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

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1. Introduction

Members of the Mycobacterium tuberculosis complex (MTC) are genetically very closely related, with a high degree of conservative interstrain 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 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 (Mt) 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 (SIT336) and SIT149. Of the total strains, 12 (22%) were unique and have not been described in SpolDB4 to date.

Conclusion: We found the high diversity of Mt in pulmonary tuberculosis patients in this setting. T3 ETH family identified as the numerous M.tuberculosis strains circulating in the community.

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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-L-Cysteine-Sodium Hydroxide (NACl–NaOH) method [4] and concentrated at 3000 × 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 7-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 are positive for M. tuberculosis by RD9 PCR were further characterized by following Standard spolotyping method as described by Kamerbeek [9]. The SpolDB4 database [3] and a web-based computer algorithm, Spotclust http://bbs insight.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. 1/7/2013, 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 ≤ 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 38–37 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 spoligotyping data while the remaining 2(4.4%) isolates did not give any pattern upon spoligotyping (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%) | 0.097 |
| 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%) | |
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 SIT149 consisted of 9 isolates; the second largest cluster was SIT336 comprising 6 isolates.

Classification of the spoligotype pattern with web-based SPOTCLUST 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 Bahdar, 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 SIT336 and SIT149 [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%) 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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