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Spoligotyping of *Mycobacterium tuberculosis* isolates from tuberculosis diagnosed patients at Dilla University Referral Hospital and other private clinics, Southern EthiopiaGebremedhin Gebrezgabiher<sup>1</sup>, Gebremedhin Romha<sup>2\*</sup>, Gobena Ameni<sup>3</sup><sup>1</sup>Samara University, College of Veterinary Medicine, P.O. Box 132, Samara, Ethiopia<sup>2</sup>Dilla University, College of Agriculture and Natural Resource, Department of Animal and Range Science, P.O. Box 419, Dilla, Ethiopia<sup>3</sup>Aklilu Lemma Institute of Pathobiology, Addis Ababa University, P.O. Box 1176, Addis Ababa, Ethiopia

## PEER REVIEW

## Peer reviewer

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## Comments

The study is very important in which authors discovered *M. tuberculosis* strains circulating in the study area. The result of this study has also tried to indicate the way of transmission of the agent in the society of the study area. Therefore, based on this finding, control and prevention strategies could be set.

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## ABSTRACT

**Objective:** To assess *Mycobacterium tuberculosis* (*M. tuberculosis*) strains existing in Gedeo zone and the surrounding areas of the Southern Ethiopia using spoligotyping.

**Methods:** A cross sectional study was carried out from February, 2012 to June, 2013 and 97 (76 sputum and 21 fine needle aspirate) samples were taken from tuberculosis diagnosed patients at Dilla University Referral Hospital and other private clinics. Culturing, region of difference (RD9) deletion typing and spoligotyping techniques were employed to isolate *M. tuberculosis* strains.

**Results:** Growth of mycobacteria was observed in 35.1% (34/97). Speciation of isolates showed that 91.2% (31/34) of the isolates were *M. tuberculosis*. Further characterization led to the identification of 23 different spoligotype patterns of *M. tuberculosis* of which 61% and 39% displayed unique and cluster patterns, respectively. The most dominant shared type was spoligotype international type 53. Of the 23 strains, 12 have not been registered in the international spoligotyping database (SpolDB4). Seventy one percent of the strains belonged to the Euro-American lineage.

**Conclusions:** This study revealed the existence of both genetically diverse and clustered *M. tuberculosis* strains from tuberculosis patients in the area, suggesting reactivation of infection and recent transmission, respectively. Molecular epidemiology of *M. tuberculosis* should be done nationwide in order to set appropriate control measures.

## KEYWORDS

*Mycobacterium tuberculosis* strains, Spoligotyping, Dilla University, Southern Ethiopia

## 1. Introduction

Tuberculosis (TB) remains a major global health problem. It causes ill-health among millions of people each year and ranks second in being the cause of death from an infectious disease worldwide following HIV. Despite the availability of treatment that cures most TB cases, the latest report of World Health Organization (WHO) showed that there are an estimated 8.6 million new cases in 2012 and 1.3 million TB deaths (under 1.0 million among HIV negative people and 0.3 million HIV-associated TB deaths)[1]. The

dramatic resurgence in the incidence of TB throughout the world in the last decade warrants an increased need for more rapid methods to diagnose and prevent dissemination of this disease[2].

*Mycobacterium tuberculosis* (*M. tuberculosis*) has probably been a human pathogen for millions of years, and that cattle and other animals are likely to have acquired mycobacteria from humans rather than the reverse, has profound implications for the epidemiology and control of human TB[3]. Epidemiological links within and outside the traditional concentric circle approach are limited by the quality of the contact investigation, the skill and

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knowledge of the investigator and the information provided by the patient[4]. Therefore, molecular typing methods have permitted investigation of the outbreaks[5]. Molecular markers have also extensively been used for tracking transmission patterns within specific populations and/or defined geographical settings, and quantification of the extent of transmission that occurs within a given population[6-10].

