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A study of *Mycobacterium tuberculosis* genotypic diversity & drug resistance mutations in Varanasi, north India

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***Background & objectives*: One-fifth of the world's new tuberculosis (TB) cases and two-thirds of cases in the South East Asian region occur in India. Molecular typing of *Mycobacterium tuberculosis* isolates has greatly facilitated to understand the transmission of TB. This study was aimed to investigate the molecular epidemiology of *M. tuberculosis* genotypes in Varanasi, north India, and their association with clinical presentation among patients with pulmonary TB.**

***Methods*: *M. tuberculosis* isolates from 104 TB patients attending a tertiary referral hospital of north India were screened for susceptibility to isoniazid (INH), rifampicin (RIF), ethambutol (EMB) and streptomycin (STR) by proportion method and multiplex-allele-specific-polymerase chain reaction (MAS-PCR). These were genotyped by spoligotyping. The spoligotype patterns were compared with those in the international SITVIT2 spoligotyping database.**

***Results*: Eighty three of 104 isolates were distributed in 38 SITs, of which SIT3366 was newly created within the present study. The mass of ongoing transmission with MDR-TB isolates in Varanasi, northern India, was linked to Beijing genotype followed by the CAS1_Delhi lineage. HIV-seropositive patients had a significantly higher proportion of clustered isolates than HIV-seronegative patients and compared with the wild type(wt) isolates, the isolates with *katG*315Thr mutation were considerably more likely to be clustered.**

***Interpretation & conclusions*: This study gives an insight into the *M. tuberculosis* genetic biodiversity in Varanasi, north India, the predominant spoligotypes and their impact on disease transmission. In this region of north India, TB is caused by a wide diversity of spoligotypes with predominance of four genotype lineages: Beijing, CAS, EAI and T. The Beijing genotype was the most frequent single spoligotype and strongly associated with multi drug resistant (MDR)-TB isolates. These findings may have important implications for control and prevention of TB in north India.**

Key words Beijing genotypes - drug resistance - HIV - molecular epidemiology - SITVIT2 - tuberculosis

India accounts for one fifth of the world's new tuberculosis (TB) cases and two-thirds of cases in the South East Asia[1]. Regardless of the efforts to control TB, the disease burden of human TB is still a very serious and widespread public health problem particularly in developing countries. This situation is worsened by the appearance of multidrug-resistant (MDR), extensively drug resistant (XDR) strains of *Mycobacterium tuberculosis* and a high incidence of human immunodeficiency virus (HIV)/TB co-infection[2].

Molecular techniques in the TB field have provided new ways to study dissemination dynamics and evolutionary genetics of the pathogen, with a direct impact on TB control actions[3]. Spoligotyping is the second most widely used method for *M. tuberculosis* complex genotype after insertion sequence 6110 (IS*6110)*-based fingerprinting[4]. Spoligotyping in combination with mycobacterial interspersed repetitive units-variable-number of tandem DNA repeats (MIRU-VNTR) has been used to replace IS*6110*-restriction fragment length polymorphisms (RFLP) typing. The IS*6110*-RFLP method has been considered the gold standard for genotyping *M. tuberculosis*[7], however, this is an expensive, laborious and extensive method that requires weeks for culturing and specific software to analyse the RFLP band patterns. Moreover, this method is not applicable to isolates having either too low or zero IS*6110* copy number[5].

Spoligotyping is based on a polymorphism in the chromosomal direct repeat (DR) locus[3] and has been applied to the characterization of the *M. tuberculosis* complex. It is internationally accepted as a rapid, first line discriminatory test and the gold standard for the identification of Beijing strains of *M. tuberculosis*[6,7]. The spoligotyping method was also the basis for making of the largest genotype database for *M. tuberculosis*, containing a global distribution and phylogenetic analysis for worldwide spoligotypes[6].

A better understanding of drug-resistant TB epidemiology is essential to develop evidence-based control strategies for MDR-TB. Drug-resistance is associated with a number of factors including poor adherence to anti-TB treatment[8]. MDR-TB is a result of the step-wise accumulation of mutations in drug-resistance conferring genes. Previously, drug-resistant *M. tuberculosis* strains were thought to be less infectious and less likely to cause disease when compared to their drug-susceptible counterparts[9]. However, recent studies have shown that these are able to transmit and cause disease as often as drug-susceptible organisms[10]. Further, the prevalence of different drug resistant clones of *M. tuberculosis* varies from one area to another and studies in different geographical settings are necessary to understand the epidemiology of disease[11].

This study was undertaken to provide with an insight into the *M. tuberculosis* genotypic diversity and drug resistance mutations in Varanasi, north India. We also compared our data on a global scale to present an in-depth analysis of prevailing *M. tuberculosis* clones to understand their probable transmission dynamics.

Material & Methods

The study was conducted at the Department of Microbiology, Institute of Medical Sciences, Banaras Hindu University (BHU) and Sir SundarLal (SS) Hospital, a tertiary-care hospital of BHU. Sputum specimens were collected during the period of January 2008 to January 2010, from clinically suspected pulmonary TB patients, from Varanasi and adjoining districts of Uttar Pradesh, attending various OPDs of SS Hospital, BHU, Varanasi. Culture and drug susceptibility testing (DST) of *M. tuberculosis* was done at Department of Microbiology, Institute of Medical Sciences, Banaras Hindu University (BHU) while spoligotyping was done at the Laboratory Nuclear Medicine Section, Isotope Group, Bhabha Atomic Research Centre, Mumbai, India.

