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Genotype analysis of ofloxacin-resistant multidrug-resistant *Mycobacterium tuberculosis* isolates in a multicentered study from India

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***Background & objectives*: Drug resistance surveillance offers useful information on trends of drug resistance and the efficacy of control measures. Studies and reports of drug-resistant mutations and phenotypic assays thus become important. This study was conducted to investigate the molecular characteristics of ofloxacin (OFX)-resistant, multidrug-resistant tuberculosis (MDR-TB) isolates from different geographical regions of India and their association with strains of different genotypes. Further, the nitrate reductase assay (NRA) was tested against Mycobacteria Growth Indicator Tube (MGIT) for the determination of OFX resistance as an alternative and cost-effective method.**

***Methods*: A total of 116 *Mycobacterium tuberculosis* isolates were used to assess the mutations in the *gyrA*, *gyrB* genes and resistance levels to OFX. Mutational analysis in *gyrA* and *gyrB* genes and genotype analysis of *M. tuberculosis* isolates was done by gene-specific polymerase chain reaction (PCR) followed by DNA sequencing and spoligotyping, respectively.**

***Results*: Three (6.25%), 12 (44.44%) and 12 (29.27%) MDR-TB isolates from western, northern and southern India, respectively, were found to be OFX-resistant MDR-TB isolates. OFX resistance was observed to be significantly higher in MDR-TB cases for all study regions. Beijing genotypes from northern India were observed to be associated with OFX-resistant MDR-TB cases ($P<0.05$). Among 35 (30.15%) phenotypically OFX-resistant isolates, 22 (62.86%) had mutations in the *gyrA* gene and two (5.71%) isolates had mutations in the *gyrB* gene.**

***Interpretation & conclusions*: These results caution against the PCR-based prediction of OFX resistance patterns and highlight the need for searching other genetic loci for the detection of mutations conferring resistance to OFX in *M. tuberculosis*. Our study also showed the usefulness of NRA as an alternative method to detect OFX resistance.**

Key words Drug resistance - gyrase - multidrug-resistant tuberculosis - *Mycobacterium tuberculosis* - nitrate reductase assay - ofloxacin resistance - spoligotyping

Fluoroquinolones (FQs) such as ofloxacin (OFX), levofloxacin or moxifloxacin are important drugs for the management of multidrug-resistant tuberculosis (MDR-TB), and their resistance is associated with the condition of extensively drug-resistant (XDR)-TB. XDR-TB is defined as MDR-TB with additional resistance to any FQs and a second-line injectable drug such as kanamycin, amikacin, or capreomycin[1]. Dispersed but scanty reports are available from India about XDR-TB[2-5], but are not enough to estimate the extent and magnitude of XDR-TB. Reasons primarily include the lack of facilities for second-line drug susceptibility determination, availability of standardized protocols with desired facilities and our focus on primary care of TB patients. It is important to monitor resistance to these drugs, especially FQs like OFX. OFX remains the backbone of second-line treatment along with amikacin, important for the detection of XDR cases and crucial for overall management and control of TB[2]. Inaccurate testing of TB also facilitates the emergence of drug resistance (DR). Therefore, the accurate and rapid detection of TB and DR-TB is imperative for timely and adequate treatment to interrupt the transmission of DR/MDR- and XDR-TB[6]. Several molecular and commercial tools are available for susceptibility testing of *Mycobacterium tuberculosis*[7]. It is, however, difficult for a low resource setting to have all these sophisticated techniques and infrastructure. Earlier, the nitrate reductase assay (NRA) for the detection of first-line anti-TB drug resistance was evaluated[8,9]. NRA is a rapid, inexpensive, phenotypic method that can easily be incorporated into existing facilities of a low resource setting and is based on the metabolic ability of *M. tuberculosis* to reduce nitrate to nitrite[10]; it has been endorsed by the WHO for the rapid detection of MDR-TB[11].

This study was undertaken to do genotypic analysis of OFX-resistant and MDR-TB isolates from different regions of India to determine molecular mutations conferring drug resistance. We further evaluated NRA to detect OFX-resistant MDR-TB isolates using MGIT960 as the gold standard.

