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Spoligotyping, phenotypic and genotypic characterization of *katG*, *rpoB* gene of *M. tuberculosis* isolates from Sahariya tribe of Madhya Pradesh India

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ABSTRACT

Background: Sahariya, a primitive tribal group, residing in Gwalior and Sheopur districts of Madhya Pradesh, India, show high incidence and prevalence of pulmonary tuberculosis (PTB). The study was designed to understand the genetic diversity and phenotype — genotype association of drug resistant *M. tuberculosis* strains, infecting Sahariya tribe.
Materials and methods: A total of 103 pulmonary tuberculosis patients from Sahariya tribe were recruited from Gwalior and Sheopur districts. Sputum samples were collected and cultured on LJ slants and drug sensitivity tests were carried out. Genomic DNA was extracted, followed by spoligotyping and genotyping of drug target genes, *katG* and *rpoB*, using MAS-PCR, PCR-RFLP and DNA sequencing.
Result: Seventeen different spoligotypes were identified, in which, EAI3_IND/ST11 *M. tuberculosis* strain appeared predominant, followed by CAS1_Delhi/ST26. Results of our phenotypic drug susceptibility test identified high incidence (12.6%) of isoniazid-resistant tuberculosis, while 4.85% isolates were multi drug resistant (MDR). Further genotyping of drug target genes identified 8.7% of isoniazid-R isolates to have a mutation at *katG* codon 463, while 3.8% isolates showed mutations at two sites, *katG* codons 315 and 463. In case of MDR-TB isolates, all from CAS lineage, 3.85% had mutations on *katG* and *rpoB* genes, at codon 463 and codon 526, respectively, while 0.97% isolates were harbouring mutations at codons 315, 463 and 531.
Conclusion: Our findings have revealed that EAI3_IND strain is the predominant strain infecting Sahariya. The incidence of isoniazid-R *M. tuberculosis* strain infection is high, with an increased propensity to evolve into MDR-TB. Therefore, the TB centres should also consider isoniazid-R status of the isolates along with CBNAAT before deciding the drug regimen for the patients.

Introduction

Tuberculosis (TB) is among the deadliest infectious diseases, caused by different species of Mycobacterium complex (MTBC), which includes closely related species, such as *M. tuberculosis*, *Mycobacterium bovis*, *Mycobacterium africanum*, *Mycobacterium microti*, *Mycobacterium pinnipedii*, *Mycobacterium caprae*, *Mycobacterium canetti*, exhibiting distinct phenotypic properties and host range [1]. Global tuberculosis report published by World Health Organisation in 2018 estimated 10.0 million new incident cases of tuberculosis and about 1.3 million deaths among HIV negative patients. The high incidence cases of tuberculosis mainly occurred in South East Asia (44%), African (25%), followed by Western Pacific (18%), Eastern Mediterranean region (7.7%), Europe and America. Report also identified eight countries that account for two thirds of the global tuberculosis burden with India (27%), China (9%),

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Indonesia (8%), the Philippines (6%), Pakistan (5%), Nigeria (4%), Bangladesh (4%) and South Africa (3%) [2]. In terms of epidemiology, the importance of molecular typing of *M. tuberculosis* has been useful in the outbreak investigations in health care settings, as it helps to understand the transmission dynamics of *M. tuberculosis* strains and their distribution at a particular region/area, if so [3]. Increasing scientific evidences have concluded that *M. tuberculosis* genetic diversity has an important role in the clinical consequences. Certain genotypes are more transmissible and capable of causing disease than others due to differences in their virulence and immunogenicity as observed in animal model [4]. Certain genotypes strongly develop drug resistance during the course of anti-TB treatment, with Beijing genotype commonly associated with multi drug resistance-TB [5,11]. Studies have also shown that specific strains of *M. tuberculosis* are more prevalent in certain geographical areas. Six phylogeographical lineages illustrate the global population structure of *M. tuberculosis*: Indo-Oceanic lineage, East Asian lineage, East African-Indian lineage, Euro-American lineage, West African lineage I and West African lineage II [6]. The Indo-Oceanic lineage (Lineage I), West African lineage I (Lineage 5) and West African lineage II (Lineage 6) belong to ancient lineages, whereas, the East Asian lineage (Lineage 2), East African-Indian lineage (Lineage 3) and Euro-American lineage (Lineage 4) belong to the modern lineages in relation to the presence or absence of TbD1 genomic region, deleted in modern strains [7]. The distribution of *M. tuberculosis* clones or strains or multi-drug resistant strains at phylogenetic level, regionally, nationwide or globally, can be followed by molecular typing methods [8]. Most commonly used tools for epidemiological strain typing or molecular evolutionary studies of *M. tuberculosis* are Spoligotyping, IS6110-RFLP typing, and MIRU-VNTR typing.

Appearance of Multi drug resistant (MDR) *M. tuberculosis* strains further worsens the situation. According to WHO, resistance to at least two drugs, namely, Isoniazid (INH) and Rifampicin (RIF), causes MDR-TB. From India, only a few reports on the prevalence of drug resistant strains are available, especially from North India [9,10]. However, reports on the characterization of drug resistance phenotype and genotypes for tribal population, such as Sahariya or from North Central India, are still lacking. TB control program in India is threatened due to the growing prevalence of drug resistance. Diagnosis of drug resistant TB in patients is highly challenging due to limited resources or infrastructure for drug susceptibility testing (DST) of TB bacilli. The conventional phenotypic DST method for identification of tuberculosis is time taking before the results are declared and anti-TB treatment is initiated.