Specimens/mycobacterial isolates/phenotypic drug susceptibility testing: One hundred four clinical isolates of *M. tuberculosis* were recovered from sputum specimens from patients diagnosed with pulmonary TB on the basis of clinical symptoms, chest X-ray, and bacteriological examination. Demographic and epidemiological data were obtained on direct counselling with patients and from the medical records. Isolated cultures were identified by certain biochemical tests, such as heat-stable catalase, niacin accumulation, and susceptibility to *p*-nitro benzoic acid (PNB) followed by the amplification of a 523-bp DNA fragment specific for the IS6110 gene[12,13] present in all isolates. For this purpose, bacterial DNA was extracted by using the protocol of van Embden *et al*[5] with slight modifications. Identified *M. tuberculosis* isolates were subjected to indirect DST according to the gold standard proportion method (PM) with the recommended critical concentrations of 40 µg/ml for rifampicin (RIF), 0.2 µg/ml for isoniazid (INH), 2 µg/ml for ethambutal (EMB) and 4 µg/ml for streptomycin (STR)[14,15]. H37Rv (ATCC 27294) and a known MDR

strain were used as negative and positive controls, respectively. Among the first line anti TB drugs pyrazinamide susceptibility testing was not done.

Overall, 47 pan susceptible, 51 MDR and six other *M. tuberculosis* isolates (resistant to one or more first line anti-TB drugs) to see the association of prevailing drug-resistance with different lineages of *M. tuberculosis*. The study protocol was approved by the ethical committee of the Institute.

HIV testing: Three rapid HIV test kits (initial screening by comb AIDS, if the test was reactive then Pareekshak Triline card and AIDSCAN Trispot test were performed) based on different antigens/principles were used to test the HIV status of patients according to NACO (National AIDS Control Organization) guidelines[16].

Multiplex allele-specific (MAS)-PCR assay for detection of RIF, INH and EMB resistance determinants: A two-step MAS-PCR assay was performed to detect mutations at *rpoB* codons (516, 526 and 531), *katG* codon 315 and *embB* codon 306[17-19]. Two outer (forward & reverse) and three inner forward primers were used for three MAS-PCRs targeting three different codons of the *rpo*B gene while two outer and one inner reverse primers were used for *kat*G315 MAS PCR and two outer and two inner primers were used for *emb*B306 MAS PCR[17-19].

Spoligotyping: Spoligotyping was performed (n=104) according to the standard method of Kamerbeek *et al*[3]. *M. tuberculosis* H37Rv and deionized autoclaved water were used as positive and negative controls in each experiment. The results were documented in the form of a binary code.

Comparison of spoligotypes with an updated database: Spoligotypes in binary format were entered in the SITVIT2 database (Pasteur Institute of Guadeloupe, France), which is an updated version of the previously released SpolDB4 database[6]. At the time of the present study, SITVIT2 contained more than 3000 SITs (Spoligotype International Type) with global genotyping information on about 75,000 *M. tuberculosis* clinical isolates from 160 countries of origin. In this database, SIT designates spoligotypes shared by two or more patient isolates, as opposed to "orphan" which designates patterns reported for a single isolate. Major phylogenetic clades were assigned according to signatures provided in SpolDB4, which defined 62 genetic lineages/sub-lineages[6].

Statistical analysis: Descriptive analyses were performed using SPSS 15.0 (SPSS, Chicago, IL, USA). Binary logistic regression model was used for univariate and multivariate analyses to qualify and quantify the difference in clustering proportion between groups of subjects with different socio-demographic and clinical characteristics. The adjusted odds ratio (OR) and 95 per cent confidence interval (CI) were calculated by adjusting for the possible confounders (age and sex). Statistical significance was defined as *P* value of 0.05 or less. A cluster of *M. tuberculosis* isolates was defined as two or more isolates with identical spoligotyping patterns. A cluster of patients was defined as two or more patients with identical isolates. The geographical distribution of the clusters was assessed on the basis of the place of residence of the patients.

Results

In this study, a total of 104 isolates including drug susceptible and drug resistant both from the spectrum specimens collected from pulmonary TB patients were included. These were from 39 females (6 to 70 yr) and 65 males (16 to 75 yr). Further, 89 (85.58%) of these were HIV seronegative and 15 (14.42%) were HIV seropositive patients. Of all isolates, 47 (45.19%) were susceptible to all four first line drugs *i.e*., INH and RIF, STR and EMB while 57 (54.81) were resistant to one of these drugs. Of the 57 any drug resistant isolates, 55 (96.49%), 52 (91.23%), 41 (71.93%), 45 (78.95%) and 51 (89.47%) were INH, RIF, STR, EMB and multidrug resistant TB isolates, respectively. New and retreated cases were 56 (53.85%) and 48 (46.15%), respectively. Among 57 any drug resistant cases, 21 (36.84%) and 36 (63.12%) were primary and acquired drug resistant cases, respectively.

