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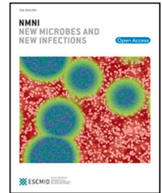

Original Article

Genetic diversity of *Mycobacterium tuberculosis* strains isolated from spiritual holy water site attendees in Northwest Ethiopia. A cross-sectional study

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A R T I C L E I N F O

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A B S T R A C T

Background: The genetic diversity of *Mycobacterium tuberculosis* complex (MTBC) strains was characterized among isolates from individuals with pulmonary tuberculosis (PTB) symptoms attended holy water sites (HWSs) in the Amhara region, Ethiopia.

Methods: A cross-sectional study was done from June 2019 to March 2020 to describe the genetic diversity and drug-resistance profiles of MTBC isolates. Sputum specimens were collected and cultured in the Löwenstein-Jensen culture medium. Line Probe Assay, MTBDR*plus* VER 2.0, and MTBDR*sl* VER 2.0 were used to detect first- and second-line anti-TB drug-resistance patterns. A spoligotyping technique was utilized to characterize the genetic diversity. Statistical analysis was performed using STATA 15.

Results: Of 560 PTB-symptomatic participants, 122 (21.8%) were culture-positive cases. Spoligotyping of 116 isolates revealed diverse MTBC sublineages, with four major lineages: Euro-American (EA) (Lineage 4), East-African-Indian (EAI) (Lineage 3), Ethiopian (ETH) (Lineage 7), East Asian (EA) (Lineage 2). The majority (96.6%) of the isolates were EA (lineage 4) and EAI, with proportions of 54.3% and 42.2%, respectively. A total of 31 spoligotype patterns were identified, 26 of which were documented in the SITVIT2 database. Of these, there were 15 unique spoligotypes, while eleven were grouped with 2-17 isolates. SIT149/T3-ETH (n = 17), SIT26/CAS1-DELHI (n = 16), SIT25/CAS1-DELHI (n = 12), and SIT52/T2 (n = 11) spoligotypes were predominant. A rare spoligotype pattern: SIT41/Turkey and SIT1/Beijing, has also been identified in North Shewa. The overall clustering rate of sub-lineages with known SIT was 76.4%.

Of the 122 culture-positive isolates tested, 16.4% were resistant to rifampicin (RIF) and/or isoniazid (INH). Multidrug-resistant TB (MDR-TB) was detected in 12.3% of isolates, five of which were fluoroquinolones (FLQs) resistant. SIT149/T3-ETH and SIT21/CAS1-KILI sublineages showed a higher proportion of drug resistance.

Conclusions: Diverse MTBC spoligotypes were identified, with the T and CAS families and EA (lineage 4) predominating. A high prevalence of drug-resistant TB, with SIT149/T3-ETH and CAS1-KILI sublineages comprising a greater share, was observed. A study with large sample size and a sequencing method with stronger discriminatory power is warranted to understand better the genetic diversity of circulating MTBC in this cohort of study, which would help to adopt targeted interventions.

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1. Introduction

Tuberculosis (TB), a disease caused by *Mycobacterium tuberculosis* complex (MTBC) bacteria, is still an important public health problem globally [1,2]. Despite major advances in diagnostic technologies, effective medicines, and major global efforts to contain the disease, an estimated 10.6 million people still develop TB each year, with over 1.3 million dying from it [3].

TB affects all nations and all age groups, but its epidemiological distribution is highly skewed towards low- and middle-income countries (LMICs) [1,2]. The nation's weak healthcare systems, HIV/AIDS pandemics, poor TB services, the advent of MDR-TB strains, and a lack of well-equipped laboratories and resources aggravated TB in LMICs [1,2]. Although Ethiopia has successfully transitioned out from the list of thirty countries with a high burden of MDR/RR-TB, it remains on the list of nations with a high burden of TB [4,5], and TB poses a significant challenge to the country's healthcare system [1,2], with an annual incidence of 119 per 100,000 people [4]. Due to the dearth of a robust TB surveillance system, limited access to diagnostic facilities, and a weak disease notification system, the true TB burden is largely unknown in Ethiopia [6].

TB disproportionately impacts the impoverished and vulnerable population groups residing in high-risk settings, like prisoners, refugees, internally displaced persons, and homeless people are at increased risk of developing TB [7–9]. Like other key population groups residing in hotspot settings [7–9], individuals who attend holy water sites (HWSs) are especially at high risk of developing TB due to the overcrowding and poor living conditions they experience in these settings, leading to high TB transmission among those cohorts of populations [10–12]. In Ethiopia, people use spiritual holy water as an alternative treatment for various types of diseases [10–12]. Particularly those who suffer from cough or TB symptoms and mental health problems, commonly seek treatment by visiting spiritual places such as HWSs and staying for an extended time in an overcrowded situation, which can exacerbate TB transmission [10,11]. Consequently, the incidence and prevalence of TB among attendees of HWSs are higher than those among the general population of Ethiopia [10]. Derseh and his colleagues reported that the prevalence of smear-positive PTB among HWS attendees was 7.4-fold higher than the prevalence in the general population of Ethiopia [10]. This can significantly affect TB control in the country by increasing the overall disease burden unless the national TB control program recognizes and gives due attention to HWSs as high-risk settings for TB transmission and plans targeted intervention [10]. Efforts aimed at preventing and controlling TB among attendees of HWSs would reduce TB transmission, thereby benefiting not only the health of attendees but also the communities, families, and thus the general public health [10].

To comprehend TB's epidemiology and transmission pattern in a particular region and to adopt control strategies, it is imperative to identify the most prevalent TB strains and define their genotypic characteristics, coverage, and frequency in the context of seven human-adapted lineages (L1-L7) of the MTBC [13,14]. In addition to the seven well-known lineages, lineages 8 and 9 [15,16] were recently reported in Central and Eastern Africa. Among these major lineages, lineage 4 is the most common; and it was further characterized and divided into ten ecological niche-based sub-lineages as globally distributed and locally limited [17,18]. Molecular genotyping tools, like spoligotyping and other PCR-based sequencing methods, help us to understand MTBC epidemiology in a specific geographical region, predict disease transmission, and identify prominent genotypes and TB strains that are likely to spread [19]. The genetic diversity and geographic distribution of MTB lineages have been studied across different settings and regions of Ethiopia [20–31] and revealed the presence of diverse MTBC sublineages. The Ethiopian-specific lineage (SIT149/T3-ETH sublineages) was reported as a predominant TB strain in the country [25,31–35], while lineage 3/CAS1-DELHI sublineages was another widespread strain in certain regions of Ethiopia [20,26,27,36]. In the current study region, lineage 3/CAS1-DELHI [20,21,37,38], and lineage 4/T3-ETH sublineages [39] were identified as the most abundant TB strains. Using spoligotyping of TB isolates from HWS attendees in northwest Ethiopia, we found that SIT149, T3-ETH, and lineage 4 were prominent TB strains, family, and lineage, respectively, while lineage 3/SIT26/CAS1-DELHI was the second predominant MTB strain. The predominance of T3-ETH and CAS1-DELHI sublineages in the country would indicate that those strains are becoming more important in TB disease transmission in Ethiopia [20,21,37,39].

