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Full Length Research Paper

Clinical and geographical profiles of *rpoB* gene mutations in *Mycobacterium tuberculosis* isolates from Hyderabad and Koraput in India

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Present study aims to study the geographical profile of *rpoB* mutations of *Mycobacterium tuberculosis* clinical isolates and their correlation with the therapeutic outcome. All the isolates were tested for drug susceptibility testing on Lowenstein-Jensen medium and PCR and DNA sequencing for *rpoB gene* mutations. Fifty two out of 101(52%) isolates were pan susceptible of which 79% were associated with cure. Thirty five isolates (35%) showed a combined resistance to isoniazid and rifampicin, out of which 63% were associated with treatment failure. Majority (94%) of the isolates from cured patients showed wild type of *rpoB* sequence; where as 77% of patients who failed the treatment were associated with mutations. Twenty eight out of 38(74%), rifampicin resistant isolates showed commonly occurring mutations such as 531, 526 and 516. Multiple silent mutations between the codons 145 -184 (out side the hot spot region) are being reported for the first time in this study. Information on the geographical profile of *rpoB* mutations in *M. tuberculosis* may therefore facilitate for an improved diagnosis of rifampicin resistance, by increasing the efficacy of the *gene* sequencing based tests.

Key words: DNA sequencing, LJ culture, *Mycobacterium tuberculosis*, *rpoB* mutations.

INTRODUCTION

India records an annual incidence of 1.9 million tuberculosis cases accounting for one-fifths of the world's new TB cases and two-thirds of the TB cases in the South-East Asia Region (TB India, 2008). The emergence and spread of MDR TB, is an increasing public problem in India with an estimated number of 110,000 cases spread across the country (TB India, 2009). MDR TB is defined as the combined resistance of *Mycobacterium tuberculosis* (*M.tb*) to isoniazid and rifampicin with or with out resistance to any other drugs. However, rifampicin resistance is considered to be more critical since it usually occurs in combination with other drugs. Hence, the detection of rifampicin resistance may be a surrogate marker for detecting MDR (Siddiqi et al, 2002).

In addition to the time tested conventional phenotypic methods, alternative genotyping based techniques are also being currently used to diagnose rifampicin resistance (Katoch, 2004). Recently, Sun et al. (2009) also demonstrated the usefulness of an oligonucleotide microarray based test to detect the *rpoB* mutations. However owing to the geographical diversity of

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Abbreviations: ***rpoB,*** β Subunit of RNA polymerase; **MDR,** multi drug resistance **DOTS,** directly observed treatment short-course; **RNTCP,** revised national tuberculosis control programmed; **LJ,** lowenstein jensen; **DST,** drug susceptibility test; **CPC,** cetyl pyridinium chloride; **PNB,** para nitro benzoic acid; **NRT,** nitrate reduction test; **RRDR,** rifampicin resistance determining region; **CTAB,** cetyl-trimethyl ammonium bromide.

M. tuberculosis clinical isolates across the world, there is a possibility of varying efficacy of genotyping based methods which may have diagnostic implications (Singh et al., 2004; Kong et al., 2006). Such diversity and its essentiality in diagnostics and new drug discovery were also reported by Sandgren et al., 2009 through a data base on drug resistance mutations in TB. Hence, the present study focuses on the geographical profile of *rpoB* mutations observed in *M. tuberculosis* clinical isolates from two DOTS clinics. Attempts were also made to cor-relate the phenotypes and genotypes of *M. tuberculosis* with therapeutic outcome in the corresponding TB patients.

MATERIALS AND METHODS

M. tuberculosis clinical isolates obtained from culture positive, pulmonary TB patients (both new and re-treatment cate-gories) enrolled between 2007 and 2008, at two DOTS clinics (one each at Hyderabad of Andhra Pradesh state and Koraput of Orissa state in India) were studied. Information on patient clinical details was recorded as per the RNTCP guidelines, and were categorized as Cat I (new =previously untreated patients) and Cat II (re-treatment= patients with a history of previous anti TB treatment for more than a month). Both the new and re-treatment patients were followed as per the DOTS guidelines (TB India, 2009). All the *M. tuberculosis* isolates that were grown on Lowenstein Jensen's (LJ), were subjected for biochemical tests, drug susceptibility test (DST), and PCR-DNA sequencing for *rpoB* mutations. The biochemical and genetic characters of the isolates were correlated with the treatment outcome, as cure, failure, death or default (lost for follow up) as defined by the DOTS guidelines (TB India, 2009).

