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Research paper

The predominance of Ethiopian specific *Mycobacterium tuberculosis* families and minimal contribution of *Mycobacterium bovis* in tuberculous lymphadenitis patients in Southwest Ethiopia

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ABSTRACT

Background: Ethiopia has an extremely high rate of extrapulmonary tuberculosis, dominated by tuberculous lymphadenitis (TBLN). However, little is known about *Mycobacterium tuberculosis* complex (MTBc) lineages responsible for TBLN in Southwest Ethiopia.

Methods: A total of 304 MTBc isolates from TBLN patients in Southwest Ethiopia were genotyped primarily by spoligotyping. Isolates of selected spoligotypes were further analyzed by 15-loci mycobacterial interspersed repetitive unit–variable number tandem repeat (MIRU-VNTR) (n = 167) and qPCR-based single nucleotide polymorphism (n = 38). Isolates were classified into main phylogenetic lineages and families by using the reference strain collections and identification tools available at *MIRU-VNTRplus* data base. Resistance to rifampicin was determined by Xpert MTB/RIF.

Results: The majority of isolates (248; 81.6%) belonged to the Euro-American lineage (Lineage 4), with the ill-defined T and Haarlem as largest families comprising 116 (38.2%) and 43 (14.1%) isolates respectively. Of the T family, 108 isolates were classified as being part of the newly described Ethiopian families, namely Ethiopia_2 (n = 44), Ethiopia_3 (n = 34) and Ethiopia_H_{37}Rv-like (n = 30). Other sub-lineages included URAL (n = 18), S (n = 17), Uganda I (n = 16), LAM (n = 13), X (n = 5), TUR (n = 5), Uganda II (n = 4) and unknown (n = 19). Lineage 3 (Delhi/CAS) was the second most common lineage comprising 44 (14.5%) isolates. Interestingly, six isolates (2%) were belonged to Lineage 7, unique to Ethiopia. Lineage 1 (East-African Indian) and Lineage 2 (Beijing) were represented by 3 and 1 isolates respectively. *M. bovis* was identified in only two (0.7%) TBLN cases. The cluster rate was highest for Ethiopia_3 isolates showing clonal similarity with isolates from North Ethiopia. Lineage 3 was significantly associated with rifampicin resistance.

Conclusions: In TBLN in Southwest Ethiopia, the recently described Ethiopia specific Lineage 4 families were predominant, followed by Lineage 3 and Lineage 4-Haarlem. The contribution of *M. bovis* in TBLN infection is minimal.

1. Introduction

Tuberculosis (TB) is caused by a group of bacteria called *Mycobacterium tuberculosis* complex (MTBc) (Pfyffer, 2015), which comprises closely related species responsible for strictly human and zoonotic TB (Davies, 2003). The majority of TB in humans is caused by *M. tuberculosis* with a small proportion of TB disease caused by *M. bovis* and *M. africanum* (LoBue et al., n.d.). The MTBc comprises seven human-adapted phylogenetic lineages (Lineage 1 to Lineage 7), with various geographical coverage and frequency, and comprising multiple sub-lineages (Coscolla and Gagneux, 2014). Ethiopia is among the 30 high-burden countries for TB, TB/HIV and multidrug-resistant TB (MDR-TB) in the world, with an estimated incidence rate of 192 per 100,000 populations in 2015 (World Health Organization, 2016). In

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Ethiopia, extrapulmonary tuberculosis (EPTB) accounts for > 32% of all forms of TB (World Health Organization, 2015). Tuberculous lymphadenitis (TBLN) is the most common form of EPTB and accounts for 80% of all EPTB cases (Berg et al., 2015; Biadglegne et al., 2015).

Whether or not the high rate of TBLN in Ethiopia is linked to a specific MTBc lineage has not been systematically investigated. Various findings support the idea of a longstanding host-pathogen co-evolution (Hershberg et al., 2008; Comas et al., 2013; Gagneux, 2012) and found a correlation between human genetic polymorphisms and specific *M. tuberculosis* lineages (Caws et al., 2008; Hanekom et al., 2007). Association between MTBc lineages and EPTB have been reported in some studies (Caws et al., 2008; Kong et al., 2007; Kong et al., 2005), such as an association between Lineage 3 and EPTB (Lari et al., 2009). On the other hand, Coscolla and Gagneux reviewed nearly one hundred published reports and failed to find a clear association between MTBc lineages and TB disease presentation (Coscolla and Gagneux, 2011).

In Southwest Ethiopia, there is limited information on the genetic diversity of circulating MTBc strains. Recently, a new phylogenetic lineage called Lineage 7 was reported by researchers in Woldiya, Northern Ethiopia (Tessema et al., 2013; Firdessa et al., 2013). Studies also described new Ethiopian specific families within Lineage 4, namely Ethiopia_2, Ethiopia_3 and Ethiopia_H_{37}Rv-like in multiple sites in Ethiopia (Biadglegne et al., 2015; Tessema et al., 2013; Ali et al., 2016). Bovine tuberculosis is an endemic disease in Ethiopia and a recognized problem in cattle with an overall prevalence ranging from 1% to 15% (Berg et al., 2009; Shitaye et al., 2007; Gumi et al., 2011; Romha et al., 2014) and reaches a high prevalence (up to 50%) in intensive dairy farms (Firdessa et al., 2012). It would seem plausible that zoonotic transmission of *M. bovis* could explain the high prevalence of TBLN. In the present study, we investigated the genetic diversity of MTBc isolates from TBLN patients and the role of newly described Ethiopian specific families and *M. bovis* in this disease presentation.