Although the molecular epidemiology of MTBC lineages/sublineages has been studied in different settings and regions of Ethiopia, no study has examined the genetic distribution of MTB strains and their drug susceptibility profiles among isolates from PTB-suspected individuals who attended HWSs in the country. To implement and strengthen regional and national TB prevention and control measures in the HWSs, it is paramount to investigate and understand the circulating TB strains, genetic diversity, and anti-TB drug resistance patterns of isolates among individuals who attended HWSs. Therefore, this study aimed to characterize the genetic diversity, distribution, and drug susceptibility profiles of MTB strains isolated from PTB symptomatic adult HWS attendees in the Amhara region, Ethiopia.

2. Methods

2.1. Study period and setting

A cross-sectional study was carried out from June 2019 to March 2020 at nine conveniently selected HWSs found across nine administrative zones in the Amhara region. The Amhara region is located in the Northwestern parts of Ethiopia between 9°20' and 14°20' North latitude and 36°20' and 40°20' East longitude (Fig. S1). Bahir-Dar is the regional capital, located 567 km from the national capital, Addis Ababa. Nine HWSs were conveniently chosen across nine administrative zones (one from each zone) based on their consistent popularity for holy water treatment, ability to accommodate a large number of attendees, and where many people visit and stay for an extended time [10] (Fig. S1).

2.2. Study population, participants, and recruitment

The study population included all HWS attendees who attended the sites during the data collection period. All PTB symptomatic adult HWS attendees (≥18 years of age) who fulfilled the inclusion criteria were included, whereas attendees under the age of 18 years and those who were seriously ill to provide necessary information or unable to produce sputum specimens were excluded from the study. Besides, individuals who reported taking anti-TB drug treatment during the sample collection time were excluded.

After obtaining consent from study participants, an interviewer-administered questionnaire was used to collect socio-demographic data. Individuals with a persistent cough lasting two weeks or longer and other symptoms, such as fever, night sweating, shortness of breath, loss of appetite, chest pain, fatigue, coughing up sputum containing blood, unexplained weight loss, and a history of contact with active TB patients, were screened and included [6].

2.3. Sputum specimen collection and culture procedures

The sputum specimens were obtained from PTB-symptomatic individuals using sterile, leak-proof screw-capped universal falcon tubes with a 50 ml capacity. The sputum samples were transported in triple-packaged ice pack carriers to ensure the cold chain was maintained and transported to the Amhara Public Health Institute (APHI), the regional research and referral laboratory center, Bahir-Dar, Ethiopia. Mycobacterial cultures were processed following standard procedures. Briefly, the sputum was treated or decontaminated with N-acetyl-L-cysteine (NALC-NaOH) and neutralized with a phosphate buffer solution

(pH 6.8). The resulting mixture was then centrifuged to prepare the culture inoculums. Then, the sediment was inoculated into Löwenstein-Jensen (LJ) slant culture tubes and incubated at 37 °C, and the growth of MTB colonies was checked weekly for up to eight weeks. Ziehl-Neelsen (ZN) acid-fast bacilli (AFB) smear microscopy was used to confirm positive LJ culture results [40]. The MPT64 antigen test ("Capilia TB-Neo version 6.0, TAUNS Laboratories, Inc. Japan") was used to identify MTBC species from non-TB mycobacteria (NTM) [41]. The quality and sterility of the LJ culture medium were maintained, and each set of tests included MTB H37Rv strains as a positive control and molecular-grade water as a negative control.

2.4. Specimen preparation

Following subculture, *Mycobacterium* suspensions were prepared from MTB colonies grown on an LJ solid medium and transferred into 1.5 ml of PrimeStore Molecular Transport Medium ("PS-MTM; Longhorn Vaccine & Diagnostics, San Antonio, TX, USA"). The preparation of MTB suspensions from a positive LJ medium relied on the culture state [42]. The MTB suspension contained in the PrimeStore tubes was transferred by air to South Africa without refrigeration, where genotypic procedures were conducted.

Additionally, some culture-positive specimens that were processed and archived at the regional referral laboratory center were accessed to compare MTB spoligotypes with our collected samples. The specimens were submitted/collected from TB patients attended various health facilities in the region for additional confirmatory testing, genotypic and phenotypic drug susceptibility testing (DST), or treatment follow-up (sputum culture conversion testing). The preparation of MTB suspensions from intact slopes and dried-out LJ culture medium, genomic DNA extraction, and spoligotyping for culture-positive isolates accessed from the regional referral laboratory center was carried out in a similar fashion.

2.5. DNA extraction

The PrimeXtract^{TM} kit ("Longhorn Vaccines and Diagnostics, San Antonio, TX, USA") was utilized to extract the MTB genomic DNA from all LJ culture-positive isolates 43,44. The extracted MTB genomic DNA quality and concentration were measured and calculated by using a spectrophotometer at optimal densities of 260 nm and 280 nm.

2.6. Drug susceptibility testing

The Line Probe Assays, second-generation GenoType®MTBDR*plus* VER2.0 [43], and GenoType®MTBDR*sl* VER2.0 [44] ("Hain Lifescience, Germany") assays were used to determine RIF and INH, FLQs, and second-line injectable drugs (SLIDs) susceptibility of the isolates, respectively. The entire procedure followed the manufacturer's protocol ("Hain Lifescience, Germany") [45,46].

2.7. Spoligotyping

All 122 culture-positive isolates were characterized by spoligotyping, as previously described [47], and as per instructions from the spoligotype kit supplier (Ocimum Biosolutions Company, IJsselstein, The Netherlands). In brief, the direct repeat (DR) region of the isolate was amplified by PCR using oligonucleotide primers derived from the DR region. The amplified PCR product was hybridized to a set of 43 immobilized oligonucleotides covalently bound to a membrane ("Animal and Plant Health Agency, Great Britain"), each corresponding to one of the unique spacer DNA sequences within the DR locus of the MTB strain. "The presence and absence of spacers were visualized on film as black and white squares, which were later converted to binary codes (1/0) for analysis". Known strains, *Mycobacterium bovis* BCG and *Mycobacterium tuberculosis* H37Rv strains were used as positive controls, and a sterile Qiagen water (Qiagen Company, Germany) as a negative control.

2.8. Data analysis and isolate characterization

The laboratory data were recorded using a prepared MS Excel spreadsheet. STATA 15 ("Stata Corp, College Station, TX, USA") was utilized for statistical analysis and the results were summarized using descriptive statistics. "The spoligotype patterns recorded in the Excel spreadsheet were converted to binary and octal formats using the SIT-VITWEB website" (http://www.pasteur-guadeloupe.fr:8081/SITVIT_ONLINE/tools.jsp) [48] and retrieved the shared international spoligotypes (SIT) number and associated lineages/sub-lineages using the updated version of SPOLDB4 and SITVITWEB (http://www.pasteur-guadeloupe.fr:8081/SITVIT2/batch.jsp). Strains that matched a pre-existing spoligotype pattern in the database were assigned an SIT number; those without SIT numbers were classified as "new" or orphan strains. Spoligo-patterns not in the database were assigned to the most probable families, subfamilies, and variants using SpolLineages [49]. "Conformal Bayesian Network (CBN) major lineages and SNP-based lineages were determined using online tools RUN TB-lineage" [50] (https://tbinsight.cs.rpi.edu/run_tb_lineage.html) and SpolLineages (http://www.pasteur-guadeloupe.fr:8081/SpolLineages/spol.jsp), respectively. The CBN refers to both the West African (L6 & 5) and Ethiopian (L7) lineages as *Mycobacterium africanum* [51]. To circumvent this, we used SpolLineages, checked the CBN grouping of major lineages, and examined lineage classification as generalists or specialists [17,18].

