Genotyping of mycobacterium tuberculosis isolated from pulmonary tuberculosis patients among people living with HIV in Addis Ababa: Cross-sectional study

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ABSTRACT

Background: Tuberculosis is a serious infection that is common in people living with HIV and increases the mortality and morbidity from the diseases. The study of genetic diversity among strains of *M. tuberculosis* has a great impact in studying pathogenicity and transmissibility, design for vaccines production, identification of nominee genes for drug targets, and improving molecular diagnostic techniques. The aim of this study was to characterize *Mycobacterium tuberculosis* (Mtb) isolated from suspected pulmonary tuberculosis among people living with HIV.

Method: A total of 143 sputum samples was collected and transported to Akililu Lemma TB laboratory. The collected samples were processed for culture using Lowenstein-Jensen medium. For 45 culture positive isolates, genotyping of mycobacterial DNA was performed by spoligotyping and isolates were assigned to families using the SpolDB4 and the model-based program 'SPOTCLUST'. Categorical data were analyzed by Chi-square test.

Result: A high level of diversity was found among the 45 isolates. Twenty six different Spoligo patterns were obtained. The T (46.7%), Family33 (44.4%) and Central Asian (CAS): (4.4%) families were the dominant isolates comprising 91.5% of the total strains. Of 44% of the Euro-American, 6/20(30%) and 9/20(45%), identified were lineage belonged to Spoligo-International-Type (SIT_{336}) and SIT_{149}. Of the total strains, 12 (22%) were unique and have not been described in SpolDB4 to date.

Conclusion: We found the high diversity of Mtb in pulmonary tuberculosis patients in this setting. T_3_ETH family identified as the numerous *M.tuberculosis* strains circulating in the community.

1. Introduction

Members of the *Mycobacterium tuberculosis* complex (MTC) are genetically very closely related, with a high degree of conservative inter-strain DNA homology, and only a difference estimated as less than 0.05% between species, subspecies and strains ([15], Magdalena et al., 1998 and Cole and Barrell, 1998). The study of genetic diversity among strains of *M. tuberculosis* has implications in pathogenicity and transmissibility, the design and preparation of vaccines, identification of candidate genes for drug targets, and improving molecular diagnostic techniques [10,15]. Such genetic variation may be a major factor in the emergence of many mycobacterial strain variants involved in causing global TB epidemics; and some are useful for epidemiological studies and for classification of the organisms into groups or constructing phylogeny [10,15], Arnold, 2007, Sreevatsan et al., 1997). Molecular epidemiology uses such changes to find the causative agent, the strain, and trace back the source of TB-outbreaks in different geographical localities to combat the disease [16]. Now a day, the recent molecular genotyping methods such as MIRU-VNTR typing, IS6110 RFLP and spoligotyping revealed a highly diverse population structure of *M.tuberculosis* with at least five major geographically associated lineages including African (Uganda, Cameroon and S-type), Asian (Beijing and CAS), Latin American-Mediterranean (LAM), African-European populations (X-type, Ghana and Haarlem) and East African- Indian (EAI) lineages that can also be further subdivided into well-defined genotypes [8,19]. Molecular typing techniques have been extensively used to speciate strains of Mtb involved in TB infections, studying the molecular epidemiology of Mtb, providing insights into dissemination

dynamics, evolutionary genetics, and detection of suspected outbreaks and person-to-person transmission [1].

2. Methods and materials

2.1. Study population and design

A cross-sectional study was conducted from January to July 2014 in two hospitals (tertiary-level) and one health center, located in Addis Ababa. The site is chosen for their facility and burden of TB/HIV co-infection. A total population included in this study was 143 TB/HIV co-infected persons. Patients were referred for study inclusion by attending clinicians if the patient was suspected to have HIV-TB co-infection. The study included adult above 18 years of age and HIV seropositive suspected pulmonary TB in the study area. Then clinical information documented for enrolled patients i.e. demographic information, past history of TB, symptoms and vital signs, HIV status and renal function.

2.2. Data collection procedure and isolation

Samples were collected from suspected pulmonary TB patients attending the health facilities. All study participants provided three consecutive sputa for their regular diagnostic activities. The same day, sputum smears were examined for the presence of acid fast bacilli (AFB) by an experienced laboratory technician using the standard Ziehl-Neelsen method [17]. Sputum samples were kept at $-20\,°C$ till transported within a maximum of 2 days to Aklilu Lemma Pathobiology Research Institute (ALPBio) for culture. At the ALPBio, sputum specimens (4–8 ml) were processed by the standard N-Acetyl-ʟ-Cysteine-Sodium Hydroxide (NALC−NaOH) method [4] and concentrated at $3000 \times g$ for 15 min. The sediment, regardless of the original sample volume, was reconstituted to 2.5 ml with phosphate buffer pH 6.8, to make the inoculums for the smears and cultures. Two Lowenstein-Jensen slants, one containing 0.75% glycerol and the other containing 0.6% pyruvate, were inoculated with the sediment and incubated at $37\,°C$. Cultures were considered negative when no colonies were seen after 8 weeks incubation period.