According to Ravan *et al.*[11], patients infected with the same *M. tuberculosis* strain indicated vast transmission of a single strain in the society, and a susceptible strain can be changed into mono drug resistant and multidrug-resistant strain in the transition period. Besides, evaluating molecular epidemiology from a population genetics perspective also enables public health researchers to determine which patient factors might be associated with clusters of related strains and how interventions might be formulated to prevent those strains from being transmitted in the future[12].

Spoligotyping is among the molecular tools that are useful for screening and epidemiologic control of TB dissemination, particularly when results are required quickly, such as in outbreaks, or in the management of transmission of multidrug-resistant TB, especially in restricted high-risk situations such as prisons, schools, and hospitals[6,13]. Although Ethiopia is among the countries with the highest incidence of TB[1], only few studies have tried to report *M. tuberculosis* strains through molecular characterization[14-16]. As there is lack of information on the epidemiology of *M. tuberculosis* on molecular basis nationwide especially in Southern Ethiopia, the aim of this study is therefore to assess *M. tuberculosis* strains in human TB patients of the study area.

## 2. Materials and methods

### 2.1. Description of the study site and population

Dilla University Referral Hospital and the private higher clinics, namely, Dr. Muluneh and Selam Higher clinics were situated at Dilla Town, Gedeo Zone of the Southern Ethiopia. Dilla is the capital town of Gedeo Zone and is located at 359 km from Addis Ababa, the capital of Ethiopia. It is located in the escarpment of the Rift Valley at 5° to 7° N latitude and 38° to 40° E longitude and its elevation ranges from 1 200 m to 3 175 m above sea level. Mean annual temperature and rainfall range from 17 °C to 22.4 °C and 1 200 to 1 800 mm, respectively[17]. The study populations were TB patients from Gedeo Zone and the surrounding areas such as Kebado and Abaya districts of Sidama and Borena zones, respectively who were being admitted at Dilla University Referral Hospital and the aforementioned private clinics for TB treatment.

### 2.2. Study design and sampling method

A cross sectional study was carried out from February, 2012 to June, 2013 to assess *M. tuberculosis* strains in human TB cases. A human TB case was defined, in this work, as a TB patient diagnosed at Dilla University Referral Hospital TB clinic and the two private higher clinics by physicians based on clinical findings as a new case of active pulmonary (PTB) and/or extra-pulmonary (EPTB). Confirmation was made for active PTB and EPTB cases by Ziehl-Neelsen staining of sputum and cytology of fine needle aspirate samples, respectively. During the study period, a total of 76 sputum and 21 fine needle aspirate (FNA) samples were collected from 97 TB patient individuals, and followed by culturing and molecular

characterization of isolates. The research was evaluated and approved by the Institutional Review Board (IRB) of the Akililu Lemma Institute of Pathobiology (ALIPB), Addis Ababa University. The reference number of the approval letter is IRB/01/2012-13. Consent was obtained from the patients.

### 2.3. Study methodology

#### 2.3.1. Sample collection and transportation

Sample collection was made as part of the routine diagnostic procedure on TB cases by medical experts before the first antimicrobial therapy. After obtaining consent from the participants, sputum samples from suspected PTB patients and FNA samples from suspected TB lymphadenitis patients were collected by trained physicians. Particularly FNA samples were collected from TB lymphadenitis patients with a maximum care and safety using a 10 mL sterile syringe fitted with a 23 gauge needle, one and half inches long by a pathologist. FNA samples were collected in cryo tube and were kept in phosphate buffer saline of 7.2 pH. All the three spot-morning-spot sputum sample of each patient was pooled together in a sterile plastic sputum cup. Then, samples were sent to ALIPB special TB laboratory in a cold chain (4 °C) and stored there at -20 °C until it was processed for mycobacteria isolation and identification.

#### 2.3.2. Mycobacteriological culturing

All specimens collected from TB suspected individual (sputum and FNA) were processed and prepared for mycobacterial culture at the ALIPB special TB laboratory in a biological safety cabinet according to the standard methods described previously[18].