A UPGMA-based dendrogram was constructed utilizing the MIRU-VNTRplus identification database to determine isolate molecular clustering of MTB isolates (Fig. 1). Two or more strains with the same spoligotype pattern were considered a "cluster", while single patterns were "unique" to this study.

***M. tuberculosis* lineages and sublineages spatial distributions:** The MTBC lineage and sub-lineages were geographically mapped using QGIS v3.22.6. UNOCHA's website (https://data.humdata.org/dataset/cod-ab-eth) was used to retrieve the shape files of the study sites from which the isolates were collected (Fig. 2).

3. Results

3.1. Characteristics of culture-positive TB cases

Of 560 PTB-symptomatic participants, 122 (21.8%) were culture-positive. The proportion of culture-positive cases in males and females was 21.8% each, with 28.5% in the young age group (18–33 years of age). Besides, most culture-positive cases (25.2%) were rural dwellers, 19.1% were farmers, and 24.4% were from households with more than five family members. About 34.0% of culture-positive cases were detected in the North Shewa zone study site. Age, residence, and sample collection site were associated with culture-positive TB ($p < 0.05$) (Table 1).

3.2. Drug resistance patterns of Mycobacterium tuberculosis isolates

Genotypic DST was conducted on 122 culture-positive isolates. Of 122 tested isolates, 83.6% were susceptible to both RIF and INH, whereas 16.4% were resistant to at least one of the two drugs. INH-or RIF-resistance was detected in 16.4% and 12.3% of isolates, respectively. Five of the 20 INH-resistant isolates were INH-mono-resistant, whereas one isolate was INH-hetero-resistant. MDR-TB (resistant to both RIF and INH) was detected in 12.3% of cases, five of which were FLQs-resistant TB (Table S1).

3.3. Genetic diversity of Mycobacterium tuberculosis lineages/sub-lineages

Spoligotyping was utilized to genotype 122 culture-positive MTB

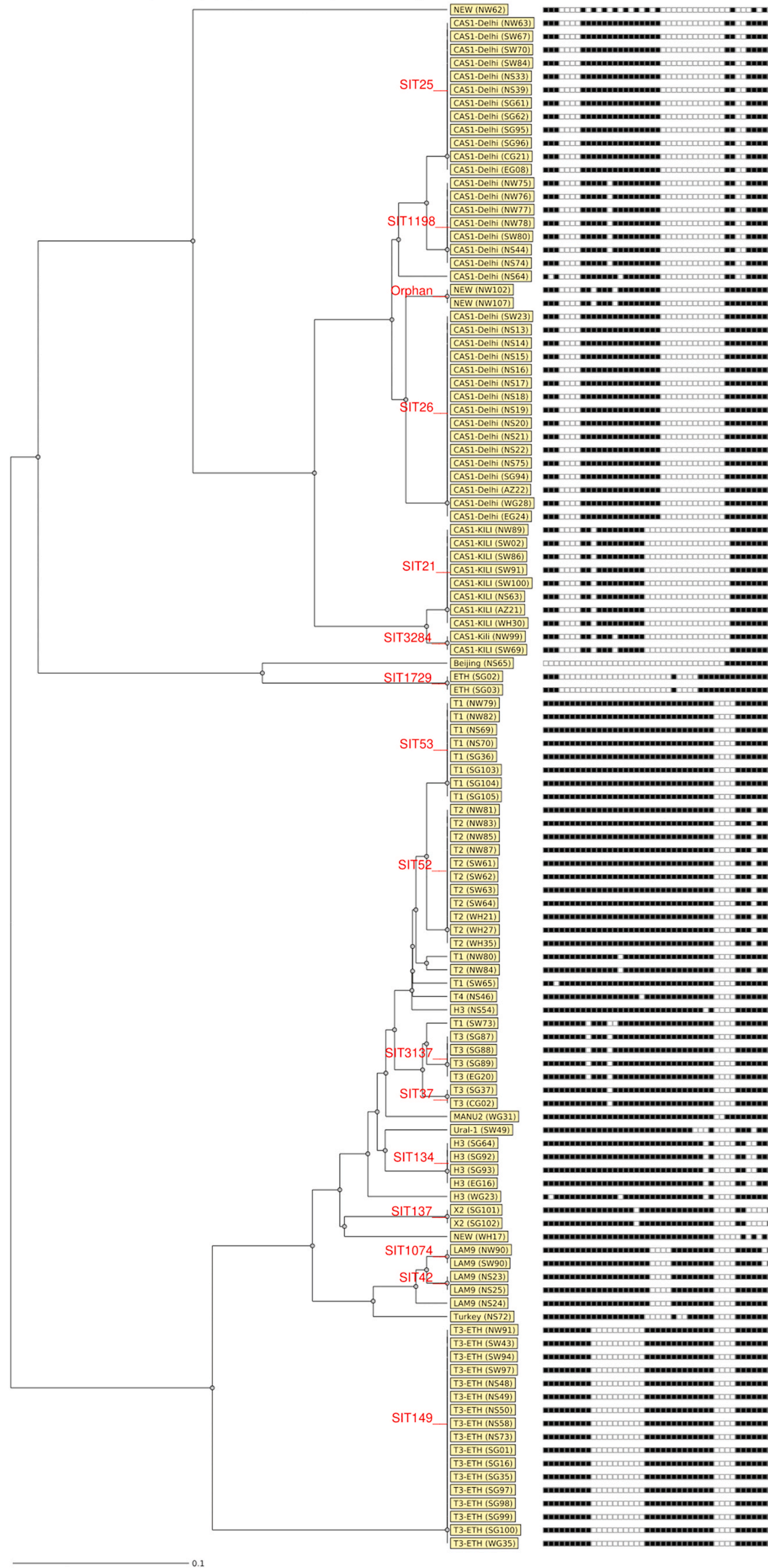

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Fig. 1. A dendrogram (phylogenetic tree) showing spoligotyping results of 116 Mycobacterium tuberculosis isolates from PTB suspects attending holy water sites. The number represents the spoligo-international type (SIT) for the corresponding spoligotype/sublineages identified in the database. "Orphan"/"New" indicates strains not found in the SITVIT2 database. The sample codes and the assigned number are indicated in the brackets. The sample codes description/abbreviations: (NW: North Wello; SW: South Wello; NS: North Shewa; SG: South Gondar; CG: Central Gondar; WG: West Gojjam; AZ: Awi zone; EG: East Gojjam; WH: Wag-Hamra).

