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# Spoligotyping of *Mycobacterium tuberculosis* isolates at a tertiary care hospital in India

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**Abstract**

OBJECTIVE Spoligotyping is a valuable genotyping tool to study the genetic diversity and molecular epidemiology of *Mycobacterium tuberculosis* (*M. tb*). The aim of this study was to analyse different spoligotype patterns of *M. tb* strains isolated from patients with tuberculosis from different parts of India.

MATERIALS AND METHODS A total of 163 *M. tb* isolates were spoligotyped between January 2014 and January 2015. About 47% ($n$ = 77) were from patients with extrapulmonary tuberculosis; of these, 10 were MDR, and seven were Pre-XDR. Of the 86 *M. tb* isolates from patients with pulmonary tuberculosis, 25 were MDR, and 25 were Pre-XDR.

RESULTS We found 61 spoligo patterns, 128 clusters in the spoligotype data base (spoldb4 data base) with spoligo international type (SIT) number and 35 true unique isolates. The most pre-dominant spoligotype was EAI lineage (56), followed by Beijing (28), CAS (20), T(9), U(7), X(3), H(3), BOVIS_1 BCG(1) and LAM(1).

CONCLUSION Although our study identified EAI, CAS and Beijing strain lineages as pre-dominant, we also found a large number of orphan strains (20%) in our study. Beijing strains were more significantly associated with MDR TB than CAS and EAI lineages. Further studies on large sample sizes would help to clearly describe the epidemiology of *M. tb* in India.

**keywords** spoligotyping, Beijing strains, multidrug-resistant tuberculosis, XDR TB, India

## Introduction

Tuberculosis (TB), caused by *Mycobacterium tuberculosis* complex (MTBC), continues to be the major cause of morbidity in the world. About 23% of the world's TB cases are reported from India [1].Genotyping methods like Spacer oligonucleotide typing (Spoligotyping), Mycobacterial Interspersed Repetitive Units-Variable Number Tandem Repeats (MIRU-VNTR) and IS6110 restriction fragment length polymorphism (IS6110-RFLP) are fingerprinting tools to understand the genetic diversity of *Mycobacterium tuberculosis* (*M. tb*) isolates [2].

The genotyping of *M. tb* isolates also helps in tuberculosis control by detecting unsuspected outbreaks, laboratory cross contaminations and to distinguish exogenous reinfection from endogenous reactivation. In recent times, spoligotyping has become one of the most widely used genotyping methods. It is PCR-based and used to analyse the polymorphism in the spacer sequences, which are present in the direct repeat (DR) region of MTBC strains. Spoligotyping is simple, rapid and highly reproducible; the results are obtained in a simple digital pattern, readily named and databased *vs.* to other genotyping methods [3]. Spoligotyping can be used on isolates from both pulmonary and extra pulmonary samples.

Other commonly used DNA fingerprinting methods in India are MIRU– VNTR and IS6110 RFLP. 24 loci MIRU–VNTR is also PCR-based and more effective to study transmission of TB disease than the IS6110 RFLP method. The technical difficulty of sizing the multiple small PCR fragments in MIRU–VNTR technique and the requirement of technical expertise in performing IS6110 RFLP led us to choose spoligotyping for our study. The aim was to identify the predominant spoligotype patterns from strains isolated at the department of Clinical Microbiology, Christian Medical College and Hospital, a tertiary care hospital receiving patients with tuberculosis from different parts of India.

## Materials and methods

*Mycobacterium tuberculosis* (*M. tb*) isolates were obtained from pulmonary and extrapulmonary samples received for routine mycobacterial culture and drug

 

susceptibility testing at the Mycobacteriology Section of the Department of Clinical Microbiology at Christian Medical College, Vellore. A total of 163 MTB isolates collected over a period of one year (January 2014 to January 2015) were analysed.

### DNA extraction

*Mycobacterium tuberculosis* isolates grown in L J medium were added to 1 ml TE buffer. The cultures were killed at 80 °C for 30 min, and 50 µl of lysozyme was added and incubated overnight at 37 °C. On the following day 70 µl of 10% SDS and 6 µl of proteinase K was added and further incubated at 65 °C for 15 min. Then 100 µl of 5 M NaCl and 80 µl of CTAB were added and incubated again at 65 °C for 15 min. Following this step, 700 µl of chloroform/isoamyl alcohol (24:1) was added and subjected to centrifugation for 20 min at 10,000 g at 25 °C. The supernatant was separated into a new eppendorf tube, and 600 µl of ice cold iso-propanol was added to the tubes and incubated at −20 °C overnight to precipitate the DNA. On the third day eppendorf tubes were centrifuged at 12 000 rpm for 15 min, and the pellet was washed with 500 µl of 70% ethanol. The genomic DNA extracted was re-suspended in TE buffer and stored at −20 °C until use ([4]).The extracted DNA was run on 1% agarose gel electrophoresis to confirm the presence of DNA and quantified using nano drop technique. The amount of DNA extracted from these isolates varied from 200 to 2500 ng/ml.

