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Emergence and Molecular Characterization of Extensively Drug-Resistant *Mycobacterium tuberculosis* Clinical Isolates from the Delhi Region in India▽

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We screened 194 *Mycobacterium tuberculosis* strains isolated from tuberculosis (TB) patients in Delhi and neighboring regions in India to identify the prevalence of extensive drug resistance (XDR) in clinical isolates. Among these, 104 isolates were found to be multidrug resistant (MDR), and 6 were identified as XDR isolates, which was later confirmed by antimicrobial susceptibility testing against the respective drug screening panel. Genotyping was carried out by amplifying and sequencing the following genes: *rpoB* (rifampin), *katG* (isoniazid), *gyrA* (fluoroquinolones), and *rrs* (amikacin, kanamycin, and capreomycin). Our analyses indicated that mutations at the hot spots of these genes were positively correlated with drug resistance in clinical isolates. The key mutation observed for *rpoB* was in the codon for amino acid position 531 (S531L), and other mutations were seen in the hot spot, including those encoding Q510P, L511H, D516V, and H526Y mutations. We identified S315T and R463L substitutions encoded in the *katG* locus. An S95T substitution encoded in the *gyrA* locus was the most common mutation observed in fluoroquinolone-resistant isolates. In addition, we saw D94G and D94N mutations encoded in the QRDR region. The 16S rRNA (*rrs*) gene encoded mainly the A1401G mutation and an additional mutation, G1484T, resulting in ribosomal modifications. Taken together, the data in this report clearly establish the presence of phenotypically distinct XDR strains in India by molecular profiling and further identify specific mutational hot spots within key genes of XDR-TB strains.

In recent years, the control of tuberculosis (TB) has become a global challenge due to the emergence of multidrug-resistant tuberculosis (MDR-TB) and extensively drug-resistant tuberculosis (XDR-TB). With 9.2 million new cases and 1.7 million deaths in 2006, TB remains one of the major life-threatening diseases worldwide (22). XDR-TB isolates are resistant to isoniazid and rifampin, to any fluoroquinolone (FQ), and to at least one of the three injectable second-line drugs (amikacin, kanamycin, and capreomycin) (6). As of June 2008, XDR-TB strains have been found in 49 countries, including the United States (6, 22). Furthermore, a recent report points to an alarming increase in the number of tuberculosis patients in the South Asian subcontinent, with India being singled out as having the greatest burden of XDR-TB, with a poor prognosis and high mortality among HIV-infected individuals (4). The risk of XDR-TB spread across country borders has heightened global concern over a potentially untreatable epidemic that may jeopardize recent advances made in global TB control.

The prevalence of XDR-TB in India was reported in 2007, but no further efforts have been made to identify its genotypes or geographical spread (9). The present study was undertaken to characterize mutations prevalent in clinical isolates from India with respect to various drug target loci. We examined the drug target genes for rifampin (*rpoB*), isoniazid (*katG*), fluoroquinolones (*gyrA*), and aminoglycosides (*rrs*), which are commonly prescribed for the treatment of tuberculosis in India. The loci studied were *rpoB* (RNA polymerase B subunit), *katG* (catalase-peroxidase), *rrs* (16S rRNA), and *gyrA* (DNA gyrase A). Here we report, for the first time, the molecular characterization of XDR-TB isolates from India. This study confirms the presence of XDR-TB in India and simultaneously raises an alarm about its prevalence among TB patients, as many of them may initially have MDR-TB that slowly progresses and mutates to XDR-TB. Furthermore, the fact that some of these patients have HIV infection or the possibility of later coinfection with HIV has the potential to make this global HIV-TB epidemic untreatable with current therapies.

MATERIALS AND METHODS

***Mycobacterium tuberculosis* clinical isolates.** *Mycobacterium tuberculosis* isolates were collected from patients reporting to the Super Religare Laboratories (SRL) Reference Center in Gurgaon, India, primarily from New Delhi and its

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TABLE 1. Primers used for amplification of different loci of target genes for XDR-TB strain characterization

