Genotyping and molecular detection of multidrug-resistant *Mycobacterium tuberculosis* among tuberculosis lymphadenitis cases in Addis Ababa, Ethiopia

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Abstract

Multidrug-resistant tuberculosis (MDR-TB) has emerged as a major public health problem. Drug-resistance surveillance data show that 3.9% of new and 21% of previously treated TB cases were estimated to have had rifampicin/ multidrug-resistant tuberculosis (MDR/RR-TB) in 2015. This implies that the MDR-TB is increasing alarmingly. Hence, a better understanding of drug resistance mechanisms and genotypes associated with multidrug resistance in *M. tuberculosis* is crucial for improving diagnostic and therapeutic methods to treat individuals with MDR-TB. The aim of this study was to analyze molecular drug resistance mutations of MDR-TB isolates from the cases of TB-lymphadenitis in relation to its genetic lineages.

A cross-sectional study was conducted on culture positive cases from July to October, 2014 in Addis Ababa, Ethiopia. Sixty isolates were included to analyze drug resistance mutated gene responsible for MDR-TB in relation to its molecular genotyping. Mycobacterial culture, GenoTypeMTBDR plus and Spoligotyping were used to undertake the study.

Of 60 TBLN isolates, 8.3% were identified MDR-TB cases and one isolate was isoniazid mono-resistant. Eleven isolates in T3-ETH genetic sub lineage were sensitive to both RMP and INH, while only 2 isolates were MDR-TB. Most of the RMP- resistant isolates showed mutation in codon S531L and all isolates mutated in the *katG* gene conferring INH resistant strains had mutations in codon of S315T1.

Screening for the *rpoB* and *katG* gene mutation of tuberculosis lymphadenitis is useful in Ethiopia for an early detection and treatment of MDR-TB. Besides, there is a drug resistance variation among different lineages of Tuberculosis lymphadenitis which has important consequences for the development of efficient control strategies.

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Introduction

In 2015, there were an estimated 480 000 new cases of multidrug-resistant tuberculosis (MDR-TB) and 250 000 deaths because of rifampicin/ multidrug-resistant tuberculosis (MDR/RR-TB). Drug-resistance surveillance data show that 3.9% of new and 21% of previously treated TB cases were estimated to have had MDR/RR-TB in 2015 [1]. MDR-TB is defined as a type of TB that shows resistance at least to rifampicin and isoniazid and it threatens TB control programmes in many parts of the world [2]. It has been reported that in Ethiopia the proportion of new cases with MDR-TB is 2.7% higher among people previously treated for TB, at 14% in 2015 [3].

A better understanding of drug-resistance mechanisms in *Mycobacterium tuberculosis* infections is crucial for the

development of rapid methods for drug-resistance detection and new anti-TB drugs, which are used to treat patients with MDR-TB. Early molecular studies identified *katG* (encoding catalase peroxidase) and *rpoB* (encoding the β-subunit of RNA polymerase) genes as major targets conferring resistance of *M. tuberculosis* to isoniazid and rifampicin, respectively [4,5]. The increased incidence of MDR-TB cases, particularly in high-burden countries like Ethiopia, raised the need for implementation of rapid molecular methods as an alternative to culture-based drug susceptibility testing. Recently, WHO recommended the use of molecular techniques such as line probe assay and GeneXpert assay for rapid screening of MDR-TB to evaluate the presence of genomic mutations conferring resistance in MDR-TB [6,7].

Some published data on MDR-TB are available in Ethiopia but limited studies on molecular detection of MDR-TB lymphadenitis (TBLN) [8,9] have been conducted and no data are found regarding genotyping characterization of specific lineage in relation to MDR-TBLN because it was forgotten that extra pulmonary TB, particularly TBLN, can also form pulmonary TB. The present study, therefore, tried to assess MDR-TB in suspected TBLN by using line probe assay, which is similar to the GeneXpert assay for detection of the mutation in specific genes responsible for the drug-resistance and also to identify the relationship of specific TB lineages to MDR-TB among TBLN isolates.

Materials and methods

Patients' recruitment and study settings

A cross-sectional study was conducted on culture-positive cases from July to October 2014 in Addis Ababa, Ethiopia at Tikur Anbessa Specialized Hospital and Alem Tena Higher Clinic, these sites were purposely selected because more patients with TBLN were referred to them. A total of 65 isolates were included to analyse the gene responsible for MDR-TB in relation to its molecular genotyping at the Ethiopia Public Health Institute. This institute is well-known in Ethiopia for conducting research and the molecular TB research centre is part of it. To this effect, demographic data were collected from all patients using a pre-structured questionnaire by trained clinical nurses. Patients on anti-TB treatment and <18 years old at the time of sample collection were excluded.