2. Materials and methods

2.1. Patients and bacterial isolates

A total of 436 consecutive patients presenting with lymph node swelling from Southwest Ethiopia were tested for TB. These patients were admitted to the Jimma University Specialized Hospital from April 2013 to February 2015. Basic demographic and clinical data were collected using a structured questionnaire. Fine needle aspirate (FNA) was collected from the swollen lymph node of all patients. The first few drops of the aspirates were used for cytomorphological diagnosis and acid fast smear microscopy. The remainder was processed for Xpert MTB/RIF® and culture.

Culture was performed in MGIT960 (Becton Dickinson, USA) and/or on Löwenstein-Jensen (L-J) medium after decontamination by *N*-acetyl-cysteine –NaOH solution at Jimma University-Mycobacteriology Research Center (JU-MRC) as previously described (http://www.finddx.org/wp-content/uplo, 2006). Primary differentiation of MTBc from non-tuberculous mycobacteria (NTM) was performed by Capilia TB-Neo (TAUNS Laboratories, Japan). Resistance to rifampicin (RIF) was determined by the Xpert MTB/RIF assay (Cepheid, USA) (Helb et al., 2010). For 25 patients, Xpert MTB/RIF was not performed due to insufficient sample volume or accidental loss of the sample sediment.

2.2. DNA extraction

Each positive liquid culture was subcultured on L-J medium. Two loops of colonies were collected from 4- to 6-weeks-old L-J slants and suspended in 400 µl TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0). DNA was extracted by heating the isolates at 80 °C for 60 min (Cadmus et al., 2006) and transported to the ITM, Belgium and Genoscreen, Lille, France for further analysis.

2.3. Spoligotyping

Spoligotyping was performed using primers (DRa and DRb) according to the procedure described by Kamerbeek et al. (1997). The amplified product was hybridized to a set of 43 immobilized oligonucleotides using an in house prepared membrane. Hybridized DNA was detected by the chemiluminescence method (Amersham Biosciences, Little Chalfont, UK) and exposure to an X-ray film (Hyperfilm ECL, Amersham Biosciences). *M. tuberculosis* H_{37}Rv and *M. bovis* BCG were used as positive controls in each run.

2.4. MIRU-VNTR typing

A total of 167 isolates were selected for further analysis by 15-loci MIRU-VNTR typing at Genoscreen, Lille, France (Supply et al., 2006). These included all isolates belonging to the ill-defined T family (n = 116), some of orphans and/or isolates with unusual spoligotype patterns (n = 34), 6 isolates with spoligotype patterns of Lineage 7, 1 Beijing strain and 10 rifampicin resistant strains as identified by Xpert MTB/RIF.

2.5. qPCR-SNP analysis

Single nucleotide polymorphism (SNP) analysis was carried out at ITM, Belgium, on a selected set of isolates (n = 32) representative of the different spoligotypes with their neighboring profile and 6 isolates with spoligotype patterns of Lineage 7. Lineage specific primers and probes were used as previously described (Stucki et al., 2012). TaqMan real-time PCR was performed according to standard protocols (Applied Biosystems, Carlsbad, USA).

2.6. Phylogenetic classification

Spoligotypes in binary format and MIRU-VNTR patterns in 15-digit codes were double entered in an Excel spreadsheet and uploaded on the *MIRU-VNTRplus* data base (www.miru-vntrplus.org). Spoligotypes common to more than one strain were assigned a shared international type number (SIT) according to the updated version of the international spoligotype database SpolDB4 (Brudey et al., 2006). Spoligotyping patterns and MIRU-VNTR 15-loci profiles were used to classify the strains into main phylogenetic lineages or families by using the reference strain collection available at *MIRU-VNTRplus* (Allix-Béguec et al., 2008). The strains were classified by the simple match approach that is based on the best match with strains of the reference data base (Allix-Béguec et al., 2008; Weniger et al., 2010) using a cut-off distance of 0.17. In addition, lineage-specific SNP analysis was deployed to assign isolates into major MTBc lineages for those that could not be classified with the above described strategy.

Lineage 7 isolates were defined based on spoligotype patterns, i.e. deletion of spacer 4–24 as previously reported by Firdessa et al. (2013) and high degree of MIRU-VNTR loci similarities with other Lineage 7 strains. Classification of the new Ethiopian specific families were primarily based on characteristic spoligotype patterns: Ethiopia_2 (absence of spacer 13), Ethiopia_3 (absence of spacer 10–19) and Ethiopia_H_{37}Rv-like (absence of spacer 33–36) (Biadglegne et al., 2015; Tessema et al., 2013). In addition, phylogenetic tree based identification was carried out to classify isolates into Ethiopia_2 and Ethiopia_H_{37}Rv-like. A cluster was defined as two or more isolates harboring identical spoligotyping and MIRU-VNTR profiles. The clustering rate was calculated using the following formula: $(n_c - c) / n$, where n_c is the total number of clustered isolates, c is the number of clusters, and n is the total number of isolates.

