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Genetic diversity of the *Mycobacterium tuberculosis* complex strains from newly diagnosed tuberculosis patients in Northwest Ethiopia reveals a predominance of East-African-Indian and Euro-American lineages

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ABSTRACT

Objectives: This study described the population structure of *M. tuberculosis* complex (MTBc) strains among patients with pulmonary or lymph node tuberculosis (TB) in Northwest Ethiopia and tested the performance of culture isolation and MPT64-based speciation for Lineage 7 (L7).
Methods: Patients were recruited between April 2017 and June 2019 in North Gondar, Ethiopia. The MPT64 assay was used to confirm MTBc, and spoligotyping was used to characterize mycobacterial lineages. Line probe assay (LPA) was used to detect resistance to rifampicin and isoniazid.
Results: Among 274 MTBc genotyped isolates, there were five MTBc lineages: L1–L4 and L7 were identified, with predominant East-African-Indian (L3) (53.6%) and Euro-American (L4) (40.1%) strains, and low prevalence (2.6%) of Ethiopia L7. The genotypes were similarly distributed between pulmonary and lymph node TB, and all lineages were equally isolated by culture and recognized as MTBc by the MPT64 assay. Additionally, LPA showed that 259 (94.5%) MTBc were susceptible to both rifampicin and isoniazid, and one (0.4%) was multi-drug resistant (resistant to both rifampicin and isoniazid).
Conclusion: These findings show that TB in North Gondar, Ethiopia, is mainly caused by L3 and L4 strains, with low rates of L7, confirmed as MTBc by MPT64 assay and with limited resistance to rifampicin and isoniazid.

Background

Tuberculosis (TB) remains a major public health threat and the leading infectious cause of morbidity and mortality throughout the world (World Health Organization, 2019). According to the World Health Organization (WHO), there were an estimated 10 million incident cases of TB and 1.5 million deaths worldwide, with the African region displaying the highest annual risk of infection, aggravated by high HIV co-infection rates and the emergence of drug-resistant TB (World Health Organization, 2019). TB disease, caused by the *Mycobacterium tuberculosis* (*M. tuberculosis*) complex (MTBc), has been present in the human population since the beginning of recorded history (Guttierez *et al.*, 2005) and co-evolved with ancient hominids (Gagneux, 2012; Comas et al., 2013). Today, eight phylogenetic lineages of the MTBc have been identified worldwide, causing TB in humans (Gagneux et al., 2006; Firdessa et al., 2013; Ngabonziza et al., 2020).

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The distribution of MTBc lineages has shown significant geographical variation (Gagneux et al., 2006; Gagneux and Small, 2007), with a major impact on disease presentation, drug resistance nature and host adaptation (Ford et al., 2013; Warner et al., 2015). A high rate of lymph node TB has been reported in Ethiopia (Berg et al., 2015; Biadglegne et al., 2015; Tadesse et al., 2017), and the country uniquely harbors *M. tuberculosis* Lineage 7 (Firdessa et al., 2013; Comas et al., 2015), a lineage in-between 'ancestral' and 'modern' MTBc members. Ethiopia also has a high incidence of TB compared to neighboring countries (Berg et al., 2015; World Health Organization, 2019), although the reasons remain unclear, justifying further understanding of potential mycobacterial factors. Drug-resistant TB has also been reported as a major problem in different regions of Ethiopia (Mulisa et al., 2015; Tesfay et al., 2016; Alene et al., 2017; Shibabaw et al., 2020); however, the overall molecular epidemiology of drug-resistant TB is poorly understood in the country.

Mycobacterial strain genotyping has been used to complement epidemiological studies and provided a helpful understanding of TB transmission dynamics (Kato-Maeda et al., 2011). Genotyping studies have been performed on culture isolates in Ethiopia (Tessema et al., 2013; Yimer et al., 2015). It has recently been shown that culture selects for modern *M. tuberculosis* strains (Sanoussi et al., 2017), thus introducing culture bias in the estimation of the proportion of the different lineages (Metcalfe et al., 2017; Sanoussi et al., 2017). Also, data on the association between circulating MTBc lineages and drug resistance in Northwest Ethiopia, one of the high TB burden sub-regions (Alene et al., 2017; Shibabaw et al., 2020), remains inadequate.

This study describes the population structure of MTBc among culture isolates and specimens from TB patients with pulmonary or lymph node presentations in Northwest Ethiopia, and the performance of culture isolation and MPT64-based speciation for Lineage 7.

Methods

Study design and patients

A cross-sectional study of consecutive patients with presumptive TB was conducted between April 2017 and June 2019 in North Gondar at the University of Gondar Hospital (UoGH), Ethiopia. The hospital serves as a comprehensive specialized hospital for people living in North Gondar and the neighboring cities of South Gondar and the Tigray region. It serves as a treatment-initiating center for multi-drug resistant TB (MDR-TB), with screening facilities for patients with presumptive resistant TB. All patients aged ≥15 years who directly presented to UoGH or were referred from other health centers and who were diagnosed with new pulmonary TB or TB lymphadenitis were asked for informed consent before initiating treatment. The sample size was calculated assuming the expected prevalence of Lineage 7 to be 15.6% among pulmonary TB (Yimer et al., 2015) and 9.8% (Biadglegne et al., 2015) among TB lymphadenitis, using a 95% confidence interval (CI) and 5% absolute precision (Fenn Buderer, 1996). The sample size was 532 for pulmonary TB and 816 for TB lymphadenitis. The computed sample size was not achieved, as only 244 pulmonary TB and 161 TB lymphadenitis patients were enrolled in this study. A structured and pre-tested questionnaire was used to collect sociodemographic and clinical data.

