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Pyrazinamide Drug Resistance Patterns in Multi Drug Resistant *Mycobacterium tuberculosis* Isolates from India

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Inclusion of Pyrazinamide (PZA) in Tuberculosis (TB) chemotherapy has significantly shortened the treatment duration to 6 months. This is the only drug which is used in the treatment of both drug sensitive as well as Multi Drug Resistant (MDR) form of Tuberculosis. Although resistance to PZA is associated with poor treatment outcome Drug Susceptibility testing (DST) of PZA is not routinely performed due to technical difficulties in public health laboratories in India. The current study was undertaken to determine the proportion of PZA resistance in MDR and Non MDR TB and to propose the most feasible test that can be adopted for detecting PZA resistance. Sputum samples from 117 MDRTB suspects were tested for multi drug resistance using Line Probe Assay (LPA) and PZA DST was performed by MGIT and Pyrazinamidase enzyme activity was analyzed. 6.8% of the samples tested were MDR and almost 43% of them were resistant to Pyrazinamide. The pncA gene was sequenced for all isolates exhibiting phenotypic resistance. Nearly half of the MDRTB cases were resistant to PZA, indicating the need to customize the MDRTB regimen based on the PZADST result.

Keywords: Pyrazinamide drug susceptibility testing, Multi drug Resistant, *Mycobacterium tuberculosis*, LPA, Modified Wayne's assay.

Pyrazinamide is an important drug in anti TB treatment because of its high in vivo sterilizing activity against *Mycobacterium tuberculosis* particularly in acidic pH. Its combination with Rifampicin (R) and Isoniazid (H) in the standard TB treatment has reduced the duration of therapy from 12 to 6 months [1]. PZA, like Isoniazid and Ethionamide, is a pro drug which must be converted into its active form, Pyrazinoic acid. The conversion into an active form is mediated by the enzyme called Pyrazinamidase (PZase) encoded by the *pncA* gene [2, 3]. The literature evidences available clearly indicate that mutations in the *pncA* gene significantly affect the activity of PZase thus conferring drug resistance [4, 5, 6]. For instance, *Mycobacterium tuberculosis* isolates which were found to be resistant to Pyrazinamide had only minimal or no PZase activity. As, PZA is used for both first and second line anti TB treatment, understanding the prevalence of PZA resistance pattern is certainly helpful to improve the TB treatment regimen.

Many studies have demonstrated a high prevalence of PZA resistance among MDR-TB patients in different localities, ranging from 10% in Papua New Guinea and 25% in Turkey, to 49% in Thailand, 50% in Central Africa, 52% in South Africa, 53% in Japan, 55% in Taiwan, 77% in Pakistan and 85% in South Korea and in India [7]. As stated above, the prevalence of PZA resistance pattern in different countries ranges from 10-85% among MDR TB patients. Such variation may be due to widespread and indiscriminate use of PZA

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in anti TB treatment regimen or overestimation of the proportion of PZA resistance due to false resistant results in the PZA DST [8].

Although the CLSI guidelines 2003 have recommended the use of BACTEC Mycobacterial Growth Indicator Tube (MGIT) 960 for PZA DST, the technique does not seem to yield reliable/ reproducible results [9, 10, 11]. Results are dependent on size and quality of inoculums and the concentration of the PZA drug used in the DST [12, 13]. In addition, the culture medium has to be acidified which in turn affects the growth of *Mycobacterium tuberculosis* [14]. Due to these technical challenges many laboratories refrain from performing the PZA DST.

MDRTB treatment comprises of 6 drugs for 6-9 months in the intensive phase and 4 drugs in the continuation phase of treatment. PZA is the only drug which is used both in the treatment of drug sensitive and drug resistant form of Tuberculosis. In previously treated tuberculosis cases, the prevalence of PZA resistance is expected to be high due to prior exposure to the drug. For such cases it would be unwise to include PZA in the MDRTB treatment regimen without knowing the in vitro drug susceptibility pattern. Moreover, many clinicians expect a favorable MDRTB treatment outcome if the organism is susceptible to PZA. Hence, knowledge of the susceptibility pattern of PZA prior to initiation of MDRTB treatment is useful in guiding the clinician to determine the course of MDRTB treatment. Currently, under the Revised National TB Control Program (RNTCP), MDRTB treatment is initiated without PZA DST.

In light of the above situation, the present study was undertaken to determine the proportion of PZA drug resistance pattern in MDRTB suspects using the most reliable, phenotypic or Genotypic technique. It is believed that this data will be valuable to make policy decisions to test for PZA susceptibility before initiating MDRTB treatment.

MATERIALS AND METHODS

The study was performed at a public health reference laboratory for Tuberculosis. The laboratory caters to a population of about 10million for providing MDRTB diagnosis. For the current study, sputum samples received from a single tuberculosis centre over a period of month was only considered. A prospective study was