Gene	Primer direction	Sequence (5′–3′)	Position (nt)	Amplicon size (bp)
katG1	Forward	GTGCCCGAGCAACACCCACCCATTAC	1–26	808
	Reverse	GGCGCCATGGGTCTTACCGAAAG	812–834	
katG2	Forward	CGGCGGTCACACTTTCGGTAAG	801–822	759
	Reverse	CGGCGGTCACACTTTCGGTAAG	1540–1560	
rpoB	Forward	CACCAGCCAGCTGAGCCAATTC	1296–1317	442
	Reverse	CCATGTAGTCCACCTCAGACGAG	1716–1738	
gyrA	Forward	GATGCAGCGCAGCTACATCGAC	69–90	325
	Reverse	CTTCGGTGTACCTCATCGCCG	374–394	
rrs	Forward	GAGATAGGCGTTCCCTTGTGGC	995–1071	527
	Reverse	AAGGAGGTGATCCAGCCGCAC	1502–1522	

neighboring regions. Most of these patients were referral cases and had been through various degrees of antitubercular drug therapy. Sputum and extrapulmonary specimens were collected from patients reporting with pulmonary or extrapulmonary tuberculosis to the SRL Reference Center. The specimens were processed by standard methods (*N*-acetyl-L-cysteine-NaOH), followed by culturing in a Bactec MGIT 960 nonradiometric automatic isolation system (BD). The isolates were characterized as belonging to the *M. tuberculosis* complex by use of *p*-nitrobenzoic acid (PNBA) in the Bactec MGIT 960 system (21).

XDR characterization by phenotypic studies. A total of 194 clinical *M. tuberculosis* isolates were used for antimicrobial testing with first- and second-line drugs in the MGIT 960 system per the manufacturer's protocol to identify the XDR-TB strains. The drug susceptibility profiles were tested at SRL, Gurgaon, India. The critical concentrations (μg/ml) of antitubercular drugs used in the MGIT 960 system were as follows: rifampin, 1.0; isoniazid, 0.1; ofloxacin, 2.0; levofloxacin, 2.0; moxifloxacin, 2.0; kanamycin, 4.0; capreomycin, 2.5; amikacin, 1.0; streptomycin, 1.0; ethambutol, 5.0; and pyrazinamide, 100.0. The MICs for the XDR strains were determined for phenotypic confirmation per the method of Sood et al. (16), and dilution of the compounds was performed per Clinical and Laboratory Standards Institute (CLSI; formerly NCCLS) guidelines (12).

Genomic DNA isolation, PCR, and DNA sequencing. The isolates were cultured on Lowenstein-Jensen slants. The colonies were scraped from the slants and resuspended in 500 μl of Middlebrook 7H9 broth. Genomic DNAs were isolated by using a Qiagen DNeasy blood and tissue kit. The primers used in this study and their nucleotide positions in the corresponding genes are listed in Table 1. PCR amplification was done by using standardized protocols. The samples were resolved in a 1% agarose gel, and the specific bands were visualized in a Gel Doc system using Quantity One software. The PCR products were purified using a Qiagen PCR purification kit according to the manufacturer's instructions. The purified DNAs were eluted in sterile double-distilled water and used for the sequencing studies. Sequencing of the amplicons was carried out at Macrogen (outsourced to Biolinkk). The sequences generated with the program were compared to the respective wild-type sequences by using clone manager software.

RESULTS

A total of 194 clinical isolates were screened for the identification of XDR in *M. tuberculosis* isolates by the Bactec MGIT 960 modified proportion method per the manufacturer's protocol. These isolates were from TB patients in Delhi and neighboring regions in India. Here we report the presence of XDR in clinical isolates from this region. Among the 194 *M. tuberculosis* isolates tested for susceptibility with the MGIT 960 system, 104 were found to be multidrug-resistant strains, and 6 were characterized as XDR-TB isolates and then cross-checked with the MICs of the respective drug screening panel of antitubercular drugs (Tables 2 and 3). These 6 XDR-TB clinical isolates were critically examined with the Bactec MGIT 960 system as well as by their MICs and were confirmed to be XDR-TB strains. The MICs of rifampin against the XDR-TB strains were >16 μg/ml, and the MICs of isoniazid against these isolates were in the range of 1 to >16 μg/ml. The MICs of fluoroquinolones against these XDR clinical isolates were in the range of 8 to >16 μg/ml, and the MICs of second-line drugs (kanamycin, amikacin, and capreomycin) were in the range of 4 to >16 μg/ml (Table 3). These MIC ranges indicate that these clinical isolates are resistant to first-line drugs (rifampin and isoniazid), to fluoroquinolones (ofloxacin), and to second-line injectable drugs (kanamycin, amikacin, and capreomycin), and therefore they are categorized as XDR-TB strains. These XDR isolates represent primarily those with

