Spoligotyping and Drug Sensitivity of Mycobacterium tuberculosis isolated from Pulmonary Tuberculosis Patients in the Arsi Zone of South Eastern Ethiopia

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Abstract

Background Tuberculosis (TB) is one of the leading disease causing morbidity and mortality in different zones of Ethiopia including the Arsi Zone. However, little or no scientific information is available on the strains of Mycobacterium tuberculosis and their drug sensitivity profiles in this Zone. This study was conducted to identify the strains of M. tuberculosis and evaluate their drug sensitivity profiles. Methodology A total of 111 clinical isolates of M. tuberculosis from patients with pulmonary TB in the Arsi Zone were used for this study. The region of difference 9 (RD 9)-based polymerase chain reaction (PCR)and spoligotyping methods were used for speciation and strain identification of Mycobacterium tuberculosis respectively.The spoligotyping patterns were compared with the international SpolDB4 (SITVIT) and Run TB-Lineage used for the identification of lineages. The phenotypic drug susceptibility patterns were confirmed by BD BactecMGIT 960 SIRE test and GenoType MTBDRplus line probe assays were used for the detection the drug resistance-conferring mutations of the isolates. Result The spoligotype patterns of 83% (92/111) of the isolates were interpretable and 56 different patterns were identified. Twenty-two of these patterns were shared types while the remaining 34 were orphans. The predominant shared types were spoligotype international type (SIT) 149 and SIT53, each consisting of 12 and 11 isolates, respectively. The lineages identified were Euro-American, East-African-Indian, Mycobacterium-africanum, and Indo-Oceanic in descending order. Phenotypically, 17.2% of the 64 tested isolates were resistant to any of the four first-line drugs while 3.1% of them were multi-drug resistant (MDR). Higher (6.2%) monoresistance was observed to Streptomycin followed by Isoniazid (3.1%) while no resistance was observed either to Rifampicin or to Ethambutol. Genotypically, five (5.4%) isolates were resistant to Isoniazid and mutated at codon S315T1 of katG. On the other hand, only 1.1% of the isolates was resistant to Rifampicin and mutated at codon S531L of rpoB gene. Conclusion The proportion of orphan strains isolated in this study was high, which could suggest the presence of new strains in the Zone. Moreover, the study showed relatively high percentage of mono-resistance to any four first-line drugs warranting for the need to strengthen the control efforts.

Background

TB is the leading cause of death from a single infectious agent, ranking above HIV/AIDS(WHO, 2018).Globally, there were an estimated 10.0 million incident cases of TB and 87% of the incident cases is accounted by the 30 high TB burden countries (WHO, 2018). TB affects all countries and all age groups but, the epidemiological distribution of TB cases is heavily skewed towards low income countries (WHO, 2018).The pandemics of HIV/AIDS, public health systems deterioration and emergence of multi-drug resistance forms of TB are worsen the spread of tuberculosis in developing countries (Forrellad *et al.*„ 2013).

According to WHO (2018) report, Ethiopia is among the world 30 high TB burden countries with an estimated incidence of 164 new TB cases per 100,000 population. The country has also constituted of 2.7% of newly diagnosed and 14% of previously treated MDR-TB patients (WHO, 2018). Like other developing countries, TB/ HIV co-infection and the emergence of MDR-TB strains are becoming the

pressing challenges in the control of effort of TB in Ethiopia (Eshetie et al., 2017; Seyoum et al., 2014; WHO, 2018).Thus, all the recently existed data showed that TB and drug resistant forms of TB are remains a major public health concern in Ethiopia.

Recent advances in molecular typing techniques highlighted the substantial genetic diversity of in *M. tuberculosis* complex isolated from TB patients (Mathema *et al.,* 2006). Genetic variability can be translated into phenotypic differences in transmission capacity, virulence, and drug susceptibility, which may further give rise to the diversity in disease outcome and epidemiological variation among different geographic regions (Kato-Maeda *et al.,* 2001; Zambrano *et al.,* 2012). Earlier genotyping studies in Ethiopia have also indicated a considerable genetic variation among clinical isolates of *M. tuberculosis (*Firdessa *et al.,,* 2013; Tulu and Ameni, 2018*).*

Knowledge on the strain diversity of *M. tuberculosis* helps to elucidate the patterns and dynamics of TB transmission (Cannas *et al.,,* 2016). In addition to the possible impact of strain variation on the outcome of TB infection and disease the diversity of strains is relevant for our understanding of drug resistance (Comas *et al.,* 2015).