Fig. 1. Modified Wayne's test: 20mins after adding 1% Ferrous ammonium sulphate

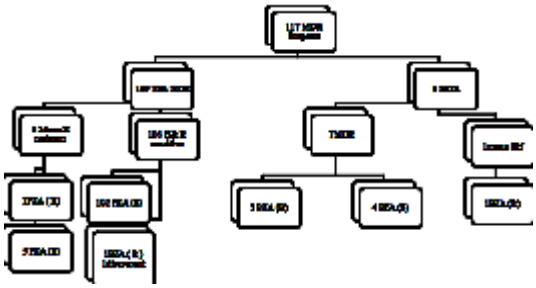

Fig. 2. Correlation of PZA DST and MDRTB

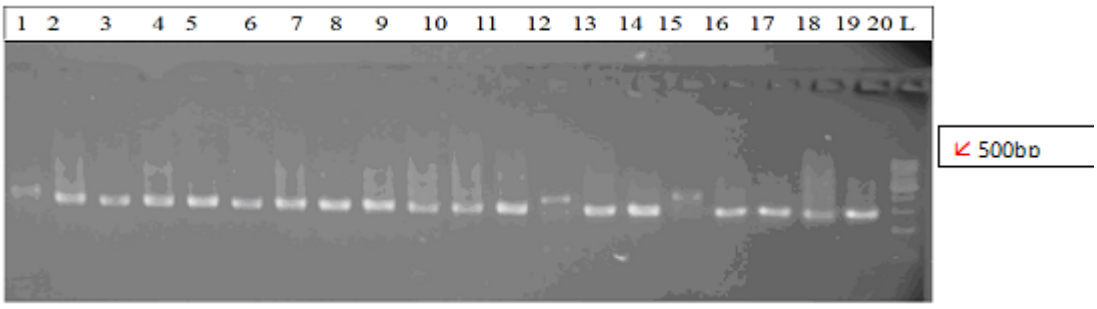

Fig. 3. Lane description of samples 1- 20, L- 100bp ladder

undertaken on 117 sputum samples. All smear positive samples were subjected to the Genotype MTBDRplus assay (Hain Lifescience, Nehren, Germany) to detect multi drug resistance. Following this, all samples were cultured and PZA DST was set up using the modified Wayne's assay and MGIT. pncA gene was sequenced for all isolates exhibiting PZA resistance in vitro.

Sample digestion and decontamination

Sputum samples were processed in a Class II Bio safety cabinet in a BSL2 laboratory. Specimens were subjected to digestion and decontamination by the N-acetyl-L-cysteine-sodium hydroxide method [15]. Following centrifugation, the pellet was suspended in 1.0 ml of phosphate buffer (pH 6.8). A concentrated smear was prepared and examined using a fluorescent microscope and graded according to International Union Against Tuberculosis and Lung Disease (IUATLD) guidelines [16]. One loop full (5mm internal diameter) of the sediment was inoculated on to Lowenstein Jensen (LJ) Medium. The remaining pellet was taken up for LPA.

Culture on Lowenstein Jensen Medium

The inoculated LJ slopes were incubated at 37^0 C for a maximum of 8 weeks and they were checked weekly for appearance of rough, tough and buff colored colonies of *Mycobacterium tuberculosis.* The growth was confirmed to be *Mycobacterium tuberculosis* by an Immuno chromatic test (SD Bioline).

Geno Type MTBDR plus assay

LPA was performed directly on samples which were smear positive. Whereas for smear negative samples, LPA for performed after obtaining growth on LJ medium. The MTBDRplus was performed according to the manufacturer's instructions [17]. The test is based on DNA strip technology and has three steps: DNA extraction, multiplex polymerase chain reaction (PCR) amplification, and reverse hybridization. 500µl of the decontaminated sediment was used for DNA extraction. The organisms present in the sediment were heat killed by placing the tubes in a water bath which was set at 95^0 C. Following this, the cells were subjected to sonication and centrifugation. The supernatant containing DNA was carefully removed. The amplification and Hybridization procedure was performed as per the manufacturer's instructions. After hybridization the nitrocellulose strips were removed and fixed on paper using a transparent cellotape. Each strip consists of 27 probes, including six controls (conjugate, amplification, M. tuberculosis complex, rpoB, katG, and inhA controls), eight rpoB wild-type (WT) and four mutant (MUT) probes. The eight rpoB WT's code for regions 506-509, 510-513, 513-517, 516-519, 518-522, 521-525, 526-529, 530-533. MUT 1 codes for D516V, MUT2A for H526Y, MUT2B for H526D and MUT3S531L. The katG WT1 codes for region 315 and MUT1 & 2 code for S315T1 and S315T2 respectively. The inhA has two wildtype probes WT1 & 2 which code for -15/-16 and -8. The strip consists of four mutations probes for inhA. MUT1 codes for C15T, MUT2 codes for A16G, MUT3A codes for T8C and MUT3B codes for T8A. Results were interpreted according to the manufacturer's instructions and reported to the concerned authorities for appropriate treatment initiation.

Modified Wayne's Enzymatic Assay

The procedure for performing modified Wayne's enzymatic assay was obtained from Singh et al., 2007. Heavy growth of *Mycobacterium tuberculosis* isolates was required for performing the test. The assay medium consisted of Modified Middlebrook 7H9 broth, 400¼g of PZA drug powder and 1.5% of agar agar. Initially the medium was autoclaved at 121 ^{0}C for 20 minutes and then allowed to cool to 60^0 C. Subsequently, the growth supplement was added to the medium. 4ml of the molten medium was then poured into Mc Cartney bottles and kept upright to solidify. After solidification, the entire batch of media was placed in the incubator at 37^0C for sterility check. Change in color of the uninoculated medium indicates contamination in the medium. Subsequently, the medium was inoculated by stabbing it 3-4 times with 2-3 loopfulls of *Mycobacterium tuberculosis* growth. The inoculated tubes were incubated at 37^0 C for 4 days. The tubes were tested for the PZase enzyme activity on the fourth day by the following technique. 1ml of freshly prepared 1% ferrous ammonium sulphate was added to the medium. The tubes were observed for 20 mins. Pink to red color band in the sub surface of the medium indicates the presence of active PZase enzyme and is able to hydrolyze the PZA drug into Pyrazinoic acid. The isolate was declared sensitive to PZA. In case of absence of a pink color band within 20

