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Molecular typing of mycobacteria isolated from tuberculous lymphadenitis cases in Addis Ababa, Ethiopia

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SUMMARY

BACKGROUND—In Ethiopia, one of the world's 22 high tuberculosis (TB) burden countries, one third of the tuberculosis (TB) cases are attributed to tuberculosis lymphadenitis (TBLN). However, information on the molecular type of the mycobacterial species and strains that cause TBLN in the country is scarce.

OBJECTIVE—To identify the species and strains of mycobacteria that cause TBLN in Ethiopia.

METHODS AND RESULTS—A cross-sectional study was conducted among 206 presumed TBLN cases to characterise positive culture isolates. *Mycobacterium tuberculosis* complex species and strains were identified using region of difference 9 deletion and SITVIT WEB, respectively. Of the 206 fine-needle aspirate samples collected, 74 (36%) were culture-positive: 73 (98.6%) of the isolates were *M. tuberculosis*, and the remaining 1.4% were *M. bovis*. Further characterisation of the 73 *M. tuberculosis* isolates led to 26 distinct spoligotype international types (SITs) and 13 newly identified patterns. The most prevalent strains were SIT149, SIT53, SIT26 and SIT37 of sublineages T3-ETH, T1, CASI-DELHI and T3, respectively; these accounted for 52.6% of the total number of strains.

CONCLUSION—TBLN was mainly caused by *M. tuberculosis* and highly clustered strains SIT149, SIT53, SIT26 and SIT37 of sublineages T3-ETH, T1, CASI-DELHI and T3, respectively.

Keywords

TBLN; FNA; recent transmission; Addis Ababa

TUBERCULOSIS (TB) REMAINS A MAJOR global health problem. In 2013, an estimated 9.0 million people worldwide developed TB and 1.5 million died from the disease.¹ The annual incidence of the disease in sub-Saharan African countries has been high since the

emergence of the human immunodeficiency virus (HIV) epidemic; 80% of TB cases occur in the 22 countries with the highest TB burden.¹ Ethiopia is one of these 22 countries.

The number of extra-pulmonary TB (EPTB) cases among the total number of new TB cases notified in Ethiopia was 42 353 (32.2%), of which more than 80% were related to tuberculous lymphadenitis (TBLN).¹ The situation has been aggravated by a high burden of multidrug-resistant TB and the endemicity of HIV in the country.² However, information about the genotypic characteristics of *Mycobacterium tuberculosis* complex (MTC) species and strains is limited.³

The scarcity of ample molecular epidemiological data from most developing countries limits our understanding of the natural history of the disease.^{4,5} Molecular typing of MTC has contributed significantly to the understanding of phylogenetic characteristics of the organism, which in turn gives new insight by providing pertinent information on the transmission dynamics of tuberculous disease.^{6,7} Recent molecular epidemiological studies conducted in central Ethiopia have shown a diversity of pulmonary TB (PTB) strains;^{8,9} however, there is less information on the molecular typing of EPTB.

The present study aimed to provide information on the genotypic diversity of TBLN cases in the capital city of Ethiopia, Addis Ababa.

METHODS AND MATERIALS

Patient recruitment and study settings

A cross-sectional study was conducted from February to October 2013 in Addis Ababa, Ethiopia, at Tikur Anbessa Specialized Hospital (TASH) and Alem Tena Higher Clinic (ATHC). A total of 206 individuals with suspected TBLN who underwent fine-needle aspiration (FNA) were included in the study. Demographic data from all patients were collected using a pre-structured questionnaire by trained clinical nurses. Patients on anti-tuberculosis treatment aged <18 years at the time of sample collection were excluded.

Sample collection and processing

Written informed consent was provided by all patients before sample collection. Briefly, FNA was performed on a swollen superficial lymph node using a sterile 21-gauge needle with an attached syringe. The overlying area was cleaned with 70% alcohol. The node was then punctured by developing a negative pressure in the syringe. Multiple in and out passes were made with the needle without exiting the node. Approximately 1–2 ml of the aspirate were collected and drops of the aspirate were placed on a clean slide for FNA cytology. The remainder was transferred into a Falcon tube containing sterile normal saline for further microbiological and molecular analysis at the Aklilu Lemma Institute of Pathobiology (ALIPB) Laboratory of Mycobacteriology, Addis Ababa, Ethiopia.