Material & Methods

Mycobacterium tuberculosis isolates from the ICMR-National AIDS Research Institute (ICMR-NARI), Pune; ICMR-National Institute for Research in Tuberculosis (ICMR-NIRT), Chennai; State TB Testing and Demonstration Centre (STDC), Pune, and Institute of Medical Sciences, Banaras Hindu University (BHU), Varanasi, were included. Information on demographic characteristics of patients and sputum mycobacteriology studies were collected from the medical files and compliance charts of the centres involved except for the southern part of India. The study was approved by the Ethics Committee of ICMR-NARI, Pune.

A total of 116 clinical isolates of *M. tuberculosis* collected from three different geographical regions of India namely Pune (western India), Varanasi (northern India) and Chennai (southern India) during 2013-2014 were studied. All isolates were freshly sub-cultured on LJ (Löwenstein-Jensen) medium (HiMedia, Mumbai) before use.

OFX was purchased from Sigma-Aldrich, USA. The stock solution was prepared at a concentration of 2 µg/ml in 0.1 N NaOH, filter sterilized and preserved at −20°C for not more than one month. To prepare Griess reagent 50 per cent (vol/vol) concentrated hydrochloric acid (HCl), 0.2 per cent (wt/vol) sulphanilamide, and 0.1 per cent (wt/vol) n-1-naphthylethylenediamine dihydrochloride were prepared in small volumes and mixed shortly before use in the ratio1:2:2, respectively.

Drug susceptibility testing (DST) against first-line drugs: BACTEC™ MGIT™ 960 Mycobacterial Detection System (Becton, Dickinson and Company, USA) using SIRE kit was used as per manufacturer's instructions for the susceptibility testing against streptomycin (STR), isoniazid (INH), rifampin (RIF) and ethambutol (EMB). *M. tuberculosis* H_{37}Rv (ATCC 27294) was used as the susceptible control.

MGIT960 for ofloxacin-drug susceptibility testing (OFX-DST): For susceptibility testing against OFX, blank tubes of medium were used wherein the final concentration of OFX was 2 µg/ml as per the Clinical and Laboratory Standards Institute (CLSI) standards[12]. The inoculum was prepared from a fresh LJ medium by following the methods used by Devasia *et al*[13]. A growth control containing no antibiotic and a sterile control without inoculation were used with every set of experiments.

Nitrate reductase assay for OFX-DST: The assay was performed as described earlier[14]. Briefly, the 7H11 medium was prepared by adding OFX at the concentration of 2 µg/ml and 1 mg/ml of sodium nitrate ($NaNO_3$). The inoculum turbidity was adjusted

to a 1 McFarland tube, and the inoculum was diluted 1:10 vol/vol in phosphate-buffered saline. For each isolate, 200 µl of the undiluted suspension was inoculated into the OFX containing tube, and 200 µl of the 1:10 dilution was inoculated into the drug-free tube. The tubes were incubated at 37°C. After seven days, 500 µl of the Griess reagent mixture was added to one drug-free tube. If any colour appeared, the reagent mixture was added in all the tubes; otherwise, the tubes were re-incubated and the procedure was repeated at days 9 and 14. An isolate was considered resistant if there was a colour change in the antibiotic tube greater than that in the 1:10-diluted growth control.

PCR amplification/DNA sequencing for detection of ofloxacin resistance determinants: Genomic DNA was extracted from *M. tuberculosis* isolates using the CTAB (cetyltrimethylammonium bromide)-NaCl method[15] and PCR assay was performed to detect mutations in *gyrA* and *gyrB* genes. Primers for *gyrA* and *gyrB* were designed by taking NC_000962 as a template which also covers the quinolone resistance determining region (QRDR) of the open reading frame (ORF). The *gyrA* gene was amplified with the forward primer (5-CCCTGCGTTCGATTGCAAA-3) and reverse primer (5-CTTCGGTGTACCTCATCGCC-3). The *gyrB* gene was amplified using the forward primer (5-ACGCGAAAGTCGTTGTGAAC-3) and reverse primer (5-CGCTGCCACTTGAGTTTGTAC-3). The amplification reaction volume of 25 µl contained 1x HiFi Buffer (Kapa Biosystems Inc., USA), 2 mM $MgCl_2$, 200 µM each of dNTP (Fermentas, USA), 10 pmol each of forward and reverse primers, 1 U of KAPA HiFi HotStart DNA Polymerase (Kapa Biosystems Inc., USA), 10 ng of DNA template and water to make the final volume. Amplification conditions were used as suggested by the manufacturer of KAPA HiFi HotStart DNA Polymerase with 66°C as annealing temperature for *gyrA* and 62°C as annealing temperature for *gyrB*. PCR products of *gyrA* and *gyrB* were 423 and 520 base pairs (bp) respectively in size. Automated sequencing was performed using the BigDye Terminator kit v3.1 (Applied Biosystems, USA) as per manufacturer's protocol. DNA sequence analysis and comparisons of *gyrA* (n=116) and *gyrB* (n=116) were carried out with SeqScape® v2.5 software from Applied BioSystems, USA.