Sahariya is a primitive tribe of North Central India, facing various survival problems, including lack of clean drinking water, poverty, lack of primary health service, illiteracy, malnutrition, etc [12]. Consequently, *M. tuberculosis* infection is a major health problem in the tribe. Several studies by our group and others have reported high incidence and prevalence of pulmonary tuberculosis along with MDR-TB in this tribe. Host genetic factors (e.g. *CCl2, CCl5* & *HLA*) were also implicated in the increased susceptibility of Sahariya to pulmonary tuberculosis [13,14,15,16]. Thus, in this report, our focus was to investigate the predominant *M. tuberculosis* strain/s responsible for pulmonary tuberculosis in Sahariya using spoligotyping and identify drug resistance gene (e.g., *katG, rpoB)* associated polymorphisms, prevalent in *M. tuberculosis* strains (clinical isolates) along with their phenotypic DST.

Materials and methods

Sample collection

Prior to sputum sampling, well informed and written consent was obtained from each participating patient of Sahariya tribe. The methods and protocols employed in this study were approved by the Institutional Ethics Committee of Jiwaji University, Gwalior. A total of n = 103 sputum samples were collected from two districts, Gwalior and Sheopur of Madhya Pradesh, where most of the Sahariya villages are located, during the years 2014–2017 (Fig. 1). The patient selection in the field was made on the basis of symptoms, such as chest pain, X-ray positive result, phlegm formation for the last 3–4 weeks, fever, hemoptysis, etc. Only those individuals positive to these symptoms were included in this study. Further confirmation of TB was made at District TB hospital, RNTCP, under the supervision of the medical officers of the respective districts. The demographic information of the population studied is given in Table 1.

Table 1
Clinical and demographic characteristics of pulmonary tuberculosis patients from Sahariya tribe.

Characteristics	MTB lineage (%)				Total
	CAS	EAI	T	Other lineages	
Gender					
Male	37	38	4	2	81
Female	9	12	1	0	22
Age group					
Adolesence 10–19	4	3	0	0	7
Younger 20–39	21	15	3	1	40
Middle 40–60	18	30	2	1	51
Elder >60	3	2	0	0	5
Category					
New cases (CAT-I)	36	39	3	3	81
Previously treated cases (CAT-II)	10	10	2	0	22

CAS — Central Asian Strain, EAI — East African Indian Strain, T — family strain.

Mycobacterium *culture and identification*

After diagnosis, the sputum samples were transported to the Center for Genomics, Jiwaji University, Gwalior, for sputum decontamination and culture on Lowenstein Jensen (LJ) media and incubated at 37 °C by modified Petroff's method, as per RNTCP protocol (www.rntcp.org.in). The growth of *M. tuberculosis* was observed after 5 to 7 weeks. Biochemical identification of species or *M. tuberculosis* complex was performed using catalase test, nitrate reduction test, tween hydrolysis test, niacin test and susceptibility to p-nitro benzoic acid (PNB) [31]. Further PCR based confirmation was done by the amplification of 245 bp DNA fragment specific for IS6110 gene using INS1 (5′-CGTGAGGGCATCGAGGTGGC-3′) and INS2 (5′-GCGTAGGCGTCGGTGACAAA-3′) primers in all clinical isolates.

Drug susceptibility test

M. tuberculosis isolates (n = 103) were subjected to drug susceptibility test (DST) for two anti-TB first line drugs, namely, Rifampicin (RIF) and Isoniazid (INH) using standard proportion method. Isoniazid and Rifampicin were incorporated at concentrations of 0.2 μg/ml and 40 μg/ml, respectively in LJ media. We used standard laboratory strain H37Rv as a control drug susceptibility control, following our laboratory standardized protocol [14]. Briefly, two third loopful of *M. tuberculosis* representing 4 mg moist weight of bacterial colony mass was scrapped and transferred to the tube containing 400 μl distilled water along with 4–6 glass beads, followed by vortexing the colonies for 30 s to produce a uniform suspension. Later-on the suspension opacity was matched with 1MacFarland standard to obtain a concentration of 1 mg/ml of tubercle bacilli. After that, we made 4 serial dilutions of the inoculum from the standard inoculum in concentrations of 10^{-1}, 10^{-2}, 10^{-3} and 10^{-4}, designated as S1, S2, S3 and S4, respectively. The

