxide, 20 mM EDTA and 0.05% Bromophenol blue and 0.05% xylene cyanol FF), followed by cooling on ice for 5 to 10 min. The denatured PCR products were loaded onto a 20% polyacrylamide gel (16 by 20 cm; containing 5% glycerol) that had been precooled to 48°C. Electrophoresis was performed in 0.53 TBE (Tris-borate-EDTA) buffer at a constant power of 5 W in a cold room overnight. The SSCP bands in the gel were visualized by silver staining [14].

DNA Sequencing: The amplified PCR product *pncA* gene from clinical isolate strains were run on 2% agarose gel and purify the PCR product using PCR purification kit (Invitrogen). The purified PCR product was directly sequenced in an automated DNA Sequencer at Bioserve in Bangalore. The nucleotide sequence obtained was analyzed using BLASTn Bioinformatics tool available at National Center for Biotechnology Information [15] to know the specificity of PCR amplification and to identify the nucleotide variation. The sequence was further subjected for BLASTx to know the amino acid changes in comparison with the wild type *Mycobacteruim tuberculosis* ($H_{37}Rv$).

RESULTS

Mycobacterial DNA was isolated from the L.J. medium slant and was subjected to PCR amplification using species specific primers, targeting the insertion sequence IS6110 (Mtb 5'G TGAGGGCATCGA GGTGG 3') (Mtb 5'CGTAGGCGTCGGTCACAAA 3') for conforming the *M.tuberculosis*. The PCR product was run on a 2% agarose gel. A clear band was formed at 123bp region confirming the presence of *M.tuberculosis* in the sputum specimen (Fig. 1). The templates of *Mycobacterium tuberculosis* clinical isolate and H37Rv wild type strain were amplified using *pncA* primers (P_1 5'GTC GGTCATGTTCGCGATCG and P_2 5'TCGGCCAGG TA GTCGCTGAT). The amplified PCR products were run on a 2% agarose gel. A clear band was formed at 222bp region, confirming the amplification of *pncA* region of *M. tuberculosis* (Fig. 2). The templates were analyzed on Bioanalyzer to purity and specificity of the PCR products and Electropherogram of PCR amplified *pncA* gene confirmed that the molecular size (222bp) of the products (Fig. 3).

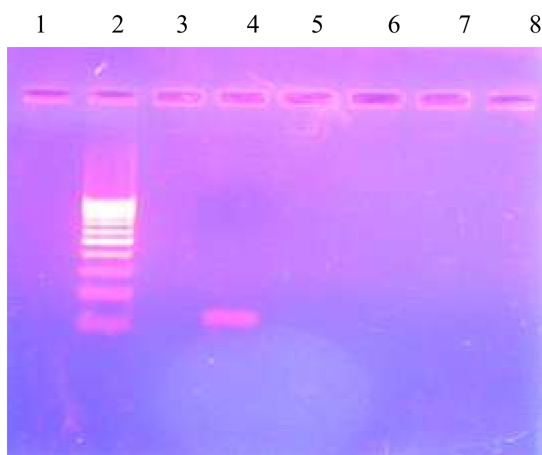

Fig. 1: Lane 4: 123 bp products amplified with IS6110 primer, Lane 2: 100 bp ladder

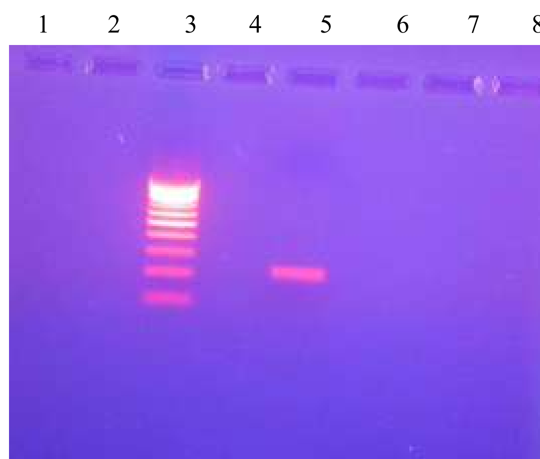

Fig. 2: Lane 3: 222bp PCR amplified *pncA* gene products, Lane 2: 100 bp ladder

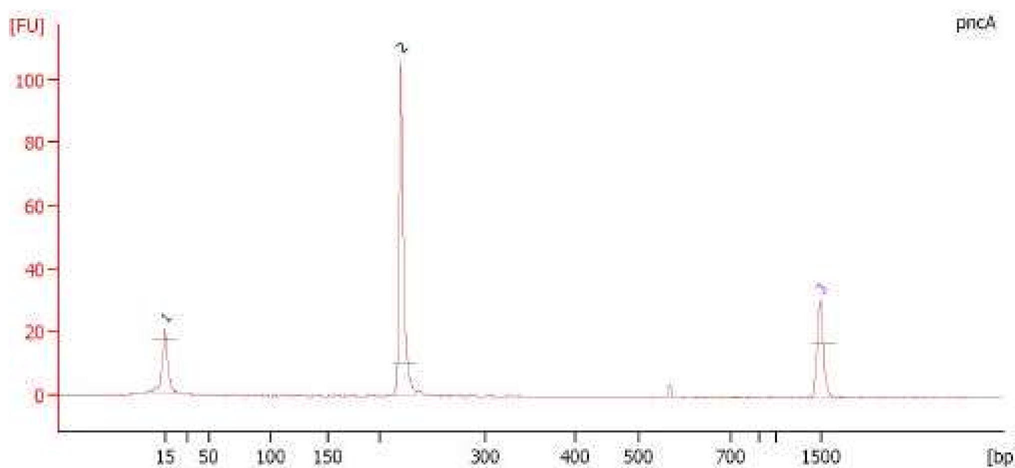

Fig. 3: Electropherogram of PCR amplified *pncA* gene (222bp)