Spoligotyping and data analysis: Spoligotyping was performed using the standard method[16] at ICMR-NIRT, Chennai. Results were matched with the SITVIT web database to get spoligotypes matched with available profiles (*http://www.pasteur-guadeloupe.fr:8081/SITVIT_ONLINE/*), and major lineages were determined.

Statistical analysis: MedCalc-free statistical calculators (*https://www.medcalc.org/calc/*) were used to calculate the statistical measures of sensitivity, specificity, accuracy, positive predictive value (PPV) and negative predictive value (NPV) of the NRA using the MGIT 960 system and DNA sequencing as the reference tests. Fisher's exact test was used to calculate exact probability.

Results

In the present study, a total of 116 *M. tuberculosis* isolates, obtained from 116 adult patients with pulmonary TB were used. Amongst 116 isolates, 45 (38.79%) and 27 (23.28%) were identified as MDR and OFX-resistant MDR-TB, respectively. There were 48 isolates from western India; 27 from northern India and 41 from southern India. The clinical characteristics of study population are given in Table I.

Performance of NRA for OFX-DST: An excellent agreement was obtained between NRA and the reference method MGIT 960 system with 100 per cent sensitivity, specificity, NPV and PPV. When the results of NRA were compared with DNA sequencing for OFX resistance detection, 90 per cent sensitivity and 97.67 per cent specificity were found with excellent agreement between the two methods.

Frequency and association of ofloxacin resistance with clinical characteristics of TB patients: OFX resistance was observed to be significantly higher in MDR-TB cases in all study regions (Table II). There were no significant differences found between OFX resistance and age, sex, HIV status and treatment history of patients based on the available data. However, a focused study to specifically assess these characteristics and their association with the development of OFX resistance may be taken up with a larger sample size. The frequency of OFX resistance in the studied isolates from the north and south India was higher compared to western India (Table II). The frequency of OFX-resistant MDR-TB cases was three (6.25%), 12 (44.44%) and 12 (29.27%) in western, northern and southern regions of India, respectively.

Table I. Clinical characteristics of the study population among phenotypically ofloxacin (OFX)-resistant and -susceptible *Mycobacterium tuberculosis* isolates from different parts of India

Total isolates = 116		
Western India (n=48)		
Characteristics	OFX-resistant (n=6)	OFX-susceptible (n=42)
Sex		
Male (36)	6	30
Female (12)	0	12
Age group (yr)		
15-44 (41)	6	35
≥45 (7)	0	7
HIV status		
HIV infected (12)	2	10
No HIV (36)	4	32
Treatment history		
Acquired drug-resistant (4)	1	3
Primary drug-resistant (44)	5	39
Northern India (n=27)		
Characteristics	OFX-resistant (n=16)	OFX-susceptible (n=11)
Sex		
Male (16)	9	7
Female (11)	7	4
Age group (yr)		
15-44 (21)	13	8
≥45 (6)	3	3
HIV status		
HIV infected (2)	0	2
No HIV (25)	16	9
Treatment history		
Acquired drug-resistant (11)	8	3
Primary drug-resistant (16)	8	8
Southern India (n=41)		
Characteristics	OFX-resistant (n=13)	OFX-susceptible (n=28)
Treatment history		
Acquired drug-resistant (35)	11	24
Primary drug-resistant (6)	2	4

Frequency of gyrA and gyrB gene mutations in OFX-resistant and sensitive clinical isolates: Our results revealed that among 35 (30.15%) phenotypically OFX-resistant isolates, 22 (62.86%) had mutations in the *gyrA* gene and two (5.71%) isolates had mutations in the *gyrB* gene. The single-nucleotide mutation sites were in codons GAC500GCC and GAC472GTC. The changes at this position are reported earlier (GAC-GCC), but the one in the present study was different GAC (codon 472 and nucleotide position 1415) to GTC (Table III). Both the isolates were from southern India. No *gyrB* mutations were observed in northern and western Indian isolates. Six of the 48 (12.5%), 16 of the 27 (59.26%) and 13 of the 41 (31.7%) isolates, from western, northern and southern India, respectively,