2.7. Ethical considerations

This study was reviewed and approved by Institutional Review

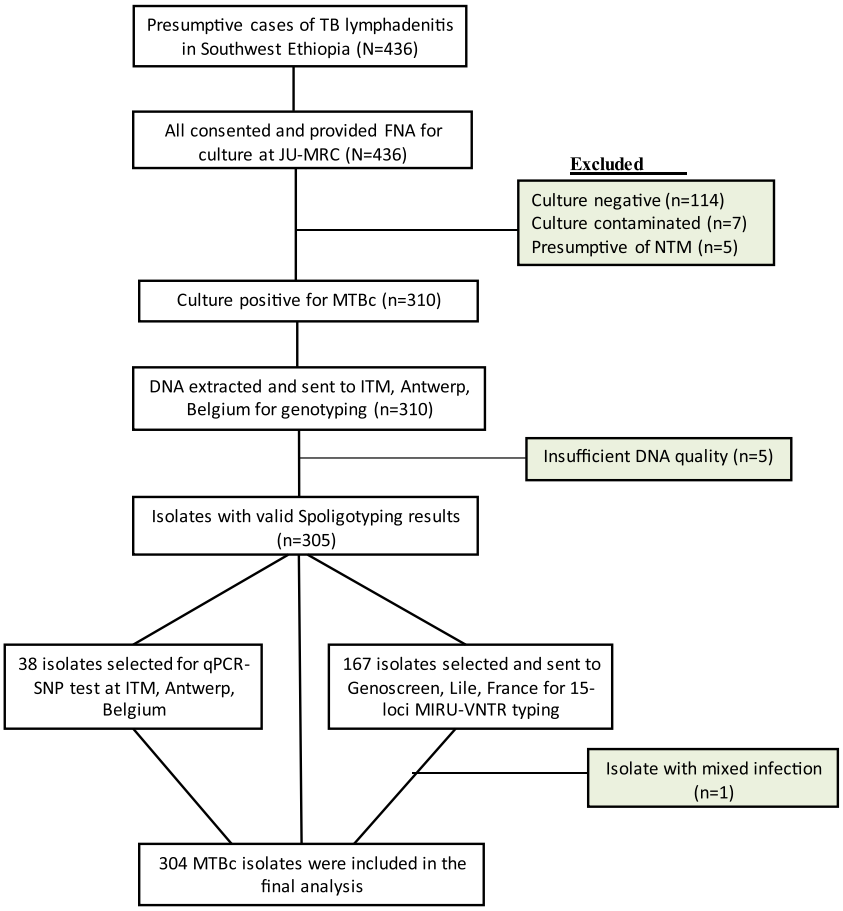


Fig. 1. Overall study flow; starting from study participant recruitment, isolation of bacteria and genotyping. FNA: fine needle aspirate, JU-MRC: Jimma University-Mycobacteriology Research Center, MTBc: *Mycobacterium tuberculosis* complex, ITM: Institute of Tropical Medicine, NTM: non-tuberculous mycobacteria, SNP: single nucleotide polymorphism.

Table 1
Demographic and clinical characteristics of TBLN patients in Southwest Ethiopia.

Patient characteristics	Total (N = 304)	Lineage 1 (n = 3)	Lineage 2 (n = 1)	Lineage 3 (n = 44)	Lineage 4 (n = 248)	Lineage 7 (n = 6)	*M. bovis* (n = 2)
Age							
< 15	33(10.9)	1(33.3)	0	5(11.4)	27(10.9)	0	0
15–30	166(54.6)	2(66.7)	0	24(54.5)	134(54.0)	5(83.3)	1(50.0)
31–60	93(30.6)	0	1(100)	12(27.3)	79(31.9)	0	1(50.0)
> 60	12(3.9)	0	0	3(6.8)	8(3.2)	1(16.7)	0
Gender							
Male	143(47)	2(66.7)	1(100)	21(47.7)	117(47.2)	2(33.3)	0
Female	161(53)	1(33.3)	0	23(52.3)	131(52.8)	4(66.7)	2(100)
HIV status							
Positive	24(7.9)	1(33.3)	0	4(9.1)	18(7.3)	0	1(50)
Negative	232(76.3)	2(66.7)	1(100)	33(75.0)	191(77.0)	4(66.7)	1(50)
Unknown	48(15.8)	0	0	7(15.9)	39(15.7)	2(33.3)	0
Site of LN swelling							
Cervical	233(76.6)	2(66.7)	1(100)	34(77.3)	189(76.2)	6(100)	1(50)
Axillary	47(15.5)	1(33.3)	0	9(20.5)	36(14.5)	0	1(50)
Inguinal	24(7.9)	0	0	1(2.3)	23(9.3)	0	0
FNA smear microscopy							
Positive	121(39.8)	1(33.3)	1(100)	24(54.5)	90(36.3)	3(50)	2(100)
Negative	183(60.2)	2(66.7)	0	20(45.5)	158(63.7)	3(50)	0
RIF resistance status							
Resistant	10(3.3)	0	0	8(18.2)	2(0.8)	0	0
Sensitive	269(88.5)	3(100)	1(100)	33(75.0)	224(90.3)	6(100)	2(100)
Not done	25(8.2)	0	0	3(6.8)	22(8.9)	0	0

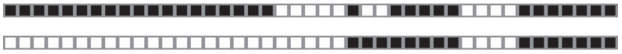
Key: numbers in bracket are reported in %. Abbreviations: TBLN = tuberculous lymphadenitis, LN = lymph node, FNA = fine needle aspirate, RIF = rifampicin.