Comparison of the spoligotypes with those in the international database: Comparison with the SITVIT2 database showed that 21 patterns (1 isolate per pattern) were not reported earlier to the database; these were classified as orphans (Table I), and corresponded to CAS1_DELHI (n=7), EAI5 (*n=4*), Manu 2 (n=3), Manu 1 (n=2), EAI3-IND (n=2), EAI4-VNM (n=1) families, respectively. The remaining two orphan isolates corresponded to an unknown pattern in one case, and an ambiguous pattern with a mixed LAM/T profile in another case. The remaining 83 isolates were distributed in 38 shared types or SITs (Table II). Twenty six (31.33%) isolates presented unique SITs, remaining 57 were clustered in 12 groups of 2 to 21 isolates each. SIT1 (Beijing clade) represented 20.39 per cent of all isolates, followed in predominance by SIT26 and SIT954 (CAS1_DELHI) which constituted 6.8 and 4.85 per cent of all isolates. Of the 38 SITs,

Table I. Orphan isolates (n=21) and corresponding spoligotyping defined lineages/sublineages found among a total of 104 *M. tuberculosis* isolates from patients residing in Varanasi, north India

Isolate no.	Year	Spoligotype description	Octal code	Suggested lineage interpretation*
IND0420081S59	2008	■■■■■■■■■■□■■■■■■■■■□□□□□□□□□□■□■■□■■■■■■	777677740013371	EAI4-VNM
IND0420101S466	2010	■■■□□□□□□□■□■■■■■■■■■□□□□□□□□□□□□■■■■■□□■■	700277740003711	CAS1-Delhi
IND0420101S485	2010	□□□■■■■■■■□□□□■■■■■■■■■□■■■□□□□■□■■□□□■■■■	077417773413071	EAI3-IND
IND04200911017	2009	■■■■□□■■■■■■■■■■■■■■■■■■■■□□□□■□■■■■■□□□□	747777777413700	EAI5
IND0420081729	2008	■■■□□□□■■□■□□■■■■■■□□■□□□□□□□□□□□□■■■■■■■■■	703237440003771	CAS1-Delhi
IND0420081732	2008	■■■■□□□■■■■■■■■■■■■■■□□□□□□□□□■□□■■■■□■■■■	743777740023671	Manu2
IND0420081861	2008	■■■■□□□■■■■■■■■■■■■■■□□□□□□□□□■□□■■■■■■■■■	743777740023771	Manu2
IND0420081862	2008	■■■■■■■■□□■□□■■■■■■■■■■■■■□□□□■□■■■□□□□□□	776237777413400	EAI5
IND0420082S23	2008	■■■■□□□■□□■■□□□□□■□□□■□□□□□□□□□□□□■■■■■■■■■	742301040003771	CAS1-Delhi
IND0420102S437	2010	■■■■□□□■■■■■■■■■■■■□□□□□□□□□□□□□□■■■■■■■■■	743777600003771	CAS1-Delhi
IND0420092S399	2009	■■■■□□□■■■■■■■■■■■■■■□□□□□□□□□□□■■■■■■■■■	743777760003771	Unknown
IND0420082S47	2008	■■■■■■■■■■□■■□□□■□□□■□□□■■□■■□■■□■■■■■■■■■	777661043333771	Manu1
IND0420082S260	2008	■■■■■■■■■■□■■■□■□■■■■□■■■■■□□□□■□■■■■■■■■■	777672757413771	EAI5
IND0420092S38	2009	■■□□□□□□□□■□■■■□■■■■■■□□□□□□□□□□□□■■■■■■■■■	600273740003771	CAS1-Delhi
IND0420082S7	2008	■□□■■□■■■■■□■■■■■■■■■■■■■■□□□□■□■■□□□■■■■	467677777413071	EAI3-IND
IND0420082K4	2008	■■■■□□□■■■■■■■■■■□□□■□□□□□□□□□□□□■■■■■■■■■	743777040003771	CAS1-Delhi
IND0420082K21	2008	■■■■□□□■■□□□■■■■■■■■■□□□□□□□□□□□□■■□□■■■■■	743077740003171	CAS1-Delhi
IND04200921466	2009	■■■■■■■■■□□□□■■■■■■■■■■■■■■□□□□■□■■■■□□■■■	777037777413631	EAI5
IND0420082761	2008	■■■■■■□□■□□□■■■■■■■■■■■■■■□■■■■□□■■■■■■■■■	771077777363771	Manu2
IND042008256S	2008	■■■■■■■■■■□□■■■■■■■□□□□□■■■■■■■□□□□■■■■■■■	777637603760771	Ambigous (LAM/T)
IND04200921058	2009	■■■■■■■■■■□□□□■■■■■■■■□□□□□■■■■■■□■■■■■■■■■	777417740773771	Manu1

*Lineage designations for orphan patterns were done manually as Expert-based interpretations using revised SpolDB4 rules

37 were matched to a pre-existing shared type in the database, whereas one SIT (SIT3366, n=3) was newly created within the study. Among the 26 isolates which were of unique SITs, seven (SIT24, 429, 794, 1327, 1346, 599, 2392), seven (SIT346, 380, 474, 591, 1373, 1390, 2457), five (SIT31, 358, 1129, 1267, 1163), two (SIT250, 621), two (SIT54, 1634) and one (SIT137) corresponded to CAS, EAI, T, Beijing, Manu and X family lineages while two (SIT27, 560) were of unknown origin.

