

Modern lineages of *Mycobacterium tuberculosis* in Addis Ababa, Ethiopia: implications for the tuberculosis control programme

*Mihret A^{1,2,3}, Bekele Y¹, Aytnew M¹, Assefa Y¹, Abebe M¹, Wassie L¹, Loxton GA³, Yamuah L¹, Aseffa A¹, Walzl G³, Howe R¹

1. Armauer Hansen Research Institute, Addis Ababa, Ethiopia
2. Department of Microbiology, Immunology and Parasitology, College of Health Sciences, Faculty of Medicine, Addis Ababa University
3. DST/NRF Centre of Excellence for Biomedical Tuberculosis Research, MRC Centre for Molecular and Cellular Biology, Division of Molecular Biology and Human Genetics, Faculty of Medicine and Health Sciences, Stellenbosch University, South Africa

Abstract

Background: The genotyping of *Mycobacterium tuberculosis* strains is important to have unique insights into the dissemination dynamics and evolutionary genetics of this pathogen and for TB control as it allows the detection of suspected outbreaks and the tracing of transmission chains.

Objective: To characterize *M. tuberculosis* isolates collected from newly diagnosed pulmonary TB patients in Addis Ababa

Methods: One hundred and ninety two sputum samples were cultured on Löwenstein-Jensen (LJ) slants and isolates were heat killed for molecular genotyping. The isolates were characterized using spoligotyping and were compared with the International SpoIDB4 database.

Result: T genotype constitutes the most predominant in our study (95, 49.5%) followed by the CAS genotype (42, 21.9%). Other genotypes found were Haarlem (H) (24, 12.5%), the LAM (3, 1.5%), the Beijing genotype (1, 0.5%); four (2.1%) isolates were designated as Unknown.

Conclusion: All the isolates belong to the modern lineage and there is high clustering in the genotype of isolates which indicated the presence of recent TB transmission. Therefore, the Tuberculosis Control Programme needs to do more in advocating and strengthening the health system for early detection and treatment of active TB cases as delay in treatment is the key factor in disease transmission.

Key words: Mycobacterium, genotype, recent transmission, spoligotyping

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Introduction

In terms of its historical and current disease burden, *Mycobacterium tuberculosis*, the aetiological agent for tuberculosis (TB), is undeniably the most successful human pathogen. Approximately one third of the world's population is infected with *M. tuberculosis* resulting in 10 million new cases and nearly two million deaths annually¹. Sub-Saharan Africa has had the highest annual TB incidence rates since the emergence of the human immunodeficiency virus (HIV) epidemic, and many of the countries in the group of 22 high-burden countries that collectively account for 80% of cases worldwide are from Africa¹.

In Ethiopia, the incidence of TB is estimated at 359 new cases per 100,000 populations and the country is rated 7th among the 22 high burden countries¹. The situation is exacerbated by HIV co-infection, with approximately 20% of cases presenting with TB also co-infected with HIV¹. The dangerous liaison between HIV/AIDS and TB is further complicated by the increasing incidence of multidrug-resistant (MDR), which renders therapy more cumbersome or virtually impossible respectively. Ethiopia is one of the 27 high MDR-TB burden countries with 1.6% and 12% in new cases and retreatment cases respectively¹.

The lack of comprehensive molecular epidemiological data from most countries in Africa, such as Ethiopia, has limited the understanding of TB disease dynamics. The genotyping of *Mycobacterium tuberculosis* strains is important to have unique insights into the dissemination dynamics and

*Correspondence author:

Adane Mihret
Armauer Hansen Research Institute
Addis Ababa, Ethiopia
E-mail: adane_mihret@yahoo.com

evolutionary genetics of this pathogen and for TB control as it allows the detection of suspected outbreaks and the tracing of transmission chains². Initial spoligotyping studies had shown that one of the *M. tuberculosis* spoligotypes, Beijing, was predominant in Beijing, China and other studies followed suggesting that it was also widely prevalent throughout the world. A few molecular epidemiology studies conducted in Addis Ababa had observed a high level of strain clustering but none of them reported the presence of Beijing strain in Addis Ababa^{3,4}.

The aim of this study was to obtain information on the genotypes of *M. tuberculosis* among isolates collected within a period of two years in Addis Ababa.

Methods

Study population

A total of 192 *M. tuberculosis* isolates were collected between July 2009 and June 2010 from smear positive new TB cases at 4 different health centers (Arada, T/Haimanot, Kirkos and W-23 health center) in Addis Ababa. Demographic, epidemiologic, and clinical information for all patients was collected using a prestructured questionnaire, including sex, age, and contact (family/close contact) data. Ethical approval was obtained from the AHRI/ALERT and the National Ethics Review Committees and written informed consent was obtained from all study subjects.