### Spoligotyping

Spoligotyping was performed at National Institute for Research In Tuberculosis (NIRT), Chennai as described by Kamerbeek *et al.* [4]. The direct repeat region in the genome of MTB complex was amplified using two primers, DRa and DRb. Chromosomal DNA of *M. tb* strains H37Rv and *Mycobacterium bovis* BCG P3 was used as positive controls, and molecular grade (MQ) water was used as negative control. The amplified products were hybridised on a membrane pre-coated with spacer–oligos that represent the spacer region of known sequence. The presence of spacers was visualised on X-ray films as black squares after incubation with streptavidin-peroxidase and ECL detection.

The drug susceptibility of *M. tb* isolates was tested by the 1% proportion method as per the Revised National TB Control Programme (RNTCP) [5]. First-line drugs are streptomycin, isoniazid, rifampicin and ethambutol. The cultures were inoculated on both drug-containing and drug-free medium and incubated at 37 °C for 6 weeks. Growth of more than 1% on drug-containing tubes was taken as indicative of drug-resistant strains. Strains resistant to isoniazid and rifampicin were considered multidrug resistant (MDR) and subjected to second-line drug susceptibility testing. Second-line drugs were ofloxacin and kanamycin. Both drug-susceptible and drug-resistant strains were included in this study.

## Results

We recovered 163 isolates of *M. tb* from 86 pulmonary samples such as sputum (78), broncho-alveolar lavage (6), endobronchial ultrasound guided –trans bronchial needle aspiration (EBUS-TBNA) (2), and 77 extrapulmonary samples such as pus (21), lymph nodes (21), CSF (6), bone tissue (6), urine (5), endometrial curetting (3), pleural tissue (3), omental tissue (3), ascitic fluid (2), bone marrow (2), pleural fluid (2), lung tissue (1), synovial tissue (1) and mucosal tissue (1). These isolates were spoligotyped, resulting in 61 spoligo patterns. A total of 128 clusters of these 163 isolates were in the spoligotype database (spoldb4 database) with spoligo international type (SIT) numbers. A total of 34 isolates were orphan strains without SIT numbers in the SpolDb4 database, and one unique strain was observed. The most pre-dominant spoligotype was EAI lineag (56), followed by Beijing (28), CAS (20), T(9), U(7), X(3), H(3), BOVIS_1 BCG(1) and LAM (1). The Sublineages found in this study are illustrated in Table 1.

### Drug susceptibility testing and spoligotype patterns

Forty-two of the 163 *M. tb* isolates were MDR; of these, 18 were Beijing strains, four were CAS, eight were EAI, nine were orphan strains, and one each was LAM, T and U. 89 strains were sensitive to all first-line ATT drugs, namely streptomycin, isoniazid, rifampicin and ethambutol. Most of these sensitive strains were orphan strains. A total of 25 strains were mono-resistant to one of the four-first-line drugs; these were Beijing, CAS, EAI, H and T strains. Two strains were resistant to isoniazid and ethambutol [EAI5 (1) and X1 (1)], five strains were resistant to streptomycin and isoniazid [Orphan (2), Beijing (1), EAI (2)],

## Discussion

This study compared 163 isolates of *M. tb* from both pulmonary and extrapulmonary specimens and found the genetic diversity to be similar in both kinds of specimens indicating that these are similar stains circulating in the

community and causing both forms of the disease. This differs from comparisons of *M. tb* isolates from both pulmonary and extrapulmonary samples in the literature, which found a higher genetic diversity in pulmonary tuberculosis patients [6].

In our study, one *Mycobacterium bovis* (Bovis_1 BCG) strain was isolated from a one-year-old child from the site of a BCG injection abscess. The patient had no previous history of TB and TB contact. HIV status was unknown due to missed follow-up.