Characteristics of the predominant spoligotypes in our study (patterns shared by two or more isolates) and their worldwide distribution in the SITVIT2 database (Table III) showed that a total of seven SITs predominated (representing 47/104 isolates, or 45.19% of all isolates); and corresponded to the following (in decreasing order): SIT1-Beijing (n = 21, 20.39%), SIT26-CAS1_DELHI (n = 7, 6.8%), SIT954- CAS1_DELHI (n = 5, 4.85%), SIT4-EAI3_IND (n = 4, 3.88%), SIT53 (n=4, 3.88%), SIT288-CAS2 (n = 3, 2.91%) and SIT3366-EAI3_IND (n = 3, 2.91%). Most of the spoligotypes (except SIT1 that predominated in East/South-East Asia, SIT53 in North/South America, and Europe, and SIT3366 that was newly described in our study) were simultaneously predominant in South Asia and North America. Note that 22.04, 20.33, 26.09 and 27.27 per cent of all cases with, respectively, SIT11, 26, 288 and 954 in the SITVIT2 database were reported from North America (22.04, 20.33, 26.09 and 27.27% from the United States, respectively). The isolates belonging to these four predominant lineages in the SITVIT2 database were also frequently reported from South Asia (43.33, 44.48, 51.09, and 63.64%, respectively, for SIT11, SIT26, SIT288, SIT954; and 35.93, 22.38, 43.48, and 54.55% being from India). Regarding SIT1, 34.52 per cent of cases with this pattern in SITVIT2 were reported from East-Asia,

Table II. Description of 38 shared-types (n=83 isolates) and corresponding spoligotyping defined lineages/sublineages starting from a total of 104 *M. tuberculosis* isolates from patients residing in Varanasi, north India

SIT*	Spoligotype description	Octal number	Clade**	Number (%) in study	% in study vs. database	Clustered vs. unique patterns***
1	□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□■■■■■■■■■	000000000003771	Beijing	21 (20.39)	0.22	Clustered
11	■□□■■■■■■■■■■■■■■■■■■■■■■■□□□□■□■■□□□■■■■	477777777413071	EAI3-IND	4 (3.88)	0.74	Clustered
24	■■■□□□□■■■■■■■■■■■■■■□□□□□□□□□□□□□■■□□□□■■■	703777740003031	CAS1-Delhi	1 (0.97)	5.26	Unique
26	■■■□□□□■■■■■■■■■■■■■■□□□□□□□□□□□□□■■■■■■■■■	703777740003771	CAS1-Delhi	7 (6.8)	0.65	Clustered
27	■■■□□□□■■■■■■■■■■■■■■□□■■■■■■■■■□□□□■■■■■■	703777747770371	Unknown	1 (0.97)	6.25	Unique
31	■■■■■■■□□□□□□■■■■■■■■■■■■■■■■■□□□□■■■□□□□	774037777760700	T	1 (0.97)	1.59	Unique
42	■■■■■■■■■■■■■■■■■■■■□□□□■■■■■■■■□□□□■■■■■■■	777777607760771	LAM9	2 (1.94)	0.07	Clustered
53	■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■□□□□■■■■■■■	777777777760771	T1	4 (3.88)	0.07	Clustered
54	■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■□□■■■■■■■■■	777777777763771	Manu2	1 (0.97)	0.47	Unique
137	■■■■■■■■■■■■■■■■■□■■■■■■■■■■■■■■□□□□■■□□□□■	777776777760601	X2	1 (0.97)	0.1	Unique
138	■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■□□□□■□■■■■■□□□□	777777777413700	EAI5	2 (1.94)	1.9	Clustered
250	□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□■■■■■■	000000000000371	Beijing	1 (0.97)	2.94	Unique
288	■■■□□□□□□■■■■■■■■■■■■□□□□□□□□□□□□■■■■■■■■■	700377740003771	CAS2	3 (2.91)	3.26	Clustered
346	■■■■■■■■■□■■■■■■■■■■■□□□□□□□□□□□□□■■■□■□■■■	777377700003531	EAI1-SOM	1 (0.97)	25	Unique
358	■■■□□■■■■■■■■■■■■■■■■■■■■■■■■■□□□□■■■■■■■	717777777760771	T1	1 (0.97)	3.7	Unique
380	■□■■■■■■■■■■■■■■■■■■■■■■■■■□□□□■□■■■■■■■■■	577777777413771	EAI5	1 (0.97)	20	Unique
429	■■■□□□□■■■■■■■■■■■■■■□□□□□□□□□□□□□■■■■■□■■■	703777740003731	CAS1-Delhi	1 (0.97)	4.76	Unique
474	■□□■■□■■■■■■■■■■■■■■■■■■■■■□□□□■□■■□□□□■■■	467777777413031	EAI5	1 (0.97)	20	Unique
498	■■■■■■■■■■■□■■■■■■■■■■■■■■■■■■■□□□□■■■■■■■	777677777760771	T1	2 (1.94)	8.7	Clustered
560	■■■■■■■■■□□□□□□□□□□□□□□□□□□□□□□□□□□□□■■■■■■	777000000000371	Unknown	1 (0.97)	6.25	Unique
591	■■■■■■■■■■■■■■■■■■■■■■□■■■■■□□□□■□■■■■■■■■■	777777757413771	EAI6-BGD1	1 (0.97)	1.64	Unique
599	■■■□□□□■■■■■■■■■■■■□□□□□□□□□□□□□□□□□■■■■■■■	703777400000771	CAS	1 (0.97)	5.88	Unique
621	□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□■□■■■■■■■	000000000002771	Beijing	1 (0.97)	3.13	Unique
794	■■■□□□□■■■■■■□■■■■■■■■□□□□□□□□□□□□□■■■■■■■■■	703757740003771	CAS1-Delhi	1 (0.97)	6.25	Unique
954	■■■□□□□■■■■■□■■■■■■■■■□□□□□□□□□□□□□■■■■■■■■■	703677740003771	CAS1-Delhi	5 (4.85)	45.45	Clustered
962	■■■■■■■■■■■■■■■■■■■■■■■■■■■□□□□■□■■□□□□■■■	777777777413031	EAI5	2 (1.94)	28.57	Clustered
1073	■■■■■■■■■■■□□■■■■■■■■■■■■■■■■■□□□□■■■■■■■	777637777760771	T1	2 (1.94)	33.33	Clustered
1129	■■■■■■■■□■■■■■■■■■■■■■■■■■■■■■□□□□■■■■■■■	776777777760771	T1	1 (0.97)	9.09	Unique
1163	■■□■■■■■■■■■□■■■■■■■■■■■■■■■■■■□□□□■■■■■■■	677737777760771	T3	1 (0.97)	6.25	Unique
1267	■■■■■■■□□□□□□■■■■■■■■■■■■■■■■■■□□□□□■■□□□□	774037777760300	T1	1 (0.97)	33.33	Unique
1327	■■■□□□□■■■■□□■■■■■■■■■□□□□□□□□□□□□□■■■■■■■■■	703637740003771	CAS1-Delhi	1 (0.97)	14.29	Unique
1346	■■■□□□□■■■■■■■■■■■□■□□□□□□□□□□□□□■■■■■■■■□	703777640003770	CAS1-Delhi	1 (0.97)	6.25	Unique
1373	■■■■■■■■■■■□■■■■■■■■■■■■■■■■■□□□□■□■■■■■□■■■	777677777413731	EAI1-SOM	1 (0.97)	25	Unique
1390	■■■■■■■■■■■■■■■■■■■■■■□□■■■■□□□□■□■■□■■■■■■	777777747413371	EAI6-BGD1	1 (0.97)	12.5	Unique
1634	■■■■■■■■■■■■■■■■■■■■■■■■■■■■■□■□□■■■■■■■■■	777777777723771	Manu2	1 (0.97)	7.69	Unique
2392	■■■□□□□■■■■■■■■■■■■■■□□□□□□□□□□□□□■■□□■■■■■	703777700003171	CAS	1 (0.97)	12.5	Unique
2457	■□□□□□■■■■■■■■■■■■■■■■■■■■□□□□■□■■□□□■■■■	407777777413071	EAI3-IND	1 (0.97)	33.33	Unique
3366*	■□□■■□□□□■■■■■■■■■■■■■■■■■■■■□□□□■□■■□□□■■■■	460777777413071	EAI3-IND	3 (2.91)	100	Clustered