On the other hand, drug-resistant TB (DR-TB) remains a significant challenge in TB treatment and control programmes worldwide(Dookie *et al.,* 2018). A study reports from Ethiopia revealed that the control effort of TB is threatened by the rapid emergence of drug-resistant strains particularly MDR-TB (Eshetie et al., 2017; Seyoum et al., 2014). A population based study conducted in Hitossa District of Arsi zone have shown high rate of primary and secondary resistance prevalence to any of the first-line anti-TB drugs and MDR-TB (Hamusse *et al.,,* 2016).

Although a few genotypic study have been undertaken for strain identification from different part of Ethiopia, little is known about the strains and lineages of *M. tuberculosis* circulating in the Arsi Zone. Moreover, the drug sensitivity profiles of these strains have not been known. Therefore, the objective of this study was to identify the strains and lineages of *M. tuberculosis* isolated from Arsi Zone, and also to evaluate their drug sensitivity profiles to the first line anti-TB drugs using phenotypic and genotypic methods.

Materials And Methods

Source of the isolates and processing

A total of 111 *M. tuberculosis* isolates were obtained from TB laboratory of the Aklilu Lemma Institute of Pathobiology. These culture positive isolates originally isolated from pulmonary TB patients from the Arsi Zone in between April 2015 and March 2016 and stored at –80^{o}C freezer at the Institute for further molecular investigation and drug susceptibility tests. All the laboratory tests were performed at TB laboratories of the Aklilu Lemma Institute of Pathobiology and the Ethiopian Public Health Institutes (EPHI). Theses, laboratories provide research services, TB diagnosis and drug susceptibility tests. The

socio-demographic data of the patients from which the isolates recovered were collected using case reporting form. The study protocol was approved and ethically cleared from Addis Ababa University, Aklilu Lemma Institute of Pathobiology Institutional Review Board (Ref. No. ALIPB/IRB/001/2017/18).

Sub-culturing of the isolates

The frozen isolates were retrieved from –80 freezer and thawed at room temperature. Thereafter the colony suspension was homogenized by gentle mixing and sub-cultured on solid LJ. The specimens were incubated at 37^{O}C and checked weekly for up to 8 weeks. For the purpose of getting fresh specimen for phenotypic drug susceptibility test, we also sub cultured the isolates on BACTEC™ MGIT™ liquid media following manufacturer's instructions and FIND manual.

DNA extraction

DNA was released from the bacterium by suspending two loop-full mycobacterial colonies into 200µL of sterile distilled water, and thereafter by heating the bacterial suspension in the water bath at 80°C for 60 minutes. Then, the solution was centrifuged 3000rpm for 2 minutes after which the supernatant was collected and stored at –20ºCfor molecular typing and/or other related investigations.

Region of difference 9 (RD–9)

RD9 deletion typing was performed on DNA of heat killed isolates to confirm the presence or absence of region of difference 9 as previously described by Brosch *et al.* (2002). The three primers used were RD9flankF, RD9IntR, and RD9flankR, each at a concentration of 100µM. The PCR amplification was made in a total of 20 µl, reaction mixture consisting of 10 µl HotStarTaq Master Mix (Qiagen, Crawley, UK), 7.1 µl distilled water, 0.3 µl each of the three primers (100 mM) and 2 µl DNA template (heat killed). The reaction was heated for 10 minutes at 95ºC for enzyme activation following with 35 cycles of 1 min of denaturation at 95ºC, 0.5 min of annealing at 61ºCand 2 min of extension at 72ºC were run and then a final extension was made at 72ºC for 10 min. Thereafter the product was removed from the thermocycler and run on agarose gel electrophoresis. For gel electrophoresis, 8 µl PCR products was mixed with 2 µl loading dye, loaded onto 1.5% agarose gel and electrophoresed at 100 V and 500 mA for 45 min. The gel was then visualized using a computerized Multi- Image Light Cabinet (VWR). *M. tuberculosis* H37Rv and*. bovis Bacillus, Calmette-Guérin* were used as positive controls while water was use as a negative control. Detection of a band size of 396 bp was considered as positive for *M. tuberculosis* whereas a band size of 575 bp was considered to correspond to either *M. bovis or M. africanum.*