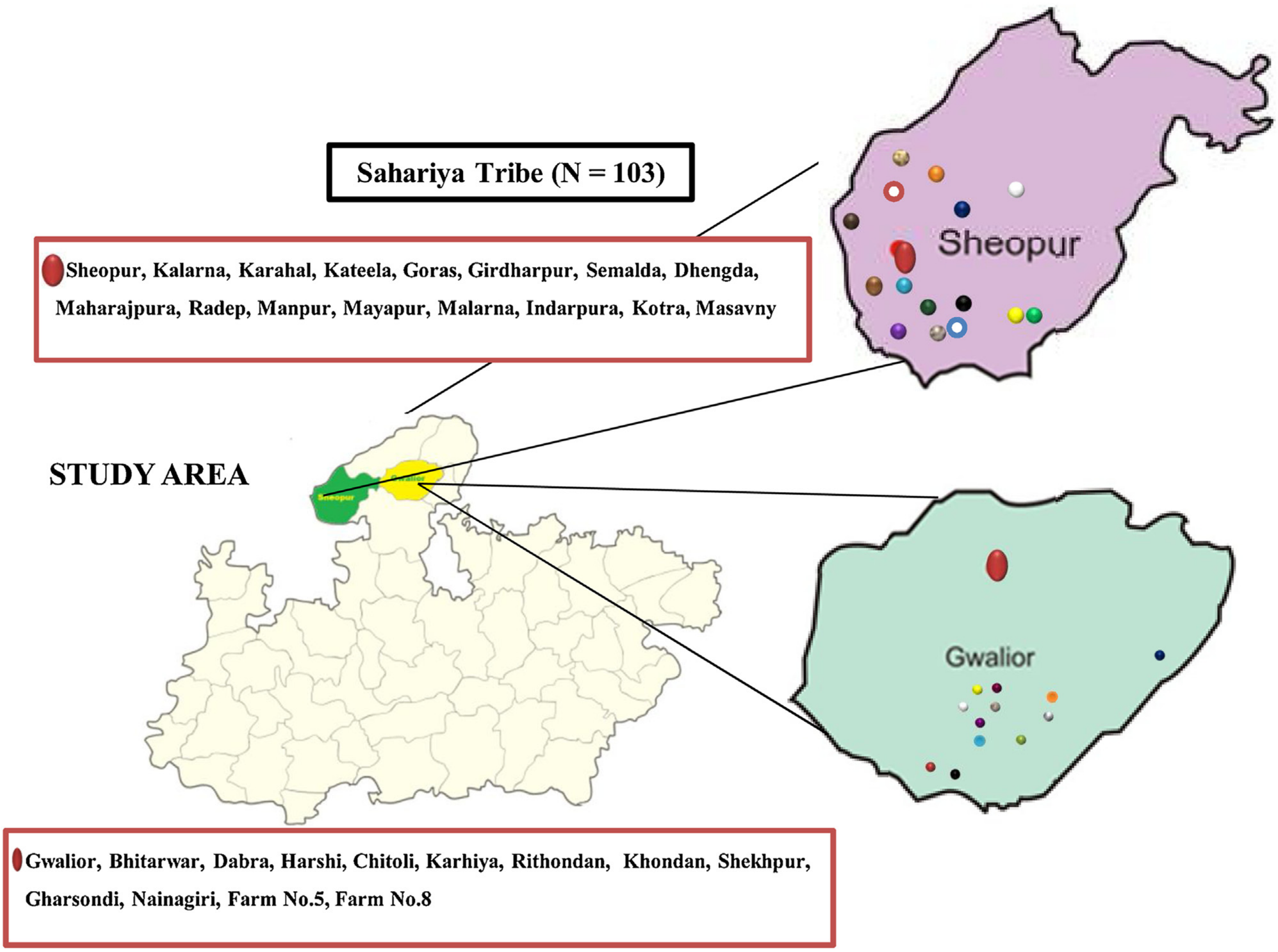


Fig. 1. Sampling sites at Gwalior and Sheopur district.

serial dilutions are inoculated in drug containing as well as drug free media and PNB slope. The reading of the growth was taken after 28 and 42 days. The result was recorded as per the resistance ratio, calculated as total number of colonies on drug containing media divided by number of colonies on drug free media and multiplied by 100. Strains with 1% bacilli resistant to two drugs, namely, isoniazid and rifampicin, were graded as multi-drug resistant (MDR) [32].

DNA isolation

DNA was extracted from *M. tuberculosis* clinical isolates growing on LJ slants using a standard protocol, which included lysis with lysozyme, proteinase K, SDS treatment and extraction with CTAB/NaCl [17].

Spoligotyping

The spoligotyping was carried out using a commercial kit (Ocimum Biosolution, India) as per manufacturer's instructions. Briefly, extracted DNA was subjected to PCR to amplify direct repeats region and interspersed known spacers region using biotinylated primers. The PCR product was hybridized to 43 immobilized oligonucleotides derived from the spacer sequence of *M. tuberculosis* H37Rv and *M. bovis* BCG. Hybridization signals were recorded by enhanced chemiluminescence detection system by exposing ECL-Hyper film (Amersham). Distilled water was used as negative control and H37Rv used as positive control. The presence and absence of spacer oligonucleotides were documented in the form of binary code that was converted into octal code. The obtained spoligo patterns were then compared with the SPOLB4 Database.

Genotyping & sequencing of drug resistance markers katG *and* rpoB

Identification and genotyping of drug resistant *M. tuberculosis* clinical isolates were performed by PCR-RFLP of *katG* (S315T & R463L) (Fig. 2A) and by multiple allele specific-PCR (MAS-PCR) of *rpoB* (S531L, H526Y/D, D516V) (Fig. 3A) [18,19]. Primer details of *katG* & *rpoB* are given in Table S1. For further confirmation of RFLP and MAS-PCR results, DNA sequencing was performed in Applied Biosystem, Genetic Analyzer Model 3500XL, using Big Dye terminator kit (ABI, USA). The electropherograms were analyzed and compared with those of reference strain H37Rv using BLAST program of NCBI.