Sample collection and processing

Fine needle aspiration (FNA) samples were collected by a pathologist from the affected nodes. Briefly, the swollen area was cleaned with 70% alcohol and then a 21-gauge needle was inserted into the mass. After removing the needle, drops of aspirate were placed on a clean slide for FNA cytology, thereafter, leftover FNA samples were added aseptically into sterile universal tubes in PBS, pH 7.2, at 4°C.

Mycobacteriological culture

Samples suspected of TBLN were processed and cultured at the Aklilu Lemma Institute of Pathobiology TB laboratory. Briefly, FNA specimens were homogenized and mixed with 0.85% normal saline and decontaminated by shaking in an equal volume of 4% NaOH for 15 min at room temperature. Then, the specimens were subjected to centrifugation at 3000 rpm for 15 minutes. After homogenization, 100 µl of the sediment was inoculated into the conventional Lowenstein−Jensen egg slant medium, containing 0.6% sodium pyruvate. The cultures were incubated at 37°C in a slanted position for 1 week and in the upright position for 4−8 weeks. the same media. Culture of the specimen was incubated at 37°C for 3−8 weeks. The media were checked for evidence of bacterial growth daily for the first week and weekly for the rest of the time until 8 weeks. Microscopic examination of the colonies was performed using Ziehl−Neelsen stain to select Acid Fast Bacilli positive isolates. Heat-killed cells were prepared by mixing two loops of colonies in 200 µl distilled water and thereafter heating at 80°C for 1 h. The heat-killed cells were used for molecular characterization.

To check for the quality of the Lowenstein−Jensen medium, a fast-growing mycobacterium complex strain was run in parallel with each batch of inoculated media. In addition to this, uninoculated Lowenstein−Jensen tubes were incubated at the same time to control for contamination.

Spoligotyping

Spoligotyping was carried out using a commercially available kit from Ocimum Biosolutions (UK) described earlier [10]. To put it briefly the direct-repeat (DR) region was amplified with primers DRa and DRb. Then, the amplified DNA was denatured and the single-stranded DNA was hybridized with 43 spacer oligonucleotides covalently bound to a membrane. For running spoligotyping the already published procedure was followed [11]. DNA from *Mycobacterium .bovis*, bacillus Calmette−Guérin and *M. tuberculosis* H37Rv were used as positive controls, whereas Qiagen water (Qiagen, Hilden, Germany) was used as a negative control. The presence of spacers was visualized on film as black squares whereas the absence of spacers was indicated by white squares following incubation with streptavidin-peroxidase and they were detected by the enhanced chemoluminescence system detection liquid (Amersham, Little Chalfont, UK). Spoligotype patterns of each strain were prepared in binary and octal format and entered into the recently released international spoligotyping database SITVITWEB [12] for detecting their spoligotype International Type (SIT) numbers. Strains for which SIT numbers could not be recovered from the database were considered orphans. Lineage and sublineage designation were according to SPOTCLUST at http://tbinsight.cs.rpi.edu/run_spotclust.html. All the steps of sample processing and PCR were carried out in a separate PCR

mix room using dedicated pipettes and autoclaved tips to prevent cross contamination. To make sure of the quality control, recent standard operating procedures for each step were followed.

Line probe assay

The MTBDRplus assay was performed directly on sputum samples and according to the manufacturer's instructions [13] at the Ethiopia Public Health Institute-TB research unit. A 500-mL portion of decontaminated FNA samples was used for DNA isolation; subsequent amplification and hybridization were based on manufacturers' recommendations [13] Each step was carried out in a separate room with unidirectional workflow between rooms. After hybridization, test strips were allowed to dry before being attached to the paper. Each strip consisted of 27 reaction zones (bands) including controls that were interpreted according to the manufacturer's instructions to determine test validity, *Mycobacterium tuberculosis* complex identification, and resistance to isoniazid and rifampicin. An internal quality control program with positive and negative controls was implemented during the study.

Statistical analysis

All demographic and laboratory data were entered, checked and analysed using SPSS version 20 (SPSS Inc., Chicago, IL, USA). The categorical data were analysed using the chi-square test or the Fisher exact test that assumes when expected value less than 5 cells. A p-value ≤0.05 was considered statistically significant.

Results

A total of 65 isolates were detected for MDR-TB among TBLN patients with GenoTypeMTBDR plus. However, valid line probe assay results were obtained for only 60 (92.3%) of the strains. The rest were not performed because of the undetectable amount of the isolates. Spoligotyping of the five undetectable strains indicated that three of them were T lineages and two of them were CAS lineages. Of the 60 isolates, 5 (8.3%) were MDR-TB, whereas only one isolate was mono-resistant to isoniazid. Of the five MDR-TB cases, 2/5 (40%) were newly detected and 3/5 (60%) were re-treated patients. The number of residents in rural and urban regions was almost similar, but all the identified MDR-TB was from the urban area (Table 1).

