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Assessing the Genetic Diversity of *Mycobacterium tuberculosis* Strains in Kerala, India: A Comprehensive Study

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ABSTRACT

Introduction: Understanding the epidemiological and clinical characteristics of different tuberculosis strains is crucial for developing improved diagnostic tools, drugs, and vaccines for tuberculosis management. This study aimed to investigate the molecular epidemiology of *Mycobacterium tuberculosis* using spoligotyping, a widely used molecular typing method, to understand the genetic diversity and transmission dynamics of *M. tuberculosis*, on isolates obtained from patients with pulmonary tuberculosis in central Kerala. **Methods**: In a prospective study at a tertiary care hospital, 404 respiratory specimens from patients with symptoms suggestive of TB were collected. Specimens underwent Ziehl-Neelsen staining, culture in liquid (BD BACTEC™ MGIT™) and solid (Lowenstein-Jensen) media, and standard drug susceptibility testing with the MGIT system. Molecular analysis involved conventional PCR amplification of genomic DNA to generate sufficient genetic material for analysis, using species-specific and primers targeting the direct repeat region, followed by spoligotyping to assess the genetic diversity of the *M. tuberculosis* strains. **Results:** Out of 404 samples from individuals with suspected pulmonary TB, Mycobacteria were cultured from 48 [11.9%] of the samples. Amongst the 48 culture-positive *M. tuberculosis* isolates, 20 (41.66%) were sensitive to all five first-line anti-TB drugs, and 3 (6.2%) were resistant to all five drugs. Spoligotyping of the 47 isolates showed that 36.1% [n=17] of the isolates belonged to the *M. tuberculosis* EAI3 (East African-Indian) family, followed by 27.6% (n=13) *M. tuberculosis* EAI5 and 21.2% (n=10) *M. tuberculosis* CAS (Central Asia). Other families observed in this study, although less prevalent, were *M. tuberculosis* Beijing, 8.5% (n=4), family 33, 4.3% (n=2), and Mycobacterium bovis-BCG family, 2.1% (n=1). **Conclusion:** This study explored the genetic diversity and distribution of circulating *M. tuberculosis* strains in central Kerala. Genotyping *M. tuberculosis* strains provides valuable insights into TB transmission and progression, which can inform the development of effective public health control strategies.

INTRODUCTION

The COVID-19 pandemic has disrupted TB services, with newly diagnosed tuberculosis cases decreasing from 7.1 million in 2019 to 5.8 million in 2020 [1]. Studies conducted during the pandemic have found a link between the pandemic and the impact on the immune system's response to TB, which may contribute to an increase in TB cases. This potential consequence poses a significant challenge to achieving the WHO's End TB Strategy goal to reduce TB incidence by 80% and TB deaths by 90% between 2015 and 2030 [2]. India bears a substantial portion of the global TB burden, accounting for approximately 27% of total cases [1]. Rapid molecular diagnostic tests, antibiotics treatment, and identifying and monitoring individuals who have been in close contact with someone who has TB are crucial steps for halting further transmission and controlling tuberculosis. In 2019, Kerala, a state in South India, reported 72 tuberculosis cases per 100,000 population, representing data from before the COVID-19 pandemic [3].

Still, the COVID pandemic substantially reduced TB

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case reporting in 2020 and 2021. *M. tuberculosis* complex (MTBC) genotyping, using methods like spoligotyping, can identify recent transmission clusters, high-risk groups such as close contacts and healthcare workers, and monitor changes in TB strain distribution [4]. It also informs strategies to combat the emergence of drug-resistant strains [5].

Studies have shown differences in virulence factors and immune system responses among different *M. tuberculosis* strains or lineages. Certain strains may exhibit varying degrees of transmissibility and disease-causing capabilities, contributing to the complex dynamics of TB transmission and pathogenesis [6]. Data from India and South Africa suggest that individuals with concurrent infection with *M. tuberculosis* and SARS-CoV-2 have an increased risk of death due to complications from both infections. Additionally, reports indicate a substantial reduction in reported TB cases and completed treatment cycles in these countries by approximately one million in 2020, amidst broader healthcare disruptions and lockdowns imposed during the pandemic [7].

Some strains of *M. tuberculosis*, such as the Beijing genotype, have been linked to the development of drug-resistant TB, although the relationship is not fully understood [8]. *M. tuberculosis* has a diverse genetic population worldwide, with six distinct strains: Indo-Oceanic, East Asian, East African-Indian, Euro-American, West African I, and West African II, though the distinctions between these strains can be ambiguous in some cases [8].