TABLE 2. XDR-TB clinical isolate susceptibilities to antimycobacterial drugs

Isolate	Origin	Source specimen	Age of patient (yr)	Gender of patient	Susceptibility to drug[a]										
					INH	RIF	OFLOX	LEVO	MOXI	KANA	CAPREO	AMIK	STREPTO	ETHAMB	PZA
625	Agra	Sputum	32	F	R	R	R	TN	R	R	R	TN	TN	TN	R
761	New Delhi	Pus	22	M	R	R	R	R	R	R	R	R	S	S	S
2403	Moradabad	Sputum	45	M	R	R	R	R	R	R	R	R	R	R	R
2301	Agra	Sputum	34	M	R	R	R	R	R	R	R	R	R	R	R
2911	New Delhi	Sputum	16	F	R	R	R	R	R	R	R	R	R	R	S
2474	New Delhi	Sputum	48	M	R	R	R	TN	TN	S	S	R	R	R	R

[a] Resistance (R) or sensitivity (S) of strains to the corresponding anti-TB drugs. TN, test not requested and therefore not performed. INH, isoniazid; RIF, rifampin; OFLOX, ofloxacin; LEVO, levofloxacin; MOXI, moxifloxacin; CAPREO, capreomycin; AMIK, amikacin; STREPTO, streptomycin; ETHAMB, ethambutol; PZA, pyrazinimide.

TABLE 3. MICs of antitubercular drugs against XDR-TB clinical isolates[a]

Drug	MIC (μg/ml) for strain					
	2911	625	2474	761	2301	H37Rv
Rifampin	16	>16	>16	>16	>16	0.25
Isoniazid	4	>16	1	4	0.5	0.25
Ethambutol	4	4	4	8	4	1
Moxifloxacin	1	1	1	1	2	0.125
Ofloxacin	8	16	8	16	8	0.5
Amikacin	>16	>16	>16	16	>16	0.5
Kanamycin	>16	>16	>16	>16	>16	2
Streptomycin	>16	16	>16	0.125	0.5	1
Cycloserine	>16	16	16	16	16	8
Capreomycin	16	16	4	>16	4	2
Ethionamide	4	4	>16	1	2	0.5
Sparfloxacin	>16	8	16	16	16	0.5

[a] Isolate 2403 was not able to grow in synthetic media, so the MICs of all these drugs with this isolate could not be studied.

acquired resistance, as either the patients had at some time been given antitubercular drug therapy or the XDR-TB isolates spread from one patient to another.

The 6 phenotypically confirmed XDR-TB isolates were further analyzed for mutations in the hot spot regions of various gene loci. The results are summarized in Table 4. On the basis of the drug susceptibility profile of each isolate, the relevant drug target genes were PCR amplified and sequenced. The corresponding genes were sequenced from all XDR-TB isolates and the standard laboratory reference strain H37Rv (ATCC 27294). Reported sequences of the genes were taken as templates for sequence analysis. No mutations were observed in the *rpoB*, *katG*, *gyrA*, and *rrs* genes of H37Rv. Sequencing results for H37Rv exactly matched the wild-type sequences of the respective genes.

A stretch of 30 amino acids at the center of the amplicon for the *rpoB* locus was studied. Amino acids 507 to 533 comprised the hot spot region for mutations. In all 6 XDR isolates, we observed mutations in the hot spot region. We identified previously reported mutations as well as certain novel mutations. The mutations observed in the hot spot region were Q510P, L511V, D516V, H526Y, and S531L (Tables 4 and 5). Amino acid 531 seemed to be the most vulnerable to mutation, as most rifampin-resistant isolates had the TCG→TTG mutation. The mutations in the hot spot region correlated well with the MICs of rifampin against XDR strains.

In the present study, we looked for mutations in the 5′ region (nucleotides [nt] 1 to 834; referred to as *katG1*) and the mid-region (nt 801 to 1560; referred to as *katG2*) of *katG*, corresponding to amino acid positions 1 to 278 and 267 to 520, respectively (Table 1). The mutations observed in the *katG* gene are summarized in Table 4. In the 5′ region, the E217G mutation was observed in XDR isolate 2301. The other strains did not show any mutations in this region, but in the mid-region of the *katG* gene, 3 mutations, S315T, D329A, and R463L, were identified; among these, the 2 key mutations were S315T and R463L. In addition to the R463L mutation, common in Indian MDR-TB isolates, four of six isolates had the S315T mutation. In one of the isolates (XDR 2474), the D329A mutation was also observed.