Results

Spoligotyping

A total of 103 *M. tuberculosis* clinical isolates from pulmonary tuberculosis cases of Sahariya tribe were studied. Among 103 cases, 81 (78.64%) were males and 22 were females (21.3%). A total of 17 different spoligo-signatures or spoligo-international types (SIT) were observed in the analyzed clinical isolates (n = 103). Out of 103, 93 isolates were observed in 7 clusters, ranging from 2-42

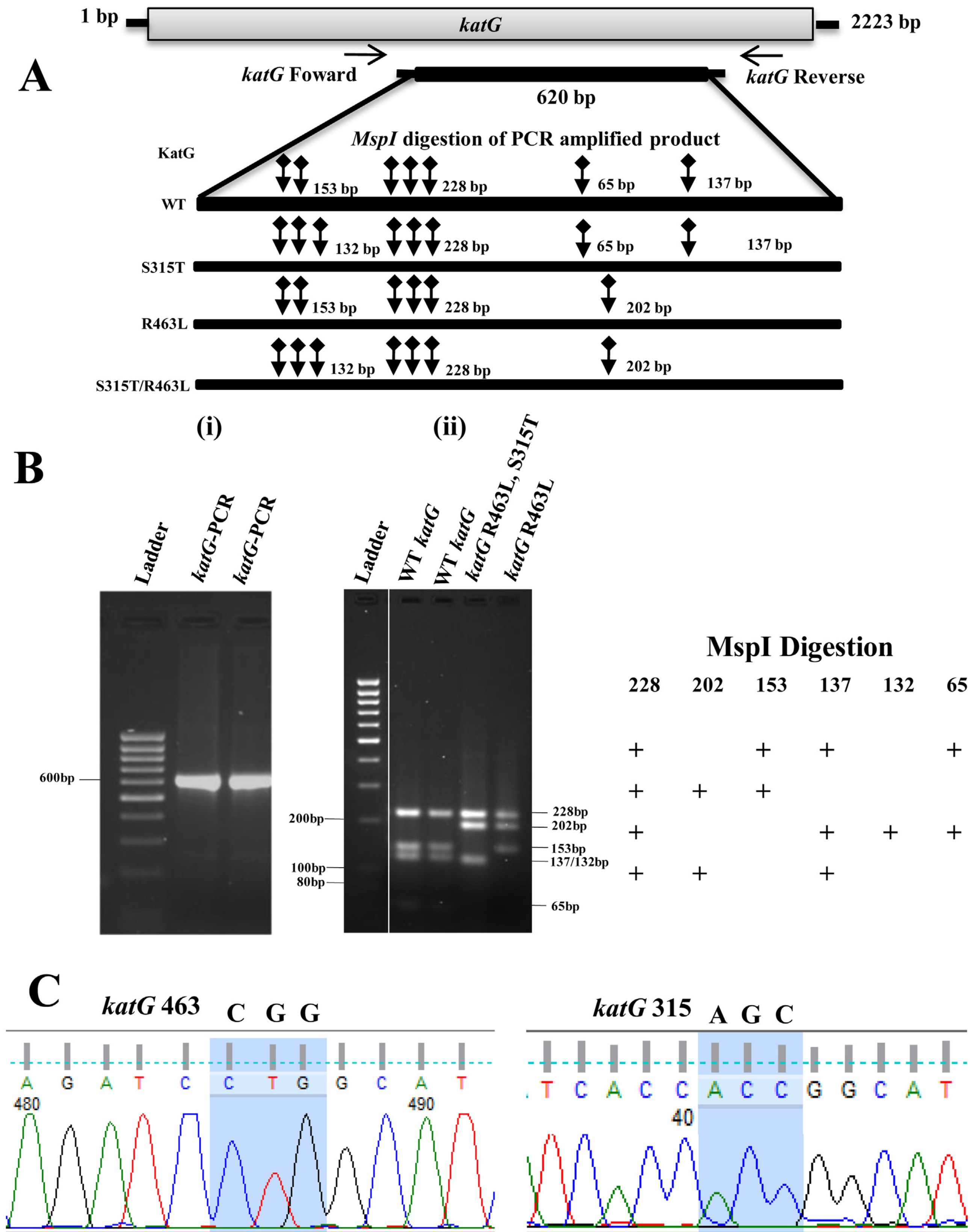


Fig. 2. PCR-RFLP and Sequencing of *katG*: A: schematic view of katG gene fragments targeted by PCR followed by restriction digestion with *MspI* for PCR-RFLP assay; B: (i) agarose gel picture of *katG* PCR amplification of 620 bp. (ii) Restriction digestion of *katG* amplicons of drug sensitive isolates (wild type); drug resistance isolates with SNP at *katG* S315T,R463L; SNP at *katG* R463L. C: Sequencing electropherogram of *katG* R463L, S315T.