As shown in Table 2, we analysed *M. tb* strains from patients from different parts of India were included in this study. Ninety-eight of our 163 patients were from North India and 65 from South India. The distribution of strain families in Northern and Southern India is presented in Table 2. Our study identified 34% of EAI strains, which is the predominant spoligotype. The prevalence of EAI strains was the same in both North and South Indian patients. However, Singh *et al.* reported in 2004 [7] that EAI strains predominate in South India, whereas the Beijing and CAS strains types do in North India. We had one EAI6 (SIT 292) strain from a patient from Jharkhand. For the first time a similar finding have been reported by Kandhakumari *et al.* in 2015 from Pudhucherry in India [2].

About 85% of the 42 MDR-TB strains came from North India (Table 3). Beijing strains were the second-largest clade in our study. We identified 18% of Beijing strains represented by SIT 1, SIT 190, SIT 255 and detected a unique Beijing-like strain (SIT 269), which we consider as pseudo-orphan, as it presented as a single strain without any clusters. The predominant Beijing strain spoligotype was SIT 1 (89%). Beijing

**Table 1** Sublineages in our study

| Sublineages | No of Strains | SIT no |
|---|---|---|
| EAI3_IND | 22 | 11,338,473,654 |
| EAI5 | 21 | 126,138,236,474,934,935,962, 1493,236,340,342,355 |
| EAI1_SOM | 6 | 1182,1251,1316,493,48 |
| EAI6_BGD1 | 7 | 1417,43,882,292,1496,591 |
| CAS1_DELHI | 14 | 1343,1590,26 |
| CAS | 4 | 1151,142,356,357 |
| CAS2 | 2 | 288 |
| T1 | 4 | 334,53,1105,1580 |
| T2 | 1 | 1077 |
| T3 | 2 | 37,1655 |
| T4 | 1 | 40 |
| X3 | 2 | 70,92 |
| X1 | 1 | 119 |
| LAM9 | 1 | 42 |

**Table 2** Distribution of different family strains in Northern and Southern part of India

| | BEIJING | CAS1_DELHI | CAS | CAS2 | EAI1_SOM | EAI3_IND | EAI5 | EAI6_BGD1 | U | Orphan (Unique) | Others | Total |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| North | 23 (23.4%) | 10 (10.2%) | 3 (3.0%) | 2 (2.0%) | 3 (3.0%) | 9 (9.1%) | 10 (10.2%) | 6 (6.1%) | 2 (2.0%) | 19 (19.3%) | 11 (11.2%) | 98 |
| South | 6 (9.2%) | 4 (6.1%) | 1 (1.5%) | 0 (0%) | 3 (4.6%) | 13 (20%) | 11 (16.9%) | 1 (1.5%) | 5 (7.6%) | 15 (23.0%) | 6 (9.2%) | 65 |
| Total | 29 | 14 | 4 | 2 | 6 | 22 | 21 | 7 | 7 | 34 | 17 | 163 |

**Table 3** Details of the MDR – TB isolates in our study

| Strains | No of MDR TB Isolates | Male | Female | HIV status | | | Pulmonary samples | Extra pulmonary samples | North India | South India |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | Positive | Negative | Unknown | | | | |
| Beijing | 18 | 15 | 3 | 1 | 15 | 1 | 14 | 4 | 16 | 2 |
| CAS | 4 | 3 | 1 | 0 | 1 | 3 | 1 | 3 | 3 | 1 |
| EAI | 8 | 7 | 1 | 0 | 6 | 2 | 3 | 5 | 7 | 1 |
| Orphan | 9 | 7 | 2 | 1 | 7 | 1 | 8 | 1 | 7 | 2 |
| LAM | 1 | 0 | 1 | 0 | 1 | 0 | 1 | 0 | 1 | 0 |
| T | 1 | 1 | 0 | 0 | 1 | 0 | 1 | 0 | 1 | 0 |
| U | 1 | 1 | 0 | 0 | 1 | 0 | 1 | 0 | 1 | 0 |
| Total | 42 | 34 | 8 | 2 | 32 | 7 | 29 | 13 | 36 | 6 |

strain was frequently (43%) associated with MDR–TB, accounting for nearly one-third of the MDR–TB isolates. Acquired MDR-TB cases outnumbered primary MDR-TB cases in our study and most of the acquired MDR-TB cases were caused by Beijing strains. About 7% of Beijing strains were susceptible to first-line ATT drugs. The emergence of this family continues to threaten TB control because of its high prevalence throughout Asia. Drug-resistant TB emerges as a result of treatment mismanagement. Low case detection and cure rates might be the reason for the rise of drug-resistant TB cases in India.