Correlation of genotyping with isoniazid and rifampicin in M. tuberculosis lineages

This study comprised six different genotypes with T and CAS dominant lineages and Haarlem, F33, F36 and LAM9 according to the database program at SPOTCLUST. Of five MDR-TBLN cases, two isolates were among the T3-ETH sublineage, two

were among the CAS lineage and only one was from other lineages (Table 2). Moreover, one isolate in the CAS lineage was isoniazid mono-resistant. The remaining 54 isolates were sensitive to both rifampicin and isoniazid among their respective lineages. All Haarlem lineages in this study were found to be sensitive to both rifampicin and isoniazid.

Analysis of rifampicin and isoniazid mutational pattern in M. tuberculosis strains

Five and six resistance-associated mutation isolates were found in *rpoB* and *katG* genes, respectively (Table 3); and five isolates (8.3%) were MDR-TB. There were no isolates that showed the mutation in the *inhA* promoter region gene. Of five rifampicin-resistant isolates, missing wild-type (WT) along with known mutations could be detected in all resistant isolates. Of these, 80% of them showed mutations in codon S531L and one isolate was in codon H526Y mutation. Among mutations in the *katG* gene conferring isoniazid-resistant strains, all resistant isolates in this study had mutations in the *katG* gene at codon S315T1.

Discussion

The emergence of drug-resistant isolates of *M. tuberculosis* poses a serious threat to global TB control. It remains a major problem for healthcare providers including those found in Ethiopia, where it accounts for high mortality. The condition has been far worsened by drug resistance. Therefore, timely genetic characterization helps for the identification of resistance mutations that will offer hope in improving MDR-TB prevention and management through the early initiation of appropriate therapy [14].

The GenoType MTBDRplus detects the mutations for RMP and INH, which is highly correlated with the sequencing [15]. As different studies showed S531L mutations conferring resistance to RMP has been detected with varying rates from 73–100% are typically found in a small region of amino acids located between position numbers 507–533 in *rpoB* gene [12,16,17]. In the present study of molecular detection of MDR-TBLN, four of five isolates with RMP resistance contained the S531L mutation; the most frequently reported resistance mutation in the Ethiopian strains [16] is similar to a previously published report from South Africa [18].

The molecular basis of resistance to INH involves mainly katG gene and the regulatory coding region of *inhA* [19,20]. *KatG* gene mutations conferring INH resistance strains is the most frequently mutated between codons 138 and 328, with 30–90% occurred in codon 315 based on geographical distribution [21]. Different research works suggest that resistance of *M. tuberculosis* to INH shows mutation at codon 315 [22,23].

TABLE 1. Distribution of anti-tuberculosis drug resistance with different sociodemographic variables

Variables		Anti-tuberculosis drug resistance			
		Rifampicin	p value	Isoniazid	p value
Sex	No. of patients		0.639		0.385
Male	30	3 (5%)		4 (6.6)	
Female	30	2 (3.3%)		2 (3.3%)	
Age (years)			0.721		0.745
18–27	32	2 (3.3%)		2 (3.3%)	
28–37	13	2 (3.3%)		2 (3.3%)	
38–47	7	1 (1.7%)		1 (1.7%)	
48–57	3	0 (0)		0 (0)	
≥58	5	0 (0)		1 (1.7%)	
Residence			0.373		0.002
Urban	29	5 (8.3%)		6 (10%)	
Rural	31	0 (0)		0 (0%)	
History of anti-tuberculosis treatment			0.826		0.019
Retreated	8	3(5%)		3(5%)	
New	52	2(3.3%)		3(5%)	
Contact history with patients with tuberculosis			0.050		0.103
Yes	13	3(5%)		3(5%)	
No	47	2(3.3%)		3(5%)	

TABLE 2. Distribution of rifampicin and isoniazid resistance with different lineages

Mycobacterium tuberculosis lineage[a] (n)	Sensitive,[b] n(%)	Anti-tuberculosis drug resistance			
		Rifampicin,[c] n (%)	p value	Isoniazid,[d] n (%)	p value
T3-ETH (13)	11 (84.6%)	2 (15.4%)	0.331	2 (15.4%)	0.485
Other T (18)	18 (100%)	0	—	0	—
CAS (13)	10 (76.9%)	2 (15.4%)	0.234	3 (23.1%)	0.474
Haarlem (9)	9 (100%)	0	—	0	—
Others (7)	6 (85.7%)	1 (14.3%)	0.128	1(14.3%)	0.179

[a]Lineages designations are according to the SPOTCLUST program (other T = T1, T2 & T3, CAS = Central Asian sub lineage and it include CASI-DELHI, CASI-KILI and CAS, Haarlem-H1, H3 and Others includes F33, F36, LAM9.
[b]Sensitive to both INH and RMP.
[c]Resistant to only RMP.
[d]Resistant to only INH.