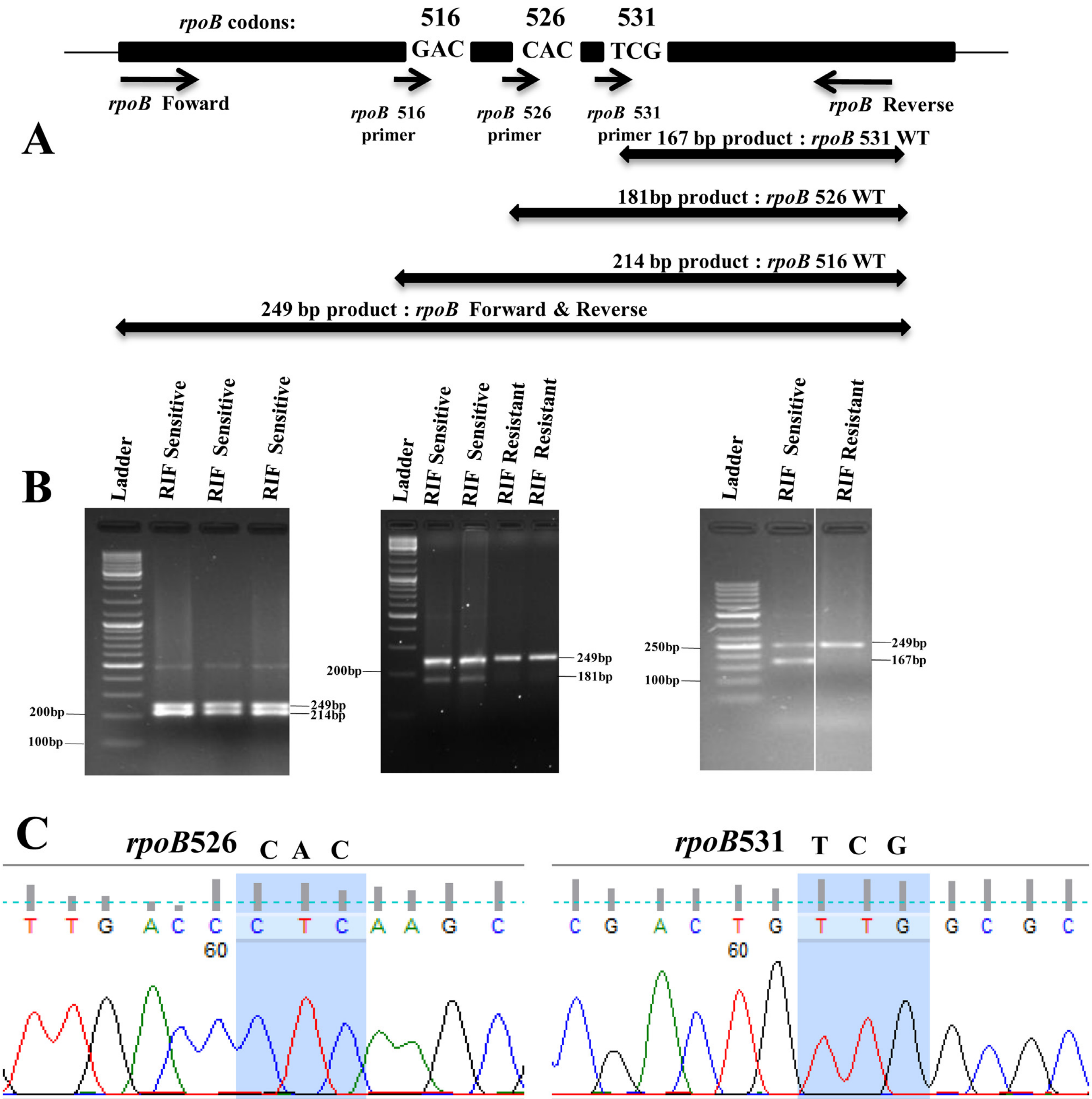


Fig. 3. MS-PCR and Sequencing of *rpoB*: A: schematic view of *rpoB* gene fragments targeted by allele specific PCR assay and dark arrows shown the primers, double headed arrows represent PCR fragments, either invariable (249 bp) or allele specific *rpoB* codon 516 (214 bp), *rpoB* codon 526 (181 bp) and *rpoB* codon 531 (167 bp); B: agarose gel picture of *rpoB*-MAS PCR amplification of 249 bp and allele specific amplification of *rpoB* codon 516, 526, 531; lane1 DNA ladder; in agarose gel, double band represent wild type *rpoB* allele in drug sensitive *M. tuberculosis* isolates; in agarose gel which shown single band of 249 bp & diapperance of allele specific PCR band are drug resistance isolates for respective codon 516, 526 and 531 of *rpoB* gene; C: sequencing electopherogram of *rpoB* codon 526 and 531.

isolates per cluster, while 10 were unique (Table 2). The major spoligotype families observed were belonging to East African Indian EAI3_IND/ST11 family of *M. tuberculosis* strains (n = 42, 40.7%), followed by Central Asian strain CAS1_Delhi/SIT26 (n = 31, 30%), CAS/ST357 family (n = 7, 6.7%) and T-family T1/ST53 (n = 4, 3.8%).

Drug susceptibility test

The drug susceptibility tests for INH and RIF were carried out for all the 103 clinical isolates. Results showed that 17.4% (n = 18) of clinical isolates showing resistance to INH or RIF + INH, which are first line anti-TB drugs. The results of our DST tests identified resistance to INH (monodrug) in 12.6% isolates and multidrug resistance in 4.85% cases. Resistance to isoniazid and rifampicin (MDR-TB) in the isolates was higher in new smear positive category patients (NSP = 4.9%, n = 4), while one MDR isolate belonged to treatment after default category (TAD = 4.5%, n = 1) (Table 3). Our results also show that Sahariya male (n = 4) has higher chance to develop MDR during the course of treatment as compared to Sahariya female (n = 1) as 80% multidrug resistant isolates were from male cases and only one isolate was from female cases.

Genotyping and sequencing of drug target

To further validate our DST observation, we performed a comprehensive molecular analysis of the drug target genes by

Table 2
Spoligotype signatures and their shared types of 103 *M. tuberculosis* clinical isolates from Sahariya tribe.