A total of 37/38 SITs matched a pre-existing shared-type in the database, whereas 1 SIT (SIT3366, n=3 isolates) was newly created within the present study.

**Clade designations according to SITVIT2 using revised SpolDB4 rules; "Unknown" designates patterns with signatures that do not belong to any of the major clades described in the database.

***Clustered strains correspond to a similar spoligotype pattern shared by 2 or more strains "within this study"; as opposed to unique strains harbouring a spoligotype pattern that does not match with another strain from this study. Unique strains matching a pre-existing pattern in the SITVIT2 database are classified as SITs, whereas in case of no match, they are designated as "orphan" (see Table I)

Table III. Description of major *M. tuberculosis* clusters in Varanasi (containing 3 or more isolates in our study), and their worldwide distribution in the SITVIT2 database

SIT (Clade) Octal number Spoligotype description	Number (%) in study	% in study vs. database	Mean Age (yr)	HIV sero-positive (%)	MDR-TB	Distribution in regions with ≥ 3% of a given SIT*	Distribution in countries with ≥ 3% of a given SIT**
1 (Beijing) 000000000003771 □□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□■■■■■■■■■■	21 (20.39)	0.22	27.47	4 (10.05)	13	ASIA-E 34.52, AMER-N 21.18, ASIA-SE 9.57, AFRI-S 8.71, ASIA-N 7.28, ASIA-S 3.85, EURO-N 3.26,	USA 20.83, CHN 19.95, JPN 12.11, ZAF 8.71, RUS 7.28, VNM 4.1,
11 (EAI3-IND) 477777777413071 ■□□■■■■■■■■■■■■■■■■■■■■■□□□□■□■■□□□■■■■	4 (3.88)	0.74	31.75	2 (50)	1	ASIA-S 43.33, AMER-N 22.04, EURO-N 12.22, EURO-W 8.52, ASIA-SE 5.0,	IND 35.93, USA 22.04, GBR 8.33, NLD 5.93, BGD 4.26, MYS 3.15, DNK 3.15,
26 (CAS1-Delhi) 703777740003771 ■■■□□□□■■■■■■■■■■■■■■□□□□□□□□□□□□■■■■■■■■■	7 (6.8)	0.65	28.43	0 (0)	4	ASIA-S 44.48, AMER-N 20.33, ASIA-W 7.8, EURO-W 7.15, AFRI-E 6.13, EURO-N 5.39, EURO-S 4.64,	IND 22.38, USA 20.33, PAK 13.37, SAU 7.24, BGD 6.96, ITA 4.46, NLD 3.99, ETH 3.53, GBR 3.25,
53 (T1) 777777777760771 ■■■■■■■■■■■■■■■■■■■■■■■■■■■■□□□□■■■■■■■■	4 (3.88)	0.07	22.25	0 (0)	2	AMER-N 18.44, AMER-S 14.23, EURO-W 11.45, EURO-S 10.24, ASIA-W 7.69, EURO-N 6.02, AFRI-S 5.61, AFRI-E 5.03, AFRI-N 3.97, ASIA-E 3.76,	USA 14.9, ITA 6.02, BRA 5.66, ZAF 5.48, TUR 3.92, AUT 3.86, MEX 3.22, PER 3.0,
288 (CAS2) 700377740003771 ■■■□□□□□□■■■■■■■■■■■■□□□□□□□□□□□□■■■■■■■■■■	3 (2.91)	3.26	26	2 (66.66)	1	ASIA-S 51.09, AMER-N 26.09, EURO-W 11.96, EURO-N 3.26,	IND 43.48, USA 26.09, NLD 8.7, BGD 4.35, GBR 3.26,
954 (CAS1-Delhi) 703677740003771 ■■■□□□□■■■■□■■■■■■■■■■□□□□□□□□□□□□■■■■■■■■■■	5 (4.85)	45.45	28	2 (40)	2	ASIA-S 63.64, AMER-N 27.27, EURO-N 9.09,	IND 54.55, USA 27.27, PAK 9.09, GBR 9.09,
3366* (EAI3-IND) 460777777413071 ■□□■■□□□□■■■■■■■■■■■■■■■■■□□□□■□■■□□□■■■■	3 (2.91)	100	36.33	1 (33.33)	0	ASIA-S 100	IND 100

