ORIGINAL RESEARCH

Molecular Epidemiology and Polymorphism Analysis in Drug-Resistant Genes in *M. tuberculosis* Clinical Isolates from Western and Northern India

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Introduction: The mechanistic details of first line drug (FLD) resistance have been thoroughly explored but the genetic resistance mechanisms of second line injectables, which form the backbone of the combinatorial drug resistant tuberculosis therapy, are partially identified. This study aims to highlight the genetic and spoligotypic differences in the second line drug (SLD) resistant and sensitive *Mycobacterium tuberculosis* (*Mtb*) clinical isolates from Mumbai (Western India) and Lucknow (Northern India).
Methods: The *rrs, eis, whiB7, tlyA, gyrA* and *gyrB* target loci were screened in 126 isolates and spoligotyped.
Results: The novel mutations were observed in *whiB7* loci (A43T, C44A, C47A, G48T, G59A and T152G in 5'-UTR; A42C, C253T and T270G in gene), *tlyA* (+CG200, G165A, C415G, and +G543) and *gyrB* (+G1359 and +A1429). Altogether, the *rrs, eis*, and *whiB7* loci harbored mutations in ~86% and ~47% kanamycin resistant isolates from Mumbai and Lucknow, respectively. Mumbai strains displayed higher prevalence of mutations in *gyrA* (~85%) and *gyrB* loci (~13%) as compared to those from Lucknow (~69% and ~3.0%, respectively). Further, spoligotyping revealed that Beijing lineage is distributed equally amongst the drug resistant strains of Mumbai and Lucknow, but EAI-5 is existed at a higher level only in Mumbai. The lineages Manu2, CAS1-Delhi and T1 are more prevalent in Lucknow.
Conclusion: Besides identifying novel mutations in *whiB7, tlyA* and *gyrB* target loci, our analyses unveiled a potential polymorphic and phylogeographical demarcation among two distinct regions.
Keywords: drug resistant tuberculosis, single nucleotide polymorphism, *rrs*, *eis*, *whiB7*, spoligotyping

Introduction

Mycobacterium tuberculosis (*Mtb*) poses an intimidating and hazardous environment, affecting millions of lives each year globally. More so, the specter of drug resistance, owing to its resilience, presents a portentous global health threat to the mankind. Multidrug Resistant Tuberculosis (MDR TB), i.e., resistance to the two primary first line drugs (FLD) Isoniazid and Rifampicin, has complicated the treatment options for tuberculosis (TB). Furthermore, extensively drug resistant TB (XDR TB), wherein the MDR mycobacteria develop additional resistance to a second line drugs (SLDs), i.e., fluoroquinolone (FQ; e.g., ofloxacin, OFX; moxifloxacin; levofloxacin) and either of the three second line injectable (SLI) aminoglycosides (kanamycin, KAN; amikacin, AMK; or capreomycin, CAP), has worsened the scenario.[1] Lately, Pre-XDR TB (resistance to either a FQ or an injectable aminoglycoside along with MDR-TB) presents another perilous threat for the high TB burden countries like India and China.[2,3] At present, the SLDs also include bedaquiline, delamanid, linezolid, and pretomanid for the treatment of drug resistant TB.[4] Though the use of SLIs is slowly being discouraged owing to their adverse side effects, they are still required in case of shorter treatment regimen and the lesser availability of newer SLDs in resource limited or developing countries like India.[5]

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The slow growth of *Mtb* and diverse drug resistance mechanisms affects its successful management. Some of the drug resistance mechanisms are due to modification in the drug target site, structural and functional changes in the efflux pumps, etc. Resistance arises partly due to treatment errors, poor adherence by patients for long treatment, toxic side effects, incorrect/inconsistent use of SLIs in patients where FLDs fail, and timely unavailability of the drug susceptibility profiles.

Attempts towards rapid measurement of phenotypic drug resistance using modern day diagnostic techniques significantly diminish erroneous treatments. Besides, the state-of-the-art TB diagnostic programs are now transitioning from phenotypic to advanced genotypic levels, emphasizing the significance of genetic mutations in resistance determination. The genetic mutation-drug resistance relationships are quite evident in case of isoniazid (*inhA, katG*) and rifampicin (*rpoB*).[6–8] The association between *rrs* and *eis* promoter mutations to SLI resistance has been widely noted.[9–12] In addition, WhiB7 transcription regulator-mediated gene regulation serves as an aid to facilitate drug resistance and is linked to low level KAN resistance by transcriptional activation of *eis*.[13,14] Loss of the methylation activity of the *tlyA* methyltransferase due to mutations disrupts the drug-ribosomal interaction conferring CAP resistance.[15,16] The *gyrAB* quinolone resistance determining region (QRDR) mutations have also been attributed to FQ resistance in diverse geographical regions.[17–19] Nonetheless, a clearer picture regarding the association of genetic mutations in *eis, whiB7, tlyA*, and *gyrB* loci in SLI and FQ resistance is required.

Single Nucleotide Polymorphisms (SNP) and genotypic co-analyses of drug resistance and specific lineages are increasingly being undertaken globally.[20–26] Most of the reports correlating the drug resistant mutations with specific genotypes in India are based on the FLD and streptomycin (STR) resistance.[27–30] The Indian geographical diversity with respect to lineage prevalence has been evaluated only to a limited extent, therefore, the true representation is lacking in global scenario.[31–33] Although, an attempt by the recent review about the major distribution of different lineages in India has assigned Manu/Beijing/CAS to Western, Beijing to North-Eastern, CAS/Beijing to Northern and EAI to Southern India,[34] inadequate information still exists on the relationships of SLD resistant mutation with different lineages.[24,35,36] Therefore, this study aims to analyze and understand the mutational characteristics and lineages of clinical *Mtb* isolates from Mumbai, Western India and Lucknow, Northern India (Figure 1).