Board (IRB) of Jimma University, Ethiopia (Ref. No. RPGC/510/2014) and ITM Institutional Review Board, Antwerp, Belgium (Ref. No. 986/15). For participants < 18 years of age, assent was obtained, as well as consent from their parent or legal representative.

3. Results

Of 436 presumptive TBLN cases who provided FNA samples, 310 were culture positive for MTBc (one isolate per patient). For 305 of these isolates, DNA extraction and spoligotyping were successful. One

Table 2
Spoligotyping patterns, SpolDB4 family and lineages/sublineages distributions of MTBC isolates in Southwest Ethiopia (N = 304).

SIT	Spoligotype pattern	Major lineage	Spoligo family (SpolDB4)	Lineage/sublineage (MIRU-VNTR*plus*)	N
665		bovis	BOVIS1	*M. bovis*	2
1		2	BEIJING	Beijing	1
142		3	CAS	Delhi/CAS	2
952		3	CAS	Delhi/CAS	1
25		3	CAS1_DELHI	Delhi/CAS	28
26		3	CAS1_DELHI	Delhi/CAS	3
247		3	CAS1_DELHI	Delhi/CAS	1
21		3	CAS1_KILI	Delhi/CAS	6
602		3	U	Unknown	1
46		4	U (likely H)	Unknown	5
50		4	H3	Haarlem	2
121		4	H3	Haarlem	1
134		4	H3	Haarlem	1
586		4	H3	Haarlem	1
699		4	H3	Haarlem	2
764		4	H3	Haarlem	1
36		4	H3-T3	Haarlem	2
35		4	H4	Haarlem	1
777		4	H4	Haarlem	14
262		4	H4	Haarlem	1
817		4	H4	Haarlem	6
1134		4	H4	Haarlem	1
41		4	LAM_TUR	TUR	1
4		4	LAM3	LAM	1
42		4	LAM9	LAM	1
53		4	T1	Ethiopia_H37Rv-like	23
53		4	T1	Ethiopia_2	2
1688		4	T1	LAM	1
122		4	T1	UgandaI	1
102		4	T1	Ethiopia_2	1
52		4	T2	UgandaI	2
584		4	T2	UgandaI	6
73		4	T2-T3	UgandaI	2
37		4	T3	Ethiopia_2	35
37		4	T3	Ethiopia_H37Rv-like	5
37		4	T3	S	3
504		4	T3	Ethiopia_2	1
149		4	T3_ETH	Ethiopia_3	31
345		4	T3_ETH	Ethiopia_3	1
40		4	T4	Ethiopia_H37Rv-like	2
231		4	T5	UgandaII	1
119		4	X1	X	2
217		4	X1	X	2
336		4	X1	X	1
910		7	U	Ethiopia_1 (Lineage7)	5
1729		7	U	Ethiopia_1 (Lineage7)	1

SIT	Spoligotype pattern	Major lineage	Spoligo family (SpolDB4)	Lineage/sublineage (MIRU-VNTR*plus*)	N
		1	Orphan	EAI	1
		1	Orphan	EAI	1
		1	Orphan	EAI	1
		3	Orphan	Delhi/CAS	1
		3	Orphan	Delhi/CAS	1
		3	Orphan	Delhi/CAS	1
		4	Orphan	Unknown	1
		4	Orphan	Haarlem	3
		4	Orphan	Haarlem	3
		4	Orphan	UgandaI	2
		4	Orphan	Unknown	1
		4	Orphan	Unknown	1
		4	Orphan	Ethiopia_3	1
		4	Orphan	Ethiopia_3	1
		4	Orphan	URAL	1
		4	Orphan	Haarlem	1
		4	Orphan	URAL	7
		4	Orphan	URAL	1
		4	Orphan	Unknown	1
		4	Orphan	Unknown	1
		4	Orphan	Unknown	1
		4	Orphan	Unknown	2
		4	Orphan	LAM	2
		4	Orphan	LAM	2
		4	Orphan	LAM	2
		4	Orphan	S	7
		4	Orphan	S	3
		4	Orphan	Unknown	1
		4	Orphan	S	5
		4	Orphan	S	1
		4	Orphan	Unknown	1
		4	Orphan	UgandaI	1
		4	Orphan	Haarlem	4
		4	Orphan	Unknown	2
		4	Orphan	URAL	1
		4	Orphan	TUR	1
		4	Orphan	Unknown	1
		4	Orphan	UgandaII	1
		4	Orphan	Unknown	1
		4	Orphan	Unknown	1
		4	Orphan	UgandaII	1
		4	Orphan	Unknown	2
		4	Orphan	Unknown	1
		4	Orphan	LAM	1
		4	Orphan	UgandaI	1
		4	Orphan	TUR	2
		4	Orphan	URAL	1
		4	Orphan	LAM	1
		4	Orphan	LAM	1
		4	Orphan	URAL	4
		4	Orphan	Haarlem	1
		4	Orphan	Haarlem	1
		4	Orphan	LAM	1

hundred sixty seven isolates with selected spoligotypes were further analyzed by MIRU-VNTR. One isolate displayed 2 alleles in six independent MIRU-VNTR loci, which suggested a mixed infection and was excluded from further analysis. Another two isolates had no PCR amplicon at one locus, and were treated as missing data at the respective loci in the final analysis. Thus, after excluding 5 isolates with insufficient DNA quality and one isolate with mixed infection, 304 isolates were included in the final analysis (Fig. 1).

