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Characterization of Pnca Gene Responsible for Pyrazinamidase Enzyme in *Mycobacterium tuberculosis* Clinical Isolate

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Abstract: Currently the detection of drug resistance in *M. tuberculosis* is primarily based on phenotypic drug susceptibility testing, which involves time consuming culture of the slow growing *Mycobacterium tuberculosis* bacilli in the presence of antibiotics With the increased understanding of the genetic mechanisms of *M. tuberculosis* drug resistance and the advancement of *M. tuberculosis* in recent years, a number of more rapid molecular methods to detect mutations in genes implicated in *M. tuberculosis* drug resistance have been developed providing a rapid alternative These methods include direct DNA sequencing Single Strand Confirmation Polymorphism analysis Polymerase Chain Reaction based universal heteroduplex generator assay and Allele specific PCR assay.

Key words: Mycobacterium tuberculosis · Antibiotics · Drug · Resistance · Mutations

INTRODUCTION

Tuberculosis (abbreviated as TB for tubercle bacillus or Tuberculosis) is a common and often deadly infectious disease caused by *Mycobacteria*, mainly *Mycobacterium tuberculosis* which affects adults in developing country. Tuberculosis usually attacks the lungs (as pulmonary TB) but can also affect the central nervous system, the lymphatic system, the circulatory system, the genitourinary system, the gastrointestinal system, bones, joints, and even the skin. Other *Mycobacteria* such as *Mycobacterium bovis, Mycobacterium africanum, Mycobacterium canetti, and Mycobacterium microti* also cause tuberculosis, but these species are less common [1]. Most people infected with *M. tuberculosis* never develop active TB. Every year 1.8 million people in India develop tuberculosis (TB). India accounts for one-fifth of the global TB incidences are estimated to have the highest number of active TB cases the countries of the world. TB kills more adults than any other disease, accounting for almost 400,000 deaths annually. It mainly afflicts are in the economically years of their lives (15-54 years), huge social and economic disruption [2].

In recent decades, the dramatic spread of the HIV epidemic in sub-Saharan Africa has resulted in notification rates of TB increasing up to 10 times in some countries. The incidence of TB is also increasing in other high HIV prevalence countries, where the population with HIV infection and TB overlap [3].

The dramatic spread of the HIV epidemic in sub-Saharan Africa has resulted in notification rates of TB increasing up to 10 times in some countries [4]. *Mycobacterium bovis* and *Mycobacterium microti* are the causative agents of TB in animals, and can be transmitted to humans. Some particular strains isolated from goats and seals have been named *Mycobacterium caprae* and *Mycobacterium pinnipedi,* although sometimes they are identified as *M. bovis* subspecies or variants. It could be expected that the major evaluative shifts involved in adaptation to different hosts would have entailed significant microbiological differentiation. However, the above mentioned agents of TB together with the vaccine bacilli Calmette-Guérin (BCG) strains rank close to each other along a phenotypically continuous taxon [5,6]. Phenotypic differentiation is consistently clear-cut between the extreme species within the taxon, *M. tuberculosis* and *M. bovis,* but differences between species comprised within these two extremes are much less defined. Mycobacterium tuberculosis divides every 12 to 24 hours [7]. This pace is extremely slow compared to that of most cultivable bacteria, which duplicate at

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regular intervals ranging from about 15 minutes to one hour. Recently [5]. Nicely exposed the low multiplication rate of the tubercle bacillus. These authors demonstrated the small proportion of cells initiating the sepration process prior to division among *tubercle bacilli* growing either in broth or inside macrophages [8]. The microscopic, conventional and other immunological methods have lower in specificity and sensitivity. The conventional methods for identifying *Mycobacterium tuberculosis* is a time consuming as it has higher generation time and needs much more infrastructure and care to avoid cross contamination [9]. Biochemical characterization and drug susceptibility has acquired extra time to give prompt result. Therefore the following studies were planned with the objective to identify the nucleotide variation and the corresponding changes in amino acid sequence of the protein involved in drug metabolism specifically *rpsL* gene.

MATERIALS AND METHODS

Mycobacterium Tuberculosis Isolation: The sputum sample was collected from the patient admitted in Government Hospital for Chest Disease at Puducherry and the sample was processed in Department of Microbiology of this hospital for, culture and sensitivity, Isolation and identification, DNA extraction. PCR amplification and agarose gel electrophoresis.