Culture

Sputum samples from presumed pulmonary TB patients and lymph node aspirates from patients with presumed TB lymphadenitis were collected based on standard protocols, as described by the WHO and Global Laboratory Initiative (GLI) (Global Laboratory Initiative, 2014). In the TB culture laboratory, the standard cultivation method on Löwenstein-Jensen (LJ) medium (Global Laboratory Initiative, 2014) was carried out for isolating mycobacterial species. Before decontamination, an aliquot of the sputum was preserved in 95% ethanol, as previously described (Williams et al., 1995). Briefly, an overnight stand and liquefied sputum sample was added into 1 mL of 95% ethanol in a 2 mL screw-capped cryovial tube (final concentration of 50%), vortexed, and kept at ambient temperature. Subsequently, the mixtures were centrifuged at 13,000 xg for 5 min, the supernatant was discarded, and the sediment (250 μL) was transported to ITM, Antwerp, Belgium. All aliquots were stored at 2–8 °C in Antwerp until DNA extraction.

Confirmation of Mycobacterium tuberculosis *complex*

Preliminary identification of the isolates obtained from positive cultures as being mycobacterial species was performed based on morphological characteristics on LJ media, and detection of acid-fast bacilli (AFB) (with cording) using Ziehl-Neelsen (ZN) smear microscopy (Global Laboratory Initiative, 2014). The MPT64-based SD Bioline rapid test (BD Diagnostic System, Sparks, MD) confirmed mycobacterial isolates with MTBc, as per manufacturer's instructions. Also, spoligotyping (Kamerbeek et al., 1997) was used as a reference standard identification for further confirmation of the MPT64-positive and MPT64-negative isolates. Isolates were considered as MTBc positive by spoligotyping when any spoligotype pattern was seen, and negative when the absence of all spacers was observed.

DNA extraction

Deoxyribonucleic acid (DNA) was extracted from LJ cultures using the GenoLyse® DNA extraction kit (Hain Life Science, Nehren, Germany) and stored at −20 °C until shipment for spoligotyping in Antwerp. Also, DNA from sputum specimens was extracted by the Promega Maxwell® 16 DNA extraction kit (Promega, USA) following the adapted ITM protocol with pre-treatment, and previously published protocols (Eddyani et al., 2015). Briefly, each sputum sample (200 μL) was pretreated with 20 μL proteinase K and 200 μL in-house lysis buffer, and homogenized overnight at 60 °C in a shaking incubator (200 rpm). Subsequently, 330 μL of Maxwell® 16 lysis buffer was added to the pre-treated sputum, vortexed, and transferred to the cartridge well (maximum 750 μL); the DNA was then eluted in 50 μL elution buffer in a Maxwell® 16 LEV device (model AS3000). A *Mycobacterium bovis* (*M. bovis*) BCG inactivated suspension and molecular biological (Milli-Q) water were included as positive and negative controls, respectively.

Genotypic drug susceptibility testing

The MTBDR*plus* LPAs, including PCR amplification and hybridization procedures, were conducted according to the manufacturer's guidelines (Hain Lifescience, 2013). The resulting strips were taped to LPA worksheets and interpreted using WHO guidelines (World Health Organization, 2016). Internal quality control was ensured by using H37Rv DNA as positive and distilled water as a negative control in each run.

Molecular genotyping

Spoligotype analysis was performed on in-house prepared membranes after PCR amplification for the direct repeat regions (Kamerbeek et al., 1997). Both *M. tuberculosis* H37Rv and *M. bovis* BCG reference strains were included as positive controls, and distilled water as a negative control in each run.

Genotyping and phylogenetic analyses

The publicly available international multimarker database of the Pasteur Institute of Guadeloupe (SITVIT2) (http://www.pasteur-guadeloupe.fr:8081/SITVIT2/) (Couvin et al., 2019a) was used to assign genotypes. Mycobacterial lineages and families were defined according to signatures provided in SITVIT2 (Demay et al., 2012; Couvin et al., 2019a) and based on TBlineage, Spotclust and MIRU-VNTR*plus* websites. In addition, spolTools (http://spoltools.emi.unsw.edu.au/) were used to analyze MTBc spoligotype data for probable relationships among genotypes using Spoligoforests (spolTools; hierarchical layout) (Tang et al., 2008).

Statistical analysis

Descriptive statistics like frequency distributions and percentages were conducted using STATA® 15.1 (StataCorp, USA). Proportions of LPA and spoligotype results for both MTBc lineages were compared using the two-sample proportion test analysis. Odds ratios (OR) and 95% confidence levels (CIs) were calculated to measure the association of MTBc lineages or families with patients' sociodemographics and disease presentation using binary logistic regression. Additionally, two-sided Pearson's Chi-square test was used to assess associations of geographic MTBc lineage distribution in study districts. A p-value of < 0.05 was considered statistically significant.

Ethical review

Institutional permission to perform this study was obtained from the Institutional Research and Ethical review committees of the University of Gondar, Ethiopia, and also from the Institute of Tropical Medicine (ITM, Antwerp) and University of Antwerp (UZA), Belgium before the commencement of research activities. Voluntarily signed informed consent was obtained from each patient and no patient personal identifiers were used in this study.