Mycobacterium culture and DNA extraction

The modified Petroff's method was used to digest and decontaminate the sputum specimens. An aliquot of 100 µl of the sample was then inoculated on two Löwenstein-Jensen (LJ) slants. Bacterial growth was read every week. Cultures with no growth after the eighth week were considered negative. *M. tuberculosis* isolates were identified using PCR-based genotyping of these isolates with previously described methods for RD9 deletions¹. Mycobacterial genomic DNA was extracted by heating the isolates at 80°C for 60 min and was stored at -20°C until it was subjected to spoligotyping.

Spoligotyping

Spoligotyping was carried out using the commercially available kit from Ocimum Biosolutions, India, using the standard method described by Kamerbeek et al⁵. Briefly, the direct-repeat (DR) region was amplified with primers DRa (biotinylated at the 5'

end) and DRb, and the amplified DNA was hybridized to inter-DR spacer oligonucleotides covalently bound to a membrane. DNA from *M. bovis*, BCG and *M. tuberculosis* H37Rv were used as positive controls, whereas autoclaved ultrapure water was used as a negative control. The amplified DNA was subsequently hybridized to a set of 43 oligonucleotide probes by reverse line blotting. The presence of spacers was visualized on film as black squares after incubation with streptavidin-peroxidase and detected with the enhanced chemoluminescence system detection liquid (Amersham, Little Chalfont, United Kingdom).

HIV testing

All participants were tested for antibodies to HIV-1 and -2 after pre- and post test counseling using rapid test (Stat pack, KHP and Unigold as a tie breaker) as per the Ethiopian National guide- line.

Database comparison

The spoligo patterns were entered and determined by comparing the spoligotyping results with already existing designations in the international spoligotyping database, SpolDB4.0 (<http://www.pasteur-guadeloupe.fr:8081/SITVITDemo>)⁵. In this database, two or more patient isolates sharing identical spoligotype patterns are defined as SIT (Spoligotype International Type) while single spoligo patterns are defined as "orphan" isolates. The SpolDB4 defines 62 genetic genotypes/subgenotypes and includes specific signatures for various *M. tuberculosis* complex members such as *M. bovis*, *M. caprae*, *M. microti*, *M. canettii*, *M. pinipedii*, and *M. africanum*, as well as including rules for defining the major genotypes/subgenotypes for *M. tuberculosis*. At the time of matching analysis, the updated SpolDB4.0 contained 39,609 patterns distributed among 2,881 shared types in 121 countries.

Result

Demographic information

Of all the total 192 patients, 26 (13.5%) were HIV positive, 136 (70.8%) were HIV negative and the remaining 30 (15.6%) had not been tested for HIV. The mean age was 28.7 years (range 18-72) and male participants were slightly higher (56.9%) in number. For the HIV-positive patients, the mean CD4 cell count at the time of presentation was 210 ±23.9 cells/µl.

Table 1: Distribution of all isolates (n=192) into different genotypes based on their spoligo pattern

Genotype*	No. of strains	% of strains
Beijing	1	0.5
CAS	42	21.9
H	24	12.5
LAM	3	1.5
T	95	49.5
U	4	2.1
Orphans	23	12

* CAS = Central Asian; LAM = Latin American-Mediterranean; H=Haaralam; U=Unknown pattern

Genetic diversity and family assignment

Among the 192 typed isolates, 169 (88%) were classified into one of the 33 shared international types (SITs) according to SpolDB4.0. The remaining 23(12%) isolates generated 20 different spoligotypes with 3 new shared spoligotypes (n=2 in each cluster) that had not been previously described in the International database.

Genotype assignment revealed that the ill defined T genotype constitute the most predominant genotype in our study, their total number being very high (95, 49.5%) followed by the CAS genotypes (42, 21.9%). Other genotypes found were Haarlem (H) (24, 13.2%), the LAM genotype (3, 1.5%), the Beijing genotype (1, 0.5%); and 4 (2.1%) isolates were designated as Unknown. The main results of the spoligotyping analysis and a representative spoligopattern are summarized in table 1 and figure 1 respectively

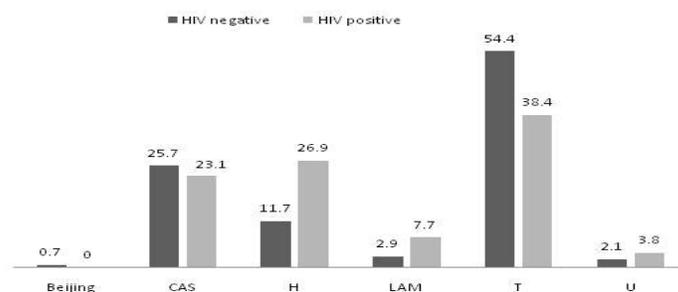
Figure 1: Representative Spoligopattern of the different genotypes of *M. tuberculosis*. The direct-repeat (DR) region of *M. tuberculosis* was amplified with primers DRa (biotinylated at the 5' end) and DRb, and the amplified DNA was hybridized to inter-DR spacer oligonucleotides covalently bound to a membrane