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gi|H37Rv|gb|M.tb  GTCGGTCATGTTCGCGATCGTCGCGGCGTCATGGACCCTATATCTGTGGCTGCCGCGTCG  60
gi|R1|gb|SRL      GTCGGTCATGTTCGCGATCGCAGTGAGCAGCCGGACCGTGTACTGGTACAGCGCTTCAC-
gi|R4|gb|SRL      GTCGGTCATGTTCGCGATCGTCGCGGCGTCATGGACCCTATATCTGTGGCTGCCGCGTCG
gi|R2|gb|SRL      GTCGGTCATGTTCGCGATCGTCGCGGCGTCATGGACCCTATATCTGTGGCTGCCGCGTCG
gi|R3|gb|SRL      GTCGGTCATGTTCGCGATCGTCGCGGCGTCATGGACCCTATATCTGTGGCTGCCGCGTCG
gi|R5|gb|SRL      GTCGGTCATGTTCGCGATCGTCGCGGCGTCATGGACCCTATATCTGTGGCTGCCGCGTCG
gi|R6|gb|SRL      GTCGGTCATGTTCGCGATCGTCGCGGCGTCATGGACCCTATATCTGTGGCTGCCGCGTCG
gi|R7|gb|SRL      GTCGGTCATGTTCGCGATCGTCGCGGCGTCATGGACCCTATATCTGTGGCTGCCGCGTCG
gi|R8|gb|SRL      GTCGGTCATGTTCGCGATCGTCGCGGCGTCATGGACCCTATATCTGTGGCTGCCGCGTCG
gi|R9|gb|SRL      GTCGGTCATGTTCGCGATCGTCGCGGCGTCATGGACCCTATATCTGTGGCTGCCGCGTCG
gi|R10|gb|SRL     GTCGGTCATGTTCGCGATCGTCGCGGCGTCATGGACCCTATATCTGTGGCTGCCGCGTCG
gi|R11|gb|south   GTCGGTCATGTTCGCGATCGTCGCGGCGTCATGGACCCTATATCTGTGGCTGCCGCGTCG
gi|R12|gb|OR      GTCGGTCATGTTCGCGATCGTCGCGGCGTCATGGACCCTATATCTGTGGCTGCCGCGTCG
gi|R13|gb|OR      GTCGGTCATGTTCGCGATCGTCGCGGCGTCATGGACCCTATATCTGTGGCTGCCGCGTCG
gi|R14|gb|OR      GTCGGTCATGTTCGCGATCGTCGCGGCGTCATGGACCCTATATCTGTGGCTGCCGCGTCG
gi|R15|gb|SMS     GTCGGTCATGTTCGCGATCGTCGCGGCGTCATGGACCCTATATCTGTGGCTGCCGCGTCG
gi|R16|gb|SMS     GTCGGTCATGTTCGCGATCGTCGCGGCGTCATGGACCCTATATCTGTGGCTGCCGCGTCG
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gi|H37Rv|gb|M.tb  GTAGAAGAAACTGCCCGGGCAGTCGCCCGAACGTATGGTGGACGTATGCGGGCGTTGATC 120
gi|R1|gb|SRL      ----------------GGGCCGT--CCAGGACGGATCG---------------------
gi|R4|gb|SRL      GTAGGCA-AACTGCCCGGGCAGTCGCCCGAACGTATGGTGGACGTATGCGGGCGTTGATC
gi|R2|gb|SRL      GTAGGAA-GACTGCCCGGGCAGTCGCCCGAACGTATGGTGGACGTATGCGGGCGTTGATC
gi|R3|gb|SRL      GTAGGCA-AACTGCCCGGGCAGTCGCCCGAACGTATGGTGGACGTATGCGGGCGTTGATC
gi|R5|gb|SRL      GTAGGCA-AACTGCCCGGGCAGTCGCCCGAACGTATGGTGGACGTATGCGGGCGTTGATC
gi|R6|gb|SRL      GTAGGCA-AACTGCCCGGGCAGTCGCCCGAACGTATGGTGGACGTATGCGGGCGTTGATC
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gi|R8|gb|SRL      GTAGGCA-AACTGCCCGGGCAGTCGCCCGAACGTATGGTGGACGTATGCGGGCGTTGATC
gi|R9|gb|SRL      GTAGGCA-AACTGCCCGGGCAGTCGCCCGAACGTATGGTGGACGTATGCGGGCGTTGATC
gi|R10|gb|SRL     GTAGGCA-AACTGCCCGGGCAGTCGCCCGAACGTATGGTGGACGTATGCGGGCGTTGATC
gi|R11|gb|south   GTAGGCA-AACTGCCCGGGCAGTCGCCCGAACGTATGGTGGACGTATGCGGGCGTTGATC
gi|R12|gb|OR      GTAGGCA-AACTGCCCGGGCAGTCGCCCGAACGTATGGTGGACGTATGCGGGCGTTGATC
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gi|R15|gb|SMS     GTAGGCA-AACTGCCCGGGCAGTCGCCCGAACGTATGGTGGACGTATGCGGGCGTTGATC
gi|R16|gb|SMS     GTAGGCA-AACTGCCCGGGCAGTCGCCCGAACGTATGGTGGACGTATGCGGGCGTTGATC
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gi|H37Rv|gb|M.tb  ATCGTCGACGTGCAGAACGACTTCTGCGAGGGTGGCTCGCTGGCGGTAACCGGTGGCGCC 180
gi|R1|gb|SRL      -TCGTGCAC------GGC------GGCCAGGGTGCCCCCGTTCGCATGA--------CGG
gi|R4|gb|SRL      ATCGTCGACGTGCAGAACGACTTCTGCGAGGGTGGCTCGCTGGCGGTAACCGGTGGCGCC
gi|R2|gb|SRL      ATCGTCGACGTGCAGAACGACTTCTGCGAGGGTGGCTCGCTGGCGGTAACCGGTGGCGCC
gi|R3|gb|SRL      ATCGTCGACGTGCAGAACGACTTCTGCGAGGGTGGCTCGCTGGCGGTAACCGGTGGCGCC
gi|R5|gb|SRL      ATCGTCGACGTGCAGAACGACTTCTGCGAGGGTGGCTCGCTGGCGGTAACCGGTGGCGCC
gi|R6|gb|SRL      ATCGTCGACGTGCAGAACGACTTCTGCGAGGGTGGCTCGCTGGCGGTAACCGGTGGCGCC
gi|R7|gb|SRL      ATCGTCGACGTGCAGAACGACTTCTGCGAGGGTGGCTCGCTGGCGGTAACCGGTGGCGCC
gi|R8|gb|SRL      ATCGTCGACGTGCAGAACGACTTCTGCGAGGGTGGCTCGCTGGCGGTAACCGGTGGCGCC
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gi|R10|gb|SRL     ATCGTCGACGTGCAGAACGACTTCTGCGAGGGTGGCTCGCTGGCGGTAACCGGTGGCGCC
gi|R11|gb|south   ATCGTCGACGTGCAGAACGACTTCTGCGAGGGTGGCTCGCTGGCGGTAACCGGTGGCGCC
gi|R12|gb|OR      ATCGTCGACGTGCAGAACGACTTCTGCGAGGGTGGCTCGCTGGCGGTAACCGGTGGCGCC
gi|R13|gb|OR      ATCGTCGACGTGCAGAACGACTTCTGCGAGGGTGGCTCGCTGGCGGTAACCGGTGGCGCC
gi|R14|gb|OR      ATCGTCGACGTGCAGAACGACTTCTGCGAGGGTGGCTCGCTGGCGGTAACCGGTGGCGCC
gi|R15|gb|SMS     ATCGTCGACGTGCAGAACGACTTCTGCGAGGGTGGCTCGCTGGCGGTAACCGGTGGCGCC
gi|R16|gb|SMS     ATCGTCGACGTGCAGAACGACTTCTGCGAGGGTGGCTCGCTGGCGGTAACCGGTGGCGCC
                  **** **      *     ** ****** * *  *    * *