Results

Patients and MTBc characteristics

A total of 405 patients were recruited, including 244 with new pulmonary TB and 161 with TB lymphadenitis (Figure 1). Most patients were male (249/405, 61.5%) and the median age was 30.0 years (IQR 24.0–40.0). The median body mass index (BMI) was 18.7 kg/m^2 (range 11.3–26.3, IQR 17.2–20.5). Among 211 TB patients with known HIV test results, 30 (14.2%) were HIV-positive. Few patients (6.7%) had been in prison or had a smoking history (2.5%). The majority of patients (69.1%) were from rural areas of the districts (Table 1).

In total, the study showed smear-positive samples among 60.7% (148/244) of pulmonary TB- and 17.4% (28/161) of TB lymphadenitis patients ($p <$ 0. 001, Table 1). Among 405 culture processed samples, a total of 280 (69.1%) were positive: 79.5% (194/244) of pulmonary specimens and 53.4% (86/161) of TB lymphadenitis patients. Most samples that did not yield an isolate had no growth in culture (45/244 from pulmonary TB and 74/161 from TB lymphadenitis), while only a few were contaminated (Figure 1). Of the 280 isolates, 274 (97.9%) were identified as MTBc by the MPT64 Ag test (Figure 1).

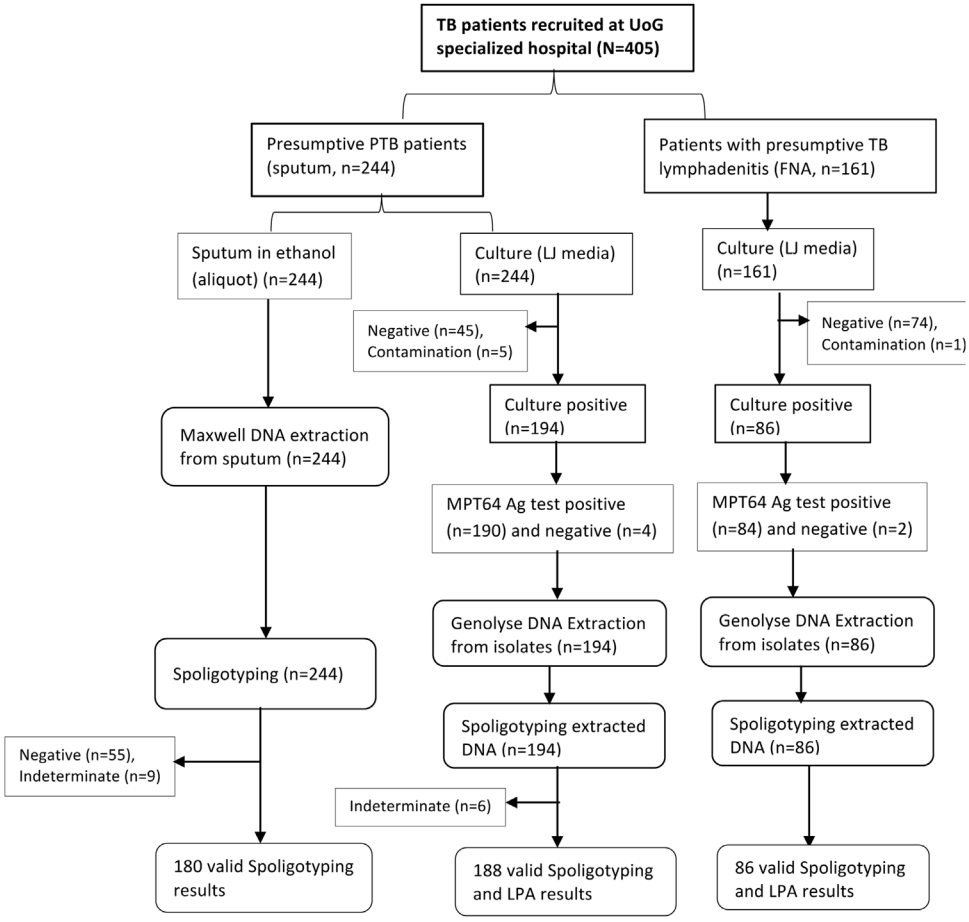

Figure 1. TB patients included in spoligotyping and line probe assay (LPA) analysis.

Table 1
Patients' characteristics by type of TB presentation.

	Total (n = 405)	Pulmonary TB (n = 244)	TB lymphadenitis (n = 161)	OR	[95% CI]	*p*-value
Gender, n (%)						
Male	249 (61.5)	160 (65.6)	89 (55.3)	0.73	0.43–1.24	0.250
Age, median (IQR)	30.0 (24–40)	28.5 (23–38)	30.0 (24–40)	1.26	0.74–2.16	0.395
BMI, median (IQR)	18.7 (17.2–20.2)	18.4 (16.8–20.0)	19.5 (18.0–20.9)	**2.06**	**1.24–3.43**	**0.005**
HIV status, n (%)						
Positive	30 (7.4)	23 (9.4)	7 (4.4)	0.67	0.21–2.14	0.495
Negative	181 (44.7)	100 (41.0)	81 (50.3)	1.41	0.83–2.39	0.201
unknown	194 (47.9)	121 (49.6)	73 (45.3)	Ref.		
Prison history, n (%)						
Yes	27 (6.7)	12 (4.9)	15 (9.3)	2.65	0.93–7.57	0.068
Smoking history, n (%)						
Yes	10 (2.5)	6 (2.5)	4 (2.5)	1.10	0.27–4.49	0.898
Patient address, n (%)						
Rural	280 (69.1)	155 (63.5)	125 (77.6)	**2.29**	**1.26–4.17**	**0.006**
Urban	125 (30.9)	89 (36.5)	36 (22.4)	Ref.		
Smear microscopy						
Positive	176 (43.5)	148 (60.7)	28 (17.4)	**0.14**	**0.08–0.22**	**0.000**

BMI, body mass index; CI, confidence interval; HIV, human immunodeficiency virus; IQR, interquartile range; OR, odds ratio; Ref., reference; TB, tuberculosis.