Webdings Format †	Octal code	ST	Family
	000000000003771	1	Beijing
	703777740000000	1264	CAS
	606777000003771	21	CAS_KILI
	77777774020771	47	H1
	000000007720771	3	H3
	777777404760771	41	LAM7_TUR
	777777607760771	42	LAM9
	77777767760771	373	T1
	777000377760771	149	T3_ETH
	777777607600031	1689	U
	703377400003771	Orphan	Orphan

† The black and white boxes indicate the presence and absence, respectively, of hybridization

Genotype assignment in HIV positive subjects also revealed that the ill defined T genotype constitute the most predominant genotype (38.5%) followed by H genotype constituting 26.9% and CAS (23.1%) (figure 2).

Figure 2: *M.tuberculosis* genotypes in HIV positive and HIV negative tuberculosis patients



Discussion

Distribution of the predominant clades of *M. tuberculosis* strains shows variation among different populations. In our study majority of the isolates (71.4%) belonged to two major genotypes: ill-defined T genotype (49.5%) and CAS genotype (21.9%) and there was no difference in the distribution of genotypes in HIV positive and HIV negative subjects. Although the T genotype is the most prevalent in this study, it is believed to be this genotype does not represent a genotype in a strict evolutionary sense and it remains as ill-defined genotype of *M. tuberculosis* that is found worldwide. This genotype includes by default strains that could not be classified in one of the established genotypes with well-established phylogeographical specificity⁶. In epidemiological language, a cluster is defined as two or more isolates obtained from different patients having identical or nearly identical genotypes. Clustered isolates have a high probability to be involved in the same chain of recent TB transmission. Epidemiological links between patients infected with strains of identical genotypes confirm that these clustered cases are involved in the same recent transmission chain. The isolates that do not belong to any cluster are often assumed to tentatively indicate the reactivation of latent infection. In our study, the genotype assignment demonstrated that a major proportion of the strains analyzed belonged to either the T (49.5%) or the Haarlem (21.9%) genotype which means they are clustered and having a high probability to be involved in the same chain of recent TB transmission. On the other hand 33 (17.2%) isolates were not clustered suggesting reactivation in these cases.

The other genotypes were LAM and Beijing comprising 5 (2.6%) and 1 (0.5%) isolates respectively. The Beijing genotype is the first report from Ethiopia. The Beijing genotype has spread globally during recent years, and is seen as an indicator strain for recent import of *M. tuberculosis* into a setting. In recent years several countries reported an increase over time of the proportion of TB due to Beijing genotype strains including countries in the region^{7,8}. Beijing *M. tuberculosis* strains have shown marked virulence in animal models of infection⁹ and it has been suggested that certain sub-genotypes of Beijing may have increased transmissibility and/or pathogenicity¹⁰. Many reports from Germany, Italy, Russia, Estonia, South Africa and Columbia documented that the isolates identified as Beijing genotype were associated with multiple drug resistance¹¹⁻¹⁵. Other studies have found Beijing strains to be associated with HIV¹⁶.

All the strains which are highly prevailed in Ethiopia (T, CAS, H, LAM and Beijing) are all members of the modern lineage and several studies showed that an immune response difference between isolates belonging to “modern” lineages with isolates belonging to “ancient” lineages. Recent studies have described a reduced immune response to Beijing and other modern strains and their association with rapid progression to severe disease, MDR and XDR tuberculosis in humans and experimental animals¹⁷⁻²⁰.

Conclusion

From the current study which we collected samples only four health centers in Addis Ababa, we have indicated that the more virulent modern lineages are

dominating in the population and there are chain of recent TB transmission in the community which the TB control needs more work in advocating and strengthening the health system for early detection and treatment of active TB cases as delay in treatment is the key factor in disease transmission. The dominance of the modern strains with their virulence and dissemination potential implies these strains are expected to be more widespread in future, also in synergy with HIV. Therefore, the tuberculosis controls programs, particularly in Africa need to impose a more effective control program in order to avoid any tuberculosis outbreaks. The other important finding of this work is the isolation of Beijing strain in Addis Ababa which was not reported before. Although we have identified only a single isolate and also it needs to be confirmed with other better molecular techniques, it is definitely an indicative of this deadly genotype is circulating in Ethiopia which alerts the TB control program to do more follow up studies in the future combined with contact-tracing and epidemiological linking in order to obtain a clear molecular-epidemiological overview of the Beijing and other genotypes.

Conflict of Interest

No conflict of interest

Acknowledgements

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