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A Preliminary Study on Molecular Characterization of *Mycobacterium tuberculosis* in Benishangul Gumuz Region, Western Ethiopia

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Authors' contributions

This work was carried out in collaboration between all authors. Author HD performed the conception of the research idea, designing and data collection, data analysis and interpretation, and manuscript drafting. Author KT performed the data analysis and interpretation, manuscript reviewing and commenting on the manuscript. Author AW managed the data collection, laboratory work and interpretation and reading of the results. Author GA performed the conception of the research idea, designing and supervising the work, and manuscript reviewing. All authors read and approved the final manuscript.

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ABSTRACT

Background: The information about the genetic diversity of *Mycobacterium tuberculosis* in certain geographic area is needed for a better understanding of epidemiology of tuberculosis and could have implications for development of new diagnostics, drugs, and vaccines. The aim of this study was to provide preliminary information on the strains of *Mycobacterium tuberculosis* circulating in Benishangul Gumuz Region.
Methods: Cross-sectional study was conducted to generate preliminary information on the genetic diversity of *Mycobacterium tuberculosis* between November 2012 and April, 2013. *Mycobacterium tuberculosis* isolates were characterized using region of difference 9 (RD9) and spoligotyping. The

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patterns of strains identified by spoligotyping were compared with the patterns registered in the SpoIDB4 database of the Pasteur Institute.

Results: Out of a total of 53 smear positive samples, 34 (64.15%) were culture positive, of which, 33 samples were confirmed to be *Mycobacterium tuberculosis* by RD9 deletion typing. Further characterization of 33 isolates using spoligotyping lead to the identification of 24 individual spoligotype patterns, among which, 18 were unique while the remaining six were found in clusters containing two to four isolates. Of the 24 patterns identified by the present study, only six patterns were registered in SpolDB4 international database, while 18 patterns were not previously registered in the database. Grouping of the 33 isolates into the lineages showed that 19 (57.6%) isolates were members of Euro-American, 6 (18.2%) isolates were Indo-Oceanic while the remaining 8 (24.2%) could not be categorized to the presently known lineages.

Conclusion: Characterization of the *Mycobacterium tuberculosis* strains revealed the identification of new strains and lineages which could not match with the existing strains and lineages suggesting the localization of these strains and lineages in this Region. Hence, further research is required to identify and document the strains and lineages circulating in the Region.

Keywords: M. tuberculosis; molecular typing; strain; lineage; Benishangul Gumuz Region.

1. INTRODUCTION

Tuberculosis (TB) is an ancient disease that currently presents an immense global health challenge. It is an infectious disease caused by the bacillus *M. tuberculosis* which typically affects the lungs (pulmonary TB), but can affect other sites as well extra pulmonary tuberculosis (EPTB).The disease spreads in the air when people who are sick with pulmonary TB expel bacteria, for example by coughing. It is estimated that one third of the world's population are infected with the tubercle bacilli [1].

Tuberculosis has been recognized as a major public health problem in Ethiopia more than half a century ago. Ethiopia is number nine out of 22 high TB burden countries that account together for 80% of all newly diagnosed TB cases in the world and one of the top five in Africa with regard to the prevalence [2]. According to the 2011 WHO report, the prevalence, incidence and mortality of all forms TB in the country is estimated to be 394, 261 and 35 per 100000 populations respectively [2]. Despite the high-TB burden in the country, very limited information is available on the genetic diversity of *M. tuberculosis* strains and lineages.

Molecular typing techniques have been extensively used to speciate strains of *M. tuberculosis* involved in TB infections, studying molecular epidemiology of *M. tuberculosis*, providing insights into dissemination dynamics, evolutionary genetics, and detection of suspected outbreaks and person-to-person transmission [3].

Although recent studies are recommending the use of robust markers such as single nucleotide polymorphisms (SNP) or large sequence polymorphisms (LSP) for a better understanding of strain lineages [4], Insertion sequence (IS) 6110 restriction fragment length polymorphism (IS6110 RFLP) has in the past served as a gold standard typing method [5,6]. However, it is costly, time consuming, and not easily standardized across laboratories.

An alternative technique is a PCR-amplification-based technique, spacer oligonucleotide typing (spoligotyping), which analyses polymorphisms of direct repeat (DR) regions. The technique is relatively simple, quick, and reliable. The method has been extensively used for simultaneous detection and typing of *M. tuberculosis* [7].

A recent study on molecular analysis of *M. tuberculosis* in Ethiopia described the diversity of strains in developed regions although it failed to address the remote settings like Benishangul Gumuz Region [8].

The present study aimed to provide a preliminary information on the genetic diversity *M. tuberculosis* isolates in new pulmonary smear positive patients in Benishangul Gumuz Region, western Ethiopia using spoligotyping and to compare the patterns obtained with those available in the international spoligotyping database, SpolDB4.0 of the Pasteur Institute of Guadeloupe.