Genotypes of the Mycobacterium tuberculosis *complex*

Spoligotyping on isolates yielded 274/280 interpretable results (Figure 1). The six MPT64-negative pulmonary isolates included four likely non-TB mycobacteria (negative spoligotypes) and two MPT64 false-negative MTBc isolates (one isolate from L1 and the other from L3). All L7 isolates were correctly classified as MTBc by MPT64 analysis.

Among the 274 spoligotype results, 68 different spoligopatterns were identified: 44 known in the SITVIT2 database and 24 orphan patterns (Table 1 and Supplementary Table 1**)**. Amongst the total 68 spoligopatterns, 38 distinct spoligotypes were identified from pulmonary TB, nine patterns from TB lymphadenitis patients, and 21 patterns were shared by both pulmonary TB and TB lymphadenitis. Five major MTBc lineages (Ls) were identified: eight (2.9%) Indo-Oceanic (L1), two (0.7%) East Asian (Beijing, L2), 147 (53.6%) East-African-Indian (L3), 110 (40.1%) Euro-American (L4), and seven (2.6%) Ethiopian lineage (L7) (Table 2**)**. Within the predominant East-African-Indian L3, the CAS1-Delhi (140, 51.1%) was the most common family, with few isolates classified as CAS1-Kili (4, 1.5%) and CAS (3, 1.1%). Euro-American L4 comprised T- (42, 15.3%), Haarlem- (28, 10.2%), T3-ETH- (20, 7.3%), LAM- (16, 5.8%), S- (2, 0.7%) and X- (2, 0.7%) families (Table 2 and Supplementary Table 1**)**. Fifty orphan spoligotypes (18.2%) were further analyzed to the nearest lineages and/or families by the TBlineage and Spotclust database and added into those identified MTBc families (Table 2**)**.

Overall, 234 (85.4%) MTBc isolates were clustered by spoligotyping, with 21 clusters comprising 2–89 isolates per cluster, while 40 isolates were singletons. The most predominant cluster was largely designated by the SIT25 (identified as CAS1-Delhi) with 89 MTBc isolates, followed by SIT53 (n = 21 isolates). The majority of MTBc clusters were from pulmonary TB (66.2%; n = 155) and rural areas (67.5%; n = 158) (Supplementary Table 1**)**.

Distribution of genotypes among direct (sputum) and indirect (isolate) spoligotyping

Direct spoligotyping was successful for 180 (73.8%) of 244 sputum specimens, while 55 (22.5%) were negative (missing all spacers; of which 47 samples were microscopy smear-negative) and nine (3.7%) had non-interpretable spoligotype results (too weak spots; of which four were smear-negative). The majority (93.3%; 168/180) of sputa with successful direct spoligotype results were also positive in culture. Of the 168 patients with both direct (sputa) and indirect (isolates) spoligotype results available, spoligotypes were concordant in 91.1% (153/168) (Supplementary Table 2). The 15 discrepant profiles differed by one spacer (n = 5), two (n = 3), three (n = 3), five (n = 3), or 28 spacers (n = 1) (Supplementary Table 2). Those with ≤5 spacers difference were identified without any change between families and/or lineage pairs, while the 28 spacer difference led to an interfamily and interlineage change (L4 T3-ETH in sputum and L3 CAS1_Delhi in culture) (Supplementary Table 3). The proportion of lineages did not significantly differ between spoligotype analysis directly on sputum versus on cultured isolates (73.8% versus 77.0%; 95% CI [-0.12–0.56]; $p = 0.476$) (Table 3). Notably, the proportion of L7 was similar in sputum and isolates (Table 3).

Spoligoforests among spoligotypes and MTBc genotypes

The spoligoforest tree was drawn by using the spoligotype data in http://www.emi.unsw.edu.au/spolTools/ (Tang et al., 2008) and presented with a Hierarchical layout in Figure 2, which shows four parent trees with linked nodes and 13 distinct nodes. The tree shows SIT26, SIT54, SIT910, and SIT35 spoligotypes as a root (i.e. these spoligotypes represent the parent spoligotypes). Six spoligotypes descended from SIT26, of which five spoligotypes consisted of small clusters (SIT952 (n = 3), SIT247 (n = 3), SIT142 (n = 2), SIT143 (n = 1), and SIT1343 (n = 1)), and one spoligotype (SIT25, named as CAS1-Delhi) with a big cluster (n = 89), whereas SIT54 (Manu family) descended into one big cluster (SIT53, named as T-family). The spoligoforest tree also indicates that SIT910 (ETH-L7) provided the SIT1720 and SIT343 spoligotypes, which were designated as the genotypes of the Ethiopian L7. It was also observed that the tree visualizes the locations of the predominant SITs such as SIT26, SIT25, SIT53, SIT149, and SIT134 in the hierarchical chain. The majority (n = 9) of the 13 distinct nodes represented orphan spoligotypes.