gi|H37Rv|gb|M.tb  GCGCTGGCCCGCGCCATCAGCGACTACCTGGCCGAA—                  216
gi|R1|gb|SRL      TAAATGTCGAACAGGATCAGCGACTACCTCCGCCGAAA
gi|R4|gb|SRL      GCGCTGGCCCGCGCCATCAGCGACTCTTGGCAAAA---
gi|R2|gb|SRL      GCGCTGGCCCGCGCCATCAGCGACTACCTGGCCGAA--
gi|R3|gb|SRL      GCGCTGGCCCGCGCCATCAGCGACTACCTGGCCGAA--
gi|R5|gb|SRL      GCGCTGGCCCGCGCCATCAGCGACTACCTGGCCGAA--
gi|R6|gb|SRL      GCGCTGGCCCGCGCCATCAGCGACTACCTGGCCGAA--
gi|R7|gb|SRL      GCGCTGGCCCGCGCCATCAGCGACTACCTGGCCGAA--
gi|R8|gb|SRL      GCGCTGGCCCGCGCCATCAGCGACTACCTGGCCGAA--
gi|R9|gb|SRL      GCGCTGGCCCGCGCCATCAGCGACTACCTGGCCGAA--
gi|R10|gb|SRL     GCGCTGGCCCGCGCCATCAGCGACTACCTGGCCGAA--
gi|R11|gb|south   GCGCTGGCCCGCGCCATCAGCGACTACCTGGCCGAA--
gi|R12|gb|OR      GCGCTGGCCCGCGCCATCAGCGACTACCTGGCCGAAA-
gi|R13|gb|OR      GCGCTGGCCCGCGCCATCAGCGACTACCTGGCCGAA--
gi|R14|gb|OR      GCGCTGGCCCGCGCCATCAGCGACTACCTGGCCGAA--
gi|R15|gb|SMS     GCGCTGGCCCGCGCCATCAGCGACTACCTGGCCGAA--
gi|R16|gb|SMS     GCGCTGGCCCGCGCCATCAGCGACTACCTGGCCGAA--
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Fig. 4. CLUSTAL O(1.2.1) multiple sequence alignment