#### 2.3.3. RD9 deletion typing

For RD9 deletion typing, a procedure used by Cadmus *et al.*[19] was followed. The primers used for RD9 deletion typing were RD9 FlankF, 5'-AAC ACG GTC ACG TTG TCG TG-3', RD9 FlankR, 5'-CAA ACC AGC AGC TGT CGT TG-3' and RD9 Internal, 5'-TTG CTT CCC CGG TTC GTC TG-3'. The mixture was heated in a thermal cycler (Applied Biosystems, PTC-100™) using an initial hot start of 95 °C for 15 min, followed by 35 cycles of 95 °C for 1 min, 55 °C for 2 min, and 72 °C for 1 min; a final extension step of 72 °C for 10 min to complete the cycle. Each PCR reaction tube contained 7.1 µL H<sub>2</sub>O Qiagen, 10 µL HotStarTaq Master Mix, 0.3 µL of each of the three primers (1.5 µmol/L final concentration), 2 µL of DNA templates of samples making the total volume 20 µL. *M. tuberculosis* H37Rv and *Mycobacterium bovis* (*M. bovis*) were used as positive controls while Qiagen water served as a negative control.

Finally, PCR products were electrophoresed in 1.5% agarose gel in 10× Tris-Acetate-EDTA running buffer with ethidium bromide at a ratio of 1:10. A 100 bp DNA (Promega Cooperation, USA) ladder and Orange 6× loading dye were also used for visual tracking of DNA migration during electrophoresis. The gel was visualized in a Multi-image™ light cabinet using Alpha Innotech version 1.2.0.1 (Alpha Innotech Corporation). 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 *Mycobacterium africanum*.

#### 2.3.4. Spoligotyping

Spoligotyping was performed as described previously[20], and according to the spoligotype kit supplier's instructions (Ocimum



### 3.3. Spoligotyping

A total of 31 samples that showed a signal with RD9 deletion typing were spoligotyped. A total of 23 different spoligotype patterns were identified (Figure 2). The dominant strain was SIT53 consisting of 22.6% of the 31 isolates. Of the total 23 patterns identified, 12 have not yet been registered in the international spoligotyping database (SpolDB4). Among the unregistered patterns, 6 strains belonged to the E-A lineage and the same 6 strains belonged to Indo-oceanic lineage. Nineteen strains (61%) displayed unique patterns; while, 12 strains (39%) showed cluster patterns (2 to 5 strains per cluster) which were classified in 4 groups. The two lineages recognized in this strain collection on the basis of spoligotyping were Euro-American and Indo-Oceanic lineages with 71% and 29%, respectively.

## 4. Discussion

In the present study, 34 of 97 (35.1%) samples were culture-positive; 39.5% (30/76) sputum and 19.0% (4/21) FNA samples. In contrast to the present study, high percentage of culture positivity (mycobacterial growth) was observed in sputum (80.8%)[15] and FNA (36.7%)[16] samples. However, low culture positivity (44.8%) had also been reported from samples collected from both PTB and ETB patients[14]. Low culture positivity in the present study could be due to the long distance of sample transportation from the study area to Addis Ababa where samples had been processed and long time sample storage might affect the viability of the organism.

A total of 31 samples that showed a signal with RD9 deletion typing were spoligotyped. Of these, 23 different spoligotype patterns were identified with two lineages, the Euro-American and Indo-Oceanic lineages. Out of the 23 different spoligotype patterns, 19 strains (61%) displayed a unique pattern while 12 strains (39%) showed a cluster patterns which were classified in 4 groups. Our study showed that diversity of the observed strains was more than the identical strains. Nineteen strains (61%) showed different spoligotype patterns. One study in Iran has reported high percentage (76%) of genetic diversity of *M. tuberculosis* strains, which is relatively similar with the present study. The same study has shown that polymorphism degree (diversity) of DNA depends on the geographic region of the separated strains; high percentage of genetic diversity in strains indicates diversity of *Mycobacterium* strains in different geographical locations[11]. The presence of genetic diversity in the present study could be due to the fact that the strains may have entered by the traders from Kenya as the study town is nearby Kenya. Rushing people with low socioeconomic conditions from different places to the study town as well as coming patients from remote areas to the hospitals and clinics under the study also could be the reasons for reactivation of infection.