Materials and Methods
Clinical Isolates of *M. tuberculosis*

Fifty-eight clinical isolates isolated from September 2015 to April 2016 were obtained from P. D. Hinduja National Hospital and Research Centre, Mumbai, Maharashtra. From these, two, eight, 20, and 28 isolates were pan-susceptible, MDR, Pre-XDR, and XDR, respectively. Sixty-eight isolates from February 2013 to March 2016 were obtained from Tertiary Care Centre Tuberculosis Laboratory, Department of Microbiology, King George Medical University (KGMU), Lucknow, Uttar Pradesh. Among these, 15, 28, and 23 isolates were MDR, Pre-XDR, and XDR, respectively and two isolates were sensitive to all the tested drugs. Out of these 68 isolates, the mutational analysis with respect to KAN resistance has been reported earlier for 58 isolates from Lucknow.[37] Remaining 10 new SLD resistant isolates were obtained in March 2016 from Lucknow and evaluated in this study. Therefore, a total of 68 isolates from Lucknow have been compared with 58 isolates of Maharashtra for analyzing the target specific mutational prevalence and lineage identification. All the isolates were isolated consecutively from different patients in the reported period and each isolate corresponded to individual TB patient. The research was conducted following the national and institutional standards in accordance with the Declaration of Helsinki.

The number of resistant and sensitive isolates for each drug included in this study is listed in Table 1. The FLD and SLD resistance profiles of all the isolates from Mumbai and Lucknow are mentioned in Supplementary Table 1. For a few strains, the complete drug susceptibility testing (DST) profile could not be determined. Hence, they were not included in the mutational occurrence calculation, and therefore, only numbers have been provided in such cases.

Figure 1 Geospatial Map of India indicating the location of two areas from where the clinical strains of *M. tuberculosis* have been obtained.

Ethical Statement

Consent was waived off by the Institutional Review board of P. D. Hinduja National Hospital and Research Centre, Mumbai, Maharashtra and the Ethics Committee of KGMU, Lucknow as deanonymized banked isolates with unique identification numbers with no link to patients were revived for the study.

Culture Growth and Determination of Minimum Inhibitory Concentration (MIC)

The mycobacterial cultures isolated and grown from the patient sputum samples at the tertiary care laboratories at KGMU, Lucknow and P.D. Hinduja National Hospital, Mumbai were inoculated and subcultured by streaking either on

Table 1 Number of Drug Resistant and Drug Sensitive Isolates for Each Drug from Mumbai and Lucknow

	Mumbai (No. of Isolates)			Lucknow (No. of Isolates)		
Drug	**R**	**S**	**ND**	**R**	**S**	**ND**
KAN	29	29	0	34	34	0
CAP	23	35	0	20	41	7
OFX	47	11	0	36	31	1

Abbreviations: KAN, Kanamycin; CAP, Capreomycin; OFX, Ofloxacin; R, resistant; S, sensitive; ND, drug susceptibility not determined.

Lowenstein Jensen (LJ) media or Middlebrook 7H9 broth (BD Difco) followed by incubation at 37°C. The contamination was checked for after 24 h and 48 h. All the procedures dealing with *Mtb* isolates were handled in the standard Biosafety Level III laboratories (BSL-III) at the respective hospitals.

The resistance to the FLDs was determined using 1% proportional method described previously.[38] The susceptibilities towards the SLIs, i.e., KAN, AMK, and CAP, were determined using Resazurin Microtiter Assay (REMA). The range of concentrations used was 1.25 µg/mL to 20 µg/mL for KAN/CAP; 0.25 µg/mL to 4.0 µg/mL for AMK; and 0.25 µg/mL to 8.0 µg/mL for OFX.[39–41] Drug cut-off points by REMA were 2.5 µg/mL for KAN/CAP, 1.0 µg/mL for AMK, and 2.0 µg/mL for OFX. MIC was defined as the minimum concentration of respective drug required to prevent the change in the color of dye from oxidized blue to reduced pink state, indicative of mycobacterial growth.

Isolation of Genomic DNA from the Clinical Isolates

The genomic DNA was isolated from all resistant and sensitive isolates as described previously.[37,42] Briefly, loopful of freshly subcultured colonies from each of the LJ medium bottles were resuspended in 200 µL of double distilled water in 1.5 mL microcentrifuge tubes (MCTs). The bacterial suspension was vortexed thrice for 10sec at 30sec intervals to allow proper dissolution of colonies and lysed by boiling at 95°C for 20 min in water bath. After cooling, 200 µL of chloroform was added and the lysates were centrifuged at 13,000 rpm for 10 min. The aqueous supernatant was transferred to fresh MCTs and used as templates for PCR.

PCR Amplification

The *Pfu* (M7741, Promega) and Q5 Polymerase (M0491, New England Biolabs) were used to amplify the target gene loci with the help of corresponding primers procured from Bioserve Biotechnologies Pvt. Ltd. The details of the primer sequences are provided in Supplementary Table 2. The cycling conditions were as follows: initial denaturation at 98°C for 30s; followed by 25 cycles of denaturation at 98°C for 20s, annealing at 55°C (*rrs*); 67°C (*eis*); 71°C (*whiB7*); 55°C (*gyrA* and *gyrB*); 65.5°C (*tlyA*) for 30s, extension at 72°C for 30s, and a final extension at 72°C for 2 min.

