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Genotyping and Drug Resistance Patterns of *M. tuberculosis* in Eastern Amhara region, Ethiopia

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Abstract

Background: Ethiopia is among the countries with the highest incidence of tuberculosis (TB) and has a early incidence of 379 cases/100,000 population. Of the high TB burden regions, Amhara is the top. So understanding the epidemiology of TB through molecular genotyping techniques such as Spoligotyping have invaluable role to combat TB. It will help to designing appropriate intervention and strengthen TB control program.

Objectives: To provide insight about the genetic biodiversity of *Mycobacterium tuberculosis*, strain specific drug susceptibility and possible associated factors in Eastern Amhara region, Ethiopia.

Methods: A facility based cross sectional study was conducted among smear positive TB patients (age ≥ 18 years old) from September 2011 to June 2012. Smear positive sputum samples were processed and decontaminated by the modified Petrof method. Primary isolation and drug susceptibility testing (DST) were carried out on egg based Lowenstein-Jensen (LJ). Genotyping of mycobacterial DNA was performed by spoligotyping and isolates were assigned to families using the SpolDB4 and the model-based program 'Spotclust'. P-values less than 0.05 were considered as statistically significant.

Result: The predominant *Mycobacterium tuberculosis* strains in the present study were ST 149/T3-ETH 49(22.6%), ST53/T1 18(8.3%) and ST50/H3 16(7.4%). T3-ETH strain showed the highest MDR-TB cases.

Conclusion: Our finding suggests that a diversity of *Mycobacterium tuberculosis* strains accompanied with high rate of drug resistance.

Keywords: Molecular typing; Drug resistance; TB; Eastern Amhara region

Introduction

Currently, tuberculosis (TB) is the second most common cause of death due to an infectious disease [1]. Globally, 9.4 million incidents and 14 million prevalent cases occurred in 2010 [2]. Africa, more specifically Sub- Saharan Africa, faces the worst TB epidemic [1].

According to 2012 the World Health Organization (WHO) report Ethiopia has been one of the 22 high TB burden countries with an incidence and prevalence rate of 300 and 470 cases per 100,000 populations respectively. Among all new TB cases notified to federal ministry of health, 30% were smear positive [3]. Among re-treatment cases 64%, 11%and 13% were relapse, treatment after failure, and treatment after default respectively [4,5].

The TB problem has been compounded by the emergence and spread of DR-TB strains both on new and previously treated cases [2,6-9,11]. Worldwide, 3.7% of new cases and 20% of previously treated cases were estimated to have multi drug resistance-tuberculosis (MDR-TB) [12].

In Ethiopia, the level of MDR- TB among new TB cases is estimated at 1.6% and 12% for previously treated cases [2,3]. The highest rate of drug resistance (DR) were reported to Streptomycin (S) (10.2%) and Ionized (H) (8.4%) [13].

In the present of such condition, understanding the epidemiology of TB through molecular genotyping techniques has an invaluable role to TB control. It can predict transmission rate and identify dominant strains of TB [14-18].

Studies showed that different lineages of *M. tuberculosis* have strain-specific differences in virulence, rate of transmission, outbreak causation, and drug resistance pattern [14-18]. For instance Beijing strains have been significantly associated with high rates of drug resistance [14-18].

In addition, it has been claimed that particular lineages of *M. tuberculosis* adapted to specific human populations [19]. Strain differences in different geographical boundaries were also well documented [20].

In Ethiopia depicted that T3_ETH and Central Asian strain (CAS) KILI were the predominant spoligotype. MDR-TB was found to be significantly associated with T3 and CAS strains [21].

In contrast to the context described above, epidemiological information regarding transmission, geographical prevalence of *Mycobacterium tuberculosis* strains as well as drug resistance profile in

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Ethiopia was very scarce, especially in rural communities such as in Eastern Amhara region.

Therefore, the aim of this study was to assess the genetic biodiversity of *Mycobacterium tuberculosis*, their drug susceptibility patterns and possible associated factors in Eastern Amhara region, Ethiopia. The information obtained from this study will provide baseline data about the genetic biodiversity and strain specific drug resistance profile in the region.

Material and Methods

Methods

Study design and period: A facility based cross-sectional descriptive study was conducted among new and re-treated pulmonary TB patients (age ≥ 18 years old) attended different health institutions from September 2011 to August 2012 in Eastern Amhara region, Ethiopia.