Mycobacteriological culture

At ALIPB, the FNA samples were processed using the standard *N*-acetyl L-cysteine–sodium hydroxide (NALC-NaOH) method.¹⁰ Briefly, FNA specimens were homogenised and mixed with 0.85% normal saline and decontaminated by shaking in an equal volume of 4% NaOH

for 15 min at room temperature. The specimens were then subjected to centrifugation at 3000 rpm for 15 min. After homogenisation, 100 µl of the sediment was inoculated into conventional Löwenstein-Jensen egg slant medium containing 0.6% sodium pyruvate. The culture was then incubated at 37°C in slant position for 1 week and in upright position for 4–8 weeks. Microscopic examination of the colonies was performed using Ziehl-Neelsen (ZN) staining to select acid-fast bacilli-positive isolates. Heat-killed cells were prepared by mixing two loopfuls of colonies in 200 µl distilled water and heating at 80°C for 1 h. The heat-killed cells were used for molecular characterisation.

Region of difference 9-based deletion typing using polymerase chain reaction

Mycobacterial species that cause TBLN were identified using polymerase chain reaction (PCR) based region of difference (RD9) and RD4 deletion typing, as described elsewhere¹¹ using RD9_FlankF, IntR and FlankR and RD4 flankF, IntR and flankR primers (the sequences have been published elsewhere).¹² PCR was performed using a standard thermocycler (VWR Thermo Cycler; VWR International, Lutterworth, UK). The RD9-based PCR reaction mixture consisted of 10 µl Hot-StarTaqMaster Mix (Qiagen, Crawley, UK), 7.1 µl Qiagen water, 0.3 µl of each of the three primers (100 mM) and 2 µl DNA template, giving a total volume of 20 µl. *M. tuberculosis* H37Rv and *M. bovis*, and Qiagen water were used respectively as positive and negative controls. The reaction mixture was then heated with the VWR Thermo Cycler PCR using the following amplification programme: 95°C for 10 min for enzyme activation, 95°C for 1 min for denaturation, 61°C for 0.5 min for annealing, 72°C for 2 min for extension, involving 35 cycles in all, with final extension at 72°C for 10 min. The product was electrophoresed using the Agarose Gel Electrophoresis System (Bio-Rad, Hemel Hempstead, UK) in 1.5% agarose gel in 1× ethylenediaminetetraacetic acid running buffer. Ethidium bromide at a ratio of 1:10, 100 base-pair DNA ladder and orange 6× loading dye were used in gel electrophoresis. The gel was visualised in a MultiImage™ Light Cabinet, version 1.2.0.1 (Alpha Innotech Corporation, Cannock, UK).

Spoligotyping

Spoligotyping was carried out using the commercially available kit from Ocimum Biosolutions (Isselstein, The Netherlands) based on the standard method described earlier.¹³ Briefly, the direct-repeat (DR) region was amplified with primers DRa and DRb; the amplified DNA was then denatured and the single-stranded DNA was hybridised with 43-spacer oligonucleotides covalently bound to a membrane. To perform spoligotyping, procedures were followed as previously published.¹¹ DNA from *M. bovis* bacille Calmette-Guérin and *M. tuberculosis* H37Rv were used as positive controls, while Qiagen water (Qiagen) was used as a negative control. The presence of spacers was visualised on film as black squares; the absence of spacers was indicated by white squares following incubation with streptavidin-peroxidase and detected using enhanced chemoluminescence system detection liquid (Amersham, Little Chalfont, UK). Lineages and sublineages were designated based on SPOTCLUST at http://tbinsight.cs.rpi.edu/run_spotclust.html (accessed November 2013), and the spoligotype patterns of each strain were prepared in binary and octal format and entered into the international spoligotyping database recently released by SITVIT WEB¹⁴ for detecting spoligotype international type (SIT) numbers. Strains for

which SIT numbers could not be recovered from the database were considered orphans. All steps involving sample processing and PCR were carried out in a separate PCR mix room using dedicated pipettes and autoclaved tips to prevent cross-contamination. To ensure quality control, recent standard operating procedures for each step were followed.

