

Identification and genotyping of the etiological agent of tuberculous lymphadenitis in Ethiopia

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Abstract

Background: In Ethiopia, little has been done to assess how *Mycobacterium bovis* has contributed to human tuberculosis, though the population routinely consumes unpasteurized milk and raw meat. The aim of this study was to determine the proportion of *M. tuberculosis* and *M. bovis* as etiological agents of tuberculous lymphadenitis (TBLN).

Methods: Patients with lymphadenopathy (n = 171) were included in a cross-sectional study at Butajira Hospital, Southern Ethiopia. Lymph node biopsies were cultured. Patients' HIV status was identified. DNA from positive cultures was tested by PCR to identify *M. bovis* and *M. tuberculosis*. Isolates were genotyped by multiplex ligation-dependent probe amplification (MLPA) assay.

Results: Among 171 patients, 156 had culture results. Of these, 107 (69%) were positive for *M. tuberculosis* complex (MTC). Six of the 10 HIV-positive patients were culture positive. *M. tuberculosis* specific sequences were identified in the DNA of each of 100 samples as assessed by RD10 targeted PCR, and each of the 95 isolates exhibited the *M. tuberculosis* specific TbD1 deletion by MLPA analysis. No *M. bovis* was identified. These results indicate that all the isolates were modern *M. tuberculosis* strains. Furthermore, MLPA studies confirmed that 42% of the isolates showed the Haarlem genotype and 12% displayed sequences compatible with INH resistance. No mutations conferring resistance to ethambutol or rifampicin were detected.

Conclusions: Our data showed that *M. tuberculosis* strains had common characteristics with strains causing pulmonary TB, which appears to be the main etiological agent of TBLN.

Key words: etiological agent, tuberculous lymphadenitis, Ethiopia

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Introduction

Tuberculosis (TB) is one of the most serious public health challenges in Ethiopia. According to a World Health Organization (WHO) report in 2008 [1], Ethiopia is number seven among the most highly affected countries on the TB-burden list. Primarily considered a pulmonary disease, TB can affect almost any organs. According to the National TB and Leprosy Control Program (NTLC) of Ethiopia [2], pulmonary TB (PTB) accounts for 63.4% of all TB cases and extra-pulmonary tuberculosis (EPTB) accounts for 36.6% of TB cases. In Ethiopia, 80% of EPTB is tuberculous lymphadenitis (TBLN) [2].

HIV has been identified as one of the most important factors contributing to an increase in the incidence of tuberculosis, particularly in Africa [1]. Treatment of co-infected patients is complicated by toxicities secondary to drug-drug interactions. HIV infection also makes TB diagnosis difficult, as the infection leads to treatment delays and hence increased morbidity and mortality rates. Drug-resistant TB is emerging as a significant problem as a result of inadequate treatment [1].

Among members of the *Mycobacterium tuberculosis* complex (MTC), *M. tuberculosis* and *M. bovis* have been implicated as the most common causes of tuberculous lymphadenitis (TBLN)

[3,4,5,6]. In Ethiopia, little has been done to identify how *M. bovis* causes human TB, despite the population's routine consumption of unpasteurized milk and raw meat. A skin testing study from Central Ethiopia reported 13.5% as the average prevalence of bovine TB in cattle and 22%, 12%, and 12% prevalence in Holstein, Zebu, and cross-bred cattle respectively [7]. Studies from Tanzania and Uganda report that *M. bovis* should be considered a pathogen of concern for people living in rural areas of Africa [8,9]. A study by Kidane *et al.* in 2002 [5] using DNA from fine needle aspirates (FNA) of TBLN patients from Butajira, southern Ethiopia reported that 17% of cases were caused by *M. bovis* and 83% by *M. tuberculosis*. However, previous studies report that the reduced yield of microorganism load compromises the interpretations of FNA in comparison to lymph node biopsies [10,11].