The QRDR region of the *gyrA* gene was sequenced to identify mutations (Fig. 1 and Table 4). The isolates showed a common mutation corresponding to the amino acid change S95T (Table 4). The second most common mutation, observed in four isolates, was D94G or D94N. Three isolates had an A90V substitution, while one isolate (XDR 2911) had two additional mutations, R128S and Y129C. All six isolates had the common mutation S95T, and 4 isolates had mutations at position 94 as well as position 95. One isolate (XDR 2911) had 4 mutations, S95T, D94G, R128S, and Y129C.

XDR-TB isolates resistant to amikacin, kanamycin, and streptomycin were tested for mutations in the *rrs* locus. The results showed two mutations, A1401T and G1484T. The A1401T mutation was a common mutation observed in 5 isolates. In XDR 761, the G1484T mutation was seen. This mutation in the *rrs* gene is the cause for the emergence of resistance to kanamycin and amikacin.

DISCUSSION

The ever-increasing burden of drug resistance is a serious concern in developing countries, particularly for patients with *M. tuberculosis* infection. This mycobacterium uses various mechanisms to evade killing by therapeutic drugs, including mutations in genes that code for drug target proteins (3, 10, 11, 13). The objective of the present study was to identify mutations in drug target genes in strains of *M. tuberculosis* prevalent among the Indian population. The findings of our study showed that many mutations in the *rpoB*, *katG*, and *gyrA* genes are similar to those reported from other parts of the world (13, 18, 19), and these common mutations might have global ramifications. In addition, a few mutations in our report clearly reflect the uniqueness of XDR-TB strains from the Delhi region. The modified proportion method (MGIT 960) as well as the MIC method revealed the existence of XDR strains in the

TABLE 4. Characterization of XDR-TB isolates through identification of mutations in drug target genes

Strain	Mutation(s) in drug target gene			
	rpoB (rifampin)	*katG* (isoniazid)	*gyrA* (FQ)	*rrs* (kanamycin, capreomycin, amikacin)
625	S531L, G566R, I569L	S315T, R463L	A90V, S95T	A1401G
761	S531L, Q510P	S315T, R463L	D94N, S95T	G1484T
2301	S531L	R463L, E217G	D94G, S95T	A1401G
2403	Q510P, L511V, S531L	S315T, R463L	A90V, S95T	A1401G
2474	H526Y	D329A, R463L	D94G, S95T	A1401G
2911	D516V	S315T, R463L	D94G, S95T, R128S, Y129C	A1401G
H37Rv	No mutation	No mutation	No mutation	No mutation

TABLE 5. XDR-TB isolates with point mutations in the *rpoB* gene

XDR strain	Rifampin MIC (μg/ml)	Mutation	Nucleotide change	Amino acid change	Mutation type
625	>16	S531L	C→T	Ser→Leu	Previously reported
761	>16	Q510P	A→C	Gln→Pro	This study
		S531L	C→T	Ser→Leu	Previously reported
2301	>16	S531L	C→T	Ser→Leu	Previously reported
2403		Q510P	A→C	Gln→Pro	This study
		L511H	T→A	Leu→His	Previously reported
		S531L	C→T	Ser→Leu	Previously reported
2474	>16	H526Y	C→T	His→Tyr	Previously reported
2911	16	D516V	A→T	Asp→Val	This study

Delhi region. Since no proper phenotypic and genotypic study is available from other parts of India, we cannot rule out the possibility of the existence of similar XDR strains throughout India. Since India remains a hot spot for TB (1), it is important to have a molecular profile for these XDR-TB strains.

We undertook the present study to characterize the mutations prevalent in clinical isolates of *M. tuberculosis* from India with respect to the drug target genes *rpoB*, *katG*, *gyrA*, and *rrs*. Most of the mutations were common in reported XDR strains, thus confirming the existence of XDR-TB in India. In addition, our study identified a few novel mutations in the XDR-TB clinical isolates from the Delhi region.