S. No.	Presence or absence of spacer	Octal code	*M. tb* strain	Shared type
1	■■■■■■■■■■■■■■■■■■■□□■■■■■■■■■■■□□□□■■■■■■■	777777477760771	H37Rv	
2	■■□■□■■■□■□■■■■□■■■■■■■■■■■■■■■■■■■■■■□□□□□	656573777777600	*M. bovis*	
3	■■■□□□□■■■■■■■■■■■■■■■□□□□□□□□□□□□■■■■■■■■■	703777740003771	CAS1_Delhi (31)	ST26
4	■□□■■■■■■■■■■■■■■■■■■■■■■■■■□□□□■□■■□□□■■■■	477777777413071	EAI3_IND (42)	ST11
5	■□□■■■■■■■■■■■■■■■■■■■■■■■■■□□□□■□■■■■■■■■■	477777777413771	EAI5_IND (3)	ST126
6	■■■□□□□■■□■■■■■■■■■■■■□□□□□□□□□□□□□■■□■■■■■	703377740003171	CAS1_Delhi (2)	ST1314
7	■■■□□□□■■■■■■■■■■■■■■■□□□□□□□□□□□□□■■□□■■■■■	703777740003171	CAS1_Delhi (4)	ST25
8	■■■□□□□□□□■■■■■■■■■■■■□□□□□□□□□□□□■■■■■■■■■	700377740003771	CAS2 (1)	ST288
9	■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■□□□□■■■■■■■	777777777760711	T1 (4)	ST53
10	■□□■■■■■■■■■■■■■■■■■■■■■■■■■■□□□□□□■■■■■■■■■	477777777403771	U (1)	ST1515
11	■□□■■■■■■■■■■■■■■■□□■■■■■■■■□□□□■□■■□□□■■■■	477777177413071	EAI3_IND (1)	ST1342
12	■■■■■■■■■■■■■■□□□□□□□□□□■■■■■■■■□□□□■■■■■■■	777760007760771	T5_RUS1(1)	ST254
13	■■■□□□□■■■■■■■■■■■■■■■□□□□□□□□□□□□□□■■■■■■■	703777740000771	CAS (7)	ST357
14	■■■■■■■■■■■■■■■■■■■■■□□□□□□□□□□□□□■■■□■□■■■	777777700003531	U (1)	ST510
15	■■■■■■■■■■■■■■■■■■■■■■■■■■■■■□□□□■□■■■■■□■■■	777777777413731	EAI1_SOM (1)	ST48
16	■■■■■■■■■■■■■■■■■■■■■■■■■■□■□□□□■□■■■■■■■■■	777777776413771	EAI5 (1)	ST152
17	■■■□□□□■■■■■■■■■■■■■■■□□□□□□□□□□□□■■■□■■■■■	703777740003571	CAS1_DELHI (1)	ST289
18	■■■■■■■■■■■■■■■■■■■■■■□■■■■■□□□□■□■■□■■■■■■	777777757413371	EAI6_BGD1 (1)	ST292
19	■■■■■■■■■■■■■■■■■■■■■■□■■■■■□□□□■□■■■■■■■■■	777777757413771	EAI6_BGD1 (1)	ST591

Table 3
Prevalence of isoniazid and rifampicin drug resistance among *M. tuberculosis* isolates from treatment after default (TAD) and new smear patients (NSP) according to the drug and the type of case from Sahariya tribe.

Drug resistance	Sahariya tribe N = 103 N(%)		
	NSP (N = 81)	TAD (N = 22)	TOTAL (N = 103)
Resistance to INH	9 (11.1)	4 (18.1)	13 (12.6)
Resistance to RIF	0 (0)	0 (0)	0 (0)
MDR resistance INH + RIF	4 (4.9)	1 (4.5)	5 (4.8)

genotyping MAS-PCR, PCR-RFLP and gene sequencing. All 103 isolates were subjected to molecular analysis, in which ninety eight clinical isolates (n = 98, 95%) of *M. tuberculosis* strains displayed *katG* gene mutation; of these 98 isolates, 93 isolates harbored mutation on codon 463 (R463L) (Fig. 2B and C), while five isolates had mutations on codon 315 (A<u>C</u>C → A<u>G</u>C/S315T) and codon 463 (C<u>G</u>G → C<u>T</u>G/R463L) (Fig. 2B and C). MAS-PCR performed for 81 bp rifampicin resistance determining region of *rpoB* gene identified mutation in five isolates (n = 5, 4.85%). Of the five isolates displaying RIF resistance associated polymorphism, one isolate had a mutation at codon 531 (T<u>C</u>G → T<u>T</u>G/S531L) (Fig. 3B and C) and four isolates had mutation at codon 526 (C<u>A</u>C → C<u>T</u>C/H526L) (Fig. 3B and C). These five isolates also had mutation in *katG* gene, of which one showed double mutations (S315T, R463L), while the other four clinical isolates harbored single mutation (R463L) in *katG* gene. Thus, these isolates (n = 5, 4.85%) can be categorized as MDR isolates. Interestingly, four of these MDR *M. tuberculosis* clinical isolates were from new smear positive/Category I patients, while the remaining one clinical isolate was from retreated/Category II/TAD (Treatment after default) cases. Genotyping results of *katG* PCR-RFLP and *rpoB* MAS-PCR were further confirmed by Sanger sequencing of drug target genes and their corresponding electropherograms are shown in Figs. 2 C and 3 C. DNA sequences of *katG* (S315T, R463L) and *rpoB* (H526Y) gene mutaions have been submitted to NCBI Gene bank and their accession numbers are MK248686 and MK248687, respectively.