Molecular identification and characterization of MTC strains has seen several recent advances. Studies have shown that PCR-based assays are rapid, specific, and sensitive in differentiating species of MTC [12,13]. Parsons *et al.* in 2002 [12] reported that regions of differences (RD1 to RD16) variably deleted in other MTC members, but present in *M. tuberculosis*, could be used to differentiate species of MTC. However, PCR approaches performed directly on clinical material produces highly variable and inconsistent results [14]. Conversely, MLPA allows the simultaneous detection of multiple mutations in the genome in one assay by amplification of sequence-specific probes rather than target DNA [15]. Recently, MLPA has been adapted to characterize MTC members [16]. *M. tuberculosis* specificity is achieved by targeting 16S rRNA and IS6110 sequences. MLPA targets the most prevalent and clinically relevant drug resistance mutations as well as several markers specific for each principal genotypic group (PGG). In this study, positive cultures from biopsy were used as sources of mycobacterial DNA to identify the proportion of *M. bovis* and *M. tuberculosis* as the etiological agents of TBLN by using RD10 targeted PCR. Furthermore, isolates were analyzed by MLPA to identify the genotype of the strains and any possible drug resistance patterns that are prevalent in the area.

In the current study, we have readdressed the issue of mycobacterial identity in TBLN patients in Ethiopia by using lymph node biopsies rather than FNA aspirates to improve mycobacterial recovery, and MLPA to improve detection and specificity. In addition, we have utilized MLPA assay to ascertain

genotype and drug resistance profiles of *M. tuberculosis* isolates.

Materials and Methods

Study Area

Butajira, in the Meskenena Mareko district of Southern Ethiopia (135 km from Addis Ababa), has an estimated population of 331,832 and an estimated geographic area of 872.5 square kilometers [17].

Study Participants

Ethical approval was obtained from AHRI/ALERT Ethical Committee (Reg.No.001/05) and the National Ethical Review Committee (NERC), (RDHE/190-64/2005). The formula used to calculate the sample size based on the hypothesis was $n = (1.96/\log_e f)^2 \{[(R+1)/(RP_2) - 2]\}$, where R is the relative risk, P₂ is the proportion of the control group, and f is defined as $1.96 \times \sqrt{\{[(1-P_1)/(nP_1)] + [(1 - P_2)/(nP_2)]\}}$, n is the number of subjects in each group and P₁ is the proportion in group one. Patients with lymphadenopathy were screened using the national diagnostic algorithm for TBLN at Butajira hospital [18].

Patients who were clinically diagnosed with TBLN and signed the written consent form were included in our study. In total, 171 patients took part in the study, from October 2005 to July 2006. Patients who were critically ill, taking anti-TB drugs, had other forms of TB, were anemic, or had medical contra-indications or did not consent were excluded from the study. This study is part of a larger cross-sectional study designed to develop serological diagnostic test(s) for TBLN.

Collection of biopsy, FNA and blood specimens

Excision biopsies and FNAs were taken from all patients. Biopsies were divided into two halves - one for histology and the other for culture. The culture biopsy was placed in a tube containing 1 ml phosphate buffer saline (PBS) and the histology biopsy was fixed with 10% formalin. The FNA was used for Ziehl Neelsen (ZN) staining and PCR in some cases. Samples were stored at 4°C prior to and during transport to Armauer Hansen Research Institute (AHRI) for processing. The delay in transporting samples from our study site to the AHRI laboratory was not more than six hours.

Culture

All processing procedures were conducted using aseptic techniques in a class I safety cabinet. Biopsies

were cut into pieces and decontaminated by 1% sodium hydroxide containing 3% sodium dodecyl sulphate (SDS). The suspensions were shaken and the homogenates were centrifuged. The pellets were neutralized by 0.1% sulfuric acid containing 0.0008% bromocresol–purple. Finally, 100 µl of each suspension was inoculated in two types of Löwenstein-Jensen (LJ) media, one containing glycerol and the other pyruvate to ensure the growth of *M. bovis*. The inoculated media were incubated at 37°C for up to eight weeks, while bacterial growth was observed weekly.

HIV testing

HIV testing was performed using the diagnostic algorithm assay kit, following the manufacturer's instructions and the National guidelines for HIV screening as described by Ethiopia's Ministry of Health. The following three stages of the kit were used: Determine Lateral Flow test (ABBOTT Japan Co.Ltd, Mitato-KU, Tokyo, Japan, and AUST R7449); Capillus (Trinity Biotech PIV, Bray, Ireland); and Unigold (Trinity Biotech-PIC, Bray, Ireland). Patients who tested positive for any two of these tests were reported as positive. Appropriate positive and negative controls supplied by the manufacturers were considered at each stage of the HIV test.