Statistical analysis

All demographic and laboratory data were entered, cleared and analysed using Statistical Package for the Social Sciences, version 20 (IBM Corp, Armonk, NY, USA). Categorical data were analysed using the χ^2 test or the Fisher's exact test when the expected cell size was > 5 . $P < 0.05$ was considered statistically significant.

Ethical considerations

Ethical clearance was obtained from the ethical and review committee of the Department of Microbiology, Immunology and Parasitology, College of Health Science, Addis Ababa University, Addis Ababa, Ethiopia.

RESULTS

Sociodemographic characteristics of the study subjects

Of 206 patients with clinically suspected TBLN included in the study, 74 (36%) were culture-positive; the isolates were confirmed using ZN staining. No culture contamination was observed in the study. The study participants' ages ranged from 18 to 71 years (mean $31.4 \pm$ standard deviation 12.2). The study sample had a male to female ratio of 1:1.2 (94 [45.6%] male and 112 [54.4%] female).

Mycobacteriological culture and identification of *M. tuberculosis* complex species

Of the 206 samples, mycobacterial growth was observed in 74 (36%) after 8 weeks' incubation. According to the species identification protocol, no non-tuberculous mycobacteria were reported. MTC species were identified based on RD9 deletion typing. Nearly all (98.6%) of the culture-positive isolates were *M. tuberculosis* (Figure 1), with only 1.4% confirmed as *M. bovis*.

Demographic characteristics and phylogenetic lineage diversity

Certain sublineages were associated with specific demographic characteristics. The T3-ETH sublineage was predominantly found among female rural residents in the 18–27 years age group, although this was not statistically significant ($P > 0.05$); other T sublineages were dominant among urban residents. Interestingly, this finding shows a different distribution of sublineages depending on history of previous anti-tuberculosis treatment, as shown in Appendix Table A.*

*The appendix is available in the online version of this article, at <http://www.ingentaconnect.com/content/iuatld/ijtld/2016/00000020/00000011/art00019>

Genetic diversity and cluster analysis of *M. tuberculosis* strains

The *M. tuberculosis* strains from our TBLN patients were highly diverse and comprised nine different genotypes, together with two dominant lineages, T and Central Asian CAS. According to the database programme at SPOTCLUST, 40 (54.1%) of the analysed isolates belonged to the T sublineage, with the following distribution: T3-ETH 14 (34.1%), T1 19 (47.5%), T3 6 (14.6%) and T4 1 (2.4%). The remaining 12 (16.2%) belonged to CAS sublineages, with 7 (58.3%) CAS1-DELHI, 2 (16.7%) CAS1-KILI and 3/12 (25%) CAS. The minor sublineages included in this study were F33 ($n = 7$, 9.5%), Haarlem ($n = 7$, 9.5%) and other sublineages. One isolate was identified as *M. bovis* and confirmed as SB1176, with a spoligotype profile lacking spacers 3–7, 9, 16, 24–26, 28–37 and 39–43 (www.Mbovis.org), accounting for 1.4% of the isolates (Figure 2).

Among the 74 isolates typed, 57 (77%) were classified into one of the 26 SITs according to the SITVIT WEB database. The remaining 16/74 (21.6%) isolates were classified into 13 different spoligotype patterns that were not reported in the database. Cluster analysis based on a composite data of spoligotyping patterns showed that 45 isolates were grouped into 11 clusters; as one cluster consisted of 2–14 isolates, the clustering rate was 60.8%. Of the 11 clusters, three clusters, comprising two isolates each, were new to the database (Figure 2). The remaining 18 patterns with a unique (non-clustered) spoligotype pattern were represented as singles in the database. Euro-American lineages formed the largest cluster rate (63.3%) among the total strains. The largest number of isolates identified within these clusters were in SIT149 of sublineage T3-ETH (14 isolates), followed by SIT53 of sublineage T1 (7 isolates) and SIT26 of sublineage CAS1-DELHI (5 isolates) (Table).