2. MATERIALS AND METHODS

2.1 Study Area

The study was conducted in Benishangul Gumuz Regional State, from November 2012-April 2013. Benishangul Gumuz Regional State is one of the nine regional states established in 1994 by the constitution of Ethiopia. It is located in the western part of Ethiopia between 34° 10'N and 37° 40'E; and in the latitude 09° 17'N and 12° 06' N. It shares border with Amhara Region in the north and north east ,Sudan Republic in the west ,Gambella Region in the south ,Oromia Region in the south east.The total area of the Region is approximately 50,380 square kilometers with altitude ranging from 580 to 2731 meters above sea level. About 75% of the Region is low land, 24% is semi-high land and 1% is high land. The capital city of the Region is Asossa, located at a distance of 659 kms in west of Addis Ababa [9].

The Region has 2 hospitals ,32 health centers and 361 health posts. Among these health facilities, the two hospitals and few of the health centers routinely diagnose and treat TB based on the clinical findings,the chest x-ray and AFB results. Most of health centers and 120 health posts used for treatment. The two hospitals namely, Asossa Hospital locted in Asossa zone and Pawe Hospital located in Metekel zone are serving the majority of the population of the Region and some from Oromiya Region. Most community from Kamashi zone are served by health facilities from Oromiya [10]. The geographical location of the Region showing the skech map of the study area is indicated by Fig. 1.

2.1.1 *Mycobacterium tuberculosis* strains and DNA isolation

A total of 53 *M. tuberculosis* isolates were collected between November 2012 and April 2013 from smear positive new TB cases at 4 different health centers and in two hospitals in Benishangul Gumuz Region. We obtained institutional ethical clearance from Aklilu Lemma Institute of Pathobiology institutional review board. The participants agreed to participate in the study and signed a free informed consent form. 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 onto two Lowenstein-Jensen (LJ) slants. Bacterial growth was read every week up to 8 weeks. Cultures with no growth after the eighth week were considered negative. *M. tuberculosis* isolates were identified using PCR-based genotyping with previously described methods for RD9 deletions [3]. Mycobacterial genomic DNA was extracted by heating the isolates at 85℃ for 45 min and was stored at −20℃ until needed for spoligotyping.

2.2 RD9 Deletion Typing

RD9 deletion typing was performed on heat killed cells to confirm the presence or absence of RD9, as described earlier, [11], using RD9 flankF, IntR and flankR primers, each at a concentration of 100µM. Polymerase chain reaction (PCR) amplification was performed on each sample using standard thermocycler (VWR Thermocycler, VWR International, East Grinstead ,UK). The PCR amplification mixtures used for RD9 typing were as follows: the reaction mixture consisted of 10 µl Hot Star TaqMaster Mix (Qiagen, Crawley,UK), 7.1 µl distilled water, 0.3 µl of each of the three primers (100mM) and 2 µl DNA template (heat-killed cells), giving a total volume of 20 µl. Gel electrophoresis was used for the separation of the PCR product. For gel electrophoresis, 8µl PCR product was mixed with 2 µl loading dye, loaded on to 1.5% agarose gel and electrophoresed at 100V and 500mA for 45 min. The gel was then visualized using a computerized Multi-Image Light Cabinet (VWR). *M. tuberculosis* H37Rv, *M. bovis* bacilli Calmette-Guerin and water were included as positive and negative controls. Interpretation of the result was based on bands of different sizes, as it was previously described by [11].

2.3 Spoligotyping

Spoligotyping was performed as previously described by [7] and as per the spoligotpye kit supplier's instructions (Ocimum Biosolutions, Ijsselstein, and The Netherlands). The direct repeat (DR) region was amplified by PCR using oligonucleotide primers derived from the DR sequence. A total volume of 25 µl of the following reaction mixture was used for PCR: 12.5 µl of HotStarTaq Master Mix (Qiagen; this solution provides a final concentration of 1.5 $MgCl_2$ and 200 mM of each deoxoriboneuclotide triphosphate), 2 µl of each primer (20 µl pmol each), 5 µl suspension of heat-killed cells (approximately 10-50 ng), and 3.5 µl distilled water. The amplified product was hybridized to a set of 43 immunobilised oligonucleotides, each corresponding to one of the unique spacer DNA