2.3. Identification and characterization of MTBC

For a total of 45 bacterial isolates grown on ʟ-J media, species identification of MTBC was performed by using RD9-based PCR which is done on heat killed cells to confirm the presence or absence of RD9 using three primers namely, RD9flankF, RD9IntR, and RD9flankR. Amplification was done by standard thermo cycler (VWR Thermo cycler, UK). The PCR amplification mixture used consisted of 10 µl HotStarTaqMaster Mix (Qiagen, United Kingdom), 7.1 µl distilled water, 0.3 µl of each three primers and 2 µl of DNA template (heat-killed cells), giving a total volume of 20 µl. The PCR reaction was heated at $95\,°C$ for 15 min after which it was subjected to 35 cycles consisting of $95\,°C$ for one min, $55\,°C$ for one minute, and $72\,°C$ for 1 min. Thereafter, the reaction mixture was maintained at $72\,°C$ for 10 min following which the product was removed from the thermocycler and run on agarose gel electrophoresis. For gel electrophoresis, 8 µl PCR products were 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, *M. bovis* bacilleCalmette-Guérin, and water were included as positive and negative controls. Interpretation of the result was based on bands of different sizes, as previously described by Parsons et al. [14]. Isolates that were positive for *M. tuberculosis* by RD9 PCR were further characterized by following Standard spoligotyping method as described by Kamerbeek [9]. The SpolDB4 database [3] and a web-based computer algorithm, Spotclust http://tbinsight. cs.rpi.edu/ run_ spotclust. html, was used to assign new isolates to families, subfamilies and variants. SpolDB4 assigned names (shared

types) were used whenever a spoligotype pattern was found in the database. Patterns not found in SpolDB4 were assigned to families and subfamilies by Spotclust. Spoligotypes described only once (non-clustered) in this study and in the SpolDB4 were designated as `Orphan' (not assigned). A cluster was defined as two or more isolates from different patients with identical spoligotype patterns.

2.4. Ethical issue

The study has been approved by the Ethical Review and Research Committee (ERC) of the Department of Medical Microbiology, Immunology and Parasitology (DMIP) (Protocol No. 1/T/2013, on meeting No.23rd) of Addis Ababa University. Written informed consent was obtained from all study participants before the interview and sample collection.

2.5. Statistical analysis

Completed questionnaires were coded by numbers and the data was then transferred to SPSS version 16 for analysis. Categorical data were analyzed by Chi-square test. The level of significance was set at $p \leq 0.05$, and 95% confidence interval was used throughout.

3. Results

3.1. Socio-demographic information

A total of 143 eligible study participants were included. The majority of the participants (60.1%) were married and 61.5% of them were female. Among 143 suspected pulmonary TB HIV seropositive patients, 45(31.5%) were confirmed as pulmonary TB by culture. Of this confirmed PTB cases, 20(45.8%) were found among 28–37 age group. A total of 45 *M. tuberculosis* isolates were utilized to carry out RD9-based PCR and spoligotyping analysis of which 43(95.5%) gave valid spolygotyping data while the remaining 2(4.4%) isolates did not give any pattern upon spolygotyping (Table 1).

3.2. Spoligotype result

Of 45 clinical isolates, 30/45(66.7%) were classified into one of 17 distinct spoligotype patterns shared international types (SIT) according to SpolDB4.0. The remaining 15/45(33.3%) isolates generate 12 different spoligotypes pattern that had not been previously reported to the SpolDB4.0. Among the distinct spoligotype pattern characterized, 5 patterns corresponding to cluster with 2–9 isolates per clusters were

Table 1

Socio-demographic characteristic of the PTB suspected HIV seropositivity patients in Addis Ababa, Ethiopia ($N = 143$).

Variables categories		Frequency	%	PTB(45)	*p* value
Age	18–27	21	14.7%	9(18.6%)	0.089
	28–37	76	53.1%	20(45.8%)	
	38–47	28	19.6%	10(22%)	
	48–57	13	9%	5(11.9%)	
	> 57	5	3.5%	1(1.7%)	
Gender	Male	55	38.5%	17(37.3%)	
	Female	88	61.5%	28(62.7%)	0.229
Marital status	Single	28	19.6%	7(15.3%)	0.0971
	Married	86	60.1%	24(52.5%)	
	Divorced	12	8.4%	5(13.6%)	
	Widow	17	11.9%	9(18.6%)	
Educational status	Non educated	18	12.6%	5(11.9%)	0.741
	Elementary school	35	24.5%	10(22%)	
	High school	78	54.5%	25(54.2%)	
	Higher education	12	8.4%	5(11.9%)	