The prevalence of CAS strains was 12%, accounting for 75% of North Indian strains. This is similar to the finding of Gupta *et al.* [8], who also reported CAS to be the major clade in the North India.

There were 34 orphan strains in our study population, which were not identified in the spolD4 database. A total of 22 were PAN sensitive, and nine were MDR. Three strains were resistant to either one or two ATT drugs.

**Table 4** Description of different strain clusters in our study

| SIT(Clade) octal Number | No (%) in study | %in study *vs.* database | Mean age (year) | Male | Female |
|---|---|---|---|---|---|
| 1 Beijing 000000000003771 | 26 (15.9) | 0.69 | 31.4 | 23/26 (88.4%) | 3/26 (11.5%) |
| 11 (EAI3_IND)477777777413071 | 18 (11.0) | 7.5 | 32.6 | 14/18 (77.7%) | 4/18 (22.2%) |
| 26 (CAS1_DELHI) 703777740003771 | 12 (7.3) | 2.9 | 36.5 | 10/12 (83.3%) | 2/10 (16.3%) |
| 288 (CAS 2)700377740003771 | 2 (1.2) | 5.0 | 19.5 | 2/2 (100%) | 0/2 (0%) |
| 48 (EAI1_SOM)777777777413731 | 2 (1.2) | 0.8 | 26 | 1/2 (50%) | 1/2 (50%) |
| 473 (EAI3_IND)401777777413071 | 2 (1.2) | 28.5 | 31.5 | 2/2 (100%) | 0/2 (0%) |
| 126 (EAI5) 477777777413771 | 3 (1.8) | 8.5 | 54.6 | 3/3 (100%) | 0/3 (0%) |
| 138 (EAI5)777777777413700 | 4 (2.4) | 5.7 | 27.5 | 2/4 (50%) | 2/4 (50%) |
| 236 (EAI5) 777777777413771 | 5 (3.0) | 5.4 | 41 | 4/5 (80%) | 1/5 (20%) |
| 340 (EAI5) 474377777413771 | 2 (1.2) | 9.5 | 34 | 1/2 (50%) | 1/2 (50%) |
| 43 (EAI6_BGD1)777777747413771 | 2 (1.2) | 11.7 | 24 | 1/2 (50%) | 1/2 (50%) |
| 37 (T3) 777737777760771 | 2 (1.2) | 1.2 | 50 | 2/2 (100%) | 0/2 (0%) |
| 172 (U) 777777777740771 | 2 (1.2) | 6.2 | 25 | 1/2 (50%) | 1/2 (50%) |

These orphan strains indicate the need for more molecular epidemiological studies in India.

We found a very high level of strain clustering in this study, which indicates substantial ongoing recent TB transmission. Three large clusters were identified comprising 26, 18 and 12 patients' isolates (Table 4). Some cases had different strain types in an epidemiological cluster, which cannot be considered in the same chain of transmission. Cluster size varied between 2 and 26. The ubiquity of the Beijing strains leads to the speculation that they may be particularly transmissible or virulent.

About 85% of patients in this cluster group are from northeastern West Bengal, Assam and Nagaland. These are adjacent states, and there may be higher possibility for transmission of *M. tb* strains of similar genotype. Only 15% of cases in the cluster group were from southern states such as Tamil Nadu (Vellore), Kerala and Andhra Pradesh.

The second-largest cluster group with 18 patients was infected by EAI3_IND strains (SIT 11), which are common in southern India. About 55% of EAI3_IND strains were isolated from South Indian patients. The third cluster of 12 patients belonged to CAS1_DELHI (SIT 26) spoligotype.

Exogenous reinfection has been reported in both immunosuppressed and immunocompetent individuals [9]. Most cases were associated with drug resistant strains of *M. tb* and some exogenous reinfections by drug-sensitive organisms have been reported [9]. Only one patient in our study had a recurrent TB infection. The patient had two episodes of TB due to irregular ATT, and the time interval between the two episodes was 11 months. The first episode of TB was caused by the EAI3_IND strain and the second by CAS, reflecting that the patient had an exogenous reinfection causing recurrent TB. Both strains were PAN-sensitive.

## Conclusion

In TB-endemic countries spoligotyping is an useful method to study the epidemiology of *Mycobacterium tuberculosis*. The TB epidemic in India is caused by a wide diversity of spoligotypes with predominance of Beijing, EAI and CAS types., and larger studies are recommended to elucidate their roles in TB transmission.

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