Sputum Sample Smearing: A clean grease free slide was taken and marked with a diamond pencil on one end. The thickest purulent part of the sputum sample was taken using a clean broomstick and smeared on about two-third portion of the slide. A circular movement was used to spread the specimen.The slides were allowed to air dry and then transferred to a hot plat (80°C), to fix the smear. Ziehl Nielsen staining, Fluorescent staining, Niacin Test, Nitrate Reduction Test, Catalase Test, Tween 80 Hydrolysis Test, PNB Test,

Mycobacterium DNA Extraction: Three loopful of culture was taken in 100µl of distilled water and was homogenized. The entire homogenized samples were treated with 50 µl of lysozyme at 37 °C for overnight incubation. 70µl of 14% SDS and 6µl of Proteinase K was added to precipitate the proteins and was incubated for 15 minutes at 65°C. 10 µl of 5 M NaCl and 80 µl of CTAB/NaCl was 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 20ºCentrifuged for 10 minutes at 12000 rpm at 4 °C. 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.

PCR Amplification Without Drug: The isolated template DNA was amplified using IS6110 primer in an authorized thermal cycler (Eppendorf). This confirms the template DNA as *Mycobacterium tuberculosis*. The PCR reaction mixture was set up as follows:
Primer for *Mycobacterium IS6110* amplification

F 5'GTGAGGGCATCGAGGTGG 3' (10pmol/µl)
R 5'CGTAGGCGTCGGTCACAAA 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 74 °C for 1 minute; and a final extension of 74°C for 5 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.

Isolation of *Pnca* Gene from Clinical Isolate Strain: Mycobacterial DNA was extracted as stated above and the Taq polymerase, dNTPs, $Mgcl_2$, Milli Q water, 10x Buffer, DMSO, template, forward and reverse primer were used for amplification of each respective gene as mentioned in the below table. 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 74 °C for 1 minute; and a final extension of 74 °C for 5 minutes. The PCR was then kept at hold at 4 °C for 15 minutes.

Agarose Gel Electrophoresis: The 2 sides of the gel-casting tray were sealed with cello tape 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-casting tray. 1µl of Gel loading dye was transferred into a 5x 5 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 TAE buffer and was subjected to electrophoresis in gel boxes for 30 minutes at 100 volts. The gel was observed under UV Trans illuminator 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.

DNA Sequencing Analysis: The amplified PCR product such as *pncA* gene from clinical isolate strain were run on 2% agarose gel and elute the DNA from the gel by Gel cleanup kit. The purified PCR product was directly sequenced in an automated DNA Sequencer (Bioserve Bangalore).The nucleotide sequence obtained was analyzed using BLASTn Bioinformatics tool available at National Center for Biotechnology Information. 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 *Mycobacterium tuberculosis* (H_{37}Rv).

RESULTS

Polymerase Chain Reaction Without Drug: *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'GTGAGGGCATCGAGGTGG 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 culture (Fig. 1).

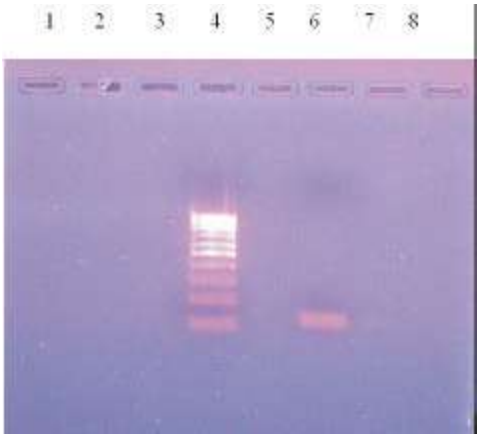

Fig. 1: 123 bp products amplified with IS6110 primer. Lane 4 100 bp ladder

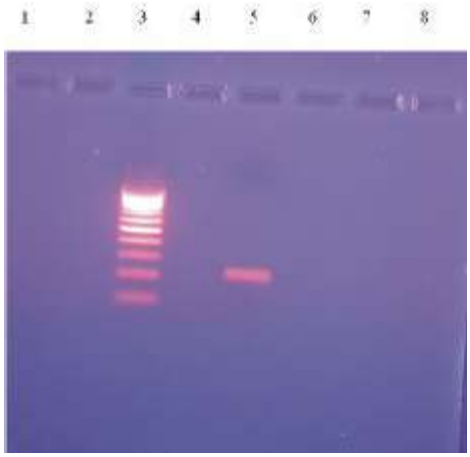

Fig. 2: PCR products of *pnc*A Lane3: 100 bp ladder. Lane-6amplification of *pncA gene*

Isolation and Amplification of *pncA* gene: Mycobacterial DNA was isolated from the *Mycobacterium tuberculosis* clinical isolate and H_{37}Rv wild type strain. The templates DNA were amplified using *pncA* primers (P_1 5'GTC GGTCATGTTCGCGATCG, and P2-5'TCGGCCAGGTAGTCGCTGAT). The amplified PCR product of H_{37}Rv and clinical isolates 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).