Spoligotyping

Spoligotyping was performed following the method previously described by Kamerbeek *et al. (*1997) and as per the spoligotype kit supplier's instructions (Ocimum Biosolutions, Ijsselstein, The Netherlands). The direct repeat (DR) region was amplified by PCR using oligonucleotide primers DRa (GGTTTTGGGTCTGACGAC) and DRb (CCGAGAGGGGACGGAAAC) which were derived from the DR sequence. DNA from known strains of *M. bovis* SB 1176 and *M. tuberculosis* H37Rv were used as positive controls, whereas Qiagen water (Qiagen company, Germany) was used as a negative control. A total volume of 25 µl reaction mixture consisting of 12.5 µl of Hot StarTaq Master Mix, 2 µl of each of the two primers (20 pmoleach), 5 µl suspension of heat-killed cells (approximately 10–50 ng), and 3.5 µl distilled water was prepared. The mixture was heated for 15 min at 96ºC and then subjected to 30 cycles of 1 min at 96ºC, 1 min at 55ºC and 30 s at 72ºC. The amplified product was hybridized to a set of 43 immobilized oligonucleotides, each corresponding to one of the unique spacer DNA sequences within the DR locus. After hybridisation, the membrane was washed twice for 10 min in 2× SSPE and 0.5% sodium dodecyl sulfate (SDS) at 60ºC and then incubated in 1:4000 diluted streptavidin-peroxidase (HotStar, Crawley, UK) for 45–60 min at 42ºC. The membrane was washed twice for 10 min in 2× SSPE and 0.5% SDS at 42ºC and rinsed with 2× SSPE for 5 min at room temperature. Then after the hybridizing, DNA was detected by the enhanced chemiluminescence method (Amersham Biosciences, Little Chalfont, UK) and by exposure to X-ray-film (Hyper film ECL, Amersham Biosciences) as specified by the manufacturer. The hybridization patterns were converted into binary and octal formats and compared with previously reported strains in the recent SITVIT2 database.

Drug Susceptibility Test

The phenotypic drug sensitivity test was done by using BACTEC MGIT 960 system (Becton Dickinson Diagnostic Systems, Sparks, MD) SIRE kit as recommended by the manufacturer instructions. The final critical drug concentrations of 0.1µg/ml for Isoniazid (INH),1.0 µg/ml for Rifampicin (RIF),1.0 µg/ml for Streptomycin (STR) and 5.0µg/ml for Ethambutol (EMB) were used as described by Siddiqi and Rüsch-Gerdes (2006). Growth was monitored by the BACTEC 960 instrument which automatically interprets results as susceptible or resistant. *M. tuberculosis* H37Rv was run per batch of DST set for quality control purpose. In this study, any drug resistance was defined as resistance to one or more first-line drugs whereas mono-resistance was defined as resistance to only one of the four first-line drugs (INH, RIF, STM, and EMB). MDR-TB was defined as *M. tuberculosis* strains that were resistant to at least INH and RIF.

GenoType® MTBDRplus assays

For the identification of mutations in the *rpoB* gene for RIF resistance and the *katG* gene for high-level INH resistance while the *inhA* gene for low-level INH resistance was performed on the heat killed mycobacterial culture according to manufacturer's instruction (Hain Lifesciences, Nehren, Germany).The DNA of the standard strain H37Rv and molecular-grade water were used as positive and negative controls, respectively.