Table II. Calculation of significance for the observation of ofloxacin (OFX) resistance in multidrug-resistant (MDR) and non-MDR cases (using MedCalc online calculator software)

Total isolates = 116						
Characteristics		OFX-resistant	OFX-susceptible	Odds ratio (OR)	*P*	95% CI
Western India (n=48)						
OFX-resistant MDR-TB	MDR (9)	3	6			
	Non-MDR (39)	3	36	6.000	0.05	0.9733 to 36.9869
Northern India (n=27)						
OFX-resistant MDR-TB	MDR (16)	12	4			
	Non-MDR (11)	4	7	5.250	0.05	0.9881 to 27.8958
Southern India (n=41)						
OFX-resistant MDR-TB	MDR (20)	12	8			
	Non-MDR (21)	1	20	30.00	0.002	3.3286 to 270.3822

CI, confidence interval

Table III. Phenotypic quinolone resistance and polymorphisms in *gyrA* and *gyrB* of *Mycobacterium tuberculosis*

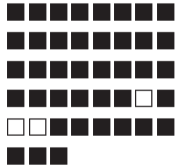
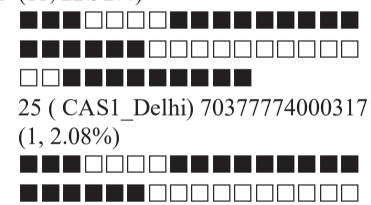
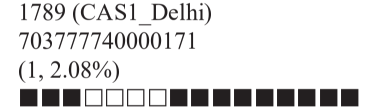
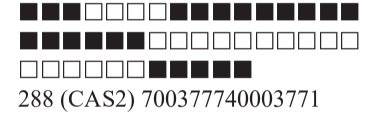
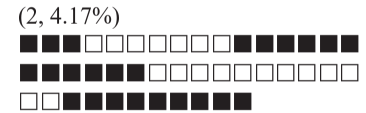
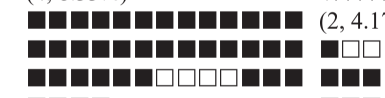
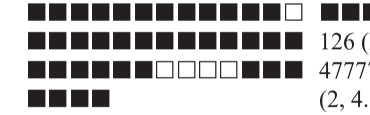
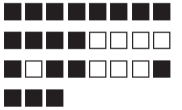
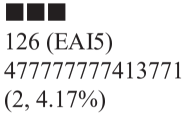
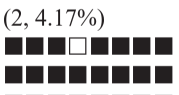
Gene	Codon mutation (nucleotide change)	Total isolates = 116					
		Western India (n=48)		Northern India (n=27)		Southern India (n=41)	
		Phenotypically quinolone-resistant (n=6), n (%)	Phenotypically quinolone-susceptible (n=42), n (%)	Phenotypically quinolone-resistant (n=16), n (%)	Phenotypically quinolone-susceptible (n=11), n (%)	Phenotypically quinolone-resistant (n=13), n (%)	Phenotypically quinolone-susceptible (n=28), n (%)
gyrA	A90V (GCG-GTG)	-	1 (2.38)	2 (12.5)	2 (18.18)	2 (15.38)	1 (3.6)
	D94G (GAC-GGC)	2 (33.33)	-	10 (62.5)	-	2 (15.38)	-
	D94N (GAC-AAC)	-	-	1 (6.25)	-	1 (7.69)	-
	D94Y (GAC-TAC)	-	-	-	-	1 (7.69)	-
	A90V+D94G (GCG-GTG+GAC-GGC)	-	-	-	-	1 (7.69)	-
gyrB	Asp500Ala (GAC-GCC)	-	-	-	-	1	-
	Asp472Val (GAC-GTC)	-	-	-	-	1	-

Polymorphisms at codons 21 and 95 of *gyrA* were excluded because these are not known to be associated with quinolone resistance; only mutations at codon 90, 91 or 94 in *gyrA* are listed here

