Molecular characterization of *Mycobacterium tuberculosis* isolated from pulmonary tuberculosis patients in Felege Hiwot Referral Hospital, northwest Ethiopia

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**KEYWORDS**
*Mycobacterium tuberculosis* complex (MTBC); SpolDB database; Spoligotyping; Tuberculosis (TB)

**Background:** Tuberculosis (TB), caused by the *Mycobacterium tuberculosis* complex (MTBC), is a serious infection in humans and animals. Ethiopia is one of the countries in Sub-Saharan Africa with the highest burden of TB. However, limited information is available on the genotypic characteristics of *M. tuberculosis* strains infecting humans. The objective of the present study was to characterize the mycobacterial species isolated from pulmonary TB patients using molecular typing.

**Materials and methods:** A cross-sectional study was conducted on 123 patients with smear-positive pulmonary TB, using Ziehl Neelsen staining and bacteriological culturing. Molecular characterizations of the mycobacterial isolates were performed using region of difference 9 (RD9) deletion typing and spoligotyping methods.

**Results:** The proportion of culture positivity was 95.9% (118/123). All the 118 isolates were confirmed to be *M. tuberculosis* by polymerase chain reaction-based RD9 deletion typing and spoligotyping methods. Further characterization of all isolates using spoligotyping resulted in the identification of 36 different spoligotype patterns. Out of these, 32 (88.9%) patterns have already been reported in the SpolDB database, whereas the remaining four (11.1%) patterns were new and

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Introduction

Tuberculosis (TB), caused by the Mycobacterium tuberculosis complex (MTBC), is a serious infection in humans and animals. M. tuberculosis belongs to a group of closely related microorganisms known as the MTBC, which is a group of genetically similar species, as defined by DNA/DNA hybridization studies. The MTBC comprises seven members, including M. tuberculosis, Mycobacterium bovis, Mycobacterium africanaum, Mycobacterium microti, Mycobacterium canettii, Mycobacterium pinnipedii, and Mycobacterium caprae. Although there is high genetic homogeneity among the members, they display divergent phenotypes, elicit different pathologies, and show some degree of host specificities.

Both preventable and curable TB remains to be among the world’s major causes of illness and death. In 1993, the World Health Organization (WHO) declared TB as a global health emergency. One-third of the world’s population (i.e., 2 billion people) carries the TB bacteria; more than 9 million of them become sick with active TB each year, and they can spread the disease to others.

Currently, according to the Global Report 2010 by WHO, Ethiopia ranks seventh among the high TB-burden countries in the world, with an estimated incidence of 378 new cases per 100,000 population per year. The estimated prevalence of all forms of TB and smear-positive TB is 579 per 100,000 and 286 per 100,000 population, respectively. Even though Ethiopia is one of the high TB-burden countries in Africa, limited information is available on the genotypic characteristics of M. tuberculosis strains infecting humans there. In addition to being used in designing a more targeted control measure, the availability of such information will help study the phylogenetic characteristics of an organism, which in turn will provide new insight into the natural history of M. tuberculosis.

In developing countries where the burden of TB is greatest, few studies have been conducted and the application of the new molecular epidemiology needs to be developed further. Molecular characterization of M. tuberculosis strain circulating in the community, for epidemiology, transmission, and control of TB, is crucial in the control of the disease. In addition, there is mounting evidence to suggest that specific strains of M. tuberculosis belonging to discrete phylogenetic clusters (lineages) may differ in virulence, pathogenesis, and epidemiologic characteristics, all of which may impact TB control and vaccine development strategies significantly.

The present study was aimed at characterizing Mycobacteria species isolated from patients with pulmonary TB from Bahir Dar, Felege Hwiwot Referral Hospital, Northwest Ethiopia, and identifying the species, strains, and predominant lineages of strains that cause TB in the population.