Geographical distribution of MTBc lineages

The majority of the 274 MTBc isolates were identified from four districts: Gondar (74, 27.0%), Dembia (50, 18.3%), Gondar Zuria (31, 11.3%), and Wegera (31, 11.3%) (Figure 3 and Supplementary Table 4**)**. Genotypes belonging to L3 and L4 were widespread in all districts, while the ancient L1 was found only in Alefa, Belesa, Dembia, Gondar, and Wegera with one or two isolates each. The only two L2 (Beijing) isolates were identified in Gondar and Belesa districts. The Ethiopian L7 was only identified in Belesa, Chilga, Dembia, Gondar, and Wegera districts with one or two isolates

Table 2
Distribution of *Mycobacterium tuberculosis* complex (MTBc) lineages and families among isolates, by TB presentation.

Lineages (Ls)	Total, n (%)	Family (n)	SIT nr	No. of total isolates, n/274 (%)	TB presentation	
					PTB, (%) n = 188	TB LN, (%) n = 86
Indo-Oceanic (L1)	8 (2.9)	Family 36 (1)	SIT4	1 (0.4)	1 (0.5)	0 (0.0)
		Manu (2)	SIT54	2 (0.7)	0 (0.0)	2 (2.3)
		Family 34 (5)	SIT56	4 (1.4)	3 (1.4)	1 (1.2)
			orphan	1 (0.4)	1 (0.5)	0 (0.0)
East Asian (L2)	2 (0.7)	Beijing (2)	SIT1	2 (0.7)	1 (0.5)	1 (1.2)
East-African-Indian (L3)	147 (53.6)	CAS1-Delhi (140)	SIT1198	4 (1.4)	3 (1.4)	1 (1.2)
			SIT1199	2 (0.7)	1 (0.5)	1 (1.2)
			SIT1343	1 (0.4)	1 (0.5)	0 (0.0)
			SIT141	1 (0.4)	1 (0.5)	0 (0.0)
			SIT142	2 (0.7)	2 (1.1)	0 (0.0)
			SIT247	3 (1.1)	1 (0.5)	2 (2.3)
			SIT25	89 (32.5)	56 (29.8)	33 (38.4)
			SIT26	10 (3.6)	9 (4.8)	1 (1.2)
			SIT952	3 (1.1)	2 (1.1)	1 (1.2)
			orphan	25 (9.1)	22 (11.7)	3 (3.5)
		CAS1-Kili (n = 4)	SIT21	4 (1.4)	2 (1.1)	2 (2.3)
		CAS (n = 3)	SIT1789	1 (0.4)	1 (0.5)	0 (0.0)
			orphan	2 (0.7)	2 (1.1)	0 (0.0)
Euro-American (L4)	110 (40.1)	Haarlem (n = 28)	SIT121	4 (1.4)	4 (2.1)	0 (0.0)
			SIT134	12 (4.4)	10 (5.3)	2 (2.3)
			SIT35	1 (0.4)	0 (0.0)	1 (1.2)
			SIT47	1 (0.4)	0 (0.0)	1 (1.2)
			SIT50	4 (1.4)	1 (0.5)	3 (3.5)
			SIT750	4 (1.4)	3 (1.4)	1 (1.2)
			SIT764	2 (0.7)	0 (0.0)	2 (2.3)
		T-family (42)	SIT1626	1 (0.4)	1 (0.5)	0 (0.0)
			SIT1688	1 (0.4)	1 (0.5)	0 (0.0)
			SIT1745	1 (0.4)	0 (0.0)	1 (1.2)
			SIT196	1 (0.4)	1 (0.5)	0 (0.0)
			SIT205	1 (0.4)	1 (0.5)	0 (0.0)
			SIT358	1 (0.4)	1 (0.5)	0 (0.0)
			SIT44	1 (0.4)	1 (0.5)	0 (0.0)
			SIT52	3 (1.1)	1 (0.5)	2 (2.3)
			SIT53	21 (7.7)	13 (6.9)	8 (9.3)
			orphan	11 (4.0)	8 (4.3)	3 (3.5)
		T3-ETH (20)	SIT149	19 (6.9)	10 (5.3)	9 (10.5)
			orphan	1 (0.4)	1 (0.5)	0 (0.0)
		LAM (16)	SIT33	1 (0.4)	1 (0.5)	0 (0.0)
			SIT41	2 (0.7)	1 (0.5)	1 (1.2)
			SIT42	1 (0.4)	1 (0.5)	0 (0.0)
			SIT59	1 (0.4)	1 (0.5)	0 (0.0)
			SIT230	1 (0.4)	1 (0.5)	0 (0.0)
			orphan	10 (3.6)	9 (4.8)	1 (1.2)
		S-family (2)	SIT34	1 (0.4)	1 (0.5)	0 (0.0)
			SIT156	1 (0.4)	1 (0.5)	0 (0.0)
		X-family (2)	SIT119	1 (0.4)	1 (0.5)	0 (0.0)
			SIT336	1 (0.4)	1 (0.5)	0 (0.0)
Ethiopian lineage (L7)	7 (2.6)	ETH1 family (7)	SIT343	1 (0.4)	1 (0.5)	0 (0.0)
			SIT910	1 (0.4)	0 (0.0)	1 (1.2)
			SIT1729	5 (1.8)	3 (1.6)	2 (2.3)

Mycobacterium tuberculosis complex lineages and families from 274 isolates from pulmonary TB (PTB) and TB lymphadenitis (TB LN) patients in Northwest Ethiopia.
L, lineage; CAS, Central Asian; LAM family, Latin-American-Mediterranean.

each. The geographic distribution of lineages did not significantly differ by district ($p > 0.05$, data not shown).