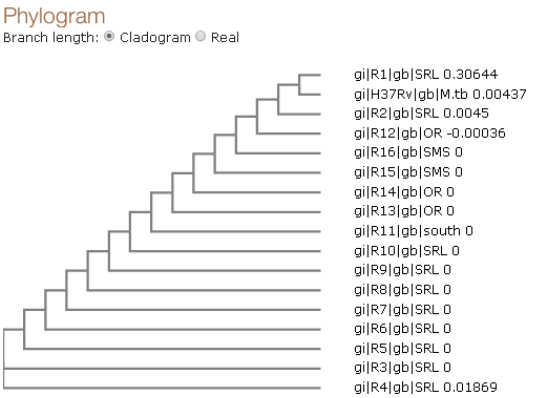
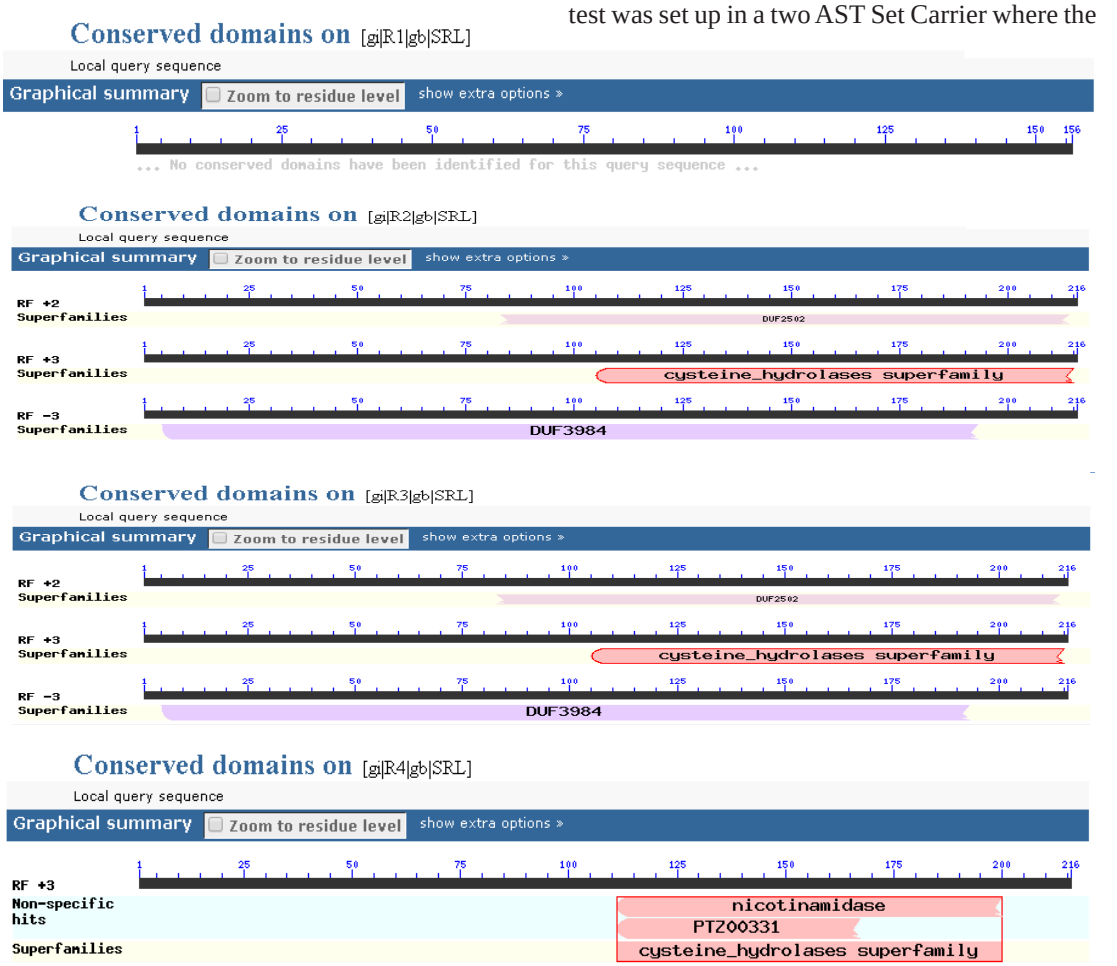