3.1. Study participants' characteristics

Of 304 TBLN patients included in the analysis, 53% (161/304) were females (Table 1). The patients' mean age was 29 years (± 13 SD) with a range of 6–78 years. Twenty four (7.9%) TBLN patients were co-infected with HIV. The majority (76.6% (233/304)) of TBLN patients were presented with cervical lymphadenopathy. FNA smear microscopy was positive in only 121 (39.8%) of culture confirmed TB patients. Rifampicin susceptibility data were available for 279 (91.8%) of all strains and revealed 10 (3.6%) RIF-resistant cases.

3.2. Distribution of different MTBc lineages and families

The population structure showed that 248 (81.6%) isolates belonged to the Euro-American lineage (Lineage 4), with the ill-defined T and Haarlem as the largest families, comprising 116 (38.2%) and 43 (14.1%) isolates respectively. The second most predominant lineage was the Delhi/CAS lineage (Lineage 3) comprising 44 isolates (14.5%). Lineage 1 (EAI), *M. bovis* and Lineage 2 (Beijing) were represented by 3,

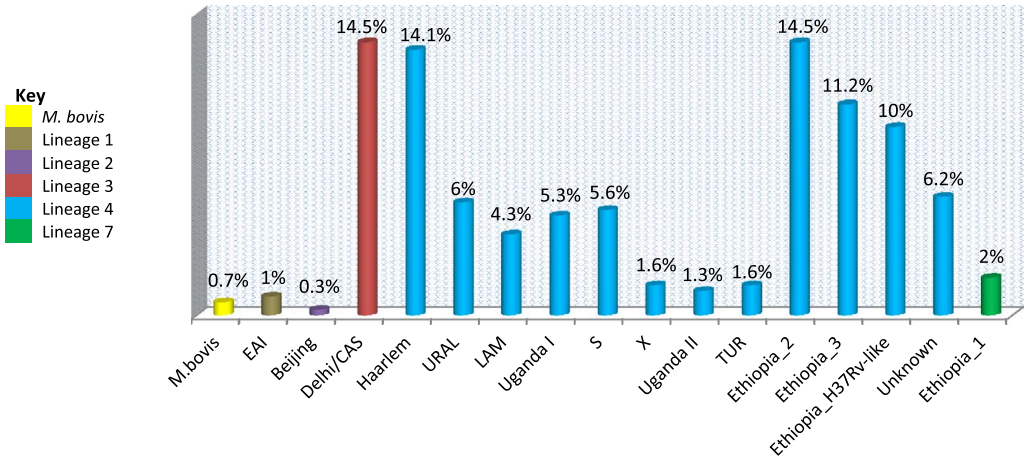


Fig. 2. Proportion of MTBc lineages/families identified from TBLN patients in Southwest Ethiopia (N = 304) based on spoligotyping, 15-loci MIRU-VNTR and qPCR-SNP analysis. L = lineage, EAI = East-African Indian, LAM = Latin American Mediterranean, CAS = Central Asian.

Table 3
Clustering rate analysis by spoligotyping and 15-loci MIRU-VNTR among selected MTBc isolates in Southwest Ethiopia (N = 166).

Genotyping methods	No. total of profiles	No. of isolates with unique profiles	No. of clusters	No. of isolates in cluster	Clustering rate (%)
Spoligotyping	43	28	15	138	74.1
MIRU-VNTR	108	84	24	82	34.9
Spoligotyping + MIRU-VNTR	117	95	22	71	29.5

2 and 1 isolates respectively. The two *M. bovis* isolates showed a typical bovine spoligotype patterns (SIT 665) lacking spacers 3–5, 9, 16, and 39–43, features that define strains of the African 1 clonal complex of *M. bovis*. Six isolates were identified as Lineage 7 (Ethiopia_1) (Table 2 and Fig. 2).

Spoligotype analysis revealed 96 different spoligotype patterns: 214 (71.4%) of 304 isolates were assigned to 42 shared types (SITs), whereas 90 (29.6%) isolates exhibited 54 patterns that did not match a SIT in the database (SpolDB4) and were termed orphans (Table 2). Among the poorly defined T-isolates, 93% (108/116) of the isolates were classified by MIRU-VNTR*plus* as being part of the newly described Ethiopian specific Lineage 4 families; namely Ethiopia_2 (n = 44), Ethiopia_3 (n = 34) and Ethiopia_H_{37}Rv-like (n = 30). Six of the 'unknown' (U) isolates (SIT910 and SIT1729) in the SpolDB4 were identified as Ethiopia_1 (Lineage 7) (Table 2). Nineteen (6.3%) isolates that could not be assigned to previously known or new phylogenetic MTBc families were assigned to the Euro-American lineage after qPCR-SNP analysis. The different lineages/families identified from TBLN patients in Southwest Ethiopia are depicted in Fig. 2.