DNA Sequence Analyses

Sequencing of purified PCR products was performed using Applied Biosystems analyzer with ABI BigDye v3.1 Cycle Sequencing kit. Chromatograms obtained were analyzed using Finch TV version 1.4.0 software (Geospiza Inc.). Mutations were detected in the respective genes by sequence alignment with wild-type *Mtb*H37Rv (NC_000962.3; GI: 448814763) (www.ncbi.nih.nlm.gov/blastn). Validations of all the mutations were done using forward as well as reverse sequencing reactions. Heterogeneous mutations in the chromatogram were comprehended as two peaks of different colors at a single peak position showing the presence of wild type and polymorphic nucleotide. The cut off called by the KB^TM Basecaller for SNP calling was 25% for mixed bases. The quality value, which is per base estimate of the Basecaller accuracy, was 20 for pure bases and ranged between 10 and 20. The relative occurrence of SNPs for each isolate were calculated by dividing the number of genotypically resistant isolates carrying the mutation by the total number of phenotypically resistant isolates. The isolates with unknown drug susceptibility (not determined [ND]) profile were excluded from mutational prevalence calculation for the respective drug.

Spoligotyping

The spoligotyping of 58 clinical isolates of *Mtb* from Mumbai (2015 to 2016) and 68 isolates from Lucknow (2013 to 2016) was carried out by amplification of the 43 spacer sequences using DRa and DRb primers (DRa, 5'-GGTTTTGGGTCTGACGAC-3' (biotinylated 5' end) and DRb, 5'-CCGAGAGGGGACGGAAAC-3') provided in the spoligotyping kit (Ocimum Biosolutions, Hyderabad). The standardized spoligotyping protocol has been described previously.[43] Briefly, the cycling conditions used for spoligotyping were: initial denaturation at 95°C for 30s; followed by 35 cycles of denaturation at 95°C for 30s, annealing at 58°C for 30s, extension at 68°C for 30s, and a final extension at 68°C for 5 min. The amplified direct repeats were hybridized using MN45 Miniblotter and the manufacturer's instructions were used for the chemiluminescent detection of the bound probes (BioRad Clarity Western ECL Blotting Substrate). The ambiguous patterns in the spoligotypes of 21 isolates were resolved using the spoligotyping services of Mapmygenome India Ltd. The results of the spoligotypic patterns were converted into binary

format; with "0" representing no hybridization of the spacer and "1" representing hybridization (presence of spacer). The genomic DNA of *Mtb*H37Rv and *Mycobacterium bovis* were used in this experiment as positive controls while reaction buffer was used as the negative control. Use of controls in each spoligotyping run ensured no cross contamination of samples or reagents.

Analyses of Spoligotypes

The SITVIT2 database was employed to identify their existence as known or orphan (not determined) spoligotypes.[44] Spoligotype results of the clinical isolates used in this study were compared to the reference patterns available in the SITVIT2 database; and Shared International Types (SITs) were assigned to those which matched with the reference spoligotype patterns. The unmatched patterns which did not exist in the database at the time of analysis were termed as "Orphan or New".

Clustering of Spoligotypes

The possible phylogenetic relationships between different isolates were deduced by clustering analysis using spolTools which generated spoligoforest trees with hierarchical layout for the two geographical locations.[45] A cluster was defined as a spoligotype pattern shared by more than one isolate. This hierarchical representation has a "ranked" design where spoligotypes derived from the inferred parent are drawn below the parent.

Correlation Between Spoligotypes and Mutation Harboring Isolates

To understand the possible correlation of spoligotypic prevalence with isolates harboring the drug resistant mutations, a region-based evaluation was carried out. For each drug category, the percentage of relative lineage distribution among mutation-bearing drug resistant strains was calculated for the corresponding target loci. The significance of association of unique SLD mutations with different observed lineages was statistically analyzed using Chi-square test. Based on the absolute numbers of respective drug resistant strains carrying distinct mutations (Supplementary Table 3), the Chi-square test criterion of expected value to be ≥5 could not be fulfilled for CAP resistance in Mumbai strains and for all the drugs in Lucknow strains. Therefore, p-value for these two categories were not considered. For other two drugs in Mumbai strains, p-values of < 0.05 were considered significant. For statistical analysis, the mutation frequencies of Manu related spoligotypes for each of the unique drugs have been combined together.

GenBank Accession Numbers

The accession numbers of novel mutation harboring loci were MN131053-MN131064 for Mumbai isolates and MN166763-MN166777 for Lucknow isolates.

Results

KAN Resistance

rrs

In Mumbai, 16 out of 29 KAN^R isolates (55.1%) exhibited A1401G and C1402T in the *rrs* hot spot. The most prevalent mutation, A1401G, was found in 15 isolates (51.7%), followed by C1402T in one isolate (3.4%), (Table 2). None of the 29 KAN^S isolates displayed any polymorphism in the *rrs* loci.

In addition to the SNPs reported earlier by our group,[37] three out of five new KAN^R isolates from Lucknow exhibited A1401G. In all, twelve (35.3%) of the 34 KAN^R isolates from Lucknow exhibited A1401G in six isolates (17.6%) and G1484T in five isolates (14.7%) (Table 2). Only one KAN^S isolate (2.9%) displayed A1401G mutation (Supplementary Table 4).

eis and Its Promoter

Three KAN^R isolates (10.3%) from Mumbai harbored mutations in either the *eis* promoter or the *eis* gene. Two (6.8%) and one isolates (3.4%) carried the reported C-14T and the G-10C promoter mutations, respectively (Table 2). The Open

Table 2 Observed SNP Distribution in Mumbai and Lucknow for *rrs, eis*, and *whiB7* Target Loci

Target Genes for Studying KAN^R	SNPs#	Relative Occurrence (%)			
		KAN^R		KAN^S	
		Mumbai	Lucknow	Mumbai	Lucknow
rrs gene	A1401G	51.7	17.6	–	2.9
	C1402T	3.4	–	–	–
	G1484T	–	14.7	–	–
eis promoter	G-10C	3.4	-	-	-
	C-14T&	6.8	2.9	-	-
	C-12T&	-	2.9	3.4	-
	G-37T&	-	2.9	-	-
eis gene	T204G&	-	2.9	-	-
	G487A	6.8	3.4	10.3	2.9
5′UTR *whiB7*	A43T, C44A, C47A, G48T	27.6	-	-	-
	T152G	13.8	-	-	-
	+G127&, A264G&	-	2.9	-	-
	ΔG177&	-	2.9	3.4	-
whiB7 gene	+CG244&, A42C, T270G	-	2.9	-	-

Notes: #Co-occurrence of single nucleotide polymorphisms (SNPs) is given in Supplementary Table 4 for the respective loci. Hyphen (-) indicates the absence of mutations in the screened isolates; &Indicates polymorphisms reported in Kaur et al, 2016; the underlined mutations are novel. All mutations were confirmed using three independent sequencing reactions.