Data Analysis

Socio-demographic data were analyze using the Statistical Package for Social Sciences (SPSS) version 20.0 (Statistical Package for the Social Sciences, Chicago, IL, USA). The descriptive statistics were used to depict the demographic variables and drug-resistant profiles of the patients which were calculated as percentages of the study populations. All the generated spoligo patterns were entered and compared with the existing international, web-based SpolDB4 (SITVIT) database http://www.pasteur-guadeloupe.fr:8081/SITVITDemo/ to assign the shared type (SIT) and the web-based algorithm SPOTCLUST http://tbinsight.cs.rpi.edu/runspotclust.html was used to assign the lineages of each isolate.

Results

Demographic characteristics of patients

Socio-demographic data were collected from 111 smear-positive pulmonary TB cases. Of these 111 cases, 64(57.7%) were males and 47(42.3%) were females. The mean age of the study patients was 32.53 years. One hundred and five (94.6%) patients had no previous history of TB treatment and six patients had a previous history of TB treatment (Table1).

Table 1: Demographic characteristics of the patients

Variable		Number (%) of patients
Gender		
	Male	64 (57.7)
	Female	47 (42.3)
Age		
	18-28	58 (52.3)
	29-39	25(22.5)
	40-49	12(10.8)
	>50	16 (14.4)
HIV status		
	Negative	61(55.0)
	Positive	4(3.6)
	Unknown	46(41.4)
Marital status		
	Single	46(41.4)
	Married	64(57.7)
	Divorced	1(0.9)
Previous history of treatment		
	New	105(94.6)
	Previously treated	6(5.4)

Speciation of *M. tuberculosis* complex

Out of one hundred and eleven isolates, 92 (86.5%) isolates had intact RD9 (396 bp) and thus were considered as *M. tuberculosis*. The remaining, 19(14.5%) isolates did not show an interpretable signal for RD9-based PCR.

Identification of Strains

The spoligotyping of 92 mycobacterial isolates produced a total of 56 different spoligotype patterns with an overall diversity of 61%. Of these 56 patterns, 22 (39.3%) patterns have been registered the International Spoligotyping Database (SpolDB4) and hence were considered as “shared types” while the remaining 34 (60.7%) patterns were not registered in the database and thus were orphans. Greater than half (51%) of the isolates were clustered into 11 different spoligotype patterns while the remaining 49% were unique or singletons. The spoligotyping results were summarized in Table 2 and 3.

The most commonly occurring clustered spoligotypes were SIT 149 and SIT53 consisting of 12 (13.0%) and 11(11.9%) isolates, respectively. The third and fourth most commonly observed shared types strains were SIT289 and SIT37, respectively containing each consisting of 5(5.4%) isolates. On the basis of TB-insight RUN TB-lineages, four major lineages namely; Euro-American, *M.-africanum*, Indo-Oceanic, and East-African-Indian were identified. Euro-American was the dominant (79.3%) Lineage followed by East-African-Indian (9.9%) Lineage.

Table-2: Representative description of 57 Shared International Types *(SIT)* and corresponding lineages of *M. tuberculosis* isolates from southeastern Ethiopia

CBN: a conformal Bayesian network; **EA**: Euro American; **IO**: Indo-Oceanic; **EAI:** East African-Indian.

Table 3: Representative description of 35 Orphan *M. tuberculosis* strains from southeastern Ethiopia.

Phenotypic Drug Resistance

Bacterial growth was checked in MGIT 960 broth for a total of 92 isolates. Of which 64 (70%) isolates showed growth, 22 (23.91%) did not show growth. On the other hand, the cultures of 6 (6.52%) isolates were contaminated and hence drug susceptibility test was conducted on 64 isolates Of the 64 isolates tested by MGIT for drug sensitivity, 11 isolates were resistant to at least to one first-line anti-TB drugs and two isolates were MDR isolates. Resistance was highest to the STR (14.1%) followed by INH resistant (10.9%) (Table 4).