3.3. Cluster analysis

By spoligotyping alone 247 (81.2%) of the 304 isolates were grouped into 49 clusters with each cluster consisting of 2 to 42 isolates. The largest cluster was observed for isolates belonging to the T3 (ST37, Ethiopia_2, n = 42 isolates), followed by strains belonging to T3_ETH (ST149, Ethiopia_3, n = 31 isolates), the third largest cluster by strains of the Dehli/CAS lineage (ST25, n = 28 isolates) and the fourth largest cluster by T1 (ST53, Ethiopia_H_{37}Rv-like, n = 25 isolates).

MIRU-VNTR typing of the selected 166 isolates showed highly diverse patterns. One hundred and eight distinct patterns were detected in this collection, including 24 cluster patterns and 84 unique patterns. The highest discrimination was achieved when combining spoligotyping and MIRU-VNTR with the overall clustering rate of 29.5% (Table 3). Seventy one of the 166 isolates were grouped into 24 clusters, each cluster comprising 2–17 isolates. MIRU-VNTR analysis was highly discriminatory for the ill-defined T3 families: 42 isolates of T3 (SIT37 and SIT504) were split into 33 different patterns i.e. five clusters comprising 14 isolates with maximum 4 isolates per cluster. Similarly, MIRU-VNTR typing of the 24 T1 (SIT53 and SIT102) isolates showed that 17 isolates produced seven clusters (maximum of 5 isolate per cluster) whereas 7 isolates had unique MIRU types. Unlike T3 (Ethiopia_2) isolates, Ethiopia_3 (T3_ETH) isolates were highly clonal and less differentiated by MIRU-VNTR typing. The largest cluster (MLVA MtbC 15–9 type 594-?) containing 17 isolates was formed by Ethiopia_3 (Fig. 3).

Of ten RIF-resistant isolates that were further typed by MIRU-VNTR, 4 (40%) had unique MIRU-VNTR patterns and the remaining 6 (60%) were clustered into two i.e. one cluster containing 4 isolates (identical spoligotype and MIRU-VNTR patterns) and the other cluster 2 isolates. The majority (80%, 8/10) of RIF-resistant isolates were belonged to the Lineage 3 (Delhi/CAS) (Fig. 4). Although the numbers are small, the risk of having RIF-resistance was 21.6 fold (95% CI, 4.4–106, *p*-value = 0.001) higher among patients with a Delhi/CAS strains compared to patients with the other. Interestingly, all RIF-resistant isolates harbored mutation at probe E binding site of *rpoB* gene (codon 447–452) as determined by Xpert MTB/RIF test.

4. Discussion

The high rate of TBLN in Southwest Ethiopia is not explained by bovine TB transmission, and the majority is caused by Ethiopian specific families in Lineage 4 of the MTBc. The MTBc lineage distribution from TBLN patients in our study is similar to the distribution of strains documented previously from both pulmonary and TBLN patients in different regions of Ethiopia (Berg et al., 2015; Firdessa et al., 2013), reflecting the absence of pathogen-specific genetic factors associated with the high rate of TBLN in Ethiopia. Our finding is also in line with the report from Coscolla & Gangeux (Coscolla and Gagneux, 2011) who found no clear association between genotypic variation and clinical phenotypes. Ethiopian specific families within the Lineage 4 also found to cause TBLN in Northern Ethiopia (Biadglegne et al., 2015), where Lineage 7 is more common. It is of note that in our dataset some 'typical' spoligotype profiles of Ethiopia_2 and Ethiopia_H_{37}Rv-like were interspersed on the phylogenetic tree when combining spoligotyping & MIRU-VNTR (Fig. 3). Considering spoligotyping alone, Ethiopia_2 (T3) isolates differed from Ethiopia_H_{37}Rv-like (T1) because the former lacks spacer 13. Isolates of Ethiopia_2 and Ethiopia_H_{37}Rv-like were genetically diverse and some of them were ambiguously classified even after MIRU-VNTR 15-loci analysis.

Lineage 3 (Delhi/CAS) is the other dominant lineage in this study.