Reading Frame (ORF) of *eis* exhibited G487A transition (Val163Ile) in two isolates, which was also observed in three of the KAN^S isolates. One KAN^S isolate (3.4%) harbored a C-12T mutation (Supplementary Table 4).

In addition to our previously reported *eis* promoter and ORF mutations,[37] the G487A mutation was displayed in one KAN^R and one KAN^S isolate of Lucknow (Supplementary Table 4).

whiB7 and 5' UTR

Nine KAN^R isolates (31%) from Mumbai exhibited five unique mutations in the *whiB7* 5′UTR, seen as heterogeneous peaks in chromatograms. Eight isolates (27.6%) co-harbored the novel A43T, C44A, C47A and G48T; and four (13.8%) isolates exhibited novel T152G mutation in the 5′UTR. One KAN^S isolate harbored the ΔG177 5′UTR deletion. Two other novel mutations were randomly distributed in KAN^R and KAN^S isolates of Mumbai. These were G59A in 5′UTR in 16 KAN^R and 7 KAN^S isolates; and C253T in *whiB7* gene (possibly leading to Arg85Cys in the WhiB7 protein) in 4 KAN^R and 21 KAN^S isolates (Supplementary Table 4).

Apart from mutations reported by our group in Kaur et al,[37] one KAN^R strain showed novel A42C (Arg14Ser) and T270G (Ala90Ala) in Lucknow strains. The G59A 5′UTR and C253T ORF novel mutations were seen in three KAN^R isolates. One strain presented coexistence of these SNPs with A42C (Arg14Ser). All polymorphisms seen in the 34 KAN^R (14.7%) isolates are listed in Table 2 and Supplementary Table 4. One KAN^S isolate displayed C253T gene mutation (Supplementary Table 4).

CAP Resistance
tlyA

From a total of 58 isolates of Mumbai, 23 were CAP[R] while 35 were CAP[S]. None of the isolates displayed any polymorphisms specific to CAP resistance. The A33G (Leu11Leu) transition reported earlier was seen in 15 CAP[R] and all the 35 CAP[S] isolates, indicating no role in determining CAP resistance.[46] Two CAP[S] isolates (5.7%) also co-harbored novel mutation G165A (Val55Val) transition (Table 3 and Supplementary Table 5). In addition, 15 CAP[R] isolates harboring *rrs* 1400 region hotspot polymorphisms A1401G and C1402T exhibited cross resistance to KAN (Supplementary Table 5).

Amongst the 68 isolates of Lucknow, 20 were CAP[R], 41 were CAP[S], while the DST profile for 7 of the isolates could not be determined (CAP[ND]). Two CAP[R] isolates harbored previously reported C64T transition leading to the Glu22Stop non-sense mutation.[15] One CAP[R] isolate co-exhibited C64T along with novel +CG200 insertion mutation (Table 3, Supplementary Table 5). Only one CAP[S] isolate showed novel C415G (Leu139Val) mutation. The A33G was seen in 10 CAP[R], 32 CAP[S] isolates and all seven CAP[ND] isolates. One CAP[ND] isolate co-displayed novel +G543 mutation along with A33G. All the six CAP[R] isolates harboring *rrs* 1400 hotspot mutations A1401G and G1484T were also cross resistant to KAN (Supplementary Table 5).

OFX Resistance
gyrA

Out of 58 isolates from Mumbai, 47 were OFX resistant (OFX[R]) while 11 were OFX sensitive (OFX[S]). Twenty-seven isolates (57.4%) carried mutations in the 94th codon (Asp94X), where Asp was substituted with Gly (19 isolates; 40.4%), Ala (six isolates; 12.8%), His (one isolate; 2.1%), or Tyr (one isolate; 2.1%). Eleven isolates (23.4%) displayed Ala90Val substitution while one isolate, each (2.1%), exhibited Gly88Cys and Ser91Pro substitutions. Forty-four OFX[R] strains (93.6%) and 11 OFX[S] strains (100%) harbored Ser95Thr (Table 4 and Supplementary Table 6).

In Lucknow, 36 isolates were OFX[R], 31 isolates were OFX[S], and one isolate was OFX[ND]. Nineteen OFX[R] isolates (52.8%) carried mutations in the 94th codon (Asp94X), where Asp was substituted with Asp94Gly (12 isolates; 33.3%), Asp94Tyr (one isolate; 2.8%), Asp94Asn (four isolates; 11.1%) or Asp94His (two isolates; 5.5%). The mutations Ser91Pro and Ala90Val were observed in one (2.8%) and seven (19.4%) OFX[R] isolates, respectively. Besides, two OFX[R] isolates (5.8%) co-exhibited Ala90Val and Asp94Gly (Table 4). Amongst 31 OFX[S] isolates of Lucknow, Asp94X was found in four isolates (13%) and Ala90Val in two isolates (6.4%). One OFX[S] isolate (3.2%) displayed co-occurrence

Table 3 Relative Distribution of *tlyA* and *rrs* Mutations in the CAP[R] and CAP[S] Isolates from Mumbai and Lucknow

Target Genes for Studying CAP Resistance	SNPs[#]	Relative Distribution			
		CAP[R]		CAP[S]	
		Mumbai (n=23)	Lucknow (n=20)	Mumbai (n=35)	Lucknow (n=41)
tlyA	A33G	15	10	35	32
	C64T	–	2	–	–
	+CG200	–	1	–	–
	G165A	–	–	2	–
	C415G	–	–	–	1
rrs	A1401G	14	2		1
	C1402T	1	–		–
	G1484T	–	4	–	–

Notes: [#]Refer to Supplementary Table 5 for co-occurrence of mutations in *tlyA* and *rrs* loci. The total number of CAP[R/S] isolates from the respective regions included in this study is represented by "n". Hyphen indicates the absence of mutations in the screened isolates; the underlined mutations are novel. All mutations were confirmed using three independent sequencing reactions.