Distribution of MTBc lineages amongst the pulmonary TB and TB lymphadenitis

Most isolates (68.6%; n = 188) were from pulmonary TB patients. In both disease presentations, L3 and L4 strains were most common, and the lineage distribution did not significantly associate with TB disease presentation (OR = 1.1, $p = 0.724$, 95% CI [0.78–1.41]) (Supplementary Table 5).

Drug susceptibility profiles

Based on the GenoType LPA results of 274 MTBc isolates, 259 (94.5%) MTBc isolates were susceptible to both rifampicin (RIF) and isoniazid (INH), while 13 (4.7%) showed INH mono-resistance, one (0.4%) RIF mono-resistance, and one (0.4%) isolate was resistant to both INH and RIF (MDR-TB) (Supplementary Table 6). All 13 INH mono-resistant isolates had the same mutation in the *katG* gene (katG MUT1), the globally widespread S315 T mutation. The rifampicin-resistant isolate showed a missing rpoBWT8 band only on the LPA strip, presumably mutation L452 P, while the MDR isolate showed the most common *rpoB* S450 L and *katG* S315 T mutations. Isoniazid mono-resistant isolates belonged to diverse families within L3 and L4, without association between resistance and any specific genotype (data not shown).

Discussion

In Ethiopia, home to a distinct MTBc population structure that includes global Lineages 1–4 and the Ethiopia-specific Lineage 7,

Table 3
Mycobacterium tuberculosis complex (MTBc) lineages and families obtained by direct (on sputum) and indirect (on isolates) spoligotyping.

Lineages (Ls)	Family	Spoligotyping results		*p* -value*	[95% CI]*
		Direct (sputa), N = 180 (%)	Indirect (isolates), N = 188 (%)		
Indo-Oceanic (L1)		5 (2.8)	5 (2.6)	0.984	[-0.19–0.20]
	Family 36	1 (0.6)	1 (0.5)		
	Family 34	4 (2.2)	4 (2.1)		
East Asian (L2)		1 (0.6)	1 (0.5)	–	–
	Beijing	1 (0.6)	1 (0.5)		
East African Indian (L3)		101 (56.1)	104 (55.3)	0.908	[-0.13–0.14]
	CAS1-Delhi	98 (54.4)	99 (52.6)		
	CAS1-Killi	1 (0.6)	2 (1.1)		
	CAS	2 (1.1)	3 (1.6)		
Euro-American (L4)		69 (38.3)	74 (39.4)	0.893	[-0.17–0.15]
	Haarlem	16 (8.9)	18 (9.6)		
	T-family	24 (13.3)	28 (14.9)		
	T3-ETH	11 (6.1)	10 (5.3)		
	LAM	13 (7.2)	14 (7.4)		
	S-family	2 (1.1)	2 (1.1)		
	X-family	3 (1.7)	2 (1.1)		
Ethiopian lineage (L7)		4 (2.2)	4 (2.1)	0.992	[-0.20–0.20]
	ETH1 family	4 (2.2)	4 (2.1)		

CAS, Central Asian; CI, confidence interval; L, lineage; LAM family, Latin-American-Mediterranean.
* *p*-value and 95% CI were calculated using the two sample proportion test.

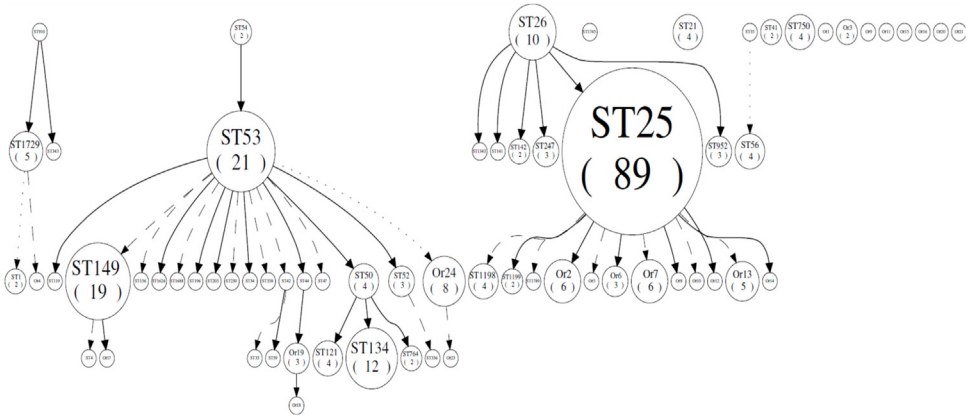

Figure 2. The spoligoforest trees were drawn using http://www.emi.unsw.edu.au/spolTools/ in Hierarchical Layout. Each spoligotype pattern represents a node in the tree with the number of isolates. Loss of spacers is represented by the edges between nodes, with the arrowheads pointing to descendant spoligotypes. Solid black lines link patterns with a maximum weight of distance. Dashed lines represent a link of weight comprised between 0.5 and 1, and dotted lines represent a link of weight <0.5 (Tang et al. 2008).