Materials and methods

Study area

The study was conducted in Bahir Dar, which is the capital city of Amhara National Regional State of Ethiopia, 565 km away from Addis Ababa. The altitude of the area is 1802 m above sea level and has an annual rain fall of 1703.4 mm; the temperature of the city ranges from 12°C to 27°C. The city has an estimated total population of 17,214,056 (8,636,875 males and 8,577,181 females). The Felege Hwiwot Referral Hospital is a tertiary-level setup that provides referral services to over 12 million inhabitants in northwest Ethiopia.

Study design

A cross-sectional study was conducted to characterize mycobacterial isolates that were recovered from pulmonary TB patients in Bahir Dar Felege Hwiwot Referral Hospital from October 2010 to June 2011. During the study period, all patients suspected of TB visiting the hospital were used as source population, whereas the study population consisted of pulmonary TB patients with positive smear for microscopic acid fast bacilli (AFB).

Sample collection and bacterial isolation

A total of 123 smear-positive sputum samples were collected and processed during the study period. Spot—morning—spot sputum samples were collected, and questionnaires were filled for each patient by trained laboratory technologists. The specimens were labeled and pooled together, kept in phosphate buffer saline (at pH 7.2 and a temperature of 4°C) in universal containers, and then transported in ice pack box to Akilu Lemma Institute of Pathobiology (ALIPB), Addis Ababa (Ethiopia), within a
week. There the samples were processed for isolation of *M. tuberculosis* according to the standard methods. In brief, the sputum samples were decontaminated by shaking in an equal volume of 4% NaOH for 15 minutes and concentrated by centrifugation at 3000 rpm for 15 minutes. The sediment was neutralized with 2N HCl, using phenol red as an indicator. Bacteriological culturing of sputum samples was performed using the conventional Löwenstein–Jensen (LJ) egg slant medium, containing 0.6% sodium pyruvate and glycerol media, for the recommended time. Then the cultures were incubated at 37°C for 4–8 weeks and examined on a weekly basis for the presence of any mycobacterial colonies.

Microscopic examinations of the cultures using the Ziehl–Neelsen staining method were performed to select AFB-positive isolates. AFB-positive isolates were prepared by mixing two loops full of colonies in 200 μL distilled water, heat-killed at 80°C for 1 hour using water bath, and stored at −20°C until molecular characterization was performed.

**Molecular typing**

Region of difference 9 (RD9) deletion typing was performed, as describe previously by Huard et al. The polymerase chain reaction (PCR) amplification mixtures used for RD9 typing consisted of 10 μL HotStarTaqMaster Mix (Qiagen, London, UK), 7.1 μL distilled H2O, 0.3 μL of each of the three oligonucleotide primers (100 mM) [RD9Ff (5′-GTG TAG GTC AGC CCC ATC C-3′), RD9Fr (5′-GCC CAA CAG CTC GAC ATC-3′), and RD9Inactive (5′- CGG TAG TCC GAC CCC TTC GTC-3′)], resulting in a total volume of 20 μL. The PCR reaction mixture was heated at 95°C for 15 minutes, after which it was subjected to 35 cycles consisting of 95°C for 1 minute, 55°C for 1 minute, and 72°C for 1 minute in the PCR thermocycler (VWR, UK). Then the reaction mixture was maintained at 72°C for 10 minutes, and 0.8 μL of the PCR product was mixed with 0.2 μL of a loading dye and loaded onto 1% agarose gel, after which it was electrophoresed at 100 V and 500 mA for 60 minutes. The gel was visualized using a computerized Multimage Light Cabinet (VWR). Interpretation of the result was based on the detection of bands of different sizes. For a band size of 396 bp, the isolate was considered as *M. tuberculosis*, whereas a band size of 575 bp was considered to correspond to either *M. bovis* or *M. africanum*. Molecular characterization of isolates using RD9 deletion typing showed all the 118 isolates were *M. tuberculosis* (Fig. 1). These isolates were further characterized using spoligotyping (Fig. 2).

**Results**

**Sociodemographic characteristics**

Out of 123 AFB-positive sputum samples, 118 isolates were culture positive (95.9%). The age of patients ranged from 15 years to 80 years (mean age, 31.85 ± 1.05 years); the male to female sex ratio was 1:1.3. Of the 118 patients, 12 (10.2%) were HIV positive and 106 (89.8%) were HIV negative (Table 1).