Drug Susceptibility test (Proportion Sensitivity test Method): Pure culture of *Mycobacterium tuberculosis* isolated from LJ medium were subjected to drug sensitivity testing using the proportion method. Individual colonies on Pyrazinamide drug slants, drug free L.J slants and drug free acid slants were counted and tabulated in (Table 2).

Table 1: Growth of *M.tuberculosis* Pyrazinamide drug slants, drug free L.J slants and drug free acid slants

sp	With drug	Drug free	L.J.Slants
S1	TNTC	TNTC	98
S2	TNTC	TNTC	27
S3	67	65	9
S4	32	19	-

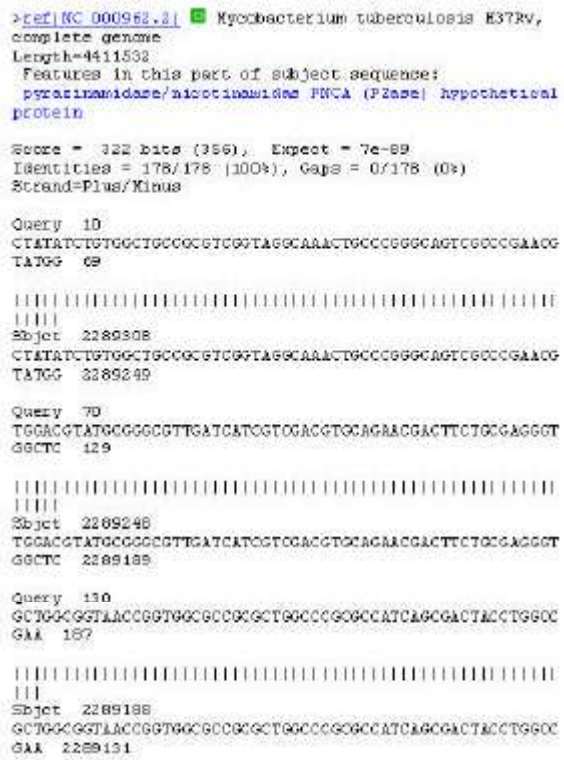

Fig. 3: BLASTx Analysis of *pncA* gene (-strand)

Percentage of drug resistant bacilli present in the bacterial population

$$= \frac{\text{No. of CFU on drugslopes} \times \text{Dilution Factor}}{\text{No. of CFU on drug-free slopes} \times \text{Dilution factor}} \times 100$$

$$\text{Percentage of Pyrazinamide resistent bacilli} = \frac{18 \times 10}{11 \times 1000} \times 100 = 1.6\%$$

Since the percentage of resistance is greater than 1%, the strain was considered to be resistant to Pyrazinamide drug.

DNA Sequencing: The desired PCR product (*pncA* gene) to be sequenced was eluted from the gel by Gel cleanup kit. The purified PCR product was directly sequenced in an automated DNA Sequencer. Sequenced DNA of mutant strain was compared with $H_{37}Rv$

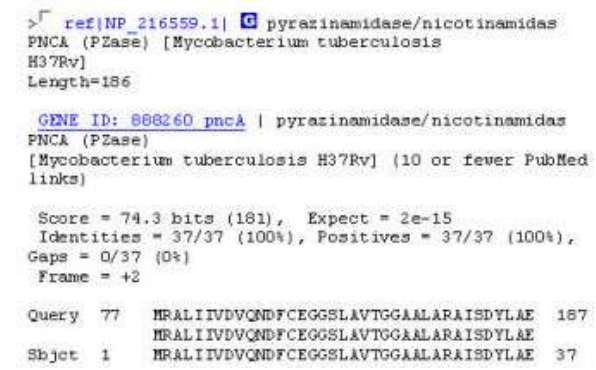

Fig. 4: BLASTn Analysis of *pncA* gene (+strand)