Table 4 Distribution of Mutations (%) Observed in *gyrA* and *gyrB* QRDRs in OFX^R/S^ Isolates of Mumbai and Lucknow

Target Genes for Studying OFX Resistance	SNP^#^	Occurrence of Mutations (%)			
		OFX^R^		OFX^S^	
		Mumbai	Lucknow	Mumbai	Lucknow
gyrA	Ser95Thr	93.6	75	100	68
	Asp94X^a^	57.4	52.8	–	13
	Ser91Pro	2.1	2.8	–	3.2
	Ala90Val	23.4	19.4	–	6.4
	Gly88Cys	2.1	–	–	–
gyrB	+G1359	2.1	–	–	–
	+A1429	2.1	–	–	–
	Arg446Leu	2.1	–	–	–
	Asn499Thr	2.1	–	–	–
	Glu501Asp	2.1	–	–	–
	Ala504Val	2.1	–	–	–
	Thr500Ala	–	2.8	–	–

Notes: ^#^Refer to Supplementary Table 6 for co-occurrence of mutations in the respective loci. ^a^Variable codons: Ala/Asn/His/Tyr/Gly; Hyphen indicates the absence of mutations in the screened isolates; the underlined mutations are novel. All mutations were confirmed using three independent sequencing reactions.

of Ser91Pro and Asp94Asn. The most frequent mutation, Ser95Thr, was seen in 27 out of 36 OFX^R^ strains (75%) and 21 out of 31 OFX^S^ (67.7%), (Table 4 and Supplementary Table 6). The Ser95Thr mutation, which fails to discriminate between resistant and sensitive samples, was widely distributed in isolates from both the regions and, therefore, excluded from the frequency calculations.

gyrB

Six OFX^R^ isolates of Mumbai collectively displayed two novel mutations, i.e., +G1359 (one isolate; 2.1%) and +A1429 (one isolate; 2.1%); along with four known mutations, namely, Ala504Val (one isolate; 2.1%), Glu501Asp (one isolate; 2.1%), Arg446Leu (one isolate; 2.1%) and Asn499Thr (one isolate; 2.1%) in the gyrB QRDR (Table 4). The *gyrB* QRDR harbored no mutations in all the 11 OFX^S^ strains (Table 4 and Supplementary Table 6).

In Lucknow, only one OFX^R^ isolate (2.8%) exhibited Thr500Ala substitution (A1498G), as shown in Table 4. All the 31 OFX^S^ strains harbored no mutations in *gyrB* QRDR (Table 4 and Supplementary Table 6).

All the six OFX^R^ strains of Mumbai carrying *gyrB* polymorphisms also co-displayed either Ala90Val or Asp94X or Ser91Pro gyrA mutations. Similarly, the OFX^R^ strain from Lucknow bearing Thr500Ala *gyrB* polymorphism co-harbored Ala90Val in *gyrA* QRDR (Supplementary Table 6).

Spoligotyping Analysis of Clinical Isolates

The international genotyping SITVIT2 database was used for analyzing the spoligotyping binary patterns. All the spoligotypic binary codes of each isolate along with their SIT numbers are listed in Supplementary Tables 7 and 8. Thirty-four and 54 different spoligotypic patterns were detected for Mumbai and Lucknow, respectively. In Mumbai, the East African Indian (EAI5) and Beijing spoligotypes were relatively more prevalent whereas the EAI3-IND, CAS, Manu, and Euro American ("T" and "X3") clades were sparsely distributed (Figure 2A). In Mumbai, the EAI5 clade comprised of SIT236 (13 isolates), SIT380 (two isolates), SIT1900 and SIT126 (one isolate each). One orphan/new SIT belonging to the EAI5 clade was unavailable in SITVIT2. One isolate each displayed SIT11 (EAI3-IND) and SIT26 (CAS1-Delhi).
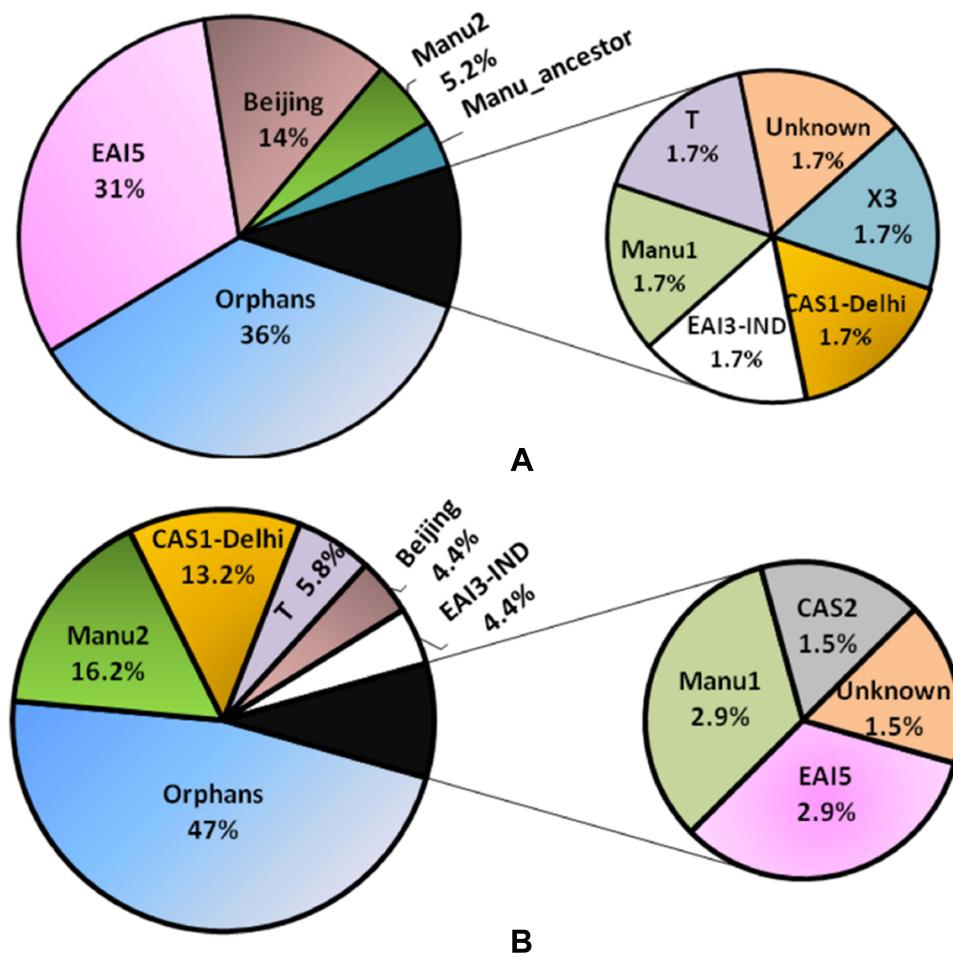
Figure 2 Percentage of different lineages for the isolates of Mumbai (**A**) and Lucknow (**B**) upon spoligotyping analyses using SITVIT2. CAS-Central Asian clade; EAI-East African Indian clade; T- ill-defined T clade; Beijing- East Asian Beijing clade; Orphans- unique patterns within any of the major clades listed in SITVIT2.