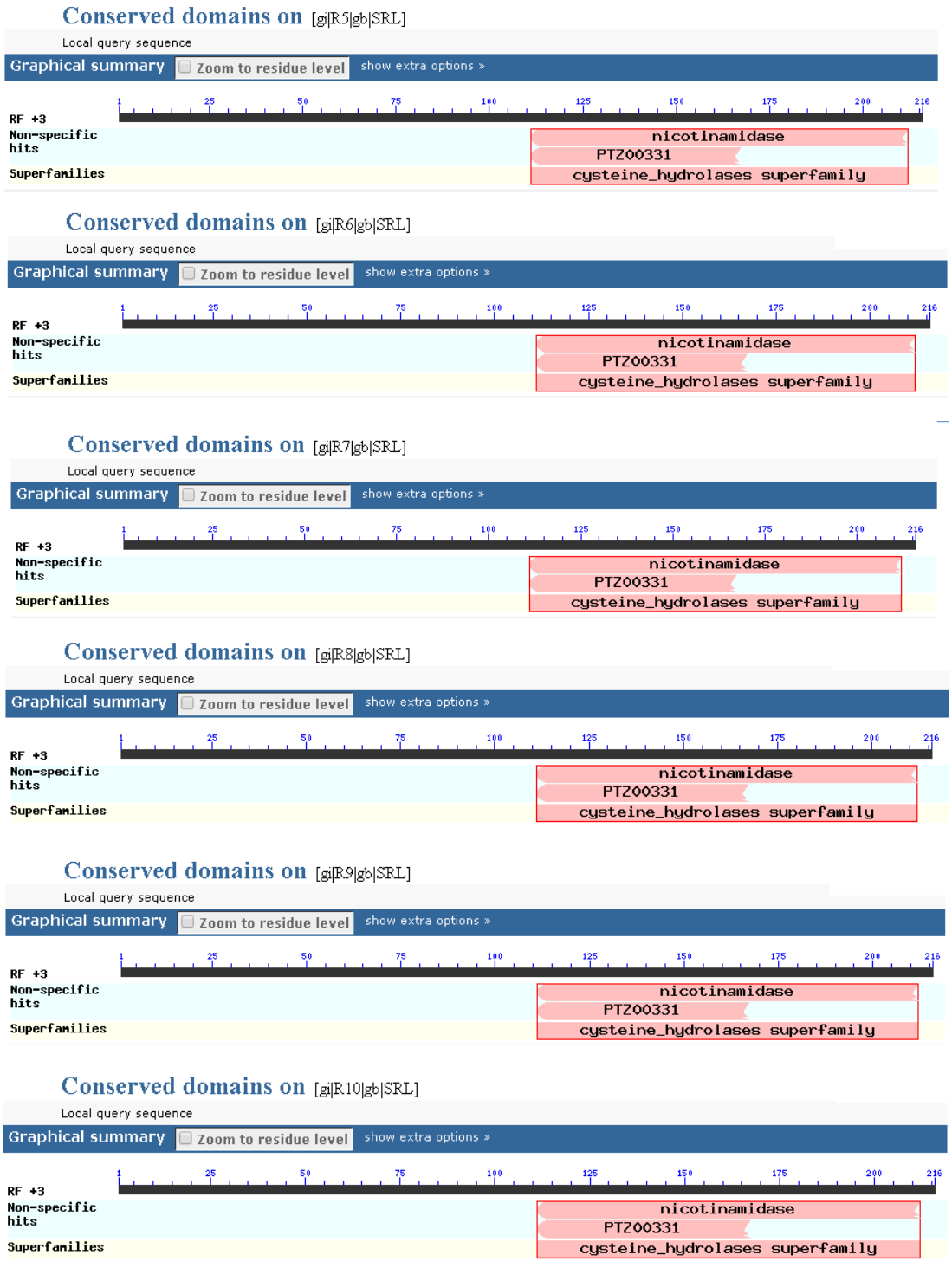
Fig. 5. ClustalW2 Phylogeny

mins, the tubes were placed at 4^0 C and observed after 4 hrs. The result was declared to be sensitive if a pink color band appeared in the medium and resistant if there was no development of pink color.

PZA Drug susceptibility testing by MGIT 960

The growth obtained from LJ medium was sub cultured onto a MGIT (Becton Dickinson, Sparks, MD) tube. A freshly positive MGIT tube was used for the PZA test. The media for performing the PZA drug susceptibility testing consists of the BACTEC MGIT 960 PZA (Becton Dickinson, Sparks, MD) medium tube (containing 7ml of Modified Middlebrook 7H9broth), the PZA drug kit (consisting of the lyophilized PZA drug, 20,000 ¼g) and growth supplement. The lyophilized drug was reconstituted with 2.5ml of sterile distilled water. As per the manufacturer's instructions, the test was set up in a two AST Set Carrier where the

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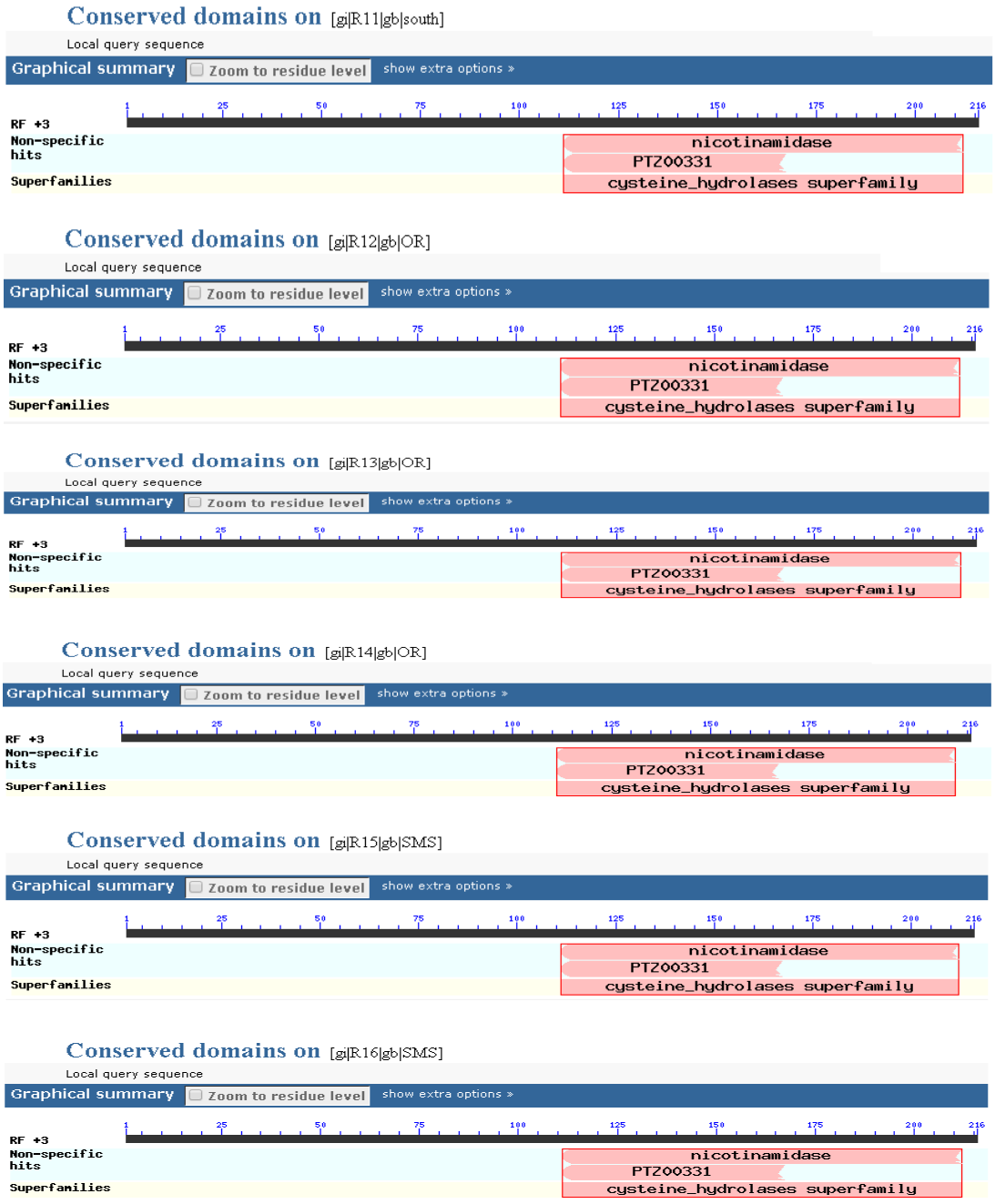