Sampling method and procedure: Fourty three public health facilities (4 hospitals, 39 health centers) in Eastern Amhara, Ethiopia were selected randomly. A stratified random sampling method was used to create different strata (referral hospital, district hospital and health centers). A separate sample unit was selected from each stratum using proportionate to size. The sample size was determined using single population proportion formula with the prevalence of smear positive *M. tuberculosis* 286/100,000 [1], 95% CI, that give a final sample size of 230.

Culture and drug susceptibility testing procedure: Smear was prepared from morning sputum of each study participants to diagnose Acid Fast Bacilli (AFB) using the national TB and leprosy guideline [5]. About 5 ml of sputum sample were transported using ice pack to Ethiopian Health and Nutrition Research Institute (EHNRI), the national TB reference laboratory of Ethiopia.

Only AFB positive samples were subjected for digestion and decontamination by modified petroff method [22]. Then 0.2 ml of the processed sputum was inoculated on to Lowenstein –Jensen media (L.J) slants. All inoculated media were incubated at 37°C. The inoculated solid L.J media was inspected two times per day for the first two weeks while 3 times per weeks for the remaining 2-4 weeks [22].

Indirect proportional method was used to test the drug susceptibility patterns of culture positive isolates [21]. H37Rv were used as positive control while Start and end control as internal quality control. All activities like reagent and media preparation were carried out as standard operating procedure [22].

Heat killing of mycobacterium suspensions: Heat killed bacterial suspension for each isolate was prepared by mixing, 2 loops full of culture in 500 µl of 1x TE buffer and the tubes were submerged in 80oC water bath for 1 hour.

Region of difference based multiplex PCR: Heat killed AFB positive samples were investigated by multiplex PCR for the presence or absence of RD4, RD9 and RD10 and, RD Flank Rev (4,9and10), RD Int. The PCR amplification was performed following the standard operational procedures. After running electrophoresis in 1.5% agarose gel, the results were interpreted as *M. tuberculosis* (RD9) present when a band of 396bp is observed.

Spoligotyping: Isolates belonging to the *M. tuberculosis* complex were further being spoligotyped as described by (1997). A total volume of 25% Micro litters (µl) and the following reaction mixtures were used for PCR amplification of DNA within the DR region of Mycobacterial isolates: 12.5 Micro –litters of Hot Starq Master Mix (Quiagen: this solution provides a final concentration of 1.5 Micro –molar Mgc l2 and 200micro-molar of each deoxynucleotides triphospates), 2 µl each primer (20 pinco-molareach), 5 µl suspension of heat killed cells (approximately 10 to 50ng), and 3.5 micro –litters distilled water. The mixture was heated for 15 min at 96°C and then subjected to 30 cycles of 1 min at 96°C, 1min at 55%, and 30 seconds at 72°C.

The amplified product was hybridized at a set of 43immobilized oligonucluotides, each corresponding to one of the unique spacer DNA sequences within the DR locus. After hybridization, the membrane was washed twice for10 min in 2x SSPE [1x SSPE is 0.18 M Nacl.10mM NaH2po4 and 1Mmedta (ph 7.7)] - 0.5% sodium dodecylsulfate at 60°C and then incubated in 1:4000-diluted streptavidin-peroxidase (Boehringer) for 45 to 60 min at 42°C. The membrane was washed twice for 10min in 2xSSPE-0.5% for 5 min at room temperature. Hybridizing DNA will be detected by enhanced chemiluminescence method (Amersham) and by exposure to x-ray film (Hyperfilm ECL, Amersham) as specified by the manufacturer. Finally the interpretation was based on presence or absence of the respective spacer sequences for each isolate. DRa: biotin-5'-CCG AGA GGG GAC GGA AAC-3' and DRb: 5'-GGT TTT GGG TCT GAC GAC-3' primeras will be used [23].

Statistical analysis

Data were entered and analyzed using Statistical Package for Social Sciences (SPSS) version 16 software. The spoligotyping results were prepared in octal and binary formats into Microsoft excel spreadsheets. Spoligo type patterns were designated as 43-character-long strings consisting of black and white squares representing the presence or the absence of an individual spacer, respectively. The spoligo patterns which were prepared in binary and octal were entered and determined by comparing the spoligotyping results with already existing designations in the international spoligotyping database, SpolDB 4.0 [24]. Bi and multivariate analysis using logistic regression model was computed. P values < 0.05 were statistically significance.

Ethical considerations

Ethical clearance was obtained from Institution of Review Board (IRB) of Medical faculty, Addis Ababa University. Informed consent was obtained from each study participant. All the data were recorded using codes. The laboratory results were communicated to treating physicians for better management of the patients.