Molecular epidemiology techniques like spoligotyping, restriction fragment length polymorphism (RFLP), and Mycobacterial interspersed repetitive units-Variable number of tandem repeats (MIRU-VNTRs), have been employed to investigate the genetic diversity and transmission dynamics of *M. tuberculosis* strains, contributing to a better understanding of their relationships [9]. These methods help researchers understand the predominant strains of *M. tuberculosis* present in a specific geographic region and how they spread among individuals [5]. Spoligotyping is a technique used to identify genetic relationships between different strains of *M. tuberculosis* based on DNA polymorphism in the direct repeat (DR) locus. It is a robust method with an intra-laboratory reproducibility over 90% in well-trained laboratories, owing to its reliance on a single PCR amplification, ability to differentiate main lineages and sublineages within the *M. tuberculosis* complex, and cost-effectiveness [10, 11].

Spoligotyping relies on a specific region of the *M. tuberculosis* genome called the DR locus. This region consists of multiple short, identical DNA sequences (DRs) interspersed with unique spacer sequences of varying lengths. The spoligotyping technique involves amplifying this entire DR locus using multiplex or nested PCR. This process essentially creates copies of the target DNA region, allowing for further analysis. The PCR products, representing different spacers and DRs, are hybridized to a membrane with 43 covalently bound synthetic oligonucleotides. These oligonucleotides represent the polymorphic spacers identified in *M. tuberculosis* $H_{37}Rv$ [spacers 1–19, 22–32, and 37–43] and *M. bovis* BCG [spacers 20-21 and 33–36]. The hybridization signals are detected by chemiluminescence through biotin labeling of one of the primers used for PCR amplification and a streptavidin-peroxidase conjugate system, which binds to biotin-labeled PCR products, and then visualized by autoradiography. Individual strains are differentiated based on their unique pattern of absent spacers within the complete set of 43 spacers [10]. The missing spacers are likely caused by deletions in the bacterial DNA through mechanisms such as homologous recombination, transposition, or other forms of genetic recombination. The DR region is a preferred site for IS*6110* insertion, which can lead to deletions and genetic variation [12]. Spoligotyping is a well-established and widely applied molecular typing technique in tuberculosis studies for investigating outbreaks, transmission dynamics, and population structure [13]. It allows researchers to simultaneously identify and distinguish between distinct genetic variants or sublineages within the *M. tuberculosis* complex [11].

Despite the known presence of drug-resistant tuberculosis (DR-TB) in Kerala, only a limited number of studies have investigated the genetic diversity and transmission dynamics of multidrug-resistant *M. tuberculosis* isolates from this southern Indian state. This contrasts with the large number of studies that have investigated the molecular epidemiology of DR-TB in northern India. Furthermore, only a few investigations have aimed to identify and assess the genetic mutations associated with drug resistance genotypes in the general population. This study employed spoligotyping to investigate the molecular epidemiology of *M. tuberculosis* strains isolated from patients with tuberculosis attending a tertiary care hospital in central region of Kerala. The findings will contribute to understanding the predominant lineages of MTBC circulating in this region.

MATERIAL AND METHODS

Study setting and sample collection. The study was conducted at a tertiary care center in the central region of Kerala. A total of 404 pulmonary and extrapulmonary specimens, excluding blood, were collected from November 2015 to October 2017. These specimens were sent for routine mycobacterial culture and drug susceptibility testing (DST) using the MGIT system, in accordance with the established protocols of the Mycobacteriology Section. Blood samples were not included in this study as they were not received for culture during the study period.

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Ethical considerations. The study adhered to ethical guidelines, and received clearance from the Institutional Ethics Committee of Pushpagiri Institute of Medical Sciences and Research Centre (PIMSRC) [PIMSRC/E1/388A/44/2015].

Patient data collection. Demographic information (age, sex, place of residence, birthplace, previous episodes of TB disease, and underlying medical conditions such as diabetes, HIV, or lung disease) were collected and recorded from the patients' medical records.

Sample processing and decontamination. The samples were processed within 48 h of collection using the *N*-acetyl-L-cysteine-NaOH digestion-decontamination procedure [14] to minimize the risk of *M. tuberculosis* contamination at the Mycobacteriology Section of the Microbiology Department. This procedure was performed in a Biosafety Level 3 [BSL3] laboratory with strict adherence to World Health Organization (WHO) guidelines for working with *M. tuberculosis* to protect personnel and prevent environmental contamination.