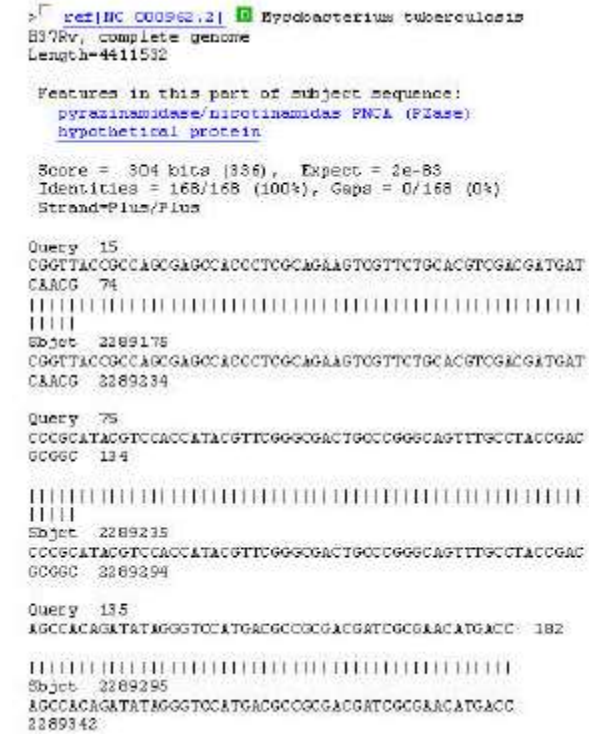

Fig. 5: BLASTx Analysis of *pncA* gene (+strand)

(wild type) strain DNA sequence using Bioinformatics tool BLASTn available at National Centre for Biotechnology Information. For detecting the mutation .Blastn and Blastx alignments of genes of interest are indicated in (Fig. 2,3,4 & 5).

>29pnCA_F
GGGGGTGGACTATATCTGTGGCTGCCGCGTCGGTAG
GCAAACTGCCCGGGCAGTCGCCCGAACGTATGGTG
GACGTATGCGGGCGTTGATCATCGTCGACGTGCAG
AACGACTTCTGCGAGGGTGGCTCGCTGGCGGTAACC
GGTGGCGCCGCGCTGGCCCGCGCCATCAGCGACTAC
CTGGCCGAA

>29pnCA_R
GGGGCCCCGGCGCACGGTTACCGCCAGCGAGCCACC
CTCGCAGAAGTCGTTCTGCACGTCGACGATGATCA
ACGCCCGCATACGTCCACCATACGTTCGGGCGACT
GCCCGGGCAGTTTGCCTACCGACGCGGCAGCCACAG
ATATAGGGTCCATGACGCCGCGACGATCGCGAACA
TGACC

DISCUSSION

Pyrazinamide (PZA) is important first-line tuberculosis (TB) drug. It also called DOTS recommended by the World Health Organization. PZA plays a unique role in achieving this shortened therapy, because PZA is believed to kill a population of semi dormant tubercle bacilli residing in an acidic environment *in vivo* that may not be affected by other TB drugs .The activity is only present at acidic pH [10]. Structurally, PZA is an analog of nicotinamide. Like isoniazid PZA is a prodrug. It requires conversion to POA by bacterial pyrazinamidase (PZase) in order to affect the tubercle bacilli Loss of PZase activity is observed in *Mycobacterium tuberculosis* strains that are resistant to PZA, and indeed, there is a very good correlation between PZA resistance and loss of this enzyme activity [11].

Pyrazinamide is a prodrug that must be activated by bacterial pyrazinamidase (PZase) to the active form pyrazinoic acid (POA), which is toxic to *Mycobacterium tuberculosis* [12]. Although a specific target for POA remains unknown, it has been suggested that POA accumulation results in a pH reduction which leads to a non-specific inhibitory effect on cellular metabolism recently, it has been shown that POA can disrupt *M. tuberculosis* membrane potential, Identification of this *pncA*-regulatory gene may be useful for designing a molecular test for better detection of PZA-resistant strains. However, these alternative resistance mechanisms have yet to be identified. The clinical *M. tuberculosis* isolated in this study had no mutation in the *pncA* gene, which was detected by direct sequencing in an automatic sequencer, and was compared with the normal wild-type $H37R_V$ strain using BLASTn sequences for detecting the mutation to confirm the drug resistance [13].

No such nucleotide variations were observed in sequenced region. There were no amino acids changes were found in the concern regions, when the same gene was compared with wild type H37RV strain using BLASTx. There is no change either in nucleotide amino acids in this strain and thus may susceptibility to Pyrazinamide drug [14].