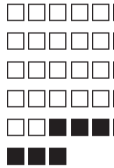
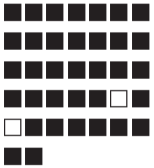
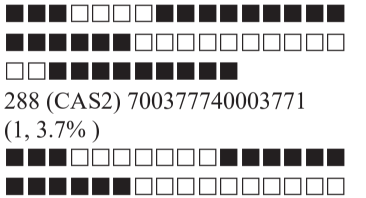
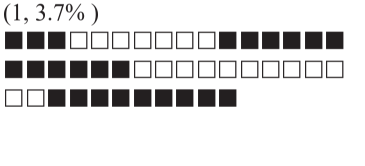
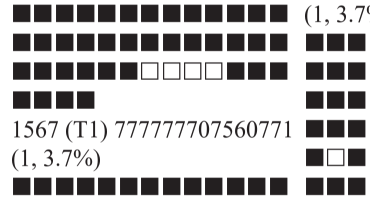
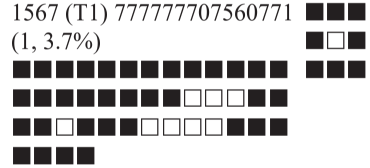
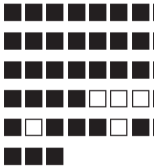
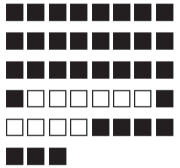
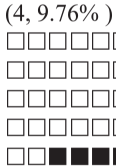
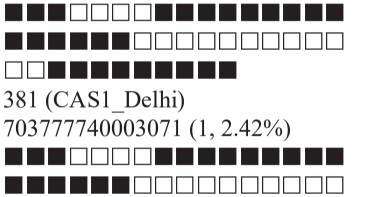
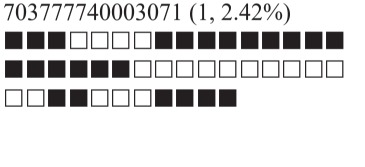
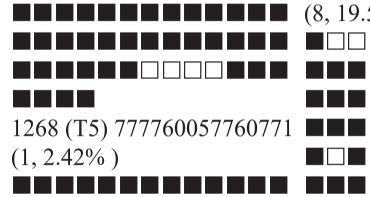
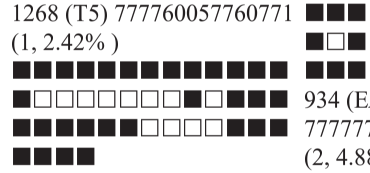
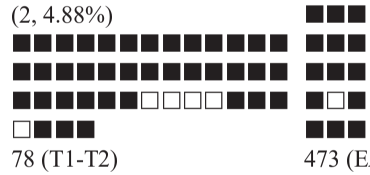
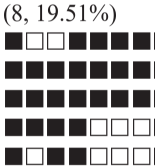
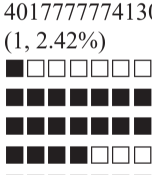
were resistant to OFX. The most predominant mutation occurred at codon D94G with nucleotide substitution from GAC-GGC in 2/6 (33.33%), 10/16 (62.5%) and 2/13 (15.38%) OFX-resistant isolates from western, northern and southern India, respectively. Further, two and one among OFX-resistant isolates from northern and southern India were found to have mutations at codons A90V (GCG-GTG) and D94N (GAC-AAC), respectively. One isolate from southern India had double mutations at codons A90V and D94G (GCG-GTG, GAC-GGC) (Table III). Four (1 each from western, southern and 2 from northern India) OFX-susceptible isolates were found to be false positive and had a mutation at codon A90V (GCG-GTG). Most of the clinical isolates (105/116, 90.52%) had an AGC-ACC and GAG-CAG polymorphisms at codons 95 and 21, respectively, but were not related to OFX resistance.

Prevalent genotypes and drug resistance: Central Asian strain (n=18, 37.5%), Beijing (n=9, 33.33%) and East African Indian (n=11, 26.83%) genotypes were predominant in western, northern and southern India, respectively (Tables IV and V). The distribution of DR according to *M. tuberculosis* genotype and geographical regions has been summarized in Table V. Only Beijing genotypes from northern India were observed to be associated with OFX-resistant MDR-TB cases (7/12, 58.33%, *P*=0.021).