*Worldwide distribution is reported for regions with more than 3% of a given SITs as compared to their total number in the SITVIT2 database. The definition of macro-geographical regions and sub-regions (*http://unstats.un.org/unsd/methods/m49/m49regin.htm*) is according to the United Nations; Regions: AFRI (Africa), AMER (Americas), ASIA (Asia), EURO (Europe), and OCE (Oceania), subdivided in: E (Eastern), M (Middle), C (Central), N (Northern), S (Southern), SE (South-Eastern), and W (Western). Furthermore, CARIB (Caribbean) belongs to Americas, while Oceania is subdivided in 4 sub-regions, AUST (Australasia), MEL (Melanesia), MIC (Micronesia), and POLY (Polynesia). Note that in our classification scheme, Russia has been attributed a new sub-region by itself (Northern Asia) instead of including it among rest of the Eastern Europe. It reflects its geographical localization as well as due to the similarity of specific TB genotypes circulating in Russia (a majority of Beijing genotypes) with those prevalent in Central, Eastern and South-Eastern Asia

**The three letter country codes are according to *http://en.wikipedia.org/wiki/ISO_3166-1_alpha-3*; countrywide distribution is only shown for SITs with ≥5% of a given SITs as compared to their total number in the SITVIT2 database

21.18 per cent from North America, but only 3.85 per cent from South Asia. The third highest proportion of predominant spoligotypes was from Western Europe. The mean age of the patients' harbouring predominant spoligotypes ranged from 22.25-36.36 years.

Distribution of predominant spoligotypes by gender, age, HIV status and DST: Almost all SITs were more prevalent in men, except SIT53, being isolated from the patients in their productive age (mean age, 27.47-36.33 yr). SIT53 was more prevalent than all other spoligotypes in women, being isolated from the patients with the age ranges from 14-20 yr (mean age, 16.33 yr) (Table III). SIT1/Beijing genotype was highly prevalent in the multidrug-resistant as well as HIV seropositive groups (13/21 isolates were MDR, four of these being isolated from HIV seropositive patients), followed by the CAS1_DELHI lineage (SIT26 and SIT654) in 6/12MDR isolates (Table III).

Association of clustered spoligotypes with clinical, epidemiological features of patients and phenotypic, genotypic DST: Binary logistic regression was applied to analyze the "clustering" of *M. tuberculosis* isolates in association with patients' demographics, clinical profiles and the bacteriologic features (Table IV). Smear-negative TB patients had a significantly higher proportion of clustered isolates than smear-positive patients (77.27 vs. 48.78%; adjusted OR, 0.248; *P*, 0.015; 95%CI, 0.080-0.765), as well as in the HIV seropositive patients in comparison to HIV seronegative patients (80.0 vs.50.56%; adjusted OR, 3.943; *P*, 0.045; 95%CI, 1.031-15.077). However, there were no significant association between clustered *M. tuberculosis* isolates and age (mean ± SD, 31.47 ±13.9), sex, treatment history and radiological findings of the patients (Table IV).

Further, drug resistant/MDR-TB and acquired drug resistant/primary drug resistant isolates were clustered in almost same proportion compared to total genotyped isolates. Compared with wild type (wt) isolates, the isolates with the *katG*315Thr mutation were more likely to be clustered (60.0 vs. 20.0%) however, the proportion was not significant. Further, among RIF and EMB resistant isolates, *rpoB*516Leu, *rpoB*526Arg, *rpoB*531Leu and *embB*306Val, *embB*306Ile alleles, respectively were not associated with clustering (Table IV).