Table 4: Drug resistance patterns to first-line antituberculosis drugs among 64 culture-positive *M. tuberculosis* isolates in southeastern Ethiopia

Resistance	Frequency	Percentage (%)
Any drug resistance	11	17.2
STR	9	14.1
INH	7	10.9
RIF	2	3.1
EMB	0	0
Mono-resistance	6	9.4
STR only	4	6.2
INH only	2	3.1
RIF only	0	0
EMB only	0	0
Two or more drug resistance		
INH + RIF	2	3.1
INH +STR	5	7.8
INH + RIF +STR	2	3.1

INH = Isoniazid, RIF = Rifampicin, EMB = Ethambutol, STR = Streptomycin

Genotypic Detection for RIF and INH Resistance

The MTBDRplus assay was performed on 92 LJ culture positive isolates. Out of 92 tested *M. tuberculosis* isolates, 86 (93.5%) isolates were susceptible to the two most effective anti TB drugs while six (6.5%) isolates were resistant to either for RIF or INH. Of the six resistant isolates, five isolates (5.4%) were INH-monoresistant and a single strain was resistant to RIF. In our current sample, there was no resistant isolate to INH and RIF i.e. no MDR isolate was detected.

The specific mutation in the five INH-resistant isolates was observed at codon S315T1 of the *katG* gene whereas mutations in the promoter region of the *inhA* gene were not

observed. In our finding single RIF resistant isolate was observed at Codon S531L of *rpoB* gene, missing WT8 with the corresponding appearance of MUT3 (Table 5).

Table-5: Mutations associated with RIF and INH drug-resistant TB with Genotype MTBDRplus VER 2.0

Gene	No of Strains	Mutation pattern	Amino acid change	result
rpoB				
	1(1.1%)	ΔWT8, MUT3	S531L	RIF r
KatG				
	5(5.4%)	ΔWT, MUT1	S315T1	INH r

INH-Isoniazid; RIF-Rifampicin; INH r- Isoniazid resistant; RIF r-Rifampicin resistant; Δ-deletion; MUT-mutant; WT-Wild type.

Association of Lineage with Drug Resistance

We observed the relationship between *M. tuberculosis* strains and their drug susceptibility patterns against the four first line anti TB drugs. In our study, the BACTEC MGIT960 DST and MTBDR*plus* 2.0 drug susceptibility analysis were identified fourteen (14) resistant *M. tuberculosis* isolates for at least one of the four first-line anti-TB drugs. All those *M. tuberculosis* strains developing resistance were belonged the most dominant Ethiopian lineage of Euro-American and significant rate of orphan strain resistance was observed to streptomycin.

Discussion

In the present study, 92 *M. tuberculosis* isolated from TB patients in the Arsi Zone were typed using RD 9-based PCR and spoligotyping. Furthermore, the isolates were tested for their drug sensitivity against the first line anti-TB drugs using MGIT960^{TM}system and GenoType MTBDRplus line probe assay.

RD 9-based PCR revealed that all the isolates were *M. tuberculosis*. This observation is consistent with the previous studies which reported *M. tuberculosis* as the main cause for pulmonary TB in most part of Ethiopia and the rest of the world (Bruchfeld *et al.*, 2002; Disassa *et al.*, 2015; Tilahun *et al.*, 2018). Consistently, a study from Nigeria reported that 85% of TB was caused by *M. tuberculosis* (Cadmus *et al.*, 2006). Further strain characterization of these 92 *M. tuberculosis* isolates by spoligotyping lead to the identification of 56 distinct patterns with 61% of genotype diversity. Overall, 51% of the isolates were clustered into 11 different spoligotype patterns whereas the remaining 49% of isolates were singletons. Comparable rates of clustering were found and reported in different regions of Ethiopia (Debebe *et al.*, 2014; Tessema *et al.*, 2013), South Africa (45%) (Mathema *et al.*, 2015) and in Zambia (37.7%) (Mulenga *et al.*, 2010). This high level of clustering strains suggests that the presence of recent human-to-human transmission in the southeastern parts of Ethiopia.The most frequently occurring clustered strains were SIT149 (13.0%) and SIT53 (11.9%), respectively. These strains werealso the most frequently circulating strains in most parts of Ethiopia (Garedew *et al.*, 2013; Getahun *et al.*, 2015; Tilahun *et al.*, 2018).