Culture and confirmation of *M. tuberculosis*. All specimens were subjected to direct smear microscopy using Ziehl-Neelsen [ZN] staining for acid-fast bacilli detection, followed by N-acetyl-L-cysteine-sodium hydroxide (NALC-NaOH) digestion and decontamination for non-sterile specimens. However, samples from sterile sites (cerebrospinal fluid (CSF), synovial fluid, and ascitic fluid) were directly inoculated after concentration by centrifugation onto BBL Mycobacteria Growth Indicator Tube (MGIT BD BACTECTM) containing 7 mL of modified Middle Brook 7H9 broth, supplemented with an enrichment supplement and a mixture of antimicrobials (polymyxin B, amphotericin B, nalidixic acid, trimethoprim, and azlocillin) to inhibit contaminants and non-target microorganisms. The MGIT tubes and Lowenstein-Jensen's medium were incubated at 37 ºC to ensure maximum recovery rates. The tubes were examined daily for up to 8 weeks of incubation using a BACTEC ™ Micro MGIT device equipped with a 365 nm wavelength UV light source fluorescence detector for signs of mycobacterial growth, including granular appearance and fluorescence. All positive tubes were confirmed for the presence of acid-fast bacilli (AFB) by Z-N staining and subcultured onto a blood agar plate to rule out contamination and confirm the purity of the isolated *M. tuberculosis* strains. The smears were examined for AFB and the characteristic cord factor of MTBC [15]. Confirmed MTBC isolates were further identified using the BD MGIT MTBC identification test [TBC ID].

Phenotypic DST for antimicrobial resistance to the first-line anti-TB drugs was performed using the BACTEC™ 960 MGIT™ system [Becton, Dickinson and Company, Franklin Lakes, NJ, USA]. The minimum inhibitory concentrations (MICs) of 1.0 µg/mL for rifampicin [RIF], 0.1 µg/mL for isoniazid [INH], 1.0 µg/mL for streptomycin [STR], 5.0 µg/mL for ethambutol [EMB], and 100 µg/mL for pyrazinamide [PZA] were employed as recommended by Becton, Dickinson and Company. The reference strain *M. tuberculosis* H37Rv strain was routinely included as a control for every batch of isolates tested, both for liquid culture-based assays and DNA extraction.

DNA extraction [10]. Bacterial DNA extraction was performed using the QIAamp DNA Mini Kit (Qiagen) according to the manufacturer's instructions. Broth culture samples were centrifuged at 6000 x g (8000 rpm) for 1 min to pellet the bacterial cells. The resulting pellet was resuspended in the minimal volume of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) and MilliQ water to ensure a sufficient amount of DNA for downstream applications, and to ensure high-quality DNA extraction using the QIAamp Mini Kit protocol.

Spoligotyping. Spoligotyping was performed on confirmed MTBC isolates to determine their genetic variation. Genomic DNA extracted from the isolates was used to amplify the highly polymorphic DR region, specifically the spacer oligonucleotides (spacers) between the DR elements. This amplification was achieved using labeled primers and the Mapmygenome Spoligotyping Kit (Hyderabad) following the manufacturer's standardized protocol with the specifically designated primers of DRa and DRb regions [10]. Conventional PCR was performed with the following conditions: an initial denaturation step at 94 ºC for 3 min, followed by 25 amplification cycles of a denaturation step at 94 ºC for 1 min, an annealing step at 55 °C for 1 min, and an extension step at 72 ºC for 30 sec [10]. Following the PCR amplification steps, a final extension step at 72 °C for 7 min was performed to ensure full-length extension of all PCR products. The PCR reactions were then stored at 4°C for subsequent spoligotyping analysis. To monitor for potential contamination, a DNA-free water control was included in each PCR run. *M. tuberculosis* H37Rv and *M. bovis*- BCG strain were included as positive amplification controls in all PCR assays to verify the presence of the expected amplicon. These strains are known to produce the specific amplicon targeted by the primers used. Following amplification, the PCR products were separated by agarose gel electrophoresis (AGE) through a 1.5% agarose gel at 100 volts (V) and 500 milliamps (mA) for 45 min. This process enables the separation and detection of the amplified DNA fragments. The amplified DNA fragments were then subjected to spoligotyping, a membrane-based technique used to identify distinct DNA sequence variations within the DR region of MTBC. In this method, spacer probes with sequences complementary to the spacer sequences between the DR regions of MTBC were hybridized into the amplified DNA fragments. The membrane was then incubated at 60 °C for 1 h, followed by two washes with a buffer solution containing 2 times the concentration of saline-sodium phosphate-EDTA (SSPE) buffer and 0.5% sodium