Culture and DST on LJ medium

Sputum specimens from Hyderabad clinic were processed by 4% NALC-NaOH method and specimens from Koraput were collected in Cetyl pyridinium chloride (CPC) and transported to the laboratory as recommended previously. (WHO, 1998; Aparna et al., 2006). A loopful of the specimen processed by either method was inoculated into two slopes of LJ medium and incubated at 37℃ up to eight weeks or till colonies appear, whichever was earlier. Colonies grown on the slopes were identified as *M. tuberculosis* by standard biochemical tests such as Para nitro benzoic acid (PNB), Nitrate reduction test (NRT) and niacin tests as described by Venkataraman and Paramasivan, 2002 and Paramasivan et al., 2002. All the isolates were tested on LJ medium, for *in vitro* DST for rifampicin (R-40 µg/mL); isoniazid (H-0.2 µg/mL), streptomycin(S-4 µg/mL) and ethambutol (E-2 µg/mL) (All drugs in pure form were obtained by Sigma, Bangalore, India), by using 1% proportion method as per the standard guidelines (RNTCP, 2009). *M. tuberculosis H37Rv*, the laboratory strain which is inherently susceptible to anti TB drugs, was used as a control (Traore et al., 2007). Drug susceptibility patterns were described as: Pan susceptible - susceptible to all drugs; MDR - resistance to H and R with or without resistance to other drugs; other resistance - resistance to one or more drugs in a pattern other than MDR.

PCR and DNA sequencing for *rpoB gene*

All the isolates were investigated for polymorphisms in the RRDR (Rifampicin Resistance Determining Region or Hot -spot) region of *rpoB gene* by PCR and DNA sequencing as described below.

DNA extraction and amplification

A loopful of *M. tuberculosis* growth on LJ slopes, was added to 250 µL of 1x TE buffer and heat killed at 80℃ for 45 min. Genomic DNA was extracted by CTAB (Cetyl-trimethyl ammonium bromide) method, using 10% CTAB -NaCl as described previously (Honore et al., 2001). Amplification of *rpoB gene* including the 81 bp hot spot region was carried out by using primers Rp4T (5'-GAGGCGATCACACCGCAGACGT-3') and Rp8T (5'-GATGTTGGGCCCCTCAGGGGTT-3') (Traore et al., 2007) in a thermocycler (Bio-Rad, California, USA). Total volume of PCR was 50 µL; containing 25 µL of 2x master mix (Bangalore Genei, Bangalore, India), 1 µL of 20 pmol of each primer, 18 µL of molecular grade water and 5 µL of genomic DNA. Amplification conditions were set for 3 min at 95℃ followed by 30 cycles of 95℃ for 30 s; 65℃ for 45 s; 72℃ for 1 min, followed by a 10 min final extension (Traore et al., 2007) . Genomic DNA of *M. tuberculosis H37Rv* was used as control. Amplified product of 255 bp was visualized on 2% agarose gel.

Sequencing

PCR products after purification by spin column (PCR purification kit, Bangalore Genei, Bangalore, India), were subjected for DNA sequencing by automated DNA sequencer with Bigdye terminator v3.1 sequencing kit (ABI 3700, Applied Biosystems), using the same primers as used for amplification. Nucleotide sequence of clinical isolates was compared with that of *H37Rv* by using *ClustalW2*, a free software program, available online at http://www.ebi.ac.uk/Tools/clustalw2/index.html. The codon num-bering system for *E. coli rpoB* was used as the reference (Ohno et al., 2003).

Amplification and sequencing for outside RR region

Isolates with *in vitro* rifampicin resistance by DST and not showing *gene* mutations in RRDR region were further tested for amplification and sequencing of a 350 bp region preceding the RRDR region by using a separate set of primers- (5'-CTTCTCCGGGTCGATGTCGTTG- 3') (5'-CGCGCTTGTCGACGTCAAACTC- 3') (Heep et al., 2001). The PCR amplification was done, by using initial denaturation at 94℃ for 5 min; 40 cycles of denaturation at 94℃ for 45 s, annealing at 64℃ for 1 min, and extension at 72℃ for 2 min; and a final extension at 72℃ for 7 min (Heep et al., 2001).

RESULTS

A total number of 101 isolates, 67 from Hyderabad and 34 from Koraput were studied; 50 from new (Cat I) and 51 from re-treatment patients (Cat II). All the isolates were tested by DST: 52 isolates were pan susceptible, 35 demonstrated MDR and 14 isolates showed other resis-tance. *In vitro* resistance of the isolates was compared with the treatment outcome of the corresponding patient. Forty one out of 52(79%) pan susceptible isolates were associated with cure and 22 out of 35(63%) of the isolates with MDR were associated with treatment failure(Table 1). PCR and DNA sequencing yielded a diverse profile of the *rpoB gene* mutations. All rifampicin susceptible isolates on LJ showed the wild type of *rpoB* sequence similar to that of *H37Rv*. Thirty out of 38(79%) rifampicin resistant isolates showed mutations at either within or outside the RRDR region. Mutations at one point

Table 1. *In vitro* resistance vs. treatment outcome.