TABLE 3. Mutations pattern identified for rifampicin- and isoniazid-resistant of *Mycobacterium tuberculosis* strains

Resistance strains[a]	Anti-tuberculosis drugs				
	Rifampicin		Isoniazid		
	Pattern of gene mutations (wild-type/mutant)	mino acid change	Pattern of gene mutations (wild-type/mutant)	Amino acid change	Result
SIT25	ΔrpoB WT8/rpoBMUT3	S531L	ΔkatG WT/ katG MUT2	S315T2	MDR
SIT149	ΔrpoBWT7/rpoBMUT2A	H526Y	ΔkatGWT/ katG MUT1	S315T1	MDR
SIT21	ΔrpoB WT8/rpoBMUT3	S531L	ΔkatGWT/ katG MUT1	S315T1	MDR
SIT149	ΔrpoB WT8/rpoBMUT3	S531L	ΔkatGWT/ katG MUT1	S315T1	MDR
Orphan	ΔrpoB WT8/rpoBMUT3	S531L	ΔkatGWT/ katG MUT1	S315T1	MDR
SIT954	—	—	ΔkatGWT/ katG MUT1	S315T1	INH[mr]

Abbreviations: INH[mr], isoniazid mono-resistance; MDR, multidrug resistance; SIT, Spoligo International Type; Δ, deletion; MUT, mutant.
Note: SIT25 belongs to the CAS_DELHI family, SIT21 belongs to the CAS1-KILI family and SIT149 belongs to the TE-ETH family according to the SITVIT WEB database.
[a]The strains designations are according to the SITVIT WEB database.

Most common mutation sites conferring INH resistance reports from Ethiopia and others were noted at S315T of the *KatG* gene [16,24], suggesting the high level of drug resistance to INH. This showed that the possible transmission of strains with a similar type of target gene of specific mutations in the community. In contrast to this study, however, in which, mutations in *InhA* gene was not occurred in any of our isolates, a high and low level of frequency of *inhA* mutation was reported in Tunisia and Canada [25,26].

A critical question in tuberculosis control is why some strains of *M. tuberculosis* are preferentially associated with multiple drug resistances [27]. Our present data showed that SIT 149 of T3-

ETH, SIT21 of CAS1-KILI and SIT25 of CAS1-DELHI were related to MDR-TB. In line with our finding, it was reported that particular sub lineages like CAS1-KILI, CAS1-DELHI and T3-ETH are highly clustered and associated with MDR-TB, in Ethiopia [28].

From TB control point of view, it is relevant to understand whether specific genotype families are over-represented among drug-resistant cases and, in particular, if these resistant strains are successfully transmitted within the community [29]. In contrast to this, the report from southern part of Ethiopia [29] indicated that Haarlem lineage was highly correlated with MDR-TB, in spite of the finding that all Haarlem lineages were sensitive to RMP and INH in our present study. Previous analyses suggest that there is substantial genetic and phenotypic diversity among isolates from the various *M. tuberculosis* lineages with significant variation in the mutation rates in drug resistance TB [30]. It was therefore observed that *M. tuberculosis* strains of different lineages differ in the total number of mutations that confer drugs resistance [27].

Increased resistance to INH may affect the success of TB control program since the drug is used both in intensive and continuous phase of anti-TB treatment [31]. In Ethiopia, there was an increased resistance to one or more first line anti-TB drugs. This might be due to the fact that there is no culture and drug susceptibility testing facility for routine diagnosis of drug resistance. Thus, drug resistant-TB is only diagnosed after prolonged treatment with first line anti-TB drugs and clinical recognition of failed treatment [32].

Although this study gave some evidence regarding the molecular MDR-TBLN pattern in the capital city Addis Ababa, it also has some limitations. Phenotypic resistance evaluation and the high discriminatory power tools was not done due to the inadequacy of the fund. In addition, as the data collected was sparse and it was carried out for short period of time, it may not thus represent the whole population of Addis Ababa.

Conclusions

Screening for the *rpoB* and *katG* gene mutation of tuberculosis is useful in Ethiopia for an early detection and treatment of MDR-TB. The information may also be used as a marker to evaluate the transmissibility of MDR-TB in the community. Besides, there is a drug resistance variation among different lineages of MTB which has important consequences for the development of efficient control strategies.

Conflict of interest

None declared.

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