DNA extraction from frozen biopsy cultures

DNA extraction was performed by the boiling method [19]. A loop-full of bacteria from positive cultures and reference strains of *M. tuberculosis* and *M. bovis* were transferred to a 1.5 ml Eppendorf tube containing 400 µl of 1xTE (Tris ethylene di-amine tetra acetic acid). The resuspended bacteria were heated for one hour at 80°C in a water bath and 2 µl from the supernatant was used for PCR.

DNA extraction from FNA

DNA was extracted as reported by van Soolingen *et al.* in 1995 [20]. Briefly, a 400 µl vial of FNA was incubated at 80°C for one hour to inactivate the bacteria. The bacteria were lysed using 10 mg/ml lysosyme (Sigma, Germany) and then digested with 10 mg/ml proteinase K (Sigma, Germany). DNA was extracted with chlorophorm-isoamyl alcohol (24:1) and precipitated with isopropanol. The DNA pellet was resuspended in 20 µl of TE buffer and stored at -20°C until used for PCR.

PCR amplification

PCR targeting the RD10 region was conducted as reported by Parsons *et al.* 2002 [12]. The RD10

primers were RD forward (RD10F): 5'-CTG-CAA-CCA-TCC-GGT-ACA-C-3', RD internal (RD10Int): 5'-GAA-GTC-GTA-ACT-CAC-CGG-GA-5', RD reverse (RD10R): 5'-AAG-CGC-TAC-ATC-GCC-AAG-3'. The two primers (RD10F) and (RD10R) are complementary to the sequences flanking the deleted region and amplify a smaller PCR product (202bp) from strains where RD10 is deleted. The third primer (RD10I) is complementary to an internal sequence of RD10 and amplifies a product of different size (308 bp) when combined with one of the flanking primers (RD10F). The two flanking primers (RD10F and RD10R) are too far apart to efficiently amplify the entire region for the strain [12]. RD10 specific sequences are deleted not only in *M. bovis*, but in other MTC, such as *M. caprae*, *M. microti*, *M. pinnipedii*, and a subset of *M. africanum*. Moreover, the assay is not specific for *M. tuberculosis* because *M. canettii* and some *M. africanum* species are RD10 intact. Therefore, the RD10 PCR technique needs to be combined with the MLPA assay, which identifies the TbD1 region and is specifically deleted in modern *M. tuberculosis* isolates. The 25µl PCR mix (Hot start PCR master mix, Qiagen GmbH) consisted of 2 µl of DNA template and 10 µM of the two flanking primers and 50 µM of the internal primer. The PCR program comprised of denaturation at 95°C for 15 minutes, then 35 cycles of denaturation at 94°C for one minute, annealing at 55°C for one minute and extension at 72°C for one minute, and a final extension at 72°C for 10 minutes in a thermocycler (Thermo-Hybaid, UK).

Analysis of PCR product

Ten µl of the amplified products and 2 µl of 6 x PCR loading dye were analyzed on a 1.8% agarose gel containing ethidium bromide. Electrophoresis was performed at 100V for one hour. One hundred bp DNA molecular weight markers were used. Amplification of a 308 bp PCR product indicates *M. tuberculosis* whereas amplification of a 202 bp product indicates *M. bovis*. However, further confirmation of MLPA through the absence and presence of TbD1 region is needed.

MLPA

MLPA analysis was performed on DNA extracts from bacteria on LJ-slants as previously described [15,16], with 10 minutes of denaturation prior to hybridization at 95°C. Briefly, 2-3 µl of each DNA sample was treated with RNase prior to analysis. MLPA-probes hybridization was done overnight (± 16 hrs) at 60°C, followed by ligation at 54°C for 15

minutes and then the ligase was inactivated at 98°C for 5 minutes. Subsequently, aliquots of the ligated products were amplified by PCR (35 cycles of 30" 95°C, 30" 60°C, 60" 72°C, followed by a final 20' 72°C) with only one primer-pair, since all probes carry the same primer-sequence.