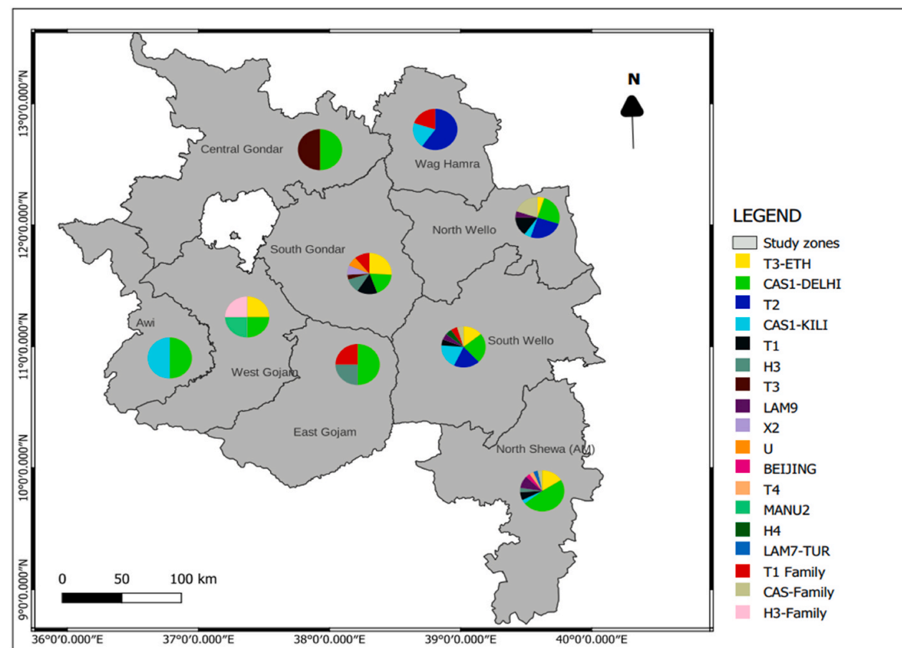

Fig. 2. Geographical location of the study area (zones) and *Mycobacterium tuberculosis* complex lineages/sublineages distribution in each study zone (n = 116 isolates); pie charts represent the number of each *Mycobacterium tuberculosis* complex lineage/sublineages among the total number of isolates in each study zones.

isolates, yielding 116 isolates with interpretable spoligotype patterns. Of 31 spoligotype patterns identified, 26 were documented in the SITVIT2 database, comprising 110 isolates. Of these, there were 15 unique spoligotypes, while eleven were grouped with 2-17 isolates that accounted for 90.0% (99/110) of all isolates with known SIT. The remaining five "new" or orphan patterns representing 5.2% (6/116) of the total isolates, were not found in the SITVIT2 database (Table 2, Table 3). The overall clustering rate of sub-lineages was 73.3% while the overall clustering with known SIT was 76.4%. The proportion of clustered isolates was higher in each study site (zones): South Gondar (92.6%), North Shewa (77.4%), South Wollo (66.7%), and North Wollo (60.0%) (Table S2). Four prominent spoligotypes were identified with SIT, comprising over one-third of the spoligotyped isolates 56/110 (50.9%): SIT149/T3-ETH (n = 17), SIT26/CAS1-DELHI (n = 16), SIT25/CAS1-DELHI (n = 12), and SIT52/T2 (n = 11) (Table 2). CAS and T were the predominant lineages/families, comprising for 39.7% and 40.5% of genotyped isolates, respectively. There were ten isolates belonging to the T superfamily (4 T1, 2 T2, 2 T3, 1 T3-ETH, 1 T4). The CAS1-DELHI and CAS1-KILI were represented by four and two isolates, respectively (Table 2, Table 3).

We identified the major MTB lineages using the CBN analysis. The analysis revealed that about 97.4% of the total 116 isolates belonged to two major lineages: EA/lineage 4 (55.2%) and EAI/lineage 3 (42.2%), whereas EA/Beijing (lineage 2) and ETH/lineage 7 were represented by one and two isolates, respectively (Table 2, Table 3). The EA/lineage 4 was the most predominant, with 42 isolates (shared type) clustered in five groups (with a clustering rate of 77.1%). In the EA (lineage 4), SIT149/T3-ETH, SIT52/T2, and SIT53/T1 subfamilies were the most frequent, with seventeen, eleven, and eight clustered strains, respectively. On the other hand, EAI/lineage 3 was the second most prevalent, with 45 isolates (shared types) clustered in five groups (with a clustering rate of 81.6%). In the EAI (lineage 3), SIT26/CAS1-DELHI, SIT25/CAS1-DELHI, and SIT21/CAS1-KILI subfamilies were the most frequent, with sixteen, twelve, and eight clustered strains, respectively. The EA/Beijing lineage was the least prevalent, with a single stain (Table 3). Additionally, using the "SNP-based lineage analysis of the SpolLineages online tool" [17,18], we found five generalist sublineages, L4.1.2/Haarlem_3 and L4.3/LAM9 (n = 4 each), L4.1.1/X2 (n = 2), L4.2.1/Ural-1 and L4.2.2.1)/TURKEY (n = 1 each) (Table 2).

3.4. Geographical distribution of lineages/sublineages of Mycobacterium tuberculosis

Euro-American/lineage 4 accounted for a substantial proportion of the MTB lineage distribution across the study administrative zones: 74.0% (South Gonar), 52.4% (South Wollo), and 50.0% (North Wollo) of the isolates collected in the respective zones of the study region. However, EAI/lineage 3 had a greater share of MTB lineage distribution in the North Shewa zone (54.8%). Euro-America and EAI were identified in all of the study zones where the isolates were collected, whereas ETH (Lineage_7), which is confined or restricted to Ethiopia, and EA (Lineage_2) lineages were detected in the South Gondar and North Shewa zone, respectively. The SIT52 spoligotype/T2 sublineages and SIT1198 spoligotype/CAS1-DELHI sublineages were reported in higher proportion in the North Wollo zone. On the other hand, SIT21/CAS1-KILI sublineages and SIT52/T2 sublineages were found in greater numbers in the South Wollo zone. In the North Shewa zone, SIT26/CAS1-DELHI sublineages accounted for the higher proportion of the total isolates, whereas SIT149/T3-ETH sublineages and SIT25/CAS1-DELHI sublineages were identified in higher proportion in the South Gondar Zone (Fig. 2 and Table S2).

The spoligotyping data showed substantial isolate clustering across study sites (zones). For instance, among the 17 SIT149/T3-ETH sublineages clustered, seven were detected in South Gondar, five in North Shewa, and three in the South Wello zone study site. Similarly, 11 of 16 clustered SIT26/CAS1-DELHI sublineages were identified from the

Table 1
Proportion of culture-positive TB cases with socio-demographic characteristics of participants (n = 560).