**Spoligotyping**

Molecular characterization of isolates using RD9 deletion typing showed all the 118 isolates were *M. tuberculosis* (Fig. 1). These isolates were further characterized using spoligotyping (Fig. 2). The spoligotyping analysis resulted in the identification of 36 different spoligotype patterns, of which 32 (88.9%) patterns have already been registered in the spoligotype databases, SpolDB4 and SITVIT. However, the remaining four (11.1%) patterns were not registered, indicating that they were new spoligotypes of *M. tuberculosis* in Ethiopia. The isolates were further grouped into 17 clusters (99 isolates) and 19 nonclustered patterns. Out of the 12 HIV-positive TB cases, 10 isolates belonged to clustered patterns and only two isolates (SIT610 and NEW) were among nonclustered patterns (Fig. 3). The most predominant spoligotypes were SIT25 and SIT53, consisting of 22 isolates and 14 isolates, respectively. SIT1729, SIT149, and SIT289 accounted for seven isolates each. Classification of the spoligotype patterns with web-based SPOTCLUST database was double entered into Microsoft Excel. The web-based SpolDB4 database (SITVIT database) is: http://www.pasteur-guadeloupe.fr:8081/SITVITDemo/ was used to assign the SIT (shared type) and the web-based algorithm SPOTCLUST (http://tbinsight.cs.rpi.edu/run_spotclust.html) was used to assign the lineages of each isolates.
The database showed 64 isolates in the Euro-American lineage and 37 isolates in the Central Asian lineage (Fig. 2).

Discussion

The present study describes the diversity of the population structure of *M. tuberculosis* clinical isolates in patients from northwest Ethiopia. All TB cases reported in this study were caused by *M. tuberculosis*. Molecular characterization of the strains of *M. tuberculosis* using spoligotyping identified 36 different spoligotype patterns, of which 17 (46%) consisted of clusters of isolates whereas 19 (54%) consisted of nonclustered single isolates. The most commonly found *M. tuberculosis* strains were SIT25 and SIT53, which were also reported earlier in the SITVIT database as the most common types in Ethiopia by other researchers. In agreement with our study, comparable prevalence of clustering was found in population-based studies from South Africa (45%), Botswana (42%), and Estonia (49%), and among randomly sampled patients from Ethiopia (42.1%). Moreover, similar findings were reported in population-based studies from Sub-Saharan Africa. None of the isolates in the other 19 (54%) spoligotype patterns corresponded to cluster strains. In agreement with other studies, our study demonstrated higher frequency of clustered isolates among HIV-positive TB patients. Interestingly, this study showed the identification of four new strains of *M. tuberculosis* belonging to the Euro-American lineage. One of these new strains was from HIV-positive TB patients, whereas the other three were from HIV-negative TB patients. In agreement with others, when the phylogenies of the isolated *M. tuberculosis* strains were compared with the current global *M. tuberculosis* distribution, most of the strains were of the Euro-American lineages, followed by those belonging to the Central Asian lineages.

In conclusion, this study has shown the presence of known and new strains of *M. tuberculosis* circulating in northwest Ethiopia and the distribution of the major phylogenetic families. Thus, it contributes to a better understanding of the molecular epidemiology of *M. tuberculosis* in Ethiopia. Generally, genotyping of *M. tuberculosis* isolates from infected individuals can play an important role in tracking the source of infection and disclosing the epidemiology of TB outbreaks. This study also provided molecular evidence for a number of patients included in our study. However, it may have been insufficient to confirm the prevalence of the most circulating *M. tuberculosis* genotypes in Ethiopia. It will be

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<th>Table 1</th>
<th>Characteristics of study participants (n = 118)</th>
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TB = tuberculosis.
It is interesting to determine whether isolates from different regions of Ethiopia exhibit similar genotypes.

Conflicts of interest

The authors declare that they have no conflicts of interest.

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References