Discussion

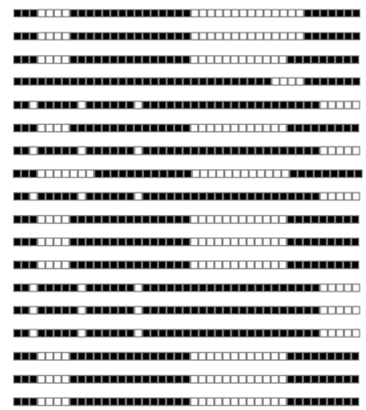
Information pertaining to genetic diversity of *M. tuberculosis* and their population structure at particular geographical area play important role in the control of TB. This helps in the surveillance of the disease and to define the origin and spread of pathogen in the community for effective control and prevention. *M. tuberculosis* infection leading to pulmonary tuberculosis is the major health issue in Sahariya tribe [14]. To our knowledge, this is the first study on the genetic diversity of mycobacterial strains circulating among the Sahariya tribe of north central India using spoligotyping method. Our study revealed EAI3_IND/ST11 and CAS1_Delhi/ST26 mycobacterial strains to be predominant in the tribal patients of Gwalior and Sheopur districts of north central India. Both these strains have also been reported as prevalent genotypes in the rural areas of Kanpur, North India, with CAS1_Delhi being 37%, followed by EAI (11%) [20]. India is a country of diverse ethnicity and geographical conditions, likely to show predominance of different *M. tuberculosis* lineages in different regions or populations. Studies so far, suggest CAS lineage to be predominant in northern part and EAI lineage in the southern part. As we move to eastern part of India, Beijing family is reported as predominant *M. tuberculosis* strain, e.g., in Assam [11]. Hospital based study from western part of India reported high percentage of Beijing genotype (23%), also associated with MDR. Predominance of Manu 1 (Manu family) and CAS 1 (Central Asian or Delhi lineage) strains, followed by Beijing strains has been reported from Hinduja Hospital, Mumbai, in rural and urban cohorts [5]. Similarly, a study from Satna district of Madhya Pradesh showed the prevalence of Manu1 strain to be the major *M. tuberculosis* genotype, followed by Manu 2, suggesting varied geographical distribution of *M. tuberculosis* strains within the state of Madhya Pradesh [21]. Thus, the distribution of *M. tuberculosis* strains seems to support the concept of demarcation between the strains prevalent from north to south and east to west in India.

Our results have shown EAI lineage family as the predominant strain infecting Sahariya tribe. The East African Indian strain EAI is an ancestral lineage (TbD1+) and comes under principal genetic group 1 [7]. Sahariya, being a primitive tribal group, may have carried EAI3_IND strain along with them during evolution and migration of *Homo sapiens* from Africa. Some reports have already suggested that humans adapted with *M. tuberculosis* complex and exhibit phylogeographic population structure with different *M. tuberculosis* lineages, associated with different human populations [22], indicating this to be a likely reason for the predominance of EAI lineage of *M. tuberculosis* strains infecting the members of Sahariya tribe.

Five multi drug resistance (n = 5, 4.85%) *M. tuberculosis* isolates were identified in this work. Out of five MDR isolates, two are from CAS1_Delhi/ST26, two from CAS/ST357 and one being CAS2/ST288. Thus, all the five clinical isolates belonged to CAS family, which suggests that the CAS clade family is more prone to developing multidrug resistance, as compared to other clades circulating in this

Table 4
Genotypic and phenotypic profile of MDR and INH-Resistant isolates along with their spoligotype signature.

Isolate code	Mutation at *katG*	Mutation at *rpoB*	INH phenotypic profile	RIF phenotypic profile	Spoligo signature	Share type/spoligo family
Gwl-404	R463L	H526L	Resistance	Resistance		ST357/CAS
Gwl-442	R463L	H526L	Resistance	Resistance		ST357/CAS
Gwl-451	R463L	H526L	Resistance	Resistance		ST26/CAS1_Delhi
Gwl-473	R463L	WT	Resistance	Susceptible		ST53/T1
Gwl-478	S315T/R463L	WT	Resistance	Susceptible		ST11/EAI3_IND
Gwl-481	R463L	H526L	Resistance	Resistance		ST26/CAS1_Delhi
Gwl-486	S315T/R463L	WT	Resistance	Susceptible		ST11/EAI3_IND
Gwl-488	S315T/R463L	S531L	Resistance	Resistance		ST288/CAS2
Gwl-504	R463L	WT	Resistance	Susceptible		ST11/EAI3_IND
Gwl-505	R463L	WT	Resistance	Susceptible		ST26/CAS1_Delhi
Gwl-514	S315T/R463L	WT	Resistance	Susceptible		ST26/CAS1_Delhi
Gwl-527	R463L	WT	Resistance	Susceptible		ST26/CAS1_Delhi
Gwl-537	S315T/R463L	WT	Resistance	Susceptible		ST11/EAI3_IND
Gwl-555	R463L	WT	Resistance	Susceptible		ST11/EAI3_IND
Gwl-562	R463L	WT	Resistance	Susceptible		ST11/EAI3_IND
Gwl-574	R463L	WT	Resistance	Susceptible		ST26/CAS1_Delhi
Gwl-584	R463L	WT	Resistance	Susceptible		ST26/CAS1_Delhi
Gwl-586	R463L	WT	Resistance	Susceptible		ST26/CAS1_Delhi



region, as shown in the dendrogram of MDR & INH-R strains (Fig. S1). We also observed that the four MDR cases were new smear positive patient (Category I), indicating that they have acquired the infection from other MDR cases of their community, while one of the MDR case was treatment defaulter patient (TAD) (Category II)/old patient. Therefore, drug resistant tuberculosis attracts more attention due to poor outcomes and the risk of transmission. Genotypic and phenotypic profile of MDR and INH-resistance isolates along with spoligotyping signature (Table 4).