Treatment outcome	*In vitro* resistance total isolates (101)					
	Isolates from new patients (50)			Isolates from previously treated patients (51)		
	MDR (7)	Other resistance (9)	Pan susceptible (34)	MDR (28)	Other resistance (5)	Pan susceptible (18)
Cure	2	6	28	1	1	13
Failure	3	2	0	19	1	1
Death*	1	1	3	4	1	1
Lost in follow up	1	0	3	4	2	3

Death - death during the treatment, which need not necessarily be due to TB.
P < 0.001.

or the other of the RRDR region were showed by 28 isolates (Table. 3). Point mutations at 531(47%), 526 (17%), 516 (13%) along with other uncommon mutations were shown by 26 isolates and multiple mutations within the hot spot region were shown by two isolates. Mutations outside the RRDR region were shown by two (5%) isolates. One of these isolates showed multiple mutations at 145, 170, 173,174, 180, 181 and 184, which is being reported for the first time (*Gene* bank accession number GQ395623) (Table 3). In comparing the mutations with clinical outcome, we found that 48 isolates (94%) out of 51 cured patients that did not show any mutations and 20 (77%) out of 26 treatment failures showed *rpoB gene* mutations. Out of 67 isolates from Hyderabad, 37 were resistant to rifampicin, of which 30 showed mutations either within or outside RRDR region. Rifampicin resistance was showed by one out of 34 *M. tuberculosis* isolates from Koraput; this isolate had no mutation in either of the regions studied.

DISCUSSION

Andhra Pradesh (AP) is one of the largest states in India with an approximate population of 80 millions. The state records 1, 14, 624 (TB India, 2009) TB patients annually with approximately 6% of them suspected to be harboring MDR (Aparna et al., 2009). AP state has recently initiated the second line anti TB treatment under the DOTS plus programme of RNTCP. The State TB control programme, also proposes to use the molecular diagnostic tests for MDR TB in near future. Orissa an adjoining state of AP is proposed to be covered by the DOTS plus programme very soon (TB India, 2009). Present study therefore, investigated the rifampicin resistance in *M. tuberculosis* isolates from TB patients in a defined area from the above two states, categorized as new and retreated as per the national TB control programme. The study records sensitivity and specificity of 74% and 100% (CI-95%), respectively, for PCR-DNA sequencing of LJ culture method (Table 2). Sensitivity was improved to 79% when the isolates were tested for mutations outside the hot-spot region. There was a significant association (p < 0.001) between *in vitro* resistance (with or with out mutation in the hot spot region) and treatment failure (Table 1).This indicates that, the conventional culture based on diagnosis of rifampicin resistance still holds well in spite of its inherent limitations such as long turn around time and requirement of specialized facility. It is very well known that *M. tuberculosis* demonstrates a high degree of geographic diversity across various parts of the world (Ruwen et al., 2005). Even in India, strains belonging to northern and southern parts of the country demonstrated a diverse evolutionary pattern (Rao et al., 2005) (our unpublished data). This diversity has also been observed in case of *rpoB* mutations.

Several authors described the presence of common as well as novel alleles for *rpoB gene* especially in the hot spot region (Mani et al., 2001; Varma et al., 2004; Siddiqi et al., 2002). There are also reports from India on mutations outside the hot spot region (Mani et al., 2001). However, there is paucity on such information from this part of the country, since most of such studies had taken place from elsewhere. Hence, we attempt to report the presence of common and novel *rpoB gene* mutations in clinical *M. tuberculosis* isolates from a defined area in India and their clinical and diagnostic implications in the context of a public health programme setting. All the isolates that are susceptible to rifampicin have shown wild type (*H37Rv*) sequence and most of the resistant isolates showed mutations within or outside the hot spot region. The common mutations observed in this region include 531 (47%), 526 (17%) and

Table 2. LJ method vs. PCR sequencing.

LJ method	PCR-sequencing results for rifampicin resistance	
	Resistance n (%)	Wild n (%)
All sensitive (52)	0	52(100)
MDR (35)	29(83)	6(17)
Other resistance (14)	1*(7)	13(93)
Grand total (101)	30(30)	71(70)

Sensitivity is 79%; specificity is 100% (CI=95%).
*Rifampicin mono resistance

Table 3. List of *rpoB gene* mutations.