knowledge of the genotypic distribution of mycobacterial strains within the country is currently limited, with prior studies suggesting that L7 is more predominant in the north of the country. Ethiopia is furthermore unique in the high proportion of TB presenting as lymphadenitis, which is not explained by pastoralist populations infected with *M. bovis* (Berg et al., 2015). This study, therefore, described the population structure of circulating MTBc strains among TB patients in Northwest Ethiopia. The predominance of the East-African-Indian L3 and Euro-American L4 was consistent with other studies in Ethiopia (Tessema et al., 2013; Biadglegne et al., 2015; Tadesse et al., 2017; Damena et al., 2019), albeit with less L7 than the expected prevalence of 9–15% (Biadglegne et al., 2015; Yimer et al., 2015). L3 and L4 have also been reported in other African countries, particularly in neighboring Sudan (Mbugi et al., 2016; Chihota et al., 2018; Couvin et al., 2019b; Shuaib et al., 2020). The current findings confirm that L3 and L4 are endemic TB strains in this study area, possibly due to co-adaptation with its human host (Gagneux, 2012; Couvin et al., 2019b), and capable of evading the host immune response and progress to rapid TB disease and transmission (de Jong et al., 2008; Tanveer et al., 2009; Portevin et al., 2011). It has been also shown that strains of evolutionarily modern L3 and L4 lineages are successful human pathogens with increased virulence, enabling their worldwide distribution (Hershberg et al., 2008; Gagneux, 2012). The current study area, North Gondar, is one of the most touristic places in Ethiopia and is also known for its mobility between the neighboring regions and bordering Sudan, which could play a role in the TB transmission dynamics in the area.

Even though this study showed a lower prevalence of L7 (2.6%) than expected, the MPT64-based rapid test efficiently identified L7 strains in culture, as compared with the lower sensitivity of this assay to detect the West African specific lineages of the MTBc strain (Ofori-Anyinam et al., 2016; N'Dira Sanoussi et al., 2018). L7 was initially identified from patients in Woldia districts of the Amhara region, Ethiopia, at a prevalence of 13% (Firdessa et al., 2013), and later in other places in Southwest and Northwest Ethiopia, with a prevalence ranging 2–15% (Biadglegne et al., 2015; Yimer et al., 2015; Tadesse et al., 2017) and representing SIT910 and SIT1729 families (designated as Ethiopia_1 in MIRU-VNTR*plus* database). This variability might be due to chance or possible human host susceptibility differences in the study area, as a previous study

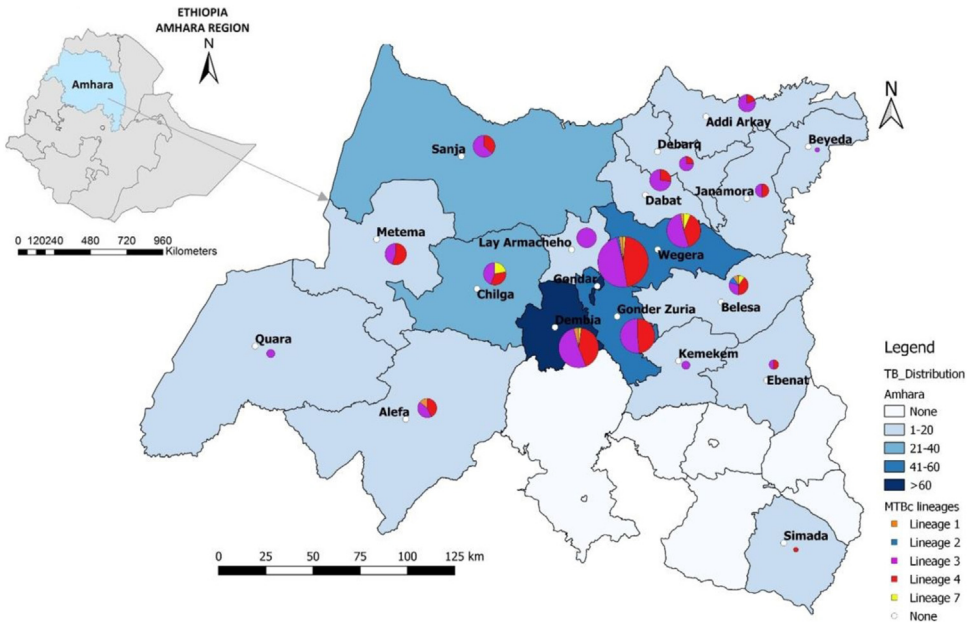

Figure 3. Distribution of TB patients and MTBc lineages in the different districts of the study area of Northwest Ethiopia. The map was created using a QGIS Desktop 3.12.0 (https://qgis.org/en/site/forusers/download.html) (Team QD, 2019) for MTBc lineage distribution. Free shapefiles were downloaded from the DIVA-GIS website: http://www.diva-gis.org/gdata at free spatial data' using country level and administrative areas.

(Yimer et al., 2015, 2016) reported that the L7 genotype was associated with slow *in vitro* growth and reflected lower fitness and virulence in TB patients than other lineages. The current study only included patients diagnosed with pulmonary TB and clinically diagnosed TB lymphadenitis patients who had direct access and/or were referred to a specialized university hospital. They might not be representative of all TB patients in the remote rural and general population of North Gondar, and may hence reflect the lower occurrence of L7 in this study. Furthermore, the small sample size of study participants might have impacted the prevalence estimates of L7. Future studies might be needed to explain the relationship between L7 virulence and clinical presentation in unbiased sampling at the first point of contact for TB diagnostics.