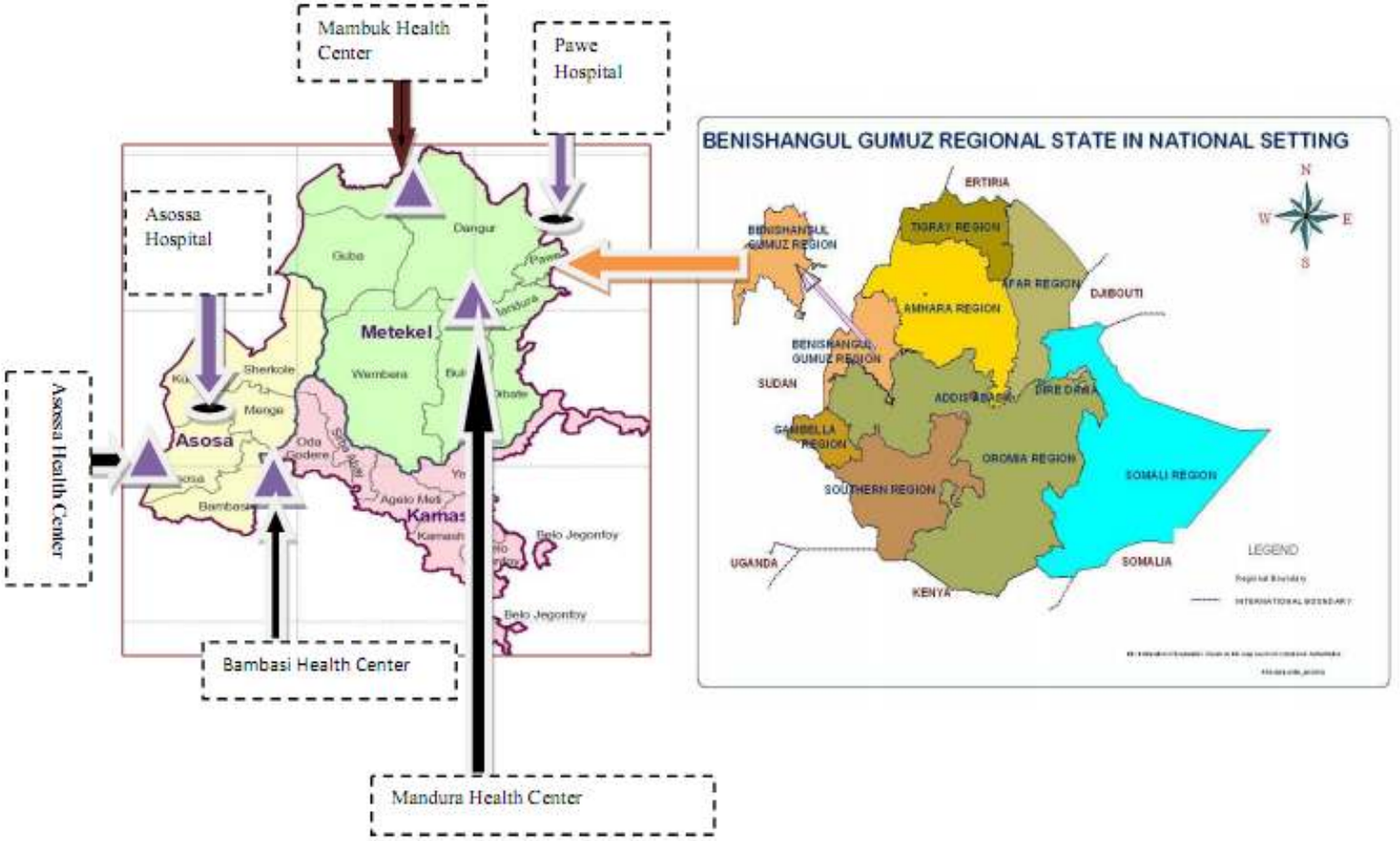


Fig. 1. Geographical location of Benishangul Gumuz Region showing the study area. The figure indicates the location of the Region in national setting and the specific study sites (two Hospitals and four health centers) addressed in the study as showed by arrows

sequences within the DR locus. Hybridized DNA was detected by the enhanced chemiluminesence method (Amersham Biosiences, UK) and by exposure to X-ray film (Hyperfilm ECL, Amersham Biosiences), as specified by the manufacturer. The presence of these spacer sequences in the *M. tuberculosis* strain was detected by reverse line blotting using an oligonucleotide-impregnated membrane. Hybridized DNA with peroxidase labeled streptavidin was detected using the ECL detection reagent.

2.4 Database Comparison

The spoligotyping results were prepared in octal and binary formats into Microsoft Excel spreadsheets; spoligotype patterns were designated as 43-character-long strings consisting of black and white squares representing the presence or the absence of an individual spacer, respectively. The spoligo patterns which were prepared in binary and octal were entered and determined by comparing the spoligotyping results with already existing designations in the international spoligotyping database, SpolDB4.0 [12] (http://www.pasteur-guadeloupe.fr:8081/SITVITDemo/). Lineage was assigned on the basis of spoligotype, using a set of rules to correlate spoligotype with lineage names that are defined by large sequence polymorphisms (LSPs) [13,14]. Spoligotype rules for each lineage corresponded to the first rule for each lineage as described by [14] which is based on the presence or absence of specific spacer sequences in the direct repeat locus (i.e., absence of 1–34 spacers for East-