Mutation codon	Amino acid and nucleotide change	No of isolates
	Mutations in RRDR region	
513	CAA (Gln)- GAA (Glu)	1
516	GAC(Asp) -GCA (Ala)	1
516	GAC(Asp) - TAC (Tyr)	2
516	GAC(Asp) - GTC (Val)	1
518	AAC(Asn) - TAC (Tyr)	1
526	CAC(His) - CTC (Leu)	1
526	CAC(His) - AAC (Asn)	2
526	CAC(His) - TAC (Tyr)	2
529	CGA (Arg) - CAA (Gln)	1
531	TCG(Ser) - TTC (Phe)	1
531	TCG(Ser) - TGG (Trp)	1
531	TCG(Ser) - TTG (Leu)	12
511,516,528	CTG(Leu) -CTA(Leu), GAC(Asp) - GTC (Val), insertion	1
513, 519, 531	Deletion, Deletion, TCG (Ser) - TTG(Leu)	1
	Mutations outside RRDR	
145,170, 173, 174, 180, 181 and 184	(T-T); (G-G); (R-R); (V-V); (V-V) ; (R-R); (G-G)	1
176	Val GTC-TTC Phenyl	1
	Total	30

516 (13%) in the order of their frequency (Table 3). In a study from China, Lu et al., 2009 reported mutations at codon 531 (51.6%) and codon 526 (32.26%). Abdelaal et al. 2009 in Egypt, also found frequencies of mutations at codons 531 (45%), 526 (30%) and 516 (20%). It indicates that, mutations at 531, 526 and 516 are common, with a slightly varied occurrence across the geographical regions. Our findings are also in agreement with similar reports from India, United States and Iran, (Negi et al., 2009; Miller et al., 1994; Kapur et al., 1994; Musser, 1995).

Certain uncommon mutations such as 511, 513, 518, 519, 528 and 529, reported from else where were also observed in the study (Van Der Zanden et al., 2003). Another interesting finding from the study was the combined occurrence of mutation at 511 along with mutations at one or the other codons as observed by Siddiqi et al. (2002). In addition to the above mentioned common mutations, we also report a *V176L,* which is a rare mutation reported by Heep et al. (2002). Occurrence of multiple silent mutations in a single isolate, between the codons145-184 (out side the RRDR region) is being reported for the first time in this study (GQ395623) (Table 3). Interestingly, the isolate was from a patient with clinically and bacteriologically confirmed treatment failure. Our observation of occurrence of no mutations in 21% of the rifampicin resistant isolates is in concurrence with similar reports from else where in India and Egypt (Negi et al., 2009; Mani et al., 2001; Abdelaal et al., 2009). The diversity in *rpoB* mutations of *M. tuberculosis* as observed in this study is an expected phenomenon (Traore et al., 2006; Hirano et al., 1999). However, the study aims at highlighting the implications of this diversity while applying relevant molecular diagnostic tests for rifampicin resistance.

Owing to the inherent limitations of conventional tests,

several new diagnostic tests for rifampicin resistance such as microscopy based methods, rapid cultures and molecular tests are being considered by the current TB control programmes (Katoch, 2004).

Molecular tests are more popular, out of all these tests, and are being tried in conjunction with the conventional tests in several settings, as they are expected to yield faster results (Wei-Lun et al., 2009). Currently, available molecular tests are designed to detect known *rpoB* polymorphisms including 531, 526 and 516 that commonly occur in *M. tuberculosis* isolates (Ozkutuk et al., 2007). However, this may not hold good for universal application across various settings, considering the geographical diversity of the mutations. For instance, a considerable proportion (17%) of isolates in the present study, that were from clinically and bacteriologically diagnosed MDR patients demonstrated uncommon mutations (513, 518 and 176 etc), not covered by the standard molecular diagnostic kits (Table 3). These patients could have missed the diagnosis had they been tested with molecular tests alone. Other reports from India demonstrating a similar finding of uncommon mutations also strengthens our finding (Mani et al., 2001). It is already demonstrated that *M. tuberculosis* strain diversity has implications in the vaccines and diagnostics for TB (Gagneux and Small, 2007). This also emphasizes the need for raising the geographical profile of the mutations for a wider and effective application of *rpoB* based diagnosis of MDR in public health settings. It may hence be apt to customize the *rpoB* detection kits so as to make them suitable to detect the locally prevalent mutations. Hence, the present study attempted to record the baseline information on the *rpoB* mutations prevailing in this part of the country, a high prevalent area for both TB and HIV infections. The study included isolates from a limited geographical coverage. However, our findings indicate the necessity to undertake similar studies covering a wider area. Information of *M. tuberculosis rpoB* genotypes prevailing in a defined area may facilitate for undertaking better and customized policies and practices for effective control of MDR TB.

Nucleotide sequence accession number

The sequence with novel mutations from out side RRDR region found in this study are deposited in *Gene* bank under the accession number GQ395623.

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