Result

Socio demographic characteristics of the study partcipants

A total of 221 smear positive respondents were enrolled in this study. Of these, 128 (57.9%) were males and 93 (42.1%) females. The mean age of the respondents was 29.6 years old. Majority of respondents 168 (76%) were married and rural residents 128 (57.9%).

Spoligotyping and drug resistance profile of *M. tuberculosis* strains

Of the 221 typed isolates, 217 (98.2%) had spoligo types result. Of these, 198/217 (91.2%) isolates had previously defined shared spoligotype numbers; while the remaining 19 (8.8%) isolates had unidentified patterns (orphan). 13 of the isolates which gave newly identified spoligotypes clustered into 3 groups of between 2 and 6 isolates.

Among the 204 isolate clusters, we found 20 minor spoligo types (including 2 to 9 isolates) and 6 major spoligotypes (>10 isolates). The most frequent strain was the ST149 49 (22.6%), ST53 18 (8.3%) and ST50 16 (7.4%).

Out of all the studied isolates, the most prevalent family was the T family (42%), the CAS family (19.4%) and Haarlem family (16.3%). In addition, strains from other family, such as the MANU2, LAM, U and X family were found. MANU 2 family strains were found only in North Shoae zone. U and X families were not found in Waghemera and North Shoae zone (Table 1).

The largest number of strains was isolated from North Shoae, North Wollo, South Wollo, Oromia and Waghmera, respectively (Table 1). There is no statistical difference in the distribution of circulating strains which belonging to T, CAS, Haarlem, LAM, U and X clade in five zones of Eastern Amhara region (P>0.05).

T3-ETH strain showed the highest (6 out of 15) MDR-TB cases. While other strains of *M. tuberculosis* such as ST 53, ST50, ST25, ST41, and STX1 showed occurrence of MDR-TB (Table 2).

Seventy of the 91 isolates (78%) belonging to four variants of the T family (T1, T2, T3, T3-ETH) showed resistance to at least one drug. 15 of the 35 (43%) Haarlem families (H3, H4) showed resistance to one or more anti-TB drugs. 9 of the 41 CAS families (CAS1-KILLI, CAS1-DELHI (two different variants)) showed that resistance to one or more anti-TB drugs (Table 2).

There was no significant association between any strain types with development of drug resistance (any resistance, mono resistance, MDR –TB) (P>0.05). In addition, analysis of cluster types also revealed that there was no association with any drug resistance, previous anti-TB drug exposure, MDR-TB and other socio-demographic variables (P>0.05) (Table 3).

Discussion

The high rate of clustering of *M. tuberculosis* strains 204 (94%) were observed in the present study. This might be an indication of rapid ongoing transmission within a communities and/or sub populations. Probably this transmission might be due to the populations in those

Family	No of strain	South Wollo	North Wollo	Waghemra	Oromia	North shoae
T family	91	18	14	19	18	22
T1	19	3	3	5	3	5
T2	8	2	4	0	1	1
T3	15	6	5	1	2	1
T3-ETH	49	7	2	13	12	15
LAM family	10	2	4	1	1	2
LAM-TUR	5	2	3	0	0	0
LAM-9	5	0	1	1	1	2
Haarlem family	35	9	14	4	5	3
H2	1	0	1	0	0	0
H3	22	5	8	3	5	1
H4	12	4	5	1	0	2
CAS family	41	12	10	3	7	9
CAS1-KILI	13	3	3	0	4	3
CAS1-DELHI	26	7	7	3	3	6
CAS	2	2	0	0	0	0
X family(X1)	13	1	0	2	2	8
MANU 2	2	0	0	0	0	2
U	6	1	2	0	1	2

Table 1: Geographical distribution of *Mycobacterium tuberculosis* strains in five zones of Eastern Amhara region, Ethiopia, September 2011 to August, 2012.

SIT No	S	E	R	H	MDR
T	32	9	13	16	8
149	21	6	10	12	6
53	6	3	2	3	2
37	4	0	1	1	0
52	1	0	0	0	0
Haarlem	7	0	2	6	2
50	6	0	2	5	2
777	1	0	0	1	0
134	0	0	0	0	0
CAS	4	1	2	2	1
25	1	1	1	1	1
21	1	0	1	0	0
26	2	0	0	1	0
LAM	1	0	1	1	1
41	1	0	1	1	1
42	0	0	0	0	0
X1	1	1	1	1	1
New/orphan	2	0	0	4	0
New/orphan	2	0	1	3	1
U	1	0	0	2	0

Key: H-Isoniazid, S- Streptomycin, R- Rifampicin , E-Ethambutol, MDR- TB -resistance to at least Isoniazid and Rifampicin

Table 2: Drug resistance patterns of predominant *M. tuberculosis* strains that cause TB in Eastern Amhara region, Ethiopia, August 2012.