A new SIT representing Manu1 was detected in one isolate. Three and two isolates represented the Manu2 (SIT54) and Manu ancestor (SIT523) clades, respectively. The East Asian Beijing lineage was represented by SIT1 in seven isolates and SIT1941 in one isolate. Amongst the isolates belonging to Euro American lineage, SIT1077 (the ill-defined T clade), SIT200 (X3 clade) and a new unknown SIT1196 were found in one isolate each. Twenty-one isolates of Mumbai for which the spoligotype pattern has not been defined in the SITVIT2 database were termed as "Orphan or New".

In Lucknow, the presence of CAS1-Delhi, Manu2, and T1 spoligotypes was more pronounced (Figure 2B). The EAI5 was observed only in two isolates, with SIT3370 and SIT380. The spoligotype pattern of two and one isolates corresponded to SIT652 and SIT11 of EAI3-IND clade. There were nine isolates of Lucknow exhibiting the CAS1-Delhi clade: SIT26 in four isolates; and SIT2696, SIT1091, SIT357, SIT1942, SIT1401 in one isolate each. The CAS2 lineage corresponding to SIT288 in SITVIT2 was displayed by one isolate. The Manu2 clade was seen in 11 isolates: SIT54 in six isolates; SIT226, SIT1484, SIT1634, SIT1247 in one isolate each; and an orphan SIT in a single isolate. The East Asian Beijing lineage (SIT1) was observed in three isolates. The T1 clade of the Euro American lineage was observed in one and three isolates represented by SIT154 and SIT53, respectively. One T1 clade member also showed an orphan SIT. The remaining 32 isolates exhibited the orphan lineages which are undefined in SITVIT2.

Distribution and Clustering of Spoligotypes

The possible phylogenetic relationships between different isolates were deduced by clustering analysis using spolTools which generated spoligoforest trees with hierarchical layout for the two geographical locations. From the 34 spoligotype
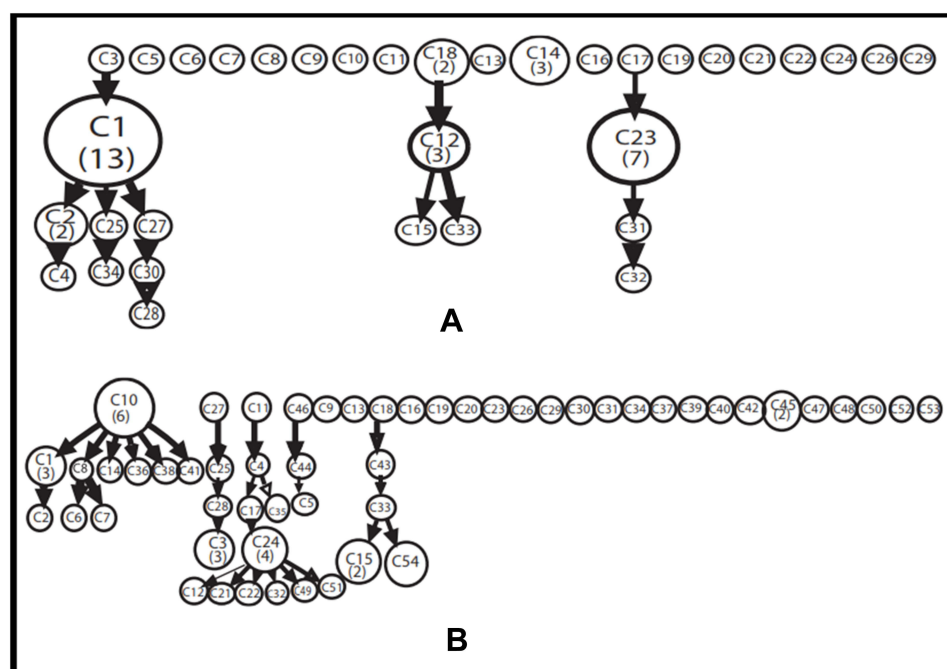
Figure 3 Cluster Analysis of different spoligotypic patterns obtained using spolTools. A hierarchical pattern of spoligoforest representation is provided here. (**A**) Mumbai; (**B**) Lucknow. The size of each node is represented by the number of isolates belonging to the cluster.

signatures found in 58 isolates of Mumbai, 30 isolates accounted for six clusters (C1, C2, C12, C14, C18 and C23) and 28 singletons displayed unshared unique spoligotypes (Figure 3A). In case of 68 isolates of Lucknow, 54 different spoligotypes corresponded to 48 singletons having only one isolate representative of a particular pattern, whereas six clusters (C1, C3, C10, C15, C24 and C45) were obtained from 20 isolates (Figure 3B).