The amplified products were first analyzed on a 2% agarose gel and then by capillary electrophoresis. DNA from confirmed *M. tuberculosis* and *M. bovis* patients was used as positive controls and distilled water was used as a negative control. The phenotypic consequences of the presence of the various MLPA products are explained in Table 3.

Quality control

For quality control, DNA from 20 positive cultures collected from biopsy were randomly selected and tested at the Royal Tropical Institute (KIT) in Amsterdam, using the Genotype MTC kit (Hain, Germany) according to the manufacturer's instructions (HAIN, Life science). All 20 DNA samples proved to be *M. tuberculosis* (data not shown).

Data analysis

Data analysis by the AHRI data management unit used Stata/SE; release 7.0. Cross-tabulation was done to compare the proportion of HIV positives among culture-positive TBLN cases with the proportion of HIV positives among culture-negative lymphadenopathy cases. Pearson Chi- square test was used as statistical tool.

Results

Culture results were available from 156 of the 171 patients as follows: 107(69%) were culture positive; 49 (31%) showed no growth; 12 were contaminated; and 3 were lost by accident. Histological examination showed granulomas in 99/107 (93%) of culture-positive patients and in 24/49 (49%) of culture-

negative patients. Seven of the culture positives could not be maintained and PCR was done using DNA extracted from the remaining 100 positive cultures. Ten (5.8%) of the 171 TBLN patients were HIV seropositive. Six of the 10 HIV seropositive were culture positive. There were no statistically significant differences between the proportions of HIV seropositivity among culture-positive TBLN patients and among culture-negative TBLN suspects (Table 1).

Table 1. Comparison of HIV status and TB Culture.

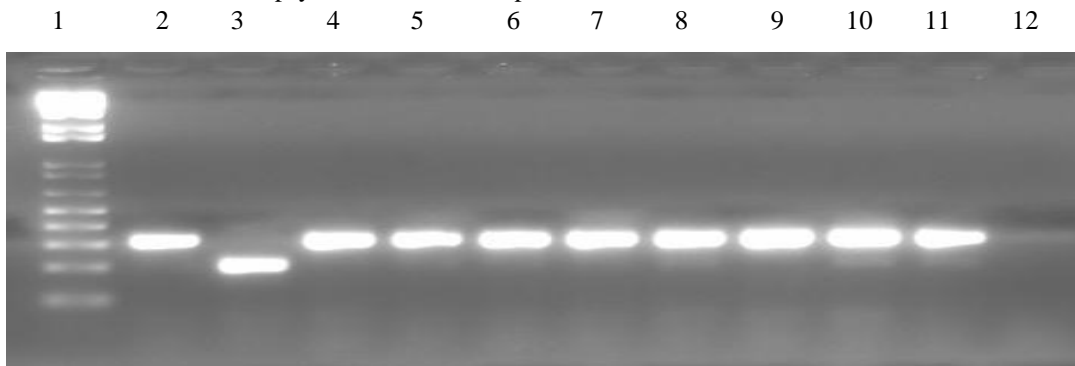
HIV	Culture		Sum
	Positive (107)	Negative (49)	
Positive (10)	6	4	10
Negative (146)	101	45	146
Sum	107	49	156

Pearson Chi-square = 0.4, P=0.546, OR=0.7 (95%CI, 0.2 - 2.5)

Figure 1 shows a representative gel picture of the RD10 PCR results using DNA extracted from biopsy cultures, and control reference strains of *M. tuberculosis* and *M. bovis*. *M. bovis* was not detected in any of the 100 positive biopsy cultures from TBLN patients.

Initially, RD10 based PCR was done using DNA extracted from 14 FNAs. Twelve out of 14 (86%) FNAs tested were positive for 308 bp bands and indicated *M. tuberculosis*. However, PCR analysis using DNA from FNAs was abandoned due to weak band intensity (result not shown). Seven (50%) among 14 of the FNAs tested by PCR had positive biopsy cultures, and all of these seven positive biopsy cultures were positive for 308 bp bands which could

Figure 1. Use of PCR-based RD10 assay to identify *M. tuberculosis* and *M. bovis* on template DNA extracted from biopsy cultures of TBLN patients.