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dodecyl sulfate (SDS) for 10 min to remove non-specifically bound probes. The membrane was incubated with streptavidin-conjugated peroxidase for 1 h and washed twice in a buffer solution with a pH of 7.4 and containing 2 times the concentration of SSPE and 0.5% SDS for 10 min. Attachment of biotin molecules to the PCR products was achieved by incorporating a biotinylated primer during the amplification step. According to the manufacturer's instructions, the biotinylated PCR products were hybridized with 43 spacer oligonucleotides covalently bound to a membrane, each with a specific sequence corresponding to a particular spacer region. The membrane was then incubated with the enhanced chemifluorescence system (ECF) (GE Healthcare, UK) substrate, a detection system that uses chemifluorescence to produce a fluorescent signal, for 10 min. Next, the resulting signal pattern indicating the binding of biotinylated PCR products to the spacer oligonucleotides was visualized using a developer solution to reveal the chemifluorescent signal and a fixer solution to stabilize the signal. Finally, the membrane was exposed to Hyperfilm, a high-sensitivity autoradiography film, to capture the chemiluminescent signal emitted from the chemifluorescent substrate bound to the hybridization pattern.

Spoligotyping results were visualized as black squares representing hybridization signals on autoradiography film, indicating the presence or absence of specific spacer oligonucleotides in the analyzed MTBC strains. These patterns were then converted into binary codes consisting of 43 digits, each representing the presence (1) or absence (0) of a corresponding spacer oligonucleotide. This binary code information was used for further analysis using the online TB-VIS tool [15] to determine the specific families and lineages of the *M. tuberculosis* strains, as different strains possess unique combinations of these 43 spacer oligonucleotides, which determine their specific families and lineages [16].

RESULTS

Of the 404 clinical samples processed, 48 (11.9%) yielded MTBC isolates. Among these isolates, 27 (56.3%) originated from patients with suspected pulmonary TB, while 21 (43.8%) originated from suspected extrapulmonary TB cases. Notably, a total of 188 specimens (46.5%) and 216 specimens (53.5%) were collected from patients suspected of having pulmonary and extrapulmonary TB, respectively.

Male patients had a mean age of 56 years (± 20 standard deviation) compared to females with a mean age of 46 years (± 22 standard deviation). The age group with the highest number of cases (61-80 years) was similar for both genders. Among the 404 processed specimens, 224 (55.4%) originated from males and 180 (44.6%) from females, with males having a higher proportion of positive cultures as shown in Figure 4.7. This trend continued among positive cultures, with 27 (56.3%) isolates from males and 21 (43.8%) from females.

Antibiotic susceptibility test results. Among the 48 *M. tuberculosis* isolates, 20 (41.7%) were susceptible to all five first-line anti-TB drugs, while three (6.2%) exhibited resistance to at least one of the five first-line anti-TB drugs. The highest rate of monoresistance was towards streptomycin [STR] [n=7, 14.9 %], followed by isoniazid [INH] [n=6, 12.5%] and pyrazinamide [PZA] [n=4, 8.3 %]. Among the total of 48 culture-positive TB cases, two [4.1%] were found to be MDR-TB. The two MDR isolates were obtained from pulmonary TB cases. Additionally, 12.5% (6/48) of the culture-positive cases displayed resistance to three or more of the five first-line anti-TB drugs.

Spolityping patterns. Among the MTBC isolates, the Indo-oceanic family was the most prevalent, followed by the East Asian lineage or Beijing family. Notably, one isolate belonged to the *M. bovis* lineage. It is important to note that the lineages of seven isolates could not be determined due to either unknown or untypeable genetic profiles or technical limitations or insufficient data. Analysis of the isolates revealed the following distribution: 36.1% (n=17) belonged to the *M. tuberculosis* East-African-Indian (EAI3) family, followed by 27.7% (n=13) in the *M. tuberculosis* EAI5 family and 21.2% (n=10) in the *M. tuberculosis* Central Asian (CAS) family. *M. tuberculosis* Beijing isolates constituted 8.5% (n=4), while the family 33 lineage and *M. bovis*-BCG family each accounted for 4.3% (n=2) and 2.1% (n=1) of the isolates, respectively (Table 1). Notably, one isolate grew in culture but failed to amplify during PCR.