Genotypes of *M. tuberculosis* infection in pulmonary disease and lymph nodes were similar, with a predominance of the CAS1-Dehli; this agrees with other studies (Srilohasin et al., 2014; Hadifar et al., 2019). In Ethiopia, like most developing countries, people living in areas with uncontrolled TB transmission (Kebede et al., 2014) are more likely to have mixed MTBc infections, in which the strains might be different in lineages and/or families, including heterogeneous drug susceptibility profiles (Shamputa et al., 2005; Cohen et al., 2012). While the current methodology was not focused on the detection of mixed infection, the patient with L3 in sputum and L4 in the derived culture may indeed have mixed infection.

Otherwise, direct (sputum) spoligotyping showed a similar distribution of MTBc families and lineages compared to using indirect (isolate) spoligotyping in this study. However, the yield of spoligotypes was slightly lower than previously reported (Sanoussi et al., 2017; Kargarpour Kamakoli et al., 2018). This difference might be due to inclusion of smear-negative sputum specimens, below the limit of detection of direct spoligotype analysis, and DNA extraction of preserved sputa pellets (aliquots) after a long period of storage in the current study. Studies indicated that direct genotyping, such as spoligotyping and MIRU-VNTR*plus*, is more successful using baseline samples with high smear grades (Bidovec-Stojkovič et al., 2014; Sanoussi et al., 2017), although the nature and quality of the sample may influence the extraction method, DNA contents and interpretability of spoligotyping results (Tønjum et al., 1996; Pathak et al., 2007). Previous studies conducted using direct spoligotyping have also reported lower detection rates of MTBc strains in patients with lower bacillary load specimens (Suresh et al., 2007; Cafrune et al., 2009), with insufficient DNA to be detected by spoligotyping.

In addition to the general dominance of L3 and L4 strains, it was also found that all drug-resistant TB was identified among L3 (4.8%, 7/147) and L4 (7.3%, 8/110) strains. Despite the population including referral patients, only one (0.4%) MDR isolate was identified in contrast to earlier reports in Ethiopia, with a range of 1.4–5.2% from newly diagnosed TB patients (Damena et al., 2019; Diriba et al., 2019; World Health Organization, 2019). However, 4.7% INH resistance with the katGS315 T mutation was found in the current study; these patients are at higher risk of acquiring RIF resistance. The current study, however, might not reflect the actual drug-resistant TB prevalence in the general population, since data were collected from the referral hospital, which was likely enriched with TB patients suspected for complicated diseases such as drug resistance. LPA might also have a lower resolution of resistant strains compared to phenotypic drug susceptibility testing, as r*poB* mutations outside the 81 bp target region might have been missed.

This study had limitations. It only used spoligotyping to characterize MTBc lineages and families, and this could have resulted in limited resolution of genotypes, precluding identification of chains of transmission. Due to resource constraints, the study only included patients attending a specialized university hospital. It also did not explore statistical analysis for clustered spoligotypes due to the unequal proportion between pulmonary and lymph node TB. Furthermore, because of the small quantity of lymph node aspirates available for only culture, direct spoligotyping from lymph node TB was unable to be compared. Phenotypic drug susceptibility testing was unable to be performed due to logistic limitations. This study provides the population structure of MTBc lineages and families circulating in Northwest Ethiopia, with limited drug resistance profiles, to inform clinicians and public health professionals.

Conclusion

This study demonstrated that the TB epidemic in Northwest Ethiopia is caused by a wide diversity of MTBc strains belonging to lineages L1–L4 and L7, with a predominance of L3-CAS1-Delhi, L4-T-family, L4-Haarlem, and L4-T3-ETH families, and low prevalence (2.6%) of Ethiopia-specific L7. The genotypes were similarly distributed between pulmonary and lymph node TB, and all lineages were equally likely to be detectable in culture and be recognized as MTBc by the MPT64 antigen test. Genotypic drug-susceptibility testing revealed relatively low INH and/or RIF resistance levels. Therefore, these findings provide new insights into phylogeographic diversity, with lower rates of L7, yet no overt sign of culture and laboratory diagnosis bias, and contribute information to scientists and public health researchers for understanding the population structure and resistance profiles of MTBc strains.

Authors' contributions

Mebrat Ejo, Ermias Diro, Florian Gehre, Leen Rigouts, and Bouke C. de Jong conceived and designed the experiments. Mebrat Ejo, Meseret Kassa, Yilak Girma, and Cecile Uwizeye performed the experiments and molecular works. Tiruzer Mekonnen and Yilkal Abebe performed a clinical diagnosis of TB lymphadenitis and taken fine-needle aspirates. Ermias Diro, Gabriela Torrea, Leen Rigouts, and Bouke C. de Jong supervised the research work. Mebrat Ejo and Bouke C. de Jong analyzed the data. Gabriela Torrea, Leen Rigouts, and Bouke C. de Jong contributed reagents/materials/analysis tools. Mebrat Ejo, Gabriela Torrea, Florian Gehre, Ermias Diro, Leen Rigouts, and Bouke C. de Jong wrote the manuscript. All authors read and approved the final manuscript before submission.

Competing interests

The authors declare that they have no competing interests.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.ijid.2020.11.129.

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