Characteristics		Culture-positive PTB		Total (n)	*p*-value
		Positive, n (%)	Negative, n (%)		
Sex	Male	67 (21.8)	241(78.2)	308	1.00
	Female	55 (21.8)	197 (78.2)	252	
Age group (year)	18–33	75 (28.5)	188 (71.5)	263	0.001
	34–49	31 (14.9)	177 (85.1)	208	
	≥50	16 (18.0)	73 (82.0)	89	
Residence	Urban	46 (17.8)	212 (82.2)	258	0.040
	Rural	76 (25.2)	226 (74.8)	302	
Marital status	Married	85 (23.9)	271 (76.1)	356	0.136
	Single*	37 (18.1)	167 (81.9)	204	
Educational status	Can't read and write	59 (23.0)	197 (77.0)	256	0.396
	Primary school	30 (18.2)	135 (81.8)	165	
	Secondary school & above	33 (23.7)	106 (76.3)	139	
Family size	1–5	57 (19.4)	237 (80.6)	294	0.153
	>5	65 (24.4)	201 (75.6)	266	
Occupation	Farmer	45 (19.1)	190 (80.9)	235	0.495
	Employed	6 (25.0)	18 (75.0)	24	
	Unemployed	23 (19.7)	94 (80.3)	117	
	Housewife	24 (25.5)	70 (74.5)	94	
	Students & others**	24 (26.7)	66 (73.3)	90	
Study area (zone)	North Wello	22 (20.6)	85 (79.4)	107	<0.001
	South Wello	22 (22.0)	78 (78.0)	100	
	North Shewa	33 (34.0)	64 (66.0)	97	
	South Gondar	28 (26.7)	77 (73.3)	105	
	Central Gondar & others***	17 (11.3)	134 (88.7)	151	

Note: *Single, divorced, and widowed; **Others: religious leaders and deacons; ***Others: Awi zone, West Gojam, East Gojam, and Wag-Hamra; PTB: Pulmonary tuberculosis; TB: Tuberculosis.

North Shewa zone study site. Four of the 12 clustered SIT25 spoligotypes/CAS1-DELHI sublineages were identified in South Gondar, while three were detected in the South Wello zone study site (Fig. 1 and Table S2). Moreover, the spoligotyping data revealed that the clustering rate of certain MTBC lineages/sublineages at each sample collecting site (zone) was higher (Table S2).

To compare with our Spoligotyped data, we retrieved some culture-positive MTB isolates from the regional referral laboratory center and spoligotyped them to further explore the distribution of MTBC lineages/sublineages in the study region. Of the 28 culture-positive MTB isolates accessed and genotyped, 23 were successfully genotyped. There were ten distinct spoligo-patterns. Thus, T3-ETH (34.8%; 8/23), CAS1-DELHI (26.1%; 6/23), and CAS1-KILI (17.4%; 4/23) sublineages were predominant. Furthermore, SIT149/T3-ETH (50.0%; 8/16), SIT26/CAS1-DELHI (25.0%; 4/16), and SIT21/CAS1-KILI (25.0%; 4/16) sublineages comprised the majority of clustered isolates. The proportion of EA (lineage 4) and EAI (lineage 3) major lineages was 56.5% and 43.5%, respectively. The SIT149/T3-ETH sublineages were the most common in EA (lineage 4), while SIT26/CAS1-DELHI and SIT21/CAS1-KILI subfamilies were prominent in EAI (lineage 3) (Table 4).

3.5. *Mycobacterium tuberculosis drug resistance profiles by lineages and subfamilies*

T3-ETH and CAS1-KILI sublineages showed a higher proportion of drug resistance than other lineages. Together, SIT21/CAS1-KILI and SIT149/T3-ETH sublineages were responsible for 85.0% of any drug resistance and INH resistance, respectively. Similarly, in 15 MDR/RR isolates, SIT21/CAS1-KILI and SIT149/T3-ETH sublineages were RIF-resistant and MDR-TB in 33.3% and 53.3%, respectively. Interestingly, four of the five FLQs-resistant TB isolates were SIT21/CAS1-Kili sublineages and were identified from the South Wollo zone study site (Table 5).

4. Discussion

To establish appropriate TB disease control measures, it is important to understand the molecular epidemiology of TB in a given region, especially in high TB burden settings, like Ethiopia. Thus, the mycobacterial genotyping technique improves our understanding of MTBC epidemiology in a certain geographical area. Although several molecular epidemiology studies have confirmed the prevalence of diverse MTBC lineages in Ethiopia, there is no evidence from high-risk settings for TB transmission, such as holy water sites (HWSs).

In this study, we characterized the genetic diversity of MTBC strains isolated from PTB-symptomatic individuals who attended HWSs found across different administrative zones of the Amhara region. Spoligotyping of 116 MTB isolates showed thirty-one distinct spoligotype patterns, of which 90.0% were grouped into eleven clusters of all isolates with known SIT. The overall sub-lineage clustering rate was 73.3%. The clustering rate of isolates in this study was similar to other reports in the same study region [20,21,52], and a multicenter molecular epidemiology study report in Ethiopia [51]. However, it was higher than the findings of earlier studies in other parts of Ethiopia [22,36,53–55]. This discrepancy may be attributable to the discriminatory power of genotyping techniques and the study population.

The current study demonstrated diverse MTBC lineages dominated by the T and CAS families, with corresponding Euro-American (EA/lineage 4) and East-African-Indian (EAI/lineage 3), which supports earlier review reports [56,57], and multicenter study report in Ethiopia [52]. Over half (55.2%) of the isolates in the current study belonged to EA/lineage 4. Consistent with our findings, comparable or higher percentages of EA lineage have been documented in various regions of Ethiopia, including the current study region [20,27,52]. Similarly, an earlier investigation conducted in prison settings across different regions of Ethiopia indicated that the lineage 4/EA lineage was the predominant strain [58]. Nevertheless, a lower proportion of lineage 4/EA lineages has been documented in the current study area [21]. However, an earlier study conducted among Ethiopian refugees found that EAI/lineage 3 was the prevalent lineage [7]. This could be the reason that people in the refugee camps were from different countries (Eritrea, Somalia, Sudan, and South Sudan), which contributed to the introduction of this lineage to Ethiopia [7]. We also found two globally dominant sublineages: L4.1.2/Haarlem (n = 6) and LAM/L4.3 (n = 5). The Haarlem/L4.1.2 sub-lineage was previously documented to be associated with a high rate of transmission clusters in Ethiopia [26]. Despite the lower proportion of isolates identified in the current study, Haarlem/L4.1.2 is reported as the third major sublineage in Ethiopia [56,57].

Several molecular epidemiology investigations in Ethiopia found that ETH (lineage 7) is prevalent across the country [56,57]. Accordingly, in the current study, two ETH (lineage 7) isolates were detected in the South Gondar zone. The ETH (lineage 7), which was first identified and reported from the Woldia area [59], is prevalent in Ethiopia's northern highlands [60,61]. ETH (lineage 7) has also been reported in different parts of Ethiopia [29,34,36,62,63]. Notably, the ETH (lineage 7), which is primarily found in Ethiopia and among immigrants from Ethiopia [14,64], is "known to progress toward disease at a slower rate than other lineages" [65]. Thus, further study is needed to understand why it is exclusive to Ethiopia and Ethiopians.