Variables	Clustered group (No)	Non clustered Group (No)	95% CI	OR	P-value
Age 18-40 >41	 176 12	 32 1	0.058-3.649	0.458	0.461
Sex Male Female	 124 12	 80 5	0.603-8.450	2.58	0.226
Previous anti-TB therapy Yes No	 64 144	 2 11	0.088-1.899	0.409	0.254
Resistance to Any one or more drugs Susceptible to all drug	 75 3	 133 10	0.142-1.993	0.532	0.349

Table 3: Clinical and epidemiologic characteristics of patients harboring clustered versus non clustered strain

five zones of Eastern Amhara region, Ethiopia have been mixed in different circumstance such as trade and traveling.

The T family, a family which has not been well defined to date was the predominant spoligotype in our study (42%) [25]. This is consistent with previous studies in different parts of the world such as in Tanzania [26], Mozambique [27] and Ethiopia [28].

Of the T family, the SIT 149 (T3-ETH) strain was the most frequent (22.6%). Ethiopia reported similar finding. It has been genotypically restricted in Ethiopia [21,28,29]. Probably this might be explained as particular lineages of *M. tuberculosis* adapted to specific human populations and a presence of strain differences in different geographical boundaries [19,20].

Similarly, The SIT 53 (T1) in our study accounted about 18 (8.3%) of the total isolates. In concordant with this, scholars showed that this strain was widespread around the world [30-32].

The CAS family, which is the second most frequent 41 (19%) spoligotype in this study (Table 1). Ethiopia reported similar findings [21,28]. This clade was also prevalent in Tanzania [24], Sudan [33], Uganda [34] and Kenya [35]. Probably this has been explaining an important trend in the *M. tuberculosis* infection pattern in East African setup.

The Haarlem family, which is the third most frequent (16.2%) spoligotype observed during this study (Table 1) [36]. Of these, H3 and H4 constituted 22 (63%) and 12 (34.3%). Studies showed that those strains have been prevalent in Europe [13,30] and Middle East [22,32,35] countries where as less frequently reported from other East African countries [26,33-35].

Moreover, the LAM7_TUR (ST41) strain contributes about 50% of the LAM families (Table 1). In line with this, it was found in Ethiopia [21,28], Tanzania [26], Uganda [34] and Kenya [24], MANU 2 family (n=2, 0.9%), reported from patients from North Shoae zone was the first report in Ethiopia (Table 1). In line with this, one study in Egypt revealed that Manu strains were occurred as an unusually high proportion [31]. The occurrence of this new strain might be the zone is near to Addis Ababa, which is a capital city of Ethiopia and currently there has been a lot of foreigners (Chinese and Japanese) who stayed in this zone due to a presence of a huge construction of road, beer and textile factories.

In our finding, there is no statistical significance difference in major circulating strains (T, H, CAS, LAM, U and X) among five zones of Eastern Amhara region (P>0.05). However, Studies showed a presence of significant difference in circulating *M. tuberculosis* strains in different territories [22, 27]. This might be explained as a presence of cosmopolitan population with a frequent migration and social mixing.

Studies showed that different *M. tuberculosis* strains such as Beijing, EAI, CAS and T3 have showen strain-specific differences in terms of development of drug resistance [14-16]. In our study, although 8/15 of the T and 6/15 of the H family developed MDR-TB, there was no statistical significance association between any strain types with the development of drug resistance (P>0.05).

Contact history, age, geographical location, and previous drug exposure [35-39] were explained as possible factors for the clustering of specific *M. tuberculosis* strain. While in the present study analysis of cluster types revealed that there was no significance association with any drug resistance, previous antitubrcle drug exposure, MDR-TB and other socio-demographic variables (P>0.05).

In conclusion, a diversity of *Mycobacterium tuberculosis* strains accompanied with high rate of drug resistance for main anti-tuberculosis drugs was observed. So, patient's adherence to anti-TB drugs (especially re-treated cases) and scaling up of DST service at district hospital level will help to reduce the development of drug resistance in the study area.

Author's Contributions

AE: conception and initiation of the study, design, implementation, analysis and Writing. KD: initiation, design, implementation, analysis and co-writing. IA, ME: Design, implementation and co-writing. AD, MW, SB and EH: implementation, and analysis. All authors read and approved the final manuscript.

Competing Interests

This work was sponsored by USAID/TB CTA, Ethiopia. No future financial aid was received from any organization for publication or other interest. There is no any competing of interest.

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