Discussion

The genetic diversity of *M. tuberculosis* genome and hence their population structure, is strongly linked to geography reinforcing the importance of localized effort to control tuberculosis. Utilizing these data for the clinical benefit of individual patients remains a challenge. Epidemiological studies are important in the surveillance of the disease to define its origin and spread in the community and for effective control and prevention. In this study, we have tried to define the genetic structure of the population of circulating *M. tuberculosis* isolates in and around Varanasi, north India. When the spoligotypes of these isolates were compared with those in the international spoligotype database of the Institute Pasteur de Guadalupe, it was found that 21 isolates were not identified in the SITVIT2 database. Of these 21 orphan isolates, only 8 cases were pan susceptible while the remaining 13 (61.90%) isolates corresponded to MDR-TB. This observation indicates the need of conducting multicenter studies to pinpoint all the prevalent *M. tuberculosis* spoligotypes in the Indian subcontinent. The remaining 83 isolates were distributed in 38 shared types or SITs. Twenty six (31.33%) isolates presented unique SITs, while the remaining 57 were clustered in 12 groups of 2 to 21 isolates each.

TB is a leading health problem in India, still our knowledge regarding the circulating strains of *M. tuberculosis* is limited. Studies done so far, suggest Central Asian family (CAS) to be the major clade in the north whereas East African Indian (EAI) predominates in the southern part of India[1,20,21]. In general, Beijing genogroup is present in low percentage throughout the country[20-23]. The emergence of this family continues to pose a serious threat to TB control due to its high virulence and frequent association with multi-drug resistance. Beijing family is highly prevalent throughout Asia and Eurasia[24], with a reported prevalence of approximately 3–11 per cent in India[20,21,25]. In our region spoligotype patterns showed that Beijing clade was the largest clade (22.11%) corresponded by SIT1, SIT250 and SIT621 and this was quite higher than the above reported frequencies. In the international database, SIT1 contains 10.7 per cent of the isolates reported. Association of Beijing strain with MDR-TB has been noted in studies carried out in United States, Estonia, Vietnam and Russia[26-28] but others have not found any association between Beijing family and drug resistance[29,30]. However, in the present study the association of Beijing strain with MDR-TB was observed at a high frequency (29.41%), accounting for nearly one third of the MDR-TB isolates. While another five of 23 Beijing genotypes were pan susceptible, two isolates were resistant to INH, STR and EMB and one

Table IV. Association of clustered *M. tuberculosis* isolates with clinical, epidemiological characteristics of patients and different drug-resistant pattern and genetic mutations[*] (n=104)

	No. of isolates/patients		Adjusted OR[b]	*P* value[c]	95% CI[b]
	Total	n (%) clustered			
Age (yr)					
Mean (Years) (±SD)	31.47 (±13.9)	30.05 (±12.8)	-	-	-
0-20	22	13 (59.09)	-	-	-
21-40	65	36 (55.38)	-	-	-
41-60	11	7 (63.64)	-	-	-
>60	6	1 (16.67)	-	-	-
Sex					
Female	39	19 (48.72)	-	-	-
Male	65	38 (58.46)	-	-	-
HIV status					
Seronegative	89	45 (50.56)	1		
Seropositive	15	12 (80.00)	3.943	0.045	1.031-15.077
Treatment history					
New	56	30 (53.57)	1		
Prior treated	48	27 (56.25)	0.954	0.908	0.425-2.139
Sputum smear test					
Negative	22	17 (77.27)	1		
Positive	82	40 (48.78)	0.248	0.015	0.080-0.765
Chest X-ray					
Negative	77	38 (49.35)	1		
Positive	27	19 (70.37)	2.338	0.081	0.902-6.061
Type of drug resistance	Any drug resistance=57	Any drug resistance=33			
Acquired	36	19 (52.78)			
Primary	21	14 (66.67)	-	-	-
Drug-resistance					
Pan-drug susceptible	47	24 (51.06)	0.905	0.817	0.386-2.119
Any drug-resistant	57	33 (57.89)	1.105	0.817	0.472-2.589
Isoniazid resistant	55	31 (56.36)	0.919	0.849	0.385-2.191
Rifampicin resistant	52	28 (53.85)	0.708	0.433	0.298-1.679
Streptomycin resistant	41	25 (60.98)	1.269	0.593	0.530-3.043
Ethambutol resistant	45	27 (60.00)	1.256	0.601	0.535-2.952
Multidrug resistant	51	27 (52.94)	0.662	0.347	0.280-1.566
Drug-resistant isolates with mutation in;					
*kat*G					
Others/wt	5	1 (20.00)			
315Thr	50	30 (60.00)	0.635	0.298	0.269-1.495
*rpo*B					
Others/wt	7	4 (57.14)			

Contd...

	No. of isolates/patients		Adjusted OR[b]	*P* value[c]	95% CI[b]
	Total	n (%) clustered			
516Leu or Trp	6	4 (66.67)	0.690	0.681	0.118-4.044
526Arg,Tyr,Asp,Gly or Asn	15	11 (73.33)	0.454	0.214	0.131-1.576
531Leu or Trp	34	16 (47.06)	1.920	0.156	0.780-4.727
526Arg, Tyr....+531Leu or trp	7	5 (71.43)	-	-	-
516Leu or Trp +531Leu or Trp	2	2 (100)	-	-	-
*emb*B					
Others/wt	20	10 (50.00)			
306Val or Leu	15	11 (73.33)	0.438	0.194	0.126-1.521
	10	6 (60.00)	0.863	0.833	0.221-3.370