Consistent with earlier research conducted in various regions of Ethiopia [25,31–35,62,66,67], the SIT149/T3-ETH sub-lineage, which has been found to be more likely in a cluster [13,20,65], was the most common in our study, followed by the SIT52/T2 spoligotype. In the current study region, SIT149/T3-ETH sublineage [39] was reported as a predominant TB strain. It is also listed in the international database as the prevalent genotype in Ethiopia [68]. A study conducted in different prison settings across Ethiopia documented that the recently identified strains, ETH_H37Rv-like and ETH_3 were predominant. The significant

Table 2
The description of successfully genotyped isolates and the corresponding spoligotyping defined lineages/sub-lineages isolated from PTB suspected attendees of holy water sites in Northwest Ethiopia (n = 116).

Octal code	Spoligotype description (Binary format)	SIT No	Sublineages (SITVIT2)	Major Lineages by CBN	**SNP-based lineages**	No. of isolates (%)
777000377760771	■■■■■■■■■□□□□□□□□□□■■■■■■■■■■■■■■□□□□■■■■■■■	149	T3-ETH	EA	EA (L4)	17 (14.7)
703777740003771	■■■□□□□■■■■■■■■■■■■■■■■□□□□□□□□□□■■■■■■■■■	26	CAS1-DELHI	EAI	EAI (L3)	16 (13.8)
703777740003171	■■■□□□□■■■■■■■■■■■■■■■■□□□□□□□□□□■■□□■■■■■	25	CAS1-DELHI	EAI	EAI (L3)	12 (10.3)
777777777760731	■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■□□□□■■■□■■■	52	T2	EA	EA (L4)	11 (9.5)
703377400001771	■■■□□□□■■□■■■■■■■■■□□□□□□□□□□□□□□□□■■■■■■■■	21	CAS1-KILI	EAI	EAI (L3)	8 (6.9)
777777777760771	■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■□□□□■■■■■■■	53	T1	EA	EA (L4)	8 (6.9)
703737740003171	■■■□□□□■■■■■□■■■■■■■■■■□□□□□□□□□□■■□□■■■■■	1198	CAS1-DELHI	EAI	EAI (L3)	7 (6.0)
776737777760771	■■■■■■■■□■■■□■■■■■■■■■■■■■■■■■■■□□□□■■■■■■■	3137	T3	EA	EA (L4)	4 (3.4)
777777777720631	■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■□■□□□□■■□□■■■	134	H3	EA	EA (L4.1.2)	4 (3.4)
777377777760771	■■■■■■■■■□■■■■■■■■■■■■■■■■■■■■■■□□□□■■■■■■■	37	T3	EA	EA (L4)	2 (1.7)
777777607760771	■■■■■■■■■■■■■■■■■■■■□□□□■■■■■■■■□□□□■■■■■■■	42	LAM9	EA	EA (L4.3)	2 (1.7)
777776777760601	■■■■■■■■■■■■■■■■■□■■■■■■■■■■■■■■□□□□■■□□□□■	137	X2	EA	EA (L4.1.1)	2 (1.7)
777777607760761	■■■■■■■■■■■■■■■■■■■■□□□□■■■■■■■■□□□□■■■■■□■	1074	LAM9	EA	EA (L4.3)	2 (1.7)
700000004177771	■■■□□□□□□□□□□□□□□□□□□□□□■□□□□■■■■■■■■■■■■■■	1729	ETH	MA**	ETH (L7)	2 (1.7)
703357740003771	■■■□□□□■■□■■■□■■■■■■■■■□□□□□□□□□□□□■■■■■■■■■	NEW	ND	EAI	EAI (L3)	2 (1.7)
703357400001771	■■■□□□□■■□■■■□■■■■■□□□□□□□□□□□□□□□□■■■■■■■■	3284	CAS1-KILI	EAI	EAI (L3)	2 (1.7)
776717777760771	■■■■■■■■□■■■□□■■■■■■■■■■■■■■■■■■□□□□■■■■■■■	NEW	T1	EA	EA (L4)	1 (0.9)
000000000003771	□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□■■■■■■■■■	1	BEIJING	EA*	EA* (L2)	1 (0.9)
777777377760771	■■■■■■■■■■■■■■■■■■□■■■■■■■■■■■■■□□□□■■■■■■■	40	T4	EA	EA (L4)	1 (0.9)
777777777720771	■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■□■□□□□■■■■■■■	50	H3	EA	EA (L4.1.2)	1 (0.9)
777777777763771	■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■□□■■■■■■■■■	54	MANU2	EA	UKN	1 (0.9)
777767777760731	■■■■■■■■■■■■■■□■■■■■■■■■■■■■■■■■□□□□■■■□■■■	117	T2	EA	EA (L4)	1 (0.9)
777767777760771	■■■■■■■■■■■■■■□■■■■■■■■■■■■■■■■■□□□□■■■■■■■	118	T1	EA	EA (L4)	1 (0.9)
677777777760771	■■□■■■■■■■■■■■■■■■■■■■■■■■■■■■■■□□□□■■■■■■■	196	T1	EA	EA (L4)	1 (0.9)
777777777420731	■■■■■■■■■■■■■■■■■■■■■■■■■■■■□□□■□□□□■■■□■■■	817	Ural-1	EA	EA (L4.2.1)	1 (0.9)
777777607760770	■■■■■■■■■■■■■■■■■■■■□□□□■■■■■■■■□□□□■■■■■■□	1933	LAM9	EA	EA (L4.3)	1 (0.9)
777777404760771	■■■■■■■■■■■■■■■■■■■□□□□□■□□■■■■■□□□□■■■■■■■	41	TURKEY	EA	EA (L4.2.2.1)	1 (0.9)
702525240001050	■■■□□□□■□■□■□■□■□■□■□■□□□□□□□□□□□□■□□□■□■□□□□	NEW	ND	EAI	EAI (L3)	1 (0.9)
503767740003171	■□■□□□□■■■■■■□■■■■■■■■■□□□□□□□□□□■■□□■■■■■	1945	CAS1-DELHI	EAI	EAI (L3)	1 (0.9)
577767777720771	■□■■■■■■■■■■■■□■■■■■■■■■■■■■■■□■□□□□■■■■■■■	NEW	H3	EA	EA (L4.1.2)	1 (0.9)
777777777760250	■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■□□□□□■□■□■□	NEW	ND	EA	EA (L4)	1 (0.9)

Abbreviations: CAS: ***Central-Asian;*** *CBN:* ***Conformal Bayesian Network;*** *EA*:* ***East-Asian;*** *EA:* ***Euro-American;*** *EAI:* ***East-African Indian;*** *ETH:* ***Ethiopian;*** *L:* ***Lineage;*** *LAM:* ***Latin-American-Mediterranean;*** *MA**:* ***Mycobacterium africanum;*** *ND:* ***Not defined;*** *PTB:* ***pulmonary tuberculosis;*** *SIT:* ***Spoligo-international type;*** *SNP:* ***Single-nucleotide polymorphism;*** *UKN:* ***Unknown.*** *"NEW"* ***indicates strains not found in the databases.***

Table 3
Clustering rate of different *Mycobacterium tuberculosis* complex lineages/sublineages (shared types) isolated from PTB suspects attended holy water sites in the Amhara region, Northwest Ethiopia (n = 116).