In this study, molecular characterization of *pncA* from a clinical isolate strain was performed. Sputum samples obtained for the study were first identified for the presence of *M. tuberculosis* by standard diagnostic procedures like AFB staining, Fluorescent staining, PCR using IS6110 amplifying primers and sputum culture [15]. The *Mycobacterium tuberculosis* was confirmed by various standard biochemical tests and it was subjected to standard sensitivity test by proportional sensitivity test method. The samples which are found to be resistant by conventional method towards the drugs such as pyrazinamide was subjected for PCR amplification of *pncA* gene [16]. The obtained sequences were subjected to sequence analysis using BLASTn to find out the specificity of amplification as well as to identify the nucleotide variations in *pncA*.The same sequences were subjected for BLASTx to found out the amino acid changes.

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REFERENCES

1. Chauhan, A., M.V. Madiraju and M. Fol, 2006. *Mycobacterium tuberculosis* cells growing inmacrophages are filamentous and deficient in FtsZ rings. J. Bacteriol., 188: 390-406.
2. Chambers, H.F., D. Moreau and D. Yajko, 1995. Can *penicillins* and other beta-lactam antibiotics is used to treat tuberculosis. Antimicrob Agents Chemother, 39: 2620-4624.
3. David, H.L., M.T. Jahan, J. Grandry and E.H. Lehmann, 1978. Numerical taxonomy of Mycobacterium africanum. Int. J. System Bacteriol., 28: 467-472.
4. Wayne, L., 1974. Simple pyrazinamidase and urease tests for routine identification of *Mycobacteria.* Am. Rev. Respir. Dis., 1: 147-151.
5. Chauhan, A., M.V. Madiraju and M. Fol, 2006. *Mycobacterium tuberculosis* cells growing inmacrophages are filamentous and deficient in FtsZ rings. J. Bacteriol., 188: 390-406.

6. Chambers, H.F., D. Moreau and D. Yajko, 1995. Can *penicillins* and other beta-lactam antibiotics is used to treat tuberculosis. Antimicrob Agents Chemother. 39: 2620-4624.
7. Amanda, M., T. Howard, V. Peter, S. Jean, P. Juan Carlos and P. Françoise, 2006. A new rapid and simple colorimetric method to detect *pyrazinamide* resistance in *Mycobacterium tuberculosis* using *nicotinamide*. Journal of Anti microbial Chemotherapy, 58: 327-331.
8. Brennan, P.J. and P. Draper, 1994. Ultra structure of *Mycobacterium tuberculosis In* Bloom BR (ed).Tuberculosis, Pathogenesis, Protection and Control. American Society for Microbiology, Washington, pp: 271-284.
9. David, H.L., M.T. Jahan, J. Grandry and E.H. Lehmann, 1978. Numerical taxonomy of *Mycobacterium africanum*. Int. J. System Bacteriol., 28: 467-472.
10. Hirano, K., M. Takahashi, Y. Kazumi, Y. Fukasawa and C. Abe, 1998. Mutation in *pncA* is a major mechanism of pyrazinamide resistance in *Mycobacterium tuberculosis*. Tuber. Lung Dis., 78: 117-122.
11. Leonid, H. and T. Sanchez, 2000. New Agar Medium for Testing Susceptibility of Mycobacterium *tuberculosis* to Pyrazinamide. Journal of Clinical Microbiology, 38: 1498-1501.
12. Harshey, R.M. and T. Ramakrishnan, 1977. Rate of ribonucleic acid chain growth in *Mycobacterium tuberculosis* H37Rv. J. Bacteriol., 129: 616-622.
13. Konno, K., F.M. Feldmann and W. McDermott, 196. Pyrazinamide susceptibility and amidase activity of tubercle bacilli. Am. Rev. Respir. Dis., 95: 461-469.
14. Mahapatra, S., J. Basu, P Brennan and D. Crick., 2005. Structure, biosynthesis, and genetics of theMycolic Acid-Arabinogalactan-Peptidoglycan complex *In* Tuberculosis and the *Tubercle bacillus*. ASM Press, Washington. pp: 275-285.
15. Mary, M. and Y. Zhang, 2006. Effects of weak acids, UV and proton motive force inhibitors on pyrazinamide activity against Mycobacterium tuberculosis *in vitro* JAC Advance Access originally published online. 82: 123-132.
16. Fischer, S.G. and L.S. Lerman, 1979. Analysis of point mutations by denaturing gradient gel electrophoresis. Proc. Natl. Acad. Sci. USA. 80: 1579-1583.