Lane 1= DNA molecular weight marker (100 bp – 1000 bp); Lane 2 = DNA from reference *M. tuberculosis* strain (H37RV); Lane 3= DNA from reference *M. bovis* strain (ATCC-19210); Lanes 4 through Lane 11 = DNA extracted from frozen bacteria of biopsy cultures from TBLN patients; Lane 12= negative control.

Table 2. Summary of MLPA results

embB-306	katG-315	inhA-15	16S rRNA	rpoB-176	rpoB-531	rpoB-526G	rpoB-526T	rpoB-522	I56110	KatG-463	gyrA-95	mutT2-58	Ogt-12	Ogt-15	Ogt-37	TbD1	Characteristics	#Strains With profile
■			■						■		■						PGG1	2
■			■						■	■	■						PGG2	41
■			■						■	■	■			□			PGG2- (Haarlem)	2
■			■						■	■	■			■			PGG2- Haarlem	29
■	■		■						■	■	■			■			PGG2- Haarlem, INH-resistant (via katG-S315T)	4
■	■		■						■	■	■						PGG2, INH-resistant (via katG-S315T)	7
■			■						■	■							PGG 3	10
■			■						■		■					■	<i>M. bovis</i> profile (pos. control)	

■ : resulted in MLPA- product (SNP present)
 □ : possibly resulted in MLPA- product (result inconclusive)
 PGG: principal Centotypic Group

MLPA- probes are depicted in the columns on the left (vertical) and indicated the targeted regions or colons (16). In the columns “characteristics”, the interpretation of the MLPA results is indicated and the last column, the number of strains resulted in specific MLPA profiles has indicated.

be *M. tuberculosis*. Six of the FNAs tested were culture negative and one FNA had no biopsy culture.

PCR-results showed that none of the patients’ samples was infected with *M. bovis*. The same was true when DNA from cultured bacteria were analyzed by MLPA (Table 2). MLPA provided additional information on the strains, such as drug resistance to isoniazid (“katG-315”, “inhA-15” mutations) and also principal genotype group (PGG). The *M. bovis* control was PGG1 with the TbD1 region. The majority of the isolates 83/95 (87%) belonged to PGG 2, and 35 (42%) of those belonged to the Haarlem genotype. Merely 2/95 (2%) strains were part of PGG 1 and 10/95 (11%) of PGG 3. INH resistance was detected in 11/95 (12%) of the strains, all of which were PGG 2 and were resistant via *katG*-S315T mutations. None of the isolates carried the TbD1 fragment. This finding excludes all other members of the MTC including *M. canettii* and *M. africanum*. Moreover, our PCR identified RD10 sequences in all the isolates tested, further evidence that all strains isolated were *M. tuberculosis*.

Discussion

This study has shown that in culture-confirmed TBLN patients, *M. tuberculosis* was the only etiological agent identified when tested by PCR, using RD10 primers system and MLPA assay. The presence of granulomas in 24 of 49 culture-negative

patients might be due to infection by either tuberculous or nontuberculous mycobacteria (NTM) which were not adequately detected by the sample culture. Our results are compatible with a study conducted in Butajira, Southern Ethiopia, which reported that in 23 patients identified with nontuberculous lymphadenitis by cytology, PCR confirmed the presence of MTC in 15 and in the remaining 8, MTC was not detected [21]. Moreover, a recent study conducted at AHRI using FNAs and biopsies of 117 TBLN patients from eastern, central and northern Ethiopia identified only *M. tuberculosis* as the etiological agent of TBLN by using the RD10 primers system [22]. In addition, MLPA results from our study clearly indicate that TbD1 was absent from all the isolates tested, confirming that all isolates are modern *M. tuberculosis* strains [23,24]. Collectively, these results suggest that *M. bovis* appears to be a rare etiological agent of TBLN in Ethiopia.