S.No	Lineage	Number of isolates (n)	Clustered strains (n)	Clustering rate (%)	Cluster number (n)	Cluster size (n)	SIT	Lineage/sublineages
1.	**EA (Lineage 2)**	1	0	0	0	0	SIT1	Beijing
2	**EAI (Lineage 3)**	49	45	81.6%	5	16	SIT26	CAS1-DELHI
						12	SIT25	CAS1-DELHI
						8	SIT21	CAS1-KILI
						7	SIT1198	CAS1-DELHI
						2	SIT3284	CAS1-KILI
3	**EA (Lineage 4)**	48	42	77.1%	5	17	SIT149	T3-ETH
						11	SIT52	T2
						8	SIT53	T1
						4	SIT3137	T3
						2	SIT37	T3
	EA (L4.1.1)	2	2	50.0%	1	2	SIT137	X2
	EA (L4.1.2)	6	4	50.0%	1	4	SIT134	H3
	EA (L4.3)	5	4	40.0%	2	2	SIT42	LAM9
						2	SIT1074	LAM9
	EA (L4.2.1)	1	0	0	0	0	SIT817	Ural-1
	EA (L4.2.2.1)	1	0	0	0	0	SIT41	TURKEY
4	**ETH (Lineage 7)**	2	2	50%	1	2	SIT1729	ETH

Abbreviations: CAS: ***Central-Asian;*** *EA*:* ***East-Asian;*** *EA:* ***Euro-American;*** *EAI:* ***East-African-Indian;*** *ETH:* ***Ethiopian;*** *LAM:* ***Latin-American-Mediterranean;*** *L:* ***Lineage;*** *SIT:* ***Spoligotype international type.***

Table 4
The description of the MTB lineages/sublineages retrieved from the regional public health research laboratory center, Amhara region, Northwest Ethiopia (n = 23).

Octal code	Spoligotype description Binary format	SIT	Lineage (SITVIT2)	Major Lineages by CBN	SNP-based lineage	No. of isolates (%)
703377400001771	■■■□□□□■■□■■■■■■■■■■□□□□□□□□□□□□□□□□■■■■■■■	21	CAS1-KILI	EAI	EAI (L3)	4 (17.4)
703777740003171	■■■□□□□■■■■■■■■■■■■■■■□□□□□□□□□□□□■■□□■■■■■	25	CAS1-DELHI	EAI	EAI (L3)	1 (4.3)
703777740003771	■■■□□□□■■■■■■■■■■■■■■■□□□□□□□□□□□□■■■■■■■■■	26	CAS1-DELHI	EAI	EAI (L3)	4 (17.4)
777777777760771	■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■□□□□■■■■■■■	53	T1	EA	EA (L4)	1 (4.3)
777776777760601	■■■■■■■■■■■■■■■■□■■■■■■■■■■■■■■■□□□□■■□□□□■	137	X2	EA	EA (L4.1.1)	1 (4.3)
777000377760771	■■■■■■■■■□□□□□□□□□□■■■■■■■■■■■■■□□□□■■■■■■■	149	T3-ETH	EA	EA (L4)	8 (34.8)
777776777760731	■■■■■■■■■■■■■■■■□■■■■■■■■■■■■■■■□□□□■■■□■■■	336	X1	EA	EA (L4.1.1)	1 (4.3)
701777740003771	■■■□□□□□■■■■■■■■■■■■■■□□□□□□□□□□□□■■■■■■■■■	1551	CAS1-DELHI	EAI	EAI (L3)	1 (4.3)
773347777761771	■■■■■■□■■□■■■□□■■■■■■■■■■■■■■■■■□□■■■■■■■■■	NEW	ND	EA	UKN	1 (4.3)
777146677763661	■■■■■■■■■□□■■□□■■□■■□■■■■■■■■■■■□□■■■■□■■□■	NEW	ND	EA	UKN	1 (4.3)

*Abbreviations: CAS: **Central-Asian;** CBN:* **Conformal Bayesian Network**; *EA: **Euro-American;** EAI: **East-African Indian;** L: **Lineage;** SIT: **Spoligo-international type.** ND: **Note defined;** SNP: **Single nucleotide polymorphism;** UKN: **Unknown;** **"NEW" indicates strains not found in the SpolDb4 database.***

Table 5
Anti-TB drug resistance profiles of different *Mycobacterium tuberculosis* lineages/sublineages (n = 20).

Lineages (n)	SIT No	Sublineages/clades	Anti-TB drug resistance pattern					
			Any drug resistance, n (%)	INHr, n (%)	INH-MR, n (%)	RIFr, n (%)	MDR, n (%)	FLQr, n (%)
EA* (L2) (1)	SIT1	BEIJING	–	–	–	–	–	–
EAI (L3) (49)	SIT21	CAS1-KILI	5 (10.2)	5 (10.2)	0 (0.0)	5 (10.2)	5 (10.2)	4 (8.2)
	SIT25	CAS1-DELHI	1 (2.0)	1 (2.0)	1(2.0)	0 (0.0)	0 (0.0)	0 (0.0)
	SIT3284	CAS1-KILI	1 (2.0)	1 (2.0)	0 (0.0)	1 (2.0)	1 (2.0)	1 (2.0)
EA (L4) (64)	SIT149	T3-ETH	12 (18.8)	12 (18.8)	4(6.3)	8 (12.5)	8 (12.5)	0 (0.0)
	SIT54	MANU2	1 (1.6)	1 (1.6)	0 (0.0)	1 (1.6)	1 (1.6)	0 (0.0)
ETH (L7) (2)	SIT1729	ETH	-	-	-	-	-	-
Total (n)			20	20	5	15	15	5

*Abbreviations: CAS: **Central-Asian;** EA: **Euro-American;** EA*: **East-Asian;** ETH: **Ethiopian;** EAI: **East-African-Indian;** **"New": Unassigned genotype; Abbreviations:** FLQr: **Fluoroquinolone-resistant;** INHr: **Isoniazid resistance;** INH-MR: **Isoniazid-monoresistance;** L: **Lineage;** MDR: **Multidrug-resistant;** RIFr: **Rifampicin resistance;** SIT: **Spoligotype international types.***

proportion and predominance of clustered T3-ETH/SIT149 sublineages in the country would indicate that those strains are becoming more important in TB disease burden and transmission in Ethiopia [20,21,37,39].

An isolate with the L4.2.2.1/Turkey/SIT41 spoligotype, specific to Turkey [69], was identified in the North Shewa zone during this study. This sub-lineage, while in modest number in this study, has previously been documented in several parts of Ethiopia [21,32,39,59,70,71]. One earlier multicenter study in Ethiopia also reported a high clustering of SIT41/Turkey (n = 11) sub-lineage [52]. The detection of this sub-lineage in the current study and earlier similar investigations in Ethiopia highlights its importance and calls for more investigation into its role in the burden of TB disease in the country.