Webdings format	SIT	Lineage	Family	No. Strain
	Orphan	Unknown	CAS	1
	Orphan	Unknown	CAS	1
	564	Indo-Oceanic	EAI4	1
	Orphan	Indo-Oceanic	Family33	1
	523	Indo-Oceanic	Family33	3
	Orphan	Indo-Oceanic	Family33	1
	1096	Indo-Oceanic	Family33	1
	Orphan	Indo-Oceanic	Family33	3
	Orphan	Indo-Oceanic	Family33	1
	415	Indo-Oceanic	Family33	1
	Orphan	Indo-Oceanic	Family33	1
	Orphan	Indo-Oceanic	Family33	1
	1149	Indo-Oceanic	Family33	1
	Orphan	Indo-Oceanic	Family33	1
	Orphan	Indo-Oceanic	Family33	1
	2731	Indo-Oceanic	Family33	2
	1690	Indo-Oceanic	Family33	1
	Orphan	Indo-Oceanic	Family33	1
	727	Euro-American	Haarlem1	1
	336	Euro-American	T1	6
	Orphan	Indo-Oceanic	T1	1
	Orphan	Indo-Oceanic	T3	1
	1123	Euro-American	T3	2
	149	Euro-American	T3_ETH	9
	249	Euro-American	T4	1
	2246	Euro-American	T4	1

Fig. 1. Spoligotype patterns of Mtb strains, family, and lineage assignment of PTB from clinical isolates according to SpolDB4.0 and SPOT-CLUST web based program. The empty boxes represent the presence of spacers, and the hyphens represent the absence of spacers. No = number of isolates, SpolDB4.0 = fourth international spoligotyping database; SIT = Spoligo International Typing, CAS = Central Asian; T = Tuscany, EAI = East-African Indian.

identified. The remaining 8 patterns represented by a unique (non-clustered) spoligotypes pattern were represented as a single in the database. Out of 15 isolates not found in spolDB4 that classified into 13 patterns, 12 were represented by a unique pattern which was true orphan according to spolDB4.0, whereas the remaining one pattern consisted of a cluster with 3 isolates per cluster were identified. The largest cluster identified in the present study was SIT_{149} consisted of 9 isolates; the second largest cluster was SIT_{336}, comprising 6 isolates.

Classification of the spoligotype pattern with web-based SPOTCL-UST database showed different families "ill-defined" (T), Central Asian (CAS), Family33, Beijing, H37Rv, Haarlem1, and EA14 were reported. Among these families, Family33 and T family consisted 20/45(44.4%) and 21/45(46.7%) isolates respectively. T family consist T1, T3, T3-ETH, and T4 with the clade accounted for 6/45(13.3%), 3/45(6.7%), 9/45(20%) and 2/45(4.4%) respectively. Other families present were CAS, 2/45(4.4%), Haarlem1 1/45(2.2%) and EA14 1/45(2.2%). This web also classified strains into different lineage: modern lineage, Euro-American 20/45(44.4%), ancestor lineage, Indo-Oceanic 23/45(51%) and 2/45(4.4%) identified were unknown lineages (Fig. 1).

4. Discussion

The present study describes the diversity of the population structure of *M.tuberculosis* clinical isolates in patients from Ethiopia. All PTB cases reported in this study were caused by *M. tuberculosis*. Out of 45 isolates, 30(66.7%) patterns have already been reported in SpolDB4 database, where the remaining 15(33.3%) patterns were not found in the SpolDB4 database. Moreover, similar findings were reported from Bahr Dar, Ethiopia [5]. Molecular characterization of the strains of *M.tuberculosis* using spoligotyping identified 26 different spoligotype patterns, of which 6(23%) consisted of clusters of isolates where 20(77%) consisted of nonclustered single isolates. In contrast with our study, comparable prevalence of clustering was found in population-based studies from South Africa (45%) [18], Botswana (42%) [12], Estonia (49%) [11], and among randomly sampled patients from Ethiopia (42.1%) [2]. The most commonly found *M.tuberculosis* strains were SIT_{336} and SIT_{149} [6,13], which were also reported earlier in the SITVIT

database as the most common types in Ethiopia by other researchers [5]. Most of the strains highly prevailed in Ethiopia (T, CAS, and H) are all members of the modern lineage [13] which agrees with our findings. The Euro-American lineage which believed introduced to Ethiopia by Europeans during the Italian invasion of Ethiopia accounted 44.4% of the total lineage identified in the present study.

In our study, the majority of the isolates (91.1%) were belonged to two major families: Family33 (44.4%) and T family(46.7%) which agree with the study conducted in Addis Ababa [13] that reported CAS family was the second predominant family. However, in our finding, CAS family accounted only 4.4% with unknown lineage and orphan. The T family, which was the most frequent spoligotype in this study, had been reported in previous studies in Ethiopia as well as elsewhere in the world [5]. Even though the clustering of isolates is an indicator of recent transmission, in our finding the problem was high in 28–37 age group (45.8%)($X^2 = 0.089$) which suggested an increased likelihood of recent TB among this age group, which may be linked to a higher prevalence of HIV infection in reproductive age [7].

5. Conclusion

The present study offers the insight into the genetic diversity of *M.tuberculosis* isolates from sputum of suspected PTB patients in Addis Ababa the capital city of Ethiopia. From the current study in which samples were collected only from two Hospitals and one health center in Addis Ababa, it can be concluded that the more virulent modern lineages are dominance. The dominance of the modern strains in the study area was given a clue that might be widespread in future. Therefore, tuberculosis controls programs, particularly in Ethiopia need to impose a more effective control program in order to avoid any tuberculosis outbreaks.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.jctube.2018.06.004.

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