The *M. tuberculosis* isolate discussed earlier was classified to lineages using the TB-VIS online tool. The isolate was identified as belonging to either the Central Asian (CAS) or East African-Indian (EAI) lineage.

Among the five multidrug-resistant (MDR) and Rifampicin-resistant (RR) *M. tuberculosis* isolates, three belonged to the *M. tuberculosis* EAI3 family, one to the *M. tuberculosis* EAI5 family, and one to the *M. tuberculosis* Beijing family.

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Table 1. Description of the major *M. tuberculosis* lineages and spoligotyping profiles [n=47]

Sl no	Spoligotype	Lineage	Family
1	1101111111111111111111111111100001011111111	Indo-Oceanic	*M. tuberculosis* EAI5
2	0011101111111111111111111111000010111111111	Indo-Oceanic	*M. tuberculosis* EAI5
3	1001111110001111111111111111000010110001111	Indo-Oceanic	*M. tuberculosis* EAI3
4	1001111111101111111111111111000010110001111	Indo-Oceanic	*M. tuberculosis* EAI3
5	1100000001001100111100000000000000111111111	Unknown	*M. tuberculosis* CAS
6	1001111111111111111111111111000010111001111	Indo-Oceanic	*M. tuberculosis* EAI5
7	1001111111111111111111111111000010111001111	Indo-Oceanic	*M. tuberculosis* EAI5
8	1111111111111111111111111111000010111110111	Indo-Oceanic	*M. tuberculosis* EAI5
9	1110000111111111111111000000000000111111111	Unknown	*M. tuberculosis* CAS
10	1100001111110000110000000000000000001000000	Indo-Oceanic	*M. tuberculosis* CAS
11	1111111111111111111111111111111110111111111	Indo-Oceanic	Family33
12	0000000000000000000000000000000000111111111	EastAsian Beijing	*M. tuberculoisis* [Beijing]
13	0110111111111111111111111111111110111110111	Indo-Oceanic	Family33
14	1001111111111111111011111110000010111001111	Indo-Oceanic	*M. tuberculosis* EAI5
15	1111111111111111111111111111000010111110110	Indo-Oceanic	*M. tuberculosis* EAI5
16	1111111111111111111110011110000010111111111	Indo-Oceanic	*M. tuberculosis* EAI5
17	1101111101111110111111111111111111111100000	M.bovis	M. *bovis*-BCG
18	1001111111111111111111111111000010110001111	Indo-Oceanic	*M. tuberculosis* EAI3
19	1001111111111111111111111111000010110001111	Indo-Oceanic	*M. tuberculosis* EAI3
20	0000000000000000000000000000000000111111111	East Asian Beijing	*M. tuberculoisis* [Beijing]
21	0110000111111111111111000000000000111111111	Unknown	*M. tuberculosis* CAS
22	1111111111111111111111111111000010111110111	Indo-Oceanic	*M. tuberculosis* EAI5
23	1111111111111111111111111111000010111110111	Indo-Oceanic	*M. tuberculosis* EAI5
24	1110000111111111111110000000000000111111111	Unknown	*M. tuberculosis* CAS
25	0000000000000000000000000000000000111111111	EastAsian Beijing	*M. tuberculosis* [Beijing]
26	1001111111111111111111111111000001011000111	Indo-Oceanic	*M. tuberculosis* EA13
27	1001111111111111111111111111000010110011	Indo-Oceanic	*M. tuberculosis* EAI3
28	0000000000000000000000000000000000111111111	EastAsian Beijing	*M. tuberculosis* [Beijing]
29	0110000111111111111111000000000000111111111	Unknown	*M. tuberculosis* CAS
30	1001111111111111010001111111000010110001111	Indo-Oceanic	*M. tuberculosis* EAI3
31	1111111111111111111111111111000010111110111	Indo-Oceanic	*M. tuberculosis* EAI5
32	1110000111111111111110000000000000111111111	Unknown	*M. tuberculosis* CAS
33	1001111111111111111111111111000010110001111	Indo-Oceanic	*M. tuberculosis* EAI3
34	1001111111111111010001111111000010110001111	Indo-Oceanic	*M. tuberculosis* EAI3
35	0110000111111111111111000000000000111111111	Unknown	*M. tuberculosis* CAS
36	0110000111111111111111000000000000111111111	Unknown	*M. tuberculosis* CAS
37	0001110111111111111111111111000010111111111	Indo-Oceanic	*M. tuberculosis* EAI5
38	1001111111111111010001111111000010110001111	Indo-Oceanic	*M. tuberculosis* EAI3
39	1001111111101111111111111111000010110001111	Indo-Oceanic	*M. tuberculosis* EAI3
40	1001111111111111010001111111000010110001111	Indo-Oceanic	*M. tuberculosis* EAI3
41	1001111111111111010001111111000010110001111	Indo-Oceanic	*M. tuberculosis* EAI3
42	1001111111111111010001111111000010110001111	Indo-Oceanic	*M. tuberculosis* EAI3
43	0001110111111111111111111111000010111111111	Indo-Oceanic	*M. tuberculosis* EAI5
44	0110000111111111111111000000000000111111111	Unknown	*M. tuberculosis* CAS
45	1001111111111111010001111111000010110001111	Indo-Oceanic	*M. tuberculosis* EAI3
46	1001111111111111010001111111000010110001111	Indo-Oceanic	*M. tuberculosis* EAI3
47	1001111111101111111111111111000010110001111	Indo-Oceanic	*M. tuberculosis* EAI3