Molecular analysis of drug resistance markers *katG*, *rpoB* (PCR-RFLP, MAS-PCR) identified 95%, n = 98 of *M. tuberculosis* strains having *katG* gene mutation at codon 463 (R463L), while 4.85%, n = 5 has double mutation at *katG* codon S315T/R463L, several reports have shown mutations in *katG* (S315T, R463L) associated with INH resistance in different geographical location around Asian continent [19,23,24,26,28]. Along with molecular analysis, we also performed phenotypic DST. Phenotypic DST for 103 isolates identified 18 clinical isolates displaying INH resistance out of which five isolates had double mutation at *katG* codon 315/463, 13 isolates has mutation at *katG* codon 463 (R463L) directly linked with INH resistance. We for the first time report the identification of mutation at *katG* codon 315 from the clinical isolates infecting this tribe. Similar type of study from this tribal group also found higher percentage of INH resistance and our previous study reported 14% of clinical isolates from Sahariya displaying resistance to INH alone (monodrug) and 6% in nontribal population [14,15]. In MDR-TB 11% isolates from Sahariya tribe have mutation at *katG* codon 463 (R463L) along with S531L *rpoB* gene mutation directly linked with phenotypic drug resistance in DST. Our previous results of isoniazid minimal inhibitory concentration method (MIC) 0.2 μg/ml and 1 μg/ml for *katG* codon 463 R463L, single mutation, confers low level resistance. Other studies have also shown INH MIC level of 1 μg/ml for R463L mutation [23,27], which is in concordance with our genotyping data presented in this study. Isoniazid has been the backbone of tuberculosis treatment for the last six decades. Russian federation and Kazakhstan reported highest percentage of INH resistance among new TB cases (NSP) (40%–43%), while other countries, such as China, Vietnam, Dominican Republic and India reported 15% to 20% of new TB cases with Isoniazid resistance [25]. The surveillance of INH resistance is important because the pathogen is also acquiring additional drug resistance during standard TB treatment. INH resistance also reduces the chance of anti-TB treatment success and increases the risk of acquiring resistance to other anti-TB first line drugs, including RIF, thereby, increasing the risk of multidrug resistant TB [29]. Our genotyping results have identified various mutations in *katG* (codon 463 C<u>G</u>G → C<u>T</u>G/R463L, codon 529 A<u>A</u>C → A<u>C</u>A/N529T and codon 529 <u>A</u>AC → <u>C</u>AC/N529H) and *rpoB* (codon 531 T<u>C</u>G → T<u>T</u>G/S531L) genes in the isolates from Sahariya tribe [23]. Apart from previously reported mutations, this study has identified a mutation in *katG* at codon 315 (A<u>C</u>C → A<u>G</u>C/S315T) and in *rpoB* at codon 526 (C<u>A</u>C → C<u>T</u>C/H526L) for the first time in this tribal population.

Our study suggests for more work to be carried out to monitor INH monodrug resistant TB prevalence in this high endemic area. In several countries, including India, due to resource limited settings, it is a common practice of detecting rifampicin (RIF) resistance mutations (CBNAAT) and classify the case as MDR, whenever a mutation is detected [30]. This method, however, misses out identifying the INH resistance, which can contribute to the progression to MDR-TB and treatment failure. Therefore, the question naturally arises, to what extent rifampicin alone can be used as a surrogate marker for MDR-TB? Genotyping for INH resistance also misleads as observed in this study that 95% clinical isolates have mutations in *katG*. Thus, it is imperative to include DST for selected isolates along with genotyping and design a treatment regimen, which can effectively kill the pathogen with minimal toxic side effects.

Conclusion

EAI3_IND/ST11 is one of the ancestral strains of *M. tuberculosis*, identification of this isolate predominantly infecting the members of Sahariya tribe indicates that this pathogenic strain also co-evolved with this tribe and thus, indirectly establishing the primitiveness of this tribe. This study also found higher prevalence of INH monodrug resistant *M. tuberculosis* strains. Such isolates have higher propensity to evolve into multi drug resistant strains as suggested by earlier reports also. Thus, it is suggested that TB Centers, along with CBNAAT, should also consider the isoniazid resistant status of the isolate before deciding appropriate drug regimen for the members of Sahariya tribe.

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Ethical approval

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Rahul Gupta: sample collection, *M. tuberculosis* culture, drug susceptibility testing, spoligotyping, DNA sequencing, DNA extraction, manuscript preparation & statistical analysis.

Rabbind Singh: Amrathal PCR-RFLP, MAS-PCR, manuscript preparation.

Ravi Prakash: biochemical identification, drug susceptibility testing.

Sanjay Jain: sample collection from Sheopur region and acid fast staining.

Pramod Kumar Tiwari: study design, drafting and revising the article.

This study was approved by Institutional Human Ethical Committee, Jiwaji University, Gwalior (Institutional Ethical Committee no. JU/IHEC/2013-A/06).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.jiph.2018.12.009.

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