Table IV. Distribution of different family strains in northern, western and southern regions of India

Regions of India (n)	SIT (Clade) Octal number (No, % in study) Spoligotype description						Orphans*
Western (48)	1 (Beijing) 000000000003771 (2, 4.17%) 	1634 (Manu2) 777777777723771 (1, 2.08%) 	26 (CAS1_Delhi) 703777740003771 (11, 22.92%)  25 (CAS1_Delhi) 703777740003171 (1, 2.08%) 427 (CAS1_Delhi) 703707740003771 (1, 2.08%)  1789 (CAS1_Delhi) 703777740000171 (1, 2.08%)  288 (CAS2) 700377740003771 (2, 4.17%)  486 (CAS) 703777740000371 (1, 2.08%)  357 (CAS) 703777740000771 (1, 2.08%) 	53 (T1) 777777777760771 (4, 8.33%)  37 (T3) 777737777760771 (2, 4.17%)  1077 (T2) 777777377760731 (2, 4.17%) 	11 (EAI3_IND) 477777777413071 (2, 4.17%)  126 (EAI5) 477777777413771 (2, 4.17%)  355 (EAI5) 477777777413031 (1, 2.08%)  48 (EAI1_SOM) 777777777413731 (1, 2.08%) 	99 (Haarlem3) 757777777720771 (2, 4.17%) 	11

Contd...

Regions of India (n)	SIT (Clade) Octal number (No, % in study) Spoligotype description						Orphans*
Northern (27)	1 (Beijing) 000000000003771 (9, 33.33%) 	1634 (Manu2) 777777777723771 (1, 3.7%) 	26 (CAS1_Delhi) 703777740003771 (2, 7.41%)  288 (CAS2) 700377740003771 (1, 3.7%) 	53 (T1) 777777777760771 (3, 11.11%)  1567 (T1) 777777707560771 (1, 3.7%) 	235 (EAI1_SOM) 777777777413531 (1, 3.7%) 	47 (Haarlem1) 777777774020771 (1, 3.7%) 	8
Southern (41)	1 (Beijing) 000000000003771 (4, 9.76%) 	0	26 (CAS1_Delhi) 703777740003771 (4, 9.76%)  381 (CAS1_Delhi) 703777740003071 (1, 2.42%) 	53 (T1) 777777777760771 (2, 4.88%)  1268 (T5) 777760057760771 (1, 2.42%)  52 (T2) 777777777760731 (2, 4.88%)  78 (T1-T2) 777777777760711 (1, 2.42%) 	11 (EAI3_IND) 477777777413071 (8, 19.51%)  934 (EAI5) 777777777413071 (2, 4.88%)  473 (EAI3_IND) 401777777413071 (1, 2.42%) 	0	15

Schematic representation of the polymorphism in repeat region of *M. tuberculosis* based on the presence of spacers. Closed boxes represent positive hybridization denoting the presence of spacer sequence while the open boxes represent the lack of hybridization and hence the absence of spacer sequence.
*Not found in SpolDB4.0 database; SIT, Spoligo-International-Type

Table V. Frequency of genotypes among drug-resistant tuberculosis patients in different geographical regions of India

Characteristics	Western India (n=48), n (%)	Northern India (n=27), n (%)	Southern India (n=41), n (%)
CAS genotype	18 (37.5)	3 (11.11)	5 (12.2)
OFX resistance	1 (2.1)	2 (7.41)	2 (4.88)
MDR-TB	2 (4.16)	2 (7.41)	2 (4.88)
OFX-resistant MDR-TB	1 (2.1)	2 (7.41)	2 (4.88)
Beijing genotype	2 (4.17)	9 (33.33)	4 (9.76)
OFX resistance	2 (100)	7 (77.78)	1 (25)
MDR-TB	2 (100)	8 (88.89)	4 (100)
OFX-resistant MDR-TB	1 (50)	7 (77.78)	1 (25)
EAI genotype	6 (12.5)	1 (3.7)	11 (26.83)
OFX resistance	0	0	1 (2.42)
MDR-TB	0	0	2 (4.88)
OFX-resistant MDR-TB	0	0	1 (2.42)

CAS, Central Asian Strain; EAI, East African Indian

Discussion

The present study was conducted with two major objectives. One being the evaluation of the performance of NRA for OFX-DST, and the other one was to find the level of concordance between genotypic assays and phenotypic assays for drug resistance. The NRA has earlier been evaluated in some settings for the detection of OFX resistance[13,17-19] including one study from India[20]. We obtained 100 per cent concordance of NRA with MGIT960-based assay, thus, NRA could be proposed as a promising tool to detect MDR-/XDR-TB isolates in low-resource settings. NRA is accurate and easy to implement in clinical diagnostic laboratories, making it a good option for rapid screening for MDR- and XDR-TB.