Table 2. Distribution of spoligotypes based on the location of TB infection (pulmonary or extrapulmonary)

Predominant spoligotypes	Location of TB infection	
	Pulmonary TB	Extra-pulmonary TB
EAI3	7 [25.9%]	10 [50%]
EAI5	9 [33.3%]	4 [20%]
CAS	5 [18.5%]	5 [25%]
Beijing	4 [14.8%]	-
Family 33	2 [7.4%]	-
M. bovis-BCG	-	1 [5%]
Total	27	20

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Table 3. Drug susceptibility pattern of the predominant spoligotype families

Predeominant spoligotypes	Pan sensitive	MDR	Monoresistant	Poly resistant
EAI3	8	3	3	3
EAI5	6	1	4	2
CAS	3	-	7	-
Beijing	-	1	1	2
Family 33	1	-	1	-
M. bovis-BCG	-	-	-	1
Total	18	5	16	8

DISCUSSION

Analyzing the frequencies and transmission patterns of various *M. tuberculosis* genotypes is crucial for developing effective strategies for TB control and vaccine development [17]. Genotyping techniques, including Spoligotyping, MIRU-VNTR, and IS6110 Restriction Fragment Length Polymorphism [IS6110-RFLP] are essential for understanding the genetic diversity of *M. tuberculosis* isolates [18]. Although RFLP typing is widely used for mycobacterial cultures, its application is limited by the slow growth rate of *M. tuberculosis*, which requires 20–40 days to produce sufficient DNA for analysis. This delay hinders its utility in clinical settings, particularly when investigating potential nosocomial transmission of tuberculosis [19]. Spoligotyping has a significant advantage over RFLP due to its ability to work with minimal DNA requirements, making it a potential tool for direct analysis of clinical specimens, potentially bypassing the need for time-consuming culturing processes [13]. However, further investigation is needed to definitively confirm the usefulness of spoligotyping for direct analysis of clinical specimens. Previous studies have reported satisfactory results when using spoligotyping on various clinical specimens. For instance, a specific study reported a specificity of 98% and a sensitivity of 96% for spoligotyping [19].

The present study revealed that the East African-Indian (EAI) family (63.8%) was the most dominant spoligotype family, accounting for 63.8% of the isolates, followed by the Central Asian strain (CAS) family (21.2%), the Beijing family (8.5%), and family 33 (4.3%). Additionally, our study detected the presence of *M. bovis*-BCG, which accounted for 2.1% of the total strains analyzed. Our results align with previous studies, showing that *M. tuberculosis* lineages 1 (Indo-Oceanic or EAI) and 3 (Central Asian or CAS) are the most prevalent in India [20]. In contrast, lineages 2 (East Asian or Beijing) and 4 (Euro-American) exhibit a wider global distribution, with a higher prevalence in Europe, Africa, and other parts of the world [20–22]. Research has revealed that lineage 3 is prevalent in northern and northwestern India, while lineage 1 is prominent in the southern region, with lower frequencies observed in other areas [8, 17, 18]. Unlike lineage 1, lineage 2 exhibits a more even distribution across India, with an overall prevalence of 17%, although it is more prevalent in certain northeastern states. This finding is consistent with the fact that most strains circulating in South India, particularly in Kerala, belong to lineage 1 [Indo-Oceanic] [23].