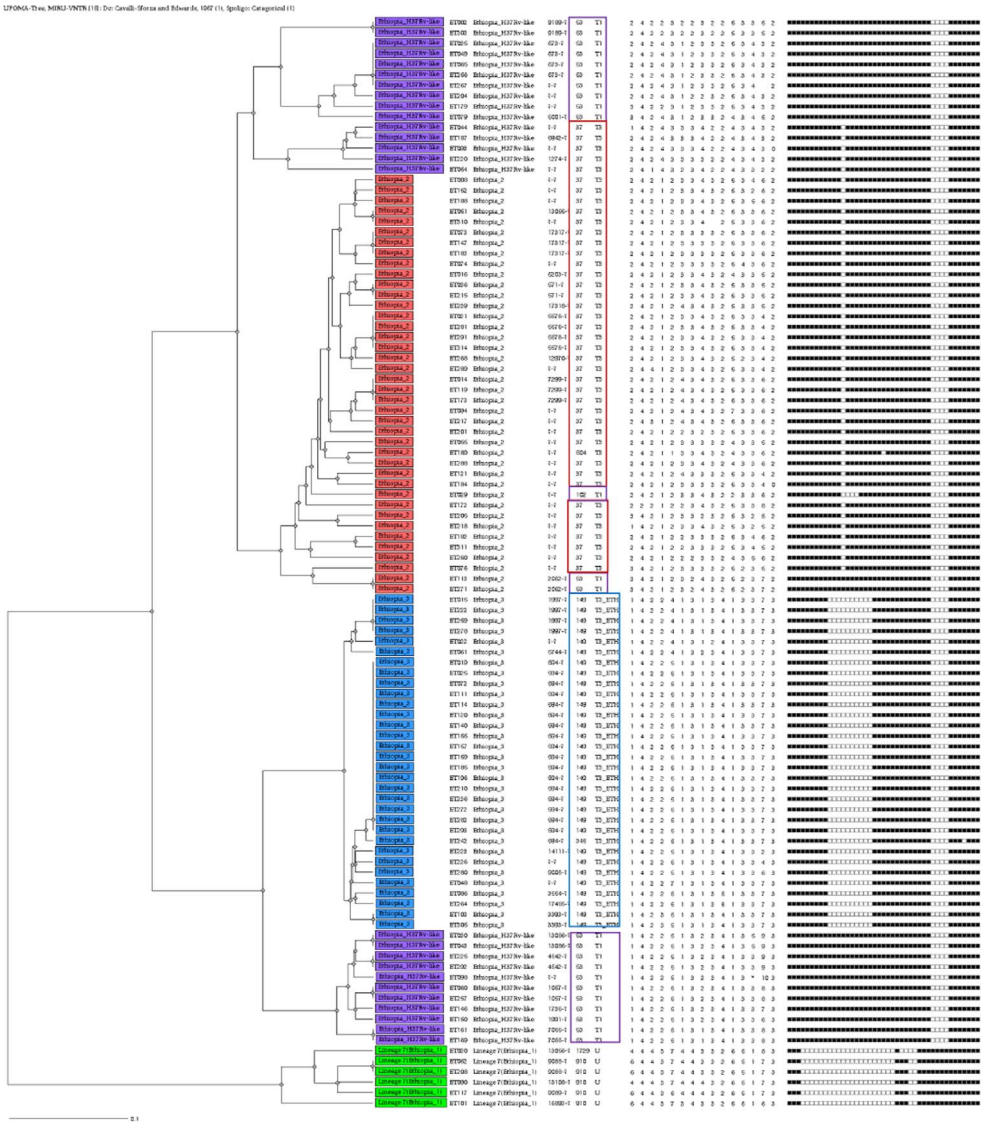


Fig. 3. UPGMA tree of selected Ethiopian specific lineages (n = 104) (41 ST37, 31 ST149, 25 ST53, 5 ST910 and 1 each from ST345, ST102, ST504 and ST1729). The tree was constructed based on spoligotyping and MIRU-VNTR results using MIRU-VNTR*plus* website. The color indicates the phylogenetic lineage/family to which Ethiopian specific MTBc lineages/families belong. Ethiopia_3 is homogeneously constituted by T3_ETH genotypes; Ethiopia_2 contains genotypes from T3 and T1; Ethiopia_H_{37}Rv-like contains genotypes from T1 and T3; Lineage 7 (Ethiopia_1) contains genotypes from U.

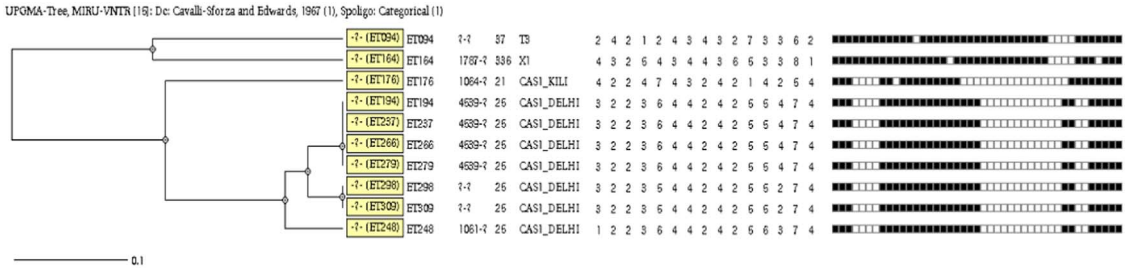


Fig. 4. UPGMA dendrogram of rifampicin resistant MTBc isolates from TBLN patients (N = 10). The tree was constructed based on spoligotyping and MIRU-VNTR results using MIRU-VNTR*plus* website. Rifampicin-resistance was determined by the Xpert MTB/RIF assay directly on the specimen or decontaminated sediment.

Previous studies (Biadglegne et al., 2015; Tessema et al., 2013; Ali et al., 2016) also confirmed the presence of Delhi/CAS across the different regions of Ethiopia, indicating the successful spread of these strains through human movement, either from Central Asia to Ethiopia or the other way around, which would be in agreement with the "out of Africa" theory postulated by Gagneux and colleagues (Comas et al., 2013). This hypothesis could be tested by genome sequencing of Asian and Ethiopian Lineage 3 isolates. In contrast to North Ethiopia where Delhi/CAS is the most prevalent lineage (Biadglegne et al., 2015; Tessema et al., 2013), a shift towards Ethiopian specific Lineage 4 strains was observed in our study. Interestingly, Lineage 3 (Delhi/CAS) was found to be significantly associated with infection of rifampicin resistant *M. tuberculosis*. As shown in Fig. 4, six of the eight RIF-resistant -Lineage 3 isolates formed two clusters (the first cluster with 4 isolate and the second with 2 isolates) and shared their RIF-resistance conferring mutations at *rpoB* gene (codon 447–452), thus possibly suggesting recent transmission of RIF-resistant strains.