Fig. 6. Blast x analysis to determine change in protein

Growth Control and PZA tubes were placed in sequence. The test has a 21 day protocol and the machine declares results as "Resistant" or "Susceptible" (MGIT procedure manual 2006).

Sequencing of pncA gene

The *pncA* gene of 20 *Mycobacterium tuberculosis* isolates was sequenced along with H37Rv strain of *M.tb*. Isolates were grown in liquid culture medium and DNA was extracted using standardized methods. The DNA was amplified using *pncA* primers (P1 5'GTC GGTCATGTTCGCGATCG, and P2-5'TCGGCCAGGTAGTCGCTGAT) [18]. The PCR reaction mixture contained DNA (100ng) 1 ¼l, Forward primer (100ng ul) 2 ¼l, Reverse primer (100ng ul) 2 ¼l, dNTPs (2.5mM each) 2 ¼l, 10x Taq pol assay buffer 5¼l , Taq Polymerase (3u ¼l) 0.5 ¼l and Water to obtain a final reaction volume of 50 ¼l. The PCR cycle parameters were set at 94^0C for 5min , 94^0C for 1 min , 57^0C for 1min , 72^0C for 1min and 72^0C for 7min. The amplified PCR product was run on 2% agarose gel and an amplicon size of 222bp was obtained. The purified PCR product was sequenced using Applied biosystems 3500 (Chromous biotech, Bangalore). The chromatograms obtained were analyzed using the

Table 1. Reason for testing vs MDR status.

Category of patient	Reason for testing	Number of MDR (mono RIF)	Number of Non MDR(mono INH)	Total number of cases
1	Failure	1	6	7
2	Retreatment case smear positive at 4 months of treatment	1	3	4
3	Contact of known MDRTB case	0	3	3
4	S+ at diagnosis, re-treatment case	4(1)	52 (4)	61
5	Any follow up S+	0	15 (2)	17
6	S- at diagnosis, re-treatment case	1	8	9
7	HIV TB case	0	7	7
8	Private referral	0	9	9

Table 3. Correlation between MDR status, PZA drug susceptibility testing, PZase enzyme activity and nucleotide changes in the pncA gene.

MDR Status	PZA DST	PZase enzyme activity	pncA mutation nucleotide change
MDR (3)	R	R	A65G (3), A66C (2), G67A(2), 67 Deletion(1), 68deletion(2), A69G(1), A206C(1), C207T (1), C208T(1), T209G(1), G211C(1), C212A(1), C213A(1), C214A(1)
MDR(4)	S	S	A65G(3), C66A(1), A66C(2), 67 Deletion(3)
Non MDR (1)	S	S	A65G, C66A, 67deletion
Non MDR (1)	S	R	A65G, C66A, 67deletion
Mono INH (5)	S	S	A65G(5), C66A(2), A66C(3), 67deletion(5)
Mono INH(1)	R	R	T21C, C22A,C24T, G26A, C27G, G28C, T29A, C30G, A31C,T32C, C38G, A40G, T43C, C44T, T45G, G48A, G49C, C50A, T51G,G52C, G55T, C56T, G57C, T58A, 61-77 deletion, 84- 85 deletion, 100-119deletion, 131-145deletion A168G, C170G, C171G, 172-179 deletion, G181T, C182A, G183A, C184aA G187tT C189G, C190A, G191A, G193A, C194G, C195G, G210C,G211C,G212C, G214AA215F
Mono Rif (1)	R	R	A65G,A66C,G67A, 68 deletion

DNA Baser- chromatogram explorer lite v4.0.0. The nucleotide sequence was analysed using and clustalW.