Association of Spoligotypes with Unique Drug Resistance

Further, to understand the possible correlation of spoligotypic prevalence with isolates harboring the drug resistant mutations, a region-based evaluation was carried out. For each drug category, the percent relative lineage distribution among mutation-bearing drug resistant strains was calculated for the corresponding target loci. The distinct differences between Mumbai and Lucknow strains were observed. In Mumbai strains, although EAI5 lineage was particularly associated with OFX, KAN and CAP resistance, Beijing lineage was observed only with strains resistant to OFX; and Manus were distributed amongst all the three drugs resistant strains harboring mutations (Table 5). The spoligotypes T1, EAI3-IND and CAS1-Delhi were associated only with OFX resistance. However, only the association of EAI5, Manu and Beijing lineages with the mutations corresponding to OFX resistance; and KAN resistance with EAI5 and Manu were found to be statistically significant with p-value of 0.02 and 0.008, respectively.

For Lucknow isolates, the mutations were found to be scattered majorly amongst Manu2 and T1 followed by EAI5, Beijing and CAS1-Delhi for OFX resistance; Beijing and CAS1-Delhi followed by Manu2, T1 and EAI3-IND for KAN resistance; and Manu2 followed by T1 and CAS1-Delhi for CAP resistance (Table 5). The statistical analysis could not be performed on Lucknow strains due to the high scattering of different strains of lineages amongst all the three drug resistant strains (Supplementary Table 3).

Discussion

Different geographical areas are expected to have diversity in the genetic nature of *Mtb* strains. Although few reports on the comparative studies on drug resistance from some highly endemic resource-limited countries are available, the SLI resistant isolates of *Mtb* from Western and Northern India have never been compared.[47,48] This study not only compares

Table 5 Percent Distribution of Major Lineages Observed Among the Respective Drug Resistance Groups in Mumbai and Lucknow

Spoligotype	% Distribution of OFX^R Isolates Carrying *gyrA* and *gyrB* Mutations		% Distribution of KAN^R Isolates Carrying *eis*, *rrs*, and *whiB7* Mutations		% Distribution of CAP^R Isolates Carrying *rrs* and *tlyA* Mutations	
	Mumbai	**Lucknow**	**Mumbai**	**Lucknow**	**Mumbai**	**Lucknow**
EAI5	37.5	8	48	0	46.7	0
Manu1	2.5	0	0	0	0	0
Manu2	7.5	16	4	6.3	6.7	22.2
Manu_ancestor	2.5	0	4	0	0	0
Beijing	12.5	8	0	12.5	0	0
T1	2.5	12	0	6.3	0	11.1
EAI3-IND	2.5	0	0	6.3	0	0
CAS1-Delhi	2.5	8	0	12.5	0	11.1
Orphan	30	48	44	56.2	46.7	55.5

Notes: Major clades: EAI-East African Indian clade; Beijing-East Asian Beijing clade; T- ill-defined T clade; CAS1-Central Asian clade; Orphan-unknown patterns within any of the major clades listed in SITVIT2.

the prevalence of mutations in SLI resistant target loci, but also elucidates the lineage specific details of the *Mtb* clinical isolates from two geographically diverse states of India.

The new antimycobacterial agents such as bedaquiline, delamanid, pretomanid etc. have been discovered recently but SLI aminoglycosides along with fluoroquinolones constitute the most used second-line drugs for the treatment of MDR-TB in India.[49] Therefore, the drug susceptibility profiles for these new drugs were not determined routinely in the strain collection centers during the stated period. Thus, this study is limited to the preexisting treatment regimen of second line injectables and fluoroquinolones.

A few studies have reported the role of *rrs* loci mutations in conferring KAN, AMK, and CAP mono-resistance or cross-resistance.[24,50–54] Our study exhibited a higher prevalence of *rrs* mutations in isolates of Mumbai than those in Lucknow (55% vs. 32%). The most reliable SNP reported globally is A1401G for tracing KAN or AMK resistance.[52,55,56] In our analysis of the *rrs* hotspot, A1401G occurred at a significantly higher frequency (~52%) for the Mumbai isolates than in Lucknow region (~18%). The prevalence of *rrs* mutations analyzed in our studies is relatively lower when compared with the global status,[19] suggesting the need to screen additional SLD-resistance-associated loci for the Indian clinical isolates. A few studies have debated on the inclusion of the G1484T polymorphism in determining CAP resistance in absence of tlyA mutations.[9,53] In our investigation, the G1484T mutation was not observed in any of the CAP^R isolates of Mumbai compared to a 20% distribution in the CAP^R strains of Lucknow, suggesting its skewed prevalence in India.