Earlier studies reported that the majority (50-90%) of FQ-resistant *M. tuberculosis* isolates carry mutations in the QRDR of the *gyrA* and a small number (3-7%) have mutations in the *gyrB* gene[21,22]. The analysis of *gyrA* and *gyrB* gene mutations in OFX-resistant and sensitive clinical isolates of *M. tuberculosis* revealed various point mutations at single or more than one position. Some mutations were found at earlier reported positions in the sequenced genes but had a different nucleotide. This showed a different amino acid on translation. The change in amino acid may have nominal or significant effect on protein structure and functions. In some cases, the observed mutations could not be correlated with phenotypic results or the phenotypes did not match the genotypes. The reason for this could be the presence of a compensating mechanism working elsewhere in the genome. In the present study, the association between *gyrA/B* mutation and OFX resistance was significant [odds ratio (OR)=32.58; *P*=0.001; 95% confidence interval (CI)=8.639-135.132]. Our data suggested that though *gyrA/B* was a sensitive marker for OFX resistance study as suggested earlier[20,21], it was not the only causative agent of OFX resistance. This was because in our study, only 68.57 per cent (24/35) of OFX-resistant isolates had mutations in *gyrA/B* gene. This situation has been reported previously also with FQs resistance[23] and can be explained by a mutation outside of the sequenced regions or another molecular alteration decreasing the accumulation of the drug inside the cell, such as an efflux mechanism[23,24]. In addition to other substitutions, we got a new substitution at *gyrA* codon 15 and 25 (nucleotide position 43 and 73, respectively) in two and three isolates, respectively, along with a large number of isolates harbouring the polymorphisms at codons 95 and 21. These substitutions were not found to be related to OFX resistance and might have evolutionary significance.

Overall, the OFX resistance was observed to be significantly higher in MDR-TB cases in all study regions and could be a surrogate marker for the emergence of OFX-resistant MDR-/XDR-TB cases. Therefore, OFX resistance may also be included along with other four first-line drugs for the initial screening of DR-TB. The frequency of OFX resistance in the studied isolates was observed to be higher in isolates

from northern and southern India compared to western India. The reason for this was not clear from the data we had and a study with a significantly higher number of isolates would be able to explain the causality of this. In all, OFX resistance was higher in all regions studied, which was in line with previous studies[5,25,26].

Our data imply that molecular techniques targeting only QRDR region to detect FQ resistance may be less specific in areas with a high prevalence of FQ-resistant MDR-TB. Larger patches of sequences covering the whole gene and regulatory regions along with other molecular alterations decreasing the accumulation of the drug inside the cell, such as an efflux mechanism should be considered. Available information on genotypes of OFX-resistant MDR-TB isolates from different geographic regions of India is scanty. Our study showed a predominance of Beijing/CAS and EAI genotypes and an association between MDR and OFX resistance-conferring mutations and the Beijing genotype. A study from north India also observed Beijing as dominant genotype along with CAS_Delhi among OFX-resistant MDR-TB isolates[5]. While correlating the association of *gyrA* mutations in various lineages another study from north India found that the association of Beijing isolates was significantly high (P=0.0006) with *gyrA* gene mutations[27]. A study conducted in Vietnam observed a significant association of Beijing genotype of *M. tuberculosis* with high-level quinolone resistance[28] while in China, Beijing genotype showed no association with FQ resistance[29,30]. These findings may be useful for the establishment of rapid molecular diagnostic methods to be implemented in India and countries with high OFX resistance. Studies with a higher number of patients over long periods are required to see the pattern of OFX resistance and to understand the epidemiology of OFX-resistant MDR- and XDR-TB in India.

One of the limitation of our study was that spoligotyping was used instead of 24 loci MIRU for genotyping of *M. tuberculosis* but the information obtained using spoligotyping could be useful as it is a first-line discriminatory test and the gold standard for the identification of Beijing strains of *M. tuberculosis.* Further, OFX-resistant MDR-TB could actually be the XDR-TB in many cases which not studied but data of DST against the injectables *i.e*., kanamycin, capreomycin and amikacin were not available. The DST only to OFX and not against aminoglycosides may not be enough to estimate the situation of XDR-TB in India. Further efforts are required to take care of these limitations with a larger number of sample.

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***Conflicts of Interest*:** None.

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