RESULTS

Of the 117 samples 2 were smear negative and 115 were smear positive. Smear negative samples were cultured and LPA was performed on culture isolates. In all 117 samples were subjected to LPA. 109 (93%) of the samples were Non MDR while 8 (6.8%) were MDR. Of the 109 Non MDR's, 6 were Mono Isoniazid resistant and 103 were sensitive to both Rifampicin & Isoniazid. Further, PZA drug resistance was detected using two methods. The performance of Modified Wayne's assay Fig.1 was evaluated against PZA DST by MGIT which was considered as the reference standard. The specificity of the test was 100% and sensitivity was 99%. The positive predictive value and negative predictive values were 86% & 100% respectively. The Modified Wayne's assay had an overall efficiency of 99% when compared with the MGIT. The PZA DST results were correlated with their MDR status and are presented in Fig.2. The reason for suspecting MDRTB in the samples referred for testing was tabulated and is found in Table.1. 52% of the samples belonged to patients who were previously treated smear positive. Of the 8 MDR's, 7 were male patients and 1 was a female patient. The MDR patients were grouped according to their age and reason for testing. This data is presented in Table.2 to find out if there was a particular age group in which the proportion of MDR's was higher. The most common mutation was the S531L which was present in 5 out of 8 MDR strains. Presence of all rpoB WT bands with a MUT 2A band was detected in one strain. 7 out of 8 MDR strains had a missing KatG WT, indicating low level Isoniazid resistance. Almost 50% (of the MDR isolates showed phenotypic resistance to PZA, however only 2% of the Non MDR's showed phenotypic resistance to PZA.

Sequencing analysis

All the 6 isolates exhibiting phenotypic resistance to PZA and 10 isolates which were PZA sensitive in phenotypic tests was taken up for pncA gene sequencing. Amplicon size of 222bp was sequenced (Fig.3). The nucleotide sequence obtained was analyzed using clustalW 2 Fig.4. The phylogeny of the samples tested was also analyzed and is presented in Fig.5. All the genetic sequences were subjected to Blast x analysis to determine if they coded for Pyrazinamidase . Results of the Blast x analysis of all the 16 isolates which were sequenced are presented in Fig.6. The correlation between MDR status, PZA drug susceptibility testing, PZase enzyme activity and nucleotide changes in the pncA gene are presented in Table.3. Of the 6 isolates which showed phenotypic resistance 3 isolates had mutations and did not code for the PZase enzyme. There was 100% agreement between phenotypic and genotypic results for isolates which were susceptible to PZA and had a functional PZase enzyme.

DISCUSSION

The prevalence of MDR among new TB cases is around 2-5% and among previously treated cases is estimated to be about 12% as per the national data [19]. However, the percentage of MDR among MDRTB suspects that were tested was around 6.8% which is similar to National data reported in 2015 [20]. Considering the fact that programmatic management of drug resistant TB services has been in place since 2012, the prevalence of MDR seems to have decreased. This indicates that the program has been successful in identifying and treating MDRTB and has been able to cut the chain of transmission. In our study, 50% of the samples were from sputum smear positive previously treated cases. Multi drug resistance was not detected in any of the contacts of an MDRTB case. Contrary to the common expectation, that the percentage of MDRTB is higher in the private sector or in HIV patients coinfected with TB, we did not find any MDRTB case among these two populations. Proportion of MDR's was the highest in male patients in the 26-35 age groups. RpoB S531L was the most common mutation observed in our study which appears as a band in MUT3 of LPA is similar to previously published data from India [21]. Low level resistance to isoniazid due to the absence of WT band in the KatG region and presence of MUT1 was found to be common. There was good correlation between the MGIT DST results and the modified Wayne's enzymatic assay in our study. Comparative studies conducted in India also showed similar results. However, these

studies have used the radiometric BACTEC460 TB system which is an older method [22, 23]. Although the culture systems used by us and by others are slightly different, the critical concentration of PZA was the same (100µgm/ml).

Almost 50% of the MDRTB cases were found to be resistant to Pyrazinamide and did not possess Pyrazinamidase activity. The pncA gene sequencing showed nucleotide substitutions and insertions. Contrary to the findings in other studies, the mutations were not dispersed through out the pncA gene in our study [24, 25]. Except for two isolates all the others showed mutations in 65, 67 and 68. 80% of the isolates showed deletion of nucleotide at position 67 of the pncA gene. Substitution of AàC was observed in half of the isolates while the other half had C àA. One of the isolates showed nucleotide deletion in 61-77, 84-85, 100-119, 131-145, 172-179 and produced an uncharacterized protein with a resistant phenotypic result. Further, some researchers have shown some degree of conservation of pncA mutations between 3-17, 67-76, 132-142 in the pyrazinamidase protein [25, 26]. These three regions are important in the formation of the active site of the enzyme [27]. The strains in our study had mutations in 2 of these conserved areas in which mutations were very common. 12% of our strains had mutations (substitutions, deletions) in regions which were not previously described. Our study has shown 87% correlation between phenotypic and genotypic results and has shown that 50% of MDRTB cases were resistant to PZA. As proposed by Ying Zhang et al., 2012, it would be prudent to classify MDRTB as PZA resistant or sensitive before initiating MDRTB treatment. This kind of pre classification of MDRTB patient will help the clinicians in forecasting patient treatment outcomes. Further, clinical studies are required to take this forward.

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