The role of *eis* promoter mutations in context to KAN resistance has already been elucidated.[9,11,46,54,57] However, these may hold little relevance if a mutation in the *eis* ORF renders the protein non-functional. Studies on the identification of *eis* ORF polymorphisms have been minimal.[24,37] No other gene alteration besides the reported mutations, T204G and G487A, were identified.[37,58] The T204G mutation reported earlier by us in Lucknow strains was found to be missing in isolates of Mumbai.[37] Another mutation G487A observed in the KAN^R and KAN^S isolates of the two regions has already been reported in KAN^S isolates of China, marking a low confidence in this mutation for justifying KAN resistance.[58] In case of *eis* promoter mutations, the relative frequencies among the isolates of Mumbai and Lucknow were comparable (~10% in Mumbai vs. ~9% in Lucknow). In accordance with previous reports, C-14T, G-10C, C-12T, and G-37T promoter mutations were found in the KAN^R isolates of both regions under study.[10,11]

However, the C-12T mutation in the KAN^S isolate supports its reported insensitivity in determining high level KAN resistance.[19,40] Considering the occurrence of mutations in *eis* promoter, the screening of specific polymorphisms in this locus may attribute low or moderate KAN resistance in Indian clinical isolates.

The over-expression of Eis protein in the absence of mutations in *eis* promoter suggests an alternate mechanism for its transcriptional regulation.[59] Apart from our previous study from Lucknow,[37] not much has been reported regarding the prevalence of mutations in the *whiB7* 5′UTR and ORF in Indian isolates. Five novel mutations in *whiB7* 5′UTR and two novel mutations in *whiB7* ORF were specific to the KAN^R isolates indicating their significant association with KAN resistance. The G59A and ΔG177 (5′UTR) and C253T (*whiB7* ORF) mutations were equally pronounced in the KAN^R and KAN^S isolates, therefore, these mutations must not be considered for KAN resistance.

Association of CAP resistance with mutations in *tlyA* 2'-O-methyltransferase and *rrs* has been discussed in the past.[15,16,60,61] The novel mutations +CG200, C415G, and +G543 were more pronounced only in isolates of Lucknow. The synonymous novel mutation in the *tlyA* ORF, G165A, seen in two of the CAP^S isolates of Mumbai was absent in all the isolates of Lucknow and might not be useful in defining CAP resistance. Gln22Stop, found in CAP^R isolates of Lucknow, has been reported as a spontaneous polymorphism linked to CAP resistance.[15] Compared to *tlyA*, the *rrs* mutations (A1401G, C1402T, and G1484T) marked a greater confidence in defining CAP resistance. The *tlyA* and *rrs* mutations were observed to be mutually exclusive in the CAP^R isolates of Mumbai and Lucknow.

The mutations in the *gyrAB* QRDR culminate into FQ resistance, hampering its usage in combating MDR-TB.[62] All the OFX resistance-associated *gyrA* mutations observed in our study have been reported previously.[10,63,64] On the other hand, two novel *gyrB* QRDR mutations, i.e., +G1359 and +A1429 apparently cause the premature stop codon generation at residues 477 and 453, respectively. Favorable survival of mycobacteria despite *gyrB* distortion/depletion has already been documented and attributed to the activation of the RecA/LexA mediated SOS response, further enhancing the drug tolerance and induction of persister cells.[65] None of the OFX^S isolates from the two states carried any polymorphism in *gyrB* QRDR, indicating its specificity towards FQ resistance. Besides, co-existence of *gyrB* with *gyrA* mutations indicates that the inclusion of novel *gyrB* mutations might be crucial in reframing the fluoroquinolone resistance in SNP based assays. This study could not comment on the association of *gyrAB* mutations with respect to newer drugs like moxifloxacin, gatifloxacin, and levofloxacin owing to the lack of availability of the respective DST profiles at the time of analysis. However, reports suggest that the mutations in codons 90 and 94 of *gyrA* are also responsible for rendering *Mtb* resistant to the newer FQs.[66,67]

Besides noteworthy differences observed in our SNP analysis, the spoligotyping evaluation also suggested region dependent differential lineage patterns. While a notably higher prevalence of EAI5 (31%) and East Asian Beijing (14%) lineage was recorded in isolates of Mumbai, the Manu2 (16%), Central Asian (CAS1-Delhi, 13%) and T (6%) spoligogroups were higher in Lucknow. Numerous studies have reported the predominance of CAS lineages in North Indian regions.[27,32,68–71] In contrast to the reported lower frequencies of Manu/SIT54 in North India,[68,70] a higher percentage of Manu2 lineage (~55%) is seen in Lucknow isolates. In our study, the predominance of EAI5 family (31%) shows a divergence from the mostly reported Manu1, CAS, and Beijing lineages from Western India.[35,72,73] The significant proportion of Orphan spoligotypes in the current study also points towards large number of unidentified spoligotypes which can only be confirmed by larger sample size and regular cluster investigation to monitor the shifting course of spoligotypes in India.

A recent study points out to the mixed or polyclonal infections in India that may occur due to infection by more than one genotypes during treatment, making drug resistance even a bigger nuisance.[74] While some reports discuss Manu ancestral lineage as an artefact due to co-infection by strains of Lineage 2 and 4, we are not in a position to comment, as this study did not examine any co-infection or polyclonal infections.[75,76]

Conclusion

On a comprehensive note, although the present study was limited by a small sample size and unknown CAP susceptibility for a few isolates, our findings reflect pronounced lineage and mutational diversity among the two distant geographical locations within India. The novel mutations observed in our study contribute to the existing pool of information on genetic polymorphisms in the drug resistant strains from India. For the isolates lacking mutations in target

loci, other determinants with unfamiliar resistance mechanisms are likely responsible. In addition, our studies showcased a higher prevalence of EAI and Beijing in Mumbai and CAS1-Delhi and Manu in Lucknow. Finally, a higher correlation of specific lineages with resistance patterns is necessary to provide a deeper insight into the chromosomally governed genotypic blueprints of resistance.

Ethical Approval

Waiver of consent was obtained from Ethics Committee of KGMU, Lucknow, Uttar Pradesh and Institutional Review Board of P. D. Hinduja National Hospital and Research Centre, Mumbai, Maharashtra, India.

Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

Disclosure

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