We analyzed 50 PZA-resistant clinical *M.tuberculosis* isolates for potential mutations in the *pncA* gene to further define the molecular basis of PZA resistance and determine the frequency of *pncA* mutations among PZA-resistant *M. tuberculosis* strains. The results of the sequence analysis of the *pncA* gene from various PZA-resistant strains are presented in Table 1. Among 50 PZA-resistant strains analyzed, 39 had *pncA* mutations including nucleotide substitutions (missense mutations) or insertions and small deletions (nonsense mutations), causing amino acid substitutions in most cases or frame shifts leading to nonsense polypeptides. Overall, 18 types of mutations were found in the 39 PZA-resistant strains and these mutations were dispersed along the *pncA* gene. However, a certain degree of conservation of *pncA* mutations was observed at the

Table 1: Characteristics of PZA-resistant clinical *M. tuberculosis* isolates

Codons	*pncA* mutation	No. of Strains	Percentage	Susceptibility pattern
137	His→Pro	2	5.1	R
138	Cys→Tyr	2	5.1	R
139	Val→Leu	2	5.1	R
142	Thr→Met	2	5.1	R
5	Ile→Ser	2	5.1	R
12	Asp→Ala	4	10.3	R
26	Ala→Gly	2	5.1	R
51	His→Gln	2	5.1	R
69	Pro→Arg	3	7.7	R
72	Cys→Arg	3	7.7	R
85	Leu→Pro	6	15.4	R
96	Lys→Asn	3	7.7	R
132	Gly→Ser	3	7.7	R
141	Gln→Pro	1	2.6	R
142	Thr→Lys	1	2.6	R
171	Ala→Pro	1	2.6	R
		39		100

Table 2: Comparison of in vitro testing (on isolates) with PCR mediated direct sequencing

PCR Mediated Direct sequencing	In vitro sensitivity tests on L.J. slants			
	PZ sensitive		PZ Resistant	
	Smear (+) ve	Smear (-) ve	Smear (+) ve	Smear (-) ve
Resistant (39)	0	0	39	0
Sensitive (11)	11	0	--	--
Total (50)	11		39	

following amino acid residues: Asp12 → Ala, Leu85 → Pro, Gly132 → Ser and Thr142 → Lys or Met. Some degree of clustering of mutations (including both missense and nonsense mutations) was found at the following regions: Gly132-Thr142 and Pro69-Leu85. Eleven of 50 PZA-resistant strains did not contain detectable *pncA* mutations. Sequencing of 20 random PZA-susceptible *M. tuberculosis* strains did not reveal any silent mutations in the *pncA* gene. The resistant pattern of clinical isolates due to mutation through the PCR mediated DNA sequencing results were compared with in vitro sensitivity tests on L.J. slants and the results are presented in Table 2. This suggests that mutations in the *pncA* gene are indicative of PZA resistance, an important feature for the detection of PZA resistance on the basis of identifying mutations in the *pncA* gene.

DISCUSSION

The present study has shown that 39 of 50 PZA-resistant clinical *M. tuberculosis* isolates and 8 mutants made in vitro had mutations in the *pncA* gene. This indicates that the *pncA* mutation is the major mechanism of PZA resistance in *M. tuberculosis*, a finding consistent with previous observations that most PZA-resistant *M. tuberculosis* strains lack PZase activity [16, 17]. The nature of the *pncA* mutations includes substitution of amino acids (23 of 39 total PZA-resistant isolates with *pncA* mutations), insertions or small deletions of nucleotides causing nonsense peptides (15 of 39 isolates) and mutations in the *pncA* promoter (1 of 39 isolates). The distribution of *pncA* mutations is dispersed along the gene. Among the eleven resistant clinical isolates that did not contain *pncA* mutations, eleven were due to false resistance (i.e., they were susceptible upon retesting), a common problem of current. The *pncA* mutations identified in various PZA-resistant strains in this study are presumed to be responsible for the PZA resistance, as previously shown by transformation studies with a functional *pncA* gene. Yet, how PZA interacts with the PZase enzyme leading to activation of PZA to POA and how mutations affect PZase activity and thus its inability to activate PZA are unknown. Site-directed mutagenesis along with crystallography studies of both wild-type and mutant PZase enzymes will provide insight into the structure-function relationship of this enzyme. This information will help us to better understand the mechanism of action of PZA and resistance to PZA. Our finding that most PZA-resistant *M. tuberculosis* strains have mutations in the *pncA* gene has implications for developing a rapid test for detecting PZA-resistant *M. tuberculosis* strains. The diversity of methods currently used in clinical laboratories for the detection of PZA resistance in *M. tuberculosis* isolates causes inconsistent results of PZA susceptibility testing [18]. Inconsistent results of PZA susceptibility testing have been reported by a number of laboratories by various methods, including the qualitative BACTEC test [19]. On the basis of our analyses of the PZA-resistant clinical isolates, there is a very good correlation between the loss of PZase activity and *pncA* mutations and PZA resistance. This feature is important for designing PCR based tests for the rapid detection of *pncA* mutations as a correlate of PZA resistance. Analysis of the *pncA* sequence has found that five PZA-resistant strains determined by the conventional method are in fact susceptible, indicating that the sequence-based test, e.g., direct sequencing by PCR, may be more accurate or reliable. We have demonstrated in this study that *pncA* mutations in PZA-resistant strains can be readily detected by the PCR followed by direct sequencing technique within short period. Thus, detection of *pncA* mutations by direct sequencing is not only fast but also will avoid the problems of current PZA susceptibility testing. This should be useful for directing the treatment of tuberculosis, reducing treatment costs and potentially limiting the spread of drug-resistant *M. tuberculosis* isolates.

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