[*]Mutation detection was done by Multiplex-allele specific-PCR; [a]cOR: crude odds ratio was calculated by comparing the clustering proportion between drug-resistant and drug-susceptible isolates in binary logistic regression model; [b]aOR: adjusted odds ratio and 95%CI were calculated by comparing the clustering proportion between drug-resistant isolate and drug-susceptible isolates, adjusted by age and sex of the subjects in a binary logistic regression model; [c]$P<0.05$

isolate was resistant to INH only. Of the 23 patients harbouring Beijing genotypes, 13 were previously treated and 10 of them were having MDR-TB Beijing genotypes. Three of them were HIV seropositive with non-MDR-TB Beijing genotypes. A study by Almeida *et al*[31] showed agreement with our finding and found 35 per cent of the Beijing genotype among MDR-TB isolates recovered in and around Mumbai, India. The authors did not report any pan susceptible Beijing isolate in their study sample. In another study from Kanpur, north India, among eight Beijing isolates, six (75%) were MDR-TB, one isolate was resistant to RIF, STR and kanamycin (KAN) and one isolate was sensitive to all six drugs tested[12]. However, the results of the present study regarding Beijing genotype should not be overinterpreted, as our samples were obtained from a tertiary care center, and sampling bias cannot be ruled out. Also, the fact that many of the Beijing strains were from patients with acquired resistance strongly suggested that a susceptible population could be present in our setting.

In our study, the second largest clade was CAS family (21.15%) represented by SIT24, SIT26, SIT288, SIT429, SIT599, SIT794, SIT954, SIT1327, SIT1346 and SIT2392 and among these the most predominant spoligotype was SIT26 (16.35%). Studies in north India showed that CAS1-DELHI strain was prevalent in 22-37% of isolates[12,20,21,23] whereas it was found at a lower frequency (7.4%) in a study from Mumbai[22] and represents only 1.7 per cent of strains in the SITVIT2 database. SIT26 represents 1.63 per cent of isolates in the updated SITVIT2 database and has been reported from 39 countries in varying numbers, with maximum number of isolates from Asian countries. A study from Pakistan also showed CAS1-DELHI type (39%) as the dominant isolate[32]. SIT26 is limited mainly to the Middle East and to Central Asia. It has also been found in regions in which frequent migration to and from the Indian subcontinent occurs, *e.g*., Saudi Arabia, Kenya, South Africa, Malaysia, Myanmar, Australia, USA and parts of Europe. The third-largest spoligotype found in our study was EAI family lineages (17.31%) with predominance of EAI3_IND (7.69%). It has also been found to be a major spoligotype in other studies from Delhi[22,23]. Further, 13 (12.5%) isolates belonged to T super family (prototype T, T1 and T3), a widespread yet poorly defined super family, needing better markers for proper characterizations[27] among which 11 (10.58%) belonged to T1 prototype. Mathuria *et al*[24] found 9.6 per cent prevalence of T1 prototype of T super family. Predominant spoligotypes, *i.e.*, SIT1, 11, 26, 53, 288 and 954, within this study were also most prevalent in North America and United States. The SIT (SIT3366, n=3) which was newly created within the study was also found predominantly in South Asia. Among these three patients with SIT3366 isolate, two were from Jaunpur and one was from Varanasi region of north India. One of these three patients had HIV seropositivity and another one had previous treatment history.

Binary logistic regression was applied to analyze the "clustering" of *M. tuberculosis* isolates in association

with patients' demographics, clinical profiles and the bacteriologic features. The isolates were more likely to be clustered in HIV seropositive patients, and the percentage of clustering was significantly higher in smear-negative TB patients as compared to smear-positive patients. However, most of these smear-negative specimens were found to be culture positive. The overall numbers of clustered isolates were also high among smear-positive patients, which may extend the transmission period of the pathogen between hosts by infecting more persons around an index case. Also, the presence of HIV is known to increase the risk of rapid progression from infection to disease, a fact that may indirectly increase TB transmission rates in the community[14].

Early TB detection and efficient treatment are necessary steps to control this infectious disease; hence the information regarding the transmission patterns of drug-resistant *M. tuberculosis* isolates is a prerequisite in decision-making for TB control. High TB burden regions[34,35] are known to harbour a higher prevalence of the *katG*315Thr mutation in INH resistant isolates compared to low TB burden areas[36]. Among drug-resistant TB patients, a considerably high clustering proportion of *katG*315Thr allele was observed in our study. This allele was associated with MDR-TB as reported elsewhere[36], and might serve as a surrogate marker to identify the recent transmission of MDR-TB and INH resistant isolates in studied areas.

TB epidemic in Varanasi, north India is caused by a wide diversity of spoligotypes with predominance of four genotype lineages: Beijing, CAS, EAI and T. The Beijing genotype was the most frequent single spoligotype and strongly associated with MDR-TB isolates in Varanasi, North India. We showed that the bulk of ongoing transmission with MDR-TB strains in Varanasi, northern India is linked to Beijing genotype followed by the CAS1_Delhi lineage. These findings have important implications for the control and prevention of tuberculosis.

Larger studies with representative sampling are needed to elucidate the actual status and role of these genotypes in the dissemination and transmission of TB in Northern India as well as elsewhere in India. This study represents a baseline study of the *M. tuberculosis* population structure in Eastern Uttar Pradesh, and may serve as an impetus for future molecular epidemiological studies in north India, and help comprehend the global tubercle bacilli genotypic diversity. Additional studies using 2nd-line typing using MIRU-VNTRs over an extended period of time and an exhaustive recruitment of patients would be beneficial to fully understand the epidemiology of TB and its transmission dynamics in this region.

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