We identified Lineage 7 in six (2%) TBLN patients in Southwest Ethiopia, similar to the 2% observed among pulmonary TB patients from South and East Ethiopia, but lower than the 8–10% reported among both pulmonary and TBLN patients in the North Eastern highlands of Ethiopia (Biadglegne et al., 2015; Tessema et al., 2013;

Firdessa et al., 2013). Lineage 7 has previously been reported in Ethiopia (Firdessa et al., 2013; Yimer et al., 2016) and among Ethiopian immigrants in Djibouti (Blouin et al., 2012), yet not elsewhere. Like *M. africanum* (lineage 5 and 6) that is localized in West Africa, Lineage 7 is limited to the Horn of Africa. The restricted geographic distribution suggests Lineage 7 strains are not as successful as modern lineages, requiring particular conditions that limit their spread. Possible explanations for this geographical restriction could be that Lineage 7 has a lower rate of progression to disease relative to other lineages (Yimer et al., 2015), with subsequent out competition by other MTBc lineages, or Lineage 7 strains have a host preference for ethnically Ethiopian people. In our study, we could not obtain detailed information on the ethnic background of the participants. Phylogenetic tree analysis clearly showed that Lineage 7 strains were located far from the recently described Ethiopian clades (Supplementary Fig. 1) and is of considerable evolutionary interest because it represents a phylogenetic branch intermediate between the ancient and modern lineages of *M. tuberculosis* (Fig. 3). However, further investigation of Lineage 7 is warranted to explain the virulence factors, pathogenesis and clinical presentations of TB disease.

A report by Kidane et al. hypothesis that the exceptionally high incidence of TBLN in Ethiopia could be due to zoonotic transmission of *M. bovis* from cattle (Kidane et al., 2002). In our study, *M. bovis* was identified only in two (0.7%) TBLN patients, similar to findings of another recent study in Ethiopia where 0.4% *M. bovis* was reported among pulmonary TB patients and none among TBLN patients (Firdessa et al., 2013). Our data indicate that the overall contribution of *M. bovis* to human TBLN is minor in Southwest Ethiopia. In general, declining rates of *M. bovis* isolation from human TB patients have been reported in Ethiopia, despite continued consumption of unpasteurized milk and milk products, which is thought to have a high risk for human infection with *M. bovis* in a setting with a high prevalence of bovine tuberculosis.

The overall clustering rate in this study was found to be 29.5%. This is in agreement with previous reports from Ethiopia in TBLN (Biadglegne et al., 2015) and pulmonary TB patients (Ali et al., 2016) that showed clustering rates of 35% and 31.2% respectively. The cluster rate in our study was significantly lower than the rate reported in Northwestern Ethiopia from pulmonary TB patients (45%) (Tessema et al., 2013), which could be due to the fact that TBLN patients are thought to be non-infectious, although the selection of isolates for MIRU-VNTR likely introduced bias in the clustering estimate. However, even in TBLN patients, the clustering remains very high for Ethiopia_3 isolates which is in agreement with another study in Northern Ethiopia (Biadglegne et al., 2015). Seventeen of our Ethiopia_3 isolates were clustered (identical spoligotype and 15-loci MIRU-VNTR profiles) with ten Ethiopia_3 isolates from Northern Ethiopia (Biadglegne et al., 2015). This indicates that Ethiopia_3 strains remain the predominant source of the most recent infection for TBLN cases, though information about possible epidemiological links between patients with clustered isolates was not available.

This study has some major limitations. First, MIRU-VNTR analysis was restricted to a selected set of MTBc isolates and 15-loci due to financial constraints, limiting the discriminatory power. Secondly, our study only included clinical cases visiting Jimma University Specialized Hospital with potential bias towards the overall TB population of this region, and epidemiological investigations of contacts, particularly among patients with clustered isolates, could not be performed. Nonetheless, our study provides valuable information on the etiology of TBLN in Southwest Ethiopia.

5. Conclusions

This study revealed a high diversity of circulating MTBc lineages responsible for TBLN in Southwest Ethiopia. The Ethiopian sub-lineages are the most dominant in TBLN patients followed by Delhi/CAS and Haarlem, yet disease presentation does not seem to be linked to the lineage type. We reported the first presence of Lineage 7 among TBLN patients in Southwest Ethiopia. Zoonotic transmission of *M. bovis* infection has been excluded as a major factor in TBLN. A more detailed genetic analysis by whole genome sequencing is warranted to clearly define the genetic variants of the poorly defined locally contained Ethiopian families.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.meegid.2017.09.016.

Conflict of interests

We authors declared that we have no competing interests.

Authors' contributions

Conceived and designed the experiments: MT, GA, LR. Performed the experiments: MT, PR, GA. Supervised the experiments: GA, LR. Contributed reagents and materials: LR, BDJ. Recruited study participants and collected clinical data: AB, MB. Analyzed data: MT, LR, CM, BDJ. Wrote the initial draft of the manuscript: MT, LR. All authors read and approved the final draft of the manuscript.

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