In the current study, EAI/lineage 3/CAS1-Delhi was the predominant sublineage, comprising 30.2% of all isolates. In line with our result, earlier studies in the Amhara region [20,21,37,38,72], and different parts of Ethiopia [26,27,32,36] documented that EAI/CAS1-Delhi sub-lineage was most prominent. Supporting our findings, an earlier study conducted among Ethiopian refugees found that EAI/lineage 3 was the prevalent lineage [7]. This could be the reason that people in the refugee camps were from different countries, which contributed to the introduction of this lineage to Ethiopia [7]. Also, a study from Sudan reported that EAI/Delhi/CAS is the predominant sub-lineage (49%) in the country [73]. The Dehli/CAS sublineage is endemic to Central Asia and the Middle East, notably in India [74]. Two possibilities could explain the existence of the Dehli/CAS lineage in Ethiopia [20]: (a) due to growing business relations between Ethiopia, India, and China may have introduced this lineage to Ethiopia; or (b) this lineage may have originated in Ethiopia and spread to Asia, which is consistent with the scenario that MTBC species originated from East Africa [75]. The predominance of these sublineages in the current study and the fact that SIT149/T3-ETH is specific to Ethiopia emphasize its importance. The high frequency of SIT149/T3-ETH and EAI/CAS1-Delhi sub-lineages could suggest that these strains are playing a larger role in the transmission of TB disease in Ethiopia, specifically in this study region.

The widely studied Beijing-sublineage (EA/lineage 2) known for its global distribution, virulent nature, and drug resistance has been reported in certain regions of Ethiopia [38,52,54,58,71]. Supporting previous findings, we identified one SIT1/Beijing sub-lineage in the North Shewa zone study area.

Although additional genotyping techniques with strong discriminatory power are necessary, our spoligotyping data revealed clustered strains across and within the study zones. For instance, among the seventeen T3-ETH/SIT149 clustered sublineages, seven were identified in South Gondar, five in North Shewa, and three in the South Wello zone. Similarly, eleven of 16 CAS1-Delhi/SIT26 clustered sub-lineages were identified in North Shewa. This pattern of distribution suggests that individuals/attendees probably visit multiple HWSs, which can lead to the acquisition and spread of the disease from one HWS to individuals who attend other sites. Another possibility is TB strains may be circulating in the HWSs that have geographical proximity. Thus, high TB strain clustering in the current study may imply recent disease transmission in HWSs, underlining the importance of TB prevention and control strategies in these high-risk congregate settings. However, the low discriminatory power of spoligotyping must be considered.

In the current study, SIT149/T3-ETH and CAS1-Kili sublineages

showed a higher proportion of drug resistance than other spoligotypes. These sub-lineages, SIT21/CAS1-Kili and SIT149/T3-ETH made up 85.0% of DR-TB isolates in this study. Consistent with our results, an earlier study in Ethiopia found that T3-ETH/ST149 and CAS1-Kili sub-lineages were common among MDR-TB isolates [32]. In another study, Bekele and his colleagues found a significant rate of drug-resistant SIT149 spoligotypes in Ethiopia [76]. In support of the above evidence, an earlier study from the Somali region of Eastern Ethiopia reported the SIT149 spoligotype was linked with MDR or monoresistance [77]. The high proportion and predominance of the SIT149/T3-ETH sub-lineage in Ethiopia may have contributed to the emergence of DR-TB, notably MDR-TB [32]. Also, studies elsewhere in Africa, Uganda [78], and Tanzania [79] reported a higher proportion of DR-TB strains among T-sublineages. The specificity of the T3-ETH sublineage to Ethiopia and its prevalence among DR-TB, specifically MDR-TB strains in our study and previous reports in Ethiopia [32,34,76] underline the relevance of this sub-lineage, especially its association with MDR-TB.

The second TB strain, SIT21/CAS1-Kili sub-lineage, accounted for 25.0% of any drug resistance and 33.0% of MDR-TB isolates in our study. Interestingly, these five FLQs-resistant SIT21/CAS1-KILI sub-lineages were identified from the South Wello zone. Consistent with our finding, one previous study in Ethiopia showed that CAS1-Kili and SIT149/T3-ETH sub-lineages were prevalent among MDR-TB isolates [32]. The emergence of drug resistance in the Ethiopian strains, SIT149/T3-ETH and CAS1-Kili/SIT21 sublineages may be due to local factors such as late diagnosis, poor compliance, incomplete contact investigations, or other unidentified factors in the TB prevention and care system. However, this study has limitations. It used only the spoligotyping method to characterize MTBC lineages/sublineages or families, which may have resulted in low discriminatory capacity and hampered identification of transmission chains.

5. Conclusions

This study revealed diverse MTBC strains, with T-superfamilies (lineage 4) and CAS (lineage 3) families being the most prevalent. CAS1-DELHI and T3-ETH sub-lineages were most prevalent in EAI (lineage 3) and EA (lineage 4), respectively. Although in low numbers, ETH (lineage 7) was detected in the current study. Genotypic DST revealed that two sublineages, SIT149/T3-ETH, and SIT21/CAS1-KILI showed a high proportion of anti-TB drug resistance.

A high clustering rate of spoligotypes was detected within and across each of the study zones; although, a genotyping tool with strong discriminatory power is warranted. This underlines the significance of strengthening the national and regional TB control program by enhancing systematic TB screening, case detection, and laboratory diagnosis among individuals attending HWSs to halt the transmission of the disease. However, it is important to conduct a large-scale investigation using genotyping tools with strong discriminatory power, such as whole genome sequencing to obtain thorough genomic information, and understand MTBC genetic diversity, clustering and transmission dynamics, and their link with anti-TB drug resistance. This would help to guide the national and regional TB control program in establishing targeted interventions for those cohorts of populations to combat TB in Ethiopia.

Data availability

The data sets analyzed during this study are available from the corresponding author upon reasonable request.

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Research ethics approval

Ethics approval was obtained from the Human Research Ethics Committee of the University of Pretoria, Faculty of Health Sciences, South Africa (Ref #: 600/2018), and the National Research Ethics Review Committee, Ethiopia (Ref #: SHE/SM/14.4/708/2019). Also, a written official permission letter was obtained from the Ethiopian Orthodox Tewahedo Church, Patriarchate Head Office, Addis Ababa (Ref #: 2478/6275/2011). A verbal and/or written consent declaration with detailed information about the study was given to participants and signed.

CRediT authorship contribution statement

Melese Abate Reta: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Project administration, Resources, Software, Validation, Visualization, Writing – original draft, Writing – review & editing. **Halima M. Said:** Formal analysis, Software, Validation, Visualization, Writing – review & editing. **Nontuthuko Excellent Maningi:** Formal analysis, Investigation, Methodology, Resources, Software, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing. **Gizachew Yismaw Wubetu:** Investigation, Methodology, Supervision, Validation, Writing – review & editing. **Mulualem Agonafir:** Formal analysis, Investigation, Methodology, Software, Validation, Visualization, Writing – review & editing. **P. Bernard Fourie:** Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Visualization, Writing – review & editing.

Declaration of competing interest

The authors declare that there are no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.nmni.2024.101235.

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