Most Ethiopian TB isolates belong to three *M. tuberculosis* lineages: lineage 1(Indo-Oceanic), lineage 3 (East-African-Indian), and lineage 4 (Euro-American lineage) (Comas *et al.*, 2015). Similarly, the Indo-Oceanic Lineage, East-African-Indian Lineage, *Mycobacterium-africanum* lineage, and the Euro-American lineage were identified in the present study. Among the lineages identified, Lineage 4 (Euro-American lineage) was the predominant Lineage consisting of 79.3% of the isolates. In agreement with our finding, the Euro-American Lineage was found the most prevalent and the widespread Lineage in Ethiopia (Comas *et al.*, 2015; Firdessa *et al.*, 2013).This lineage is prevalently reported in central highlands of Ethiopia by Bedewi *et al.* (2017) and Garedew *et al.* (2013).. Lineage 4 (Euro-American Lineage) was also the most dominant lineage in Sudanese pulmonary TB isolates (71.6%) (Khalid *et al.*, 2016). The wide occurrence of the Euro-American Lineage could due to its virulence and population movement between neighboring geographic regions, which facilitates its transmission.

The second most dominant Lineage identified in the present study was East-African-Indian Lineage. In line with our finding Bedewi *et al.* (2017) and Nuru *et al.* (2015) were reported the East-African-Indian Lineage was as the second most cause of TB in the Oromia Region and in the Amhara Region of Ethiopia, respectively. With regard to grouping into families, majority (45.7%) of the strains were members of the T1 family while 13.1% of isolates were grouped in the T3 family, and this is consistent with the findings from the Afar Region and Amhara Region (Belay *et al.*, 2014; Yimer *et al.*, 2013).

In addition to molecular typing, the 64 liquid culture confirmed *M. tuberculosis* isolates were tested for the sensitivity to the four first-line anti-TB drugs using MGIT960 phenotypic DST. Accordingly, we found 17.2% (11/64) of any drug resistance. Similar proportion of resistance was reported from Hitossa district of the Arsi Zone (Hamusse *et al.*, 2016), Jimma (Abebe *et al.*, 2012) and elsewhere in Uganda (Muwonge *et al.*, 2013). However, in contrast to this present study, a higher percentage of any drug resistance was reported in Nepal (Maharjan *et al.*, 2017) and in Myanmar (Aung *et al.*, 2015).

In the present study, the GenoType MTBDRplus assay demonstrated a mutation from 5.4% (5/92) Isoniazid resistant isolates and only 1.1% Rifampicin resistant isolate. Many research findings indicate that much of the INH-resistant strains contain mutations in codon 315 of *katG* (Lemos and Matos, 2013). In agreement with the previous reports, our finding showed that all mutation for INH resistance was found in the katGS315T1 (5.4%) whereas the mutations in the promoter region of the *inhA* gene was not observed. On the other hand, mutations in the *rpoB* gene conferring Rifampicin resistance were present in only 1.1% (1/92) of isolates. A similar trend where Rifampicin resistance was found at *rpoB* gene *S531L*by Bedewi *et al.* (2016). In this study, Euro American Lineage was associated with resistance to the first line anti-TB drugs, which is agreed with the earlier report in Ethiopia (Bedewi *et al.*, 2017)and in Papua New Guinea (Ballif *et al.*, 2012).

Conclusions

The proportion of orphan strains isolated in this study was high, which could suggest the presence of new strains in the Zone. Moreover, the study showed relatively high percentage of mono-resistance to any four first-line drugs warranting for the need to strengthen the control efforts.

Declarations

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

All the authors participated in the study design. **BH**: conception of research idea, study design, laboratory work, analysis and interpretation of result, drafting and reviewing the manuscript. **GA***: conception of research idea, study design, supervision, interpretation and drafting of manuscript. AZ: data collection, laboratory analysis and result interpretation. **BY**: Laboratory analysis and result interpretation, **KT***: conception of research idea, study design, data collection, laboratory work, analysis and interpretation of result, drafting and reviewing the manuscript. GA and KT has equal contribution. All authors read and approved the final manuscript.

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