On the other hand, clustering is a marker for recent transmission[21,22]. By using rate of recent TB transmission in a study population, one can estimate the efficacy of the TB control program[11,22]. Clustering (39%) of strains (2 to 5 strains per cluster) was observed in the present result. This is in line with the previous findings from different parts of Ethiopia that showed clustering rate of 41.2%[23] and 45.1%[24].

The two lineages recognized in this study on the basis of spoligotyping were Euro-American and Indo-Oceanic lineages

with 71% and 29%, respectively. Furthermore, the dominant strain was SIT53 consisting of 22.6% of the 31 isolates. This is supported with the findings[14,16,25] who reported that the Euro-American and Indo-Oceanic lineages, and SIT53 strains were dominant in different regions of the country. This study revealed the existence of both genetically diverse and clustered *M. tuberculosis* strains from TB patients in the area, suggesting reactivation of infection and recent transmission, respectively. Molecular epidemiology of *M. tuberculosis* should be done nationwide in order to set appropriate control measures.

### Conflict of interest statement

We declare that we have no conflict of interest.

### Acknowledgements

This research was financially partially supported by Dilla University (Grant No. CAN/09/2012), and laboratory materials and reagents were provided by Aklilu Lemma Institute of Pathobiology (ALIPB), special TB laboratory, Addis Ababa University. The authors are thankful to Dr. Girma Ababi, a pathologist at Selam Higher Clinic, Mr. Asnake Dechasa, laboratory technician at Selam Higher Clinic, Sister Meseret Habtamu, TB expert and all the laboratory technicians working at Dilla University Referral Hospital, and TB experts of ALIPB for their unreserved help during data collection and processing. We are grateful to the TB patients who were willing to involve in this study. We also appreciate Dilla University Referral Hospital, Selam and Dr. Muluneh private higher clinics for allowing us to collect sputum and FNA samples.

### Comments

#### Background

TB causes ill-health among millions of people each year and ranks second in being the cause of death from an infectious disease worldwide following human immunodeficiency virus. Ethiopia is among the countries with the highest incidence of TB. However, only few studies have tried to report *M. tuberculosis* strains through molecular characterization. The study aimed to assess *M. tuberculosis* strains existing in the areas using spoligotyping.

#### Research frontiers

The study has discovered different spoligotype patterns of *M. tuberculosis* including strains which have not been registered in the SpolDB4. Moreover, the study tried to indicate how *M. tuberculosis* (TB) could be transmitted or circulated in the society of the study area. The finding of this research contributes a lot to the field; it serves as a reference for policy makers, epidemiologists, researchers, and drug developers.

#### Related reports

The result of the present study is in agreement with the report of Ravan *et al.* (2013) who showed that high percentage of genetic diversity in strains indicates diversity of *Mycobacterium* strains in different geographical locations. On the other hand, this study is also in line with the findings of Wang *et al.* (2011) and Rodwell



et al. (2012) who reported that clustering is a marker for recent transmission. The reasons could be as author/s discussed in this manuscript.

### Innovations & breakthroughs

The study has discovered different spoligotype patterns of *M. tuberculosis* including new strains. The study also found both genetically diverse and clustered *M. tuberculosis* strains from TB patients in the area. Thereby the study suggests the way of TB transmission by discussing the topography (near to Kenya) and the socioeconomic (low) conditions of the society in the study area.

### Applications

The study revealed *M. tuberculosis* strains exist in the area. Moreover, the study already suggests the presence of reactivation of infection and recent transmission in the study area. This may help to set the appropriate control and prevention strategies in the area as well as worldwide.

### Peer review

The study is very important in which authors discovered *M. tuberculosis* strains circulating in the study area. The result of this study has also tried to indicate the way of transmission of the agent in the society of the study area. Therefore, based on this finding, control and prevention strategies could be set.

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