DISCUSSION

The low rate of cases culture-positive for TBLN ($n = 74$, 36%) identified in the study is in agreement with the 10–60% reported elsewhere.^{15–17} Several reasons could be responsible for these low rates: previous treatment with one or more antibiotic drugs, for example, could suppress and inhibit bacterial growth. In addition, the paucibacillary nature of the FNA sample and the decontamination process may have led to the suppression of growth.^{18,19}

In the present study, MTC species were isolated from TBLN cases and further identified at the species and strain levels using RD9 deletion-based PCR and spoligotyping, respectively. In line with earlier findings, 98.6% of TBLN cases were *M. tuberculosis*.^{20–22} This strain of *M. bovis* (SB1176) showed typical bovine TB, with octal number 602773761000200 and spoligotype profile lacking spacers 3–7, 9, 16, 24–26, 28–37 and 39–43. This typical form of bovine TB was first identified in a state cattle farm in Holeta District, near Addis Ababa, Ethiopia.²³ This strain typical of *M. bovis* was isolated from human samples around Sabeta, located near Holeta.

Spoligotyping was used as the primary tool for typing MTC strains because of its ease of use and straightforward coding of the database.²⁴ It was thus used to elucidate the MTC strain diversity among clinical isolates from TBLN patients and compare with the strains in the database. The genotype structure in this study was similar to that previously found in PTB isolates from the same area,⁸ and the phylogenetic structure of MTC strains among TBLN

patients resembles that observed in samples from PTB patients in Ethiopia reported earlier.²⁵ This supports the hypothesis that EPTB and PTB are caused by the same type of strains.

SIT149 strains of the T3-ETH genotype were most common among TBLN patients from rural areas. The rate of cases identified in this study ($n=14$, 19%) is in agreement with studies conducted elsewhere in Ethiopia, with reported rates ranging from 9.5% to 22.9%, although place of residence was not recorded. A long descendant branch of spoligotypes belonged to the dominant 'ill-defined' T sublineage (51.3%); this is in line with studies conducted elsewhere in Ethiopia, with rates ranging from 35.9% to 54.2%,^{8,26–28} and indicates the ongoing evolution and successful transmission of these strains in this area. Moreover, the high frequency of this strain in Denmark among Ethiopian immigrants¹⁴ and the lower frequency in other African countries²⁹ suggest that this clade might have biogeographic specificity to Ethiopia. In general, the overall dominance of the T genotype resembles the distribution pattern in Horn of Africa countries, suggesting a similar trend of transmission.³⁰

The second most prevalent sublineage was CAS, with the dominant CAS1-DELHI genotype (58.3%); this is in agreement with a study conducted in the central and Afar pastoral regions of Ethiopia.^{8,28} However, the present study differed from the study conducted in northern Ethiopia among TBLN and PTB cases, which was predominantly associated with the Delhi/CAS genotype.^{31,32} Similarly, a previously published study from Sudan showed that *M. tuberculosis* Delhi/CAS was the predominant (49%) lineage among the strains investigated.³³

CAS and CAS1 subclades are essentially localised in Central Asia, more specifically in India, the Indian subcontinent and Pakistan.³⁰ Two hypotheses could support this evidence: as mentioned above, this genotype is highly prevalent in Central Asia because of its biogeographic specificity to this part of the world, and it has been suggested that this family might have been transmitted by trade, tourism or migration from India to other parts of world such as Ethiopia.³¹ There is evidence of genotypic linkage of strains isolated from patients from a distinctive geographical region, regardless of whether the patients acquired the infection from their current locality or had experienced reactivation of disease from their native country. This suggests that *M. tuberculosis* lineages may adapt to particular genetic, cultural or environmental characteristics of the host.³⁴

In line with similar findings from previous studies conducted in Ethiopia, the Haarlem genotype was observed in 9.5% of study isolates.^{8,9} It is common in central America, Europe and the Caribbean, and has also been reported in Kenya, Malawi, Mozambique, Tanzania and West Africa.³⁰

The overall cluster rate was 60.8%, which indicates recent transmission, in line with other reports that exclusively used spoligotyping data similar to the data used in other studies.^{8,28} The high TB incidence and the increased drug resistance rates indicate an ineffective TB control programme.^{1,2} The increased worldwide prevalence of the Euro-American lineage³⁵ is a possible reason for the high clustering rate in the present study.

In conclusion, in this study TBLN was caused mainly by *M. tuberculosis*, with a high clustering rate of strains of SIT149 of sublineage T3-ETH, SIT53 of sublineage T1, SIT26 of sublineage CASI-DELHI and SIT37 of sublineage T3. The presence of these species, dominant among TBLN patients in the study area, indicates active transmission of the disease and an ineffective TB control programme in the study area.

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Competing interests: none declared.

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APPENDIX

Table A

Selected sociodemographic characteristics of study subjects by different phylogenetic sublineages of *M. tuberculosis* strains, Addis Ababa, Ethiopia

Variables	Sublineages*									
	T3-ETH	P value	Other T [†]	P value	CAS [‡]	P value	H	P value	Others [§]	P value
Sex		0.232		0.619		0.359		0.06		0.359
Male	5 (6.8)		13 (17.6)		8 (10.8)		3 (4.1)		8 (10.8)	
Female	9 (12.2)		11 (14.9)		5 (6.8)		7 (9.5)		5 (6.8)	
Age, years		0.678		0.087		0.570		0.538		0.323
18–27	8 (10.8)		12 (16.2)		6 (8.1)		6 (8.1)		7 (9.5)	
28–37	3 (4.1)		4 (5.4)		2 (2.7)		2 (2.7)		4 (5.4)	
38–47	1 (1.4)		2 (2.7)		3 (4.1)		2 (2.7)		2 (2.7)	
48–57	0 (0)		3 (4.1)		0 (0)		0 (0)		0 (0)	
58	2 (2.7)		3 (4.1)		2 (2.7)		0 (0)		0 (0)	
Place of residence		0.373		0.456		0.541		1.00		0.541
Urban	5 (6.8)		14 (19)		5 (6.8)		5 (6.8)		8 (10.8)	
Rural	9 (12.2)		10 (13.5)		8 (10.8)		5 (6.8)		5 (6.8)	
Educational level		0.166		0.262		0.858		0.268		0.793
Illiterate	6 (8.1)		6 (8.1)		4 (5.4)		3 (4.1)		3 (4.1)	
Primary	6 (8.1)		12 (16.2)		6 (8.1)		7 (9.5)		6 (8.1)	
Secondary	0 (0)		5 (6.8)		1 (1.4)		0 (0)		2 (2.7)	
Diploma	2 (2.7)		1 (1.4)		1 (1.4)		0 (0)		1 (1.4)	
Degree	0 (0)		0 (0)		1 (1.4)		0 (0)		1 (1.4)	
Marital status		0.839		0.337		0.187		0.689		0.462
Single	7 (9.5)		13 (17.6)		5 (6.8)		6 (8.1)		9 (12.2)	
Married	6 (8.1)		11 (14.9)		6 (8.1)		3 (4.1)		4 (5.4)	
Widowed	00 (0)		0 (0)		1 (1.4)		0 (0)		0 (0)	
Divorced	1 (1.4)		0 (0)		1 (1.4)		1 (1.4)		0 (0)	
History of anti-tuberculosis treatment		0.826		0.682		0.614		0.236		0.614
Yes	2 (2.7)		5 (6.8)		1 (1.4)		3 (4.1)		1 (1.4)	
No	12 (16.2)		19 (25.7)		12 (16)		7 (9.5)		12 (16.2)	

* Designated according to the web-based SPOTCLUST programme.

[†] Includes T1, T2, T3 and T4.

[‡] Includes CAS1-DELHI, CAS1-KILI and CAS.

[§] Includes X1, Ural, F33, F36, LAM.

CAS = Central Asian, H = Haarlem; LAM = Latin-American Mediterranean.

Figure 1.

Electrophoretic pattern of the amplified element of polymerase chain reaction products from clinical samples in 1.5% agarose gel, February–October 2013, Addis Ababa, Ethiopia. Lane 1 = 1 kb ladder; Lane 2 = H37Rv (*M. tuberculosis* positive control), Lane 3= Qiagen H₂O (negative control); Lane 4 = *M. bovis* (positive control); Lanes 5–12 = clinical samples from culture isolate contained DNA from mycobacteria in *M. tuberculosis* complex.

Octal code	SIT	Sublineage	Lineage	n (%)
703677740003771	954	CAS	Unknown*	1 (1.4)
77777777760771	53	T1	EA	7 (9.5)
77777777777771	523	Family33	IO	3 (4.1)
777777777560771	462	T1	EA	2 (2.7)
703777740003171	25	CAS1-DELHI	Unknown*	1 (1.4)
777736777760771	217	T1	EA	1 (1.4)
777777774000771	1952	X1	IO	1 (1.4)
777000377760771	149	T3-ETH	EA	14 (18.9)
777777404760771	41	LAM-TUR	EA	1 (1.4)
700000007177771	910	Unknown*	IO	1 (1.4)
703777740003771	26	CAS1-DELHI	Unknown*	5 (6.8)
703377400001771	21	CAS1-KILI	Unknown*	2 (2.7)
777777774020771	47	H1	EA	1 (1.4)
777777777420731	817	H4	EA	2 (2.7)
777737777760771	37	T3	EA	4 (5.4)
703777700003171	2392	CAS	Unknown*	1 (1.4)
677777777720571	699	H3	EA	1 (1.4)
777777377760771	40	T4	EA	1 (1.4)
000000007720731	830	Family36	EA	1 (1.4)
777776777760731	336	X1	EA	1 (1.4)
703760000000771	2102	CAS	IO	1 (1.4)
777707777760771	2128	T1	EA	1 (1.4)
703777740003571	289	CAS1-DELHI	Un	1 (1.4)
703777740003471	247	CAS	Un	1 (1.4)
602773761000200	SB1176	<i>M. bovis</i>	<i>M. bovis</i>	1 (1.4)
777777777420771	777	H4	EA	1 (1.4)
777377777760771	1166	T1	EA	1 (1.4)
777737377720771	Orphan	Orphan	EA	2 (2.7)
777777776200771	Orphan	Orphan	EA	1 (1.4)
777776777760661	Orphan	Orphan	EA	1 (1.4)
7773577777420771	Orphan	Orphan	EA	1 (1.4)
772737407760771	Orphan	Orphan	EA	1 (1.4)
677767777720571	Orphan	Orphan	EA	1 (1.4)
575000077760671	Orphan	Orphan	EA	1 (1.4)
777000177760771	Orphan	Orphan	EA	1 (1.4)
777347777767671	Orphan	Orphan	IO	1 (1.4)
777347777763771	Orphan	Orphan	IO	1 (1.4)
776737677760771	Orphan	Orphan	EA	1 (1.4)
773777776000771	Orphan	Orphan	IO	2 (2.7)
777347777763671	Orphan	Orphan	IO	2 (2.7)

Figure 2.

Spoligotype patterns of *M. tuberculosis* and *M. bovis* strains, lineage and sublineage assignment of TBLN from clinical isolates according to the SITVIT WEB database and SPOTCLUST programme, respectively. *Patterns with signatures that do not belong to any of the major lineages described in the database. Unique strains matching a pre-existing pattern in the SITVIT WEB database are classified as SITs; those with no match were designated 'orphans'. SIT = spoligotype international type; CAS = Central Asian; EA = Euro-American; IO = Indo-Oceanic; LAM = Latin American-Mediterranean; H = Haarlem; TBLN = tuberculous lymphadenitis.

Table
Different phylogenetic lineages of *M. tuberculosis* strains within clusters, Addis Ababa, Ethiopia

Tuberculosis lymphadenitis						
Lineages*	Total isolates	Unique strains	Cluster strains	Cluster n	(%)	Cluster size
EA [‡]	49	18	31	6	(63.3)	14
						7
						4
						2
						2
IO [‡]	12	5	7	3	(58.3)	3
						2
						2
						2
						2
Unknown [§]	12	5	7	2	(58.3)	2
						2
						5
						2
						2

* Isolated lineages are named according to the SITVIT WEB database.
[‡] Includes LAM, Haarlem-type and F36.
[‡] Includes X1, F33 and Ural.
[§] Patterns with signatures that do not belong to any of the major lineages described in the database.
EA = Euro American; IO = Indo-Oceanic; LAM = Latin American Mediterranean.