Consistent with previous studies [24, 25], the East African Indian (EAI) lineage emerged as the predominant genotype in this region of India. Singh *et al*. (2015) reported the ancient EAI lineage as predominant in South India, with a prevalence of 44% in the Chennai and 38.3% in Hyderabad [26]. This genotype, initially identified in Guinea Bissau, West Africa [27], likely migrated to the Asian mainland during the migration of modern humans out of Africa, which is supported by studies suggesting a African origin of the EAI lineage [28, 29]. Shanmugham *et al*. (2011) previously reported a high prevalence (60%) of the EAI family in the Tiruvallur district of South India, which is approximately 50 km from Chennai [17]. Our study revealed that 62.4% of the isolates belonged to the EAI lineage, which is consistent with the findings of Thomas *et al*. (2011) who identified EAI as the second most prevalent lineage in Hyderabad, India [25]. This finding suggests a geographical trend, where the prevalence of the EAI lineage decreases and the Central Asian strain (CAS) becomes more prominent as one moves northwards in India, indicating a possible spatial distribution pattern of *M. tuberculosis* lineages in the country. In contrast, studies in North India report lower EAI lineage prevalence, ranging from 3.8% in Delhi [12] to 10% overall [7], indicating a geographical variation in EAI prevalence. In our study, 7.7% (3 out of 17) of EAI strains were MDR-TB, which is higher than the 10.3% observed in non-EAI strains, indicating a disparity in MDR-TB prevalence between the two groups, contradicting previous findings [29].

The CAS family, originating primarily in the Middle East and Central Asia, exhibits a significant presence in South Asia (21.2%) and is especially prevalent in India (75%) [30, 31]. This lineage is also found in neighboring countries in this region, including Iran (19.1%) and Pakistan (56.5%) [32, 33]. The CAS lineage has been reported in various other regions, although at lower prevalence rates compared to South Asia, including Africa (5.3%), Central America (0.1%), Europe (3.3%), Far-East Asia (0.4%), and North America (3.3%) [34]. The CAS lineage, considered a more recent lineage, predominates in India's western, central, and northern regions, including urban centers such as Mumbai and Delhi, and states such as Punjab and Uttar Pradesh (including Agra), consistent with prior findings [30, 35].

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The present study identified Beijing genotypes in four isolates (14.8% of the total isolates), representing the globally predominant lineage of MTB. Notably, these genotypes are associated with increased drug resistance and virulence, which is a concern for public health [36]. The low proportion of the Beijing genotype observed in this study is consistent with the reported lower prevalence of drug-resistant tuberculosis in Kerala, India. This could potentially explain the low number of drug-resistant cases in this region.

There were 48 culture positives but in PCR amplification only 47 were amplified and identified and it is mentioned in the result section. The one which is not amplified is the M.bovis -BCG strain. Although the risk associated with this *M. bovis* isolate is likely low, it is essential to continue differentiating between BCG vaccination and *M. bovis* infection to ensure effective tuberculosis control and prevention programs, as misidentification could lead to inappropriate treatment and public health consequences. The results of this study align with previous research on *M. tuberculosis* strains in other regions of India and neighboring countries, indicating consistency in the findings. Notably, the high prevalence of the EAI strain observed in this region is consistent with previous studies indicating a potential association between this strain and lower rates of drug resistance in certain populations, which warrants further investigation to confirm this correlation. While spoligotyping offers advantages over standard typing methods, including reduced DNA requirements and easier computer-aided comparison of banding patterns, it is crucial to interpret the results with caution due to potential limitations and complexities in data analysis.

This study has some limitations, including a relatively small sample size and the absence of clustered tuberculosis episodes during the study period, which may restrict the comprehensive evaluation of spoligotyping's clinical utility in this context. Moreover, while our study identified mixed genotypes, additional longitudinal investigations are essential in this region to elucidate the complex dynamics of TB transmission and gain a deeper understanding of the local epidemiology.

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CONFLICTS OF INTEREST

The authors declare that there are no conflicts of interest associated with this manuscript.

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