The existence of common mutations in the *rpoB* gene, at codons 526 and 531, in Indian isolates and those reported previously (5, 15, 18, 20) indicates that these mutations are common for many drug-resistant strains around the globe, with the possibility of further spread. We found mutations in the hot spot region of the *rpoB* gene, encoding the residues at positions 510, 511, and 516, that correlate with those in previous studies (15, 18). Additional mutations outside the hot spot region were observed in *rpoB*, encoding the residues at positions 566 and 569. The latter mutations were detected in a significant number of drug-resistant isolates, a fact that needs to be kept in mind while designing diagnostic kits for the detection of XDR-TB. We found a definite correlation between the MIC and the type of mutation for many isolates. As reported by previous investigators (7, 8, 19), mutations at positions 526 and 531 are important in the development of high-level resistance, as isolates with these mutations frequently exhibit high MICs.

We observed that many isolates carried the R463L and S315T substitutions. The R463L mutation correlates with a study by Siddiqi et al. (15), and the S315T mutation corroborates the findings of Sun et al. very well (18). Earlier, Sreevatsan et al. (17) reported that the R463L polymorphism does not contribute to resistance *per se* but is an important marker for evolutionary genetics. The E217G mutation encoded in the 5′ region of the *katG* gene was quite uncommon and has not been reported by any other group. The MIC of isoniazid against isolates XDR 2301 and XDR 2474 was 1 μg/ml, and the mutation at position 315 was not observed. In other isolates, the mutation at position 315 was observed, and the MIC was 4 to >16 μg/ml. There appears to be a strong correlation between high-level resistance to isoniazid and mutation at position 315 in the *katG* gene product.

FQs comprise the secondary line of treatment for drug-resistant tuberculosis. One of the reasons that all of our XDR isolates were resistant to FQ may be due to its overuse in the community. In addition, FQ-resistant strains may be spreading in hospitals as well as in community settings. The most common mutation in FQ-resistant isolates in the present study was the S95T mutation, which seems to have no direct role in the development of drug resistance, as it also occurs in drug-sensitive strains (17). In reports by Siddiqi et al. (14, 15), the majority of ofloxacin-resistant MDR strains showed the S95T mutation, very few isolates showed mutations at positions 90 and 94, and they did not observe any mutation in some of the FQ-resistant isolates. Codons 89, 90, 91, 94, and 95 in the *gyrA* gene have been shown to be polymorphic (13, 23). Recently, the molecular characterization of XDR isolates in China revealed mutations at position 94 (D94N, D94G, D94A, D94H, and D94Y) and position 90 (A90V) (18). The Indian MDR isolates revealed mutations mainly at position 95 (S95T) (14, 18). The XDR isolates from our study revealed mutations at position 94 as well as position 95, which appear to have characteristic features. We also observed mutations common for the XDR isolates reported in earlier studies (15, 18, 23).

We observed two mutations (A1401G and G1484T) in the *rrs* locus, and they were associated with resistance to aminoglycosides. Earlier low-level resistance was observed due to mutations associated with the *rrs* locus, as reported by Bottger in 1994 (2). Another study by Siddiqi et al. did not find any of these mutations in 14 streptomycin-resistant isolates (15).

The emergence of XDR-TB in India is a concern, as it remains one of the major killer diseases (1). Due to inadequate monitoring and a lack of proper treatment regimens, MDR-TB and XDR-TB remain major threats to the Indian population,

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H37Rv          81 MGNYHPHGDASIYDSLVRMAQPWSLRYPLVDGQGNFGSPGNDPPAAMRYT 130
625, 2403         *********V****T************************************
761               *************NT************************************
2301, 2474        *************GT************************************
2911              *************GT*********************************SC*
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Mutation pattern of *gyrA* gene in XDR-TB isolates

FIG. 1. Mutation pattern of *gyrA* gene in XDR-TB isolates.

particularly for individuals on the lower threshold of the socioeconomic ladder. Our study provides additional information about the mutations that are common in XDR isolates from India and other parts of the world (18). To the best of our knowledge, this is the first report on XDR-TB in India with molecular characterization of target genes. Our data will be helpful in designing new molecular biology-based techniques for the diagnosis of XDR-TB. Further molecular characterization of XDR-TB strains throughout India, along with our data, will help in the design and execution of proper therapeutic interventions for patients infected with these strains.

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