Our results contrast with previous studies that suggest *M. bovis* may be responsible for a larger proportion of TBLN cases. Kidane *et al.* in 2002 [5] reported PCR-based detection of *M. bovis* in 17% of lymph node FNAs obtained from patients diagnosed as TBLN by cytology. However, studies on the potential of nucleic acid amplification techniques (NAAT) to diagnose TBLN from clinical samples reported that these techniques produce variable and inconsistent results. NAATs should be applied in conjunction with conventional methods and interpreted in the context of clinical suspicion [14]. A

Tanzanian study [8] on patients with different clinical manifestation of TB found that 16% were caused by *M. bovis*, 70.5% by *M. tuberculosis*, and 13.6% by other mycobacterial species. Moreover, the same study indicated that 26.8% of EPTB cases and only 4.3% of PTB cases were due to *M. bovis*. However, these results were based on biochemical typing which is less reliable and requires further confirmation with more sensitive and specific techniques, such as PCR.

Table 3. The MLPA-probes used in this study.

probe	target/info
embB-306	EMB resistance marker
katG-315	INH resistance marker
inhA-15	INH resistance marker
16S rRNA	16S ribosomal RNA gene, MTB-complex specific
rpoB-176	RIF resistance marker
rpoB-531	RIF resistance marker
rpoB-526G	RIF resistance marker
rpoB-526T	RIF resistance marker
rpoB-522	RIF resistance marker
IS6110	insertion element IS6110, MTB-complex specific
katG-463	genotype marker, specific for PGG 2 and 3
gyrA-95	genotype marker, specific for PGG 1 and 2
mutT2-58	genotype marker, specific for Beijing 2
ogt-12	genotype marker, specific for Beijing 2
ogt-15	genotype marker, specific for Haarlem
ogt-37	genotype marker, specific for Beijing 3 and 4
TbD1	absent in modern MTB strains

Probes were named after the gene and specific codon/region they target. A short summary of the information provided by the probes is given (15). EMB: ethambutol, INH: isoniazid, RIF: rifampicin, PGG: principal genotypic group.

Thus, using proper methods for detection is essential for conclusively identifying *M. tuberculosis*.

Differences between our results and those of Kidane *et al.* may also relate to the proportion of HIV-positive individuals in each study. HIV seropositivity was higher (27%) in Kidane's study than in ours (5.6%). Given that different regions of Ethiopia have different seroprevalences [25], it is possible that referral bias may account for these differences. This study's seroprevalence values more closely reflect overall seroprevalence in the Butajira region during the past decade [26]. Given other reported associations of HIV with *M. bovis* and *M. tuberculosis* [27,28], it is possible that HIV-infected individuals may be more prone to *M. bovis* infection

and/or transmission. However, in our study, there were only six HIV seropositives among the 100 culture-positive TBLN cases and we could not see the association of HIV with *M. bovis* infection.

This study was limited by our utilization of solid culture media, which may have been suboptimal for the growth of *M. bovis* [29,30].

However, we further analyzed *M. tuberculosis* isolates by MLPA to define genotypes and drug sensitivity profiles and the results were consistent with those found in strains causing pulmonary tuberculosis in southern Ethiopia [31]. The majority of the isolates, 83/95 (87%), belonged to PGG 2, and 35 (42%) of those belonged to the Haarlem genotype, a virulent strain that is highly prevalent in Western Europe, Brazil, and Sub-Saharan Africa [32]. INH-resistance was detected in 11/95 (12%) of the strains. However, the current MLPA assay allows identification of only the most prevalent drug-resistant mutations, so that this percentage may underestimate the total INH resistance. Nonetheless, the results obtained have revealed new information on drug resistance and genotype of TBLN isolates, prompting further research of these issues. The data presented in this study provide valuable epidemiological information.

Studies from the UK have shown that pasteurization of milk and an intensive animal TB control programme contributed to considerable reduction of bovine TB in both humans and cattle [33]. However, in developing countries there are no effective animal TB control programmes and surveillance, and the epidemiological and public health aspects of infection due to bovine TB are unknown [34]. Our data offer no evidence for transmission of the etiological agent of TBLN from cattle to human and we therefore suspect TBLN is transmitted from human to human. The absence of *M. bovis* as an etiological agent of TBLN in patients from Butajira and other parts of Ethiopia suggest that bovine TB is rare in humans. Further studies on the epidemiology, including the relationship with HIV infection, the manifestation of TBLN, and its association with certain *M. tuberculosis* lineages, such as Haarlem genotype, are warranted.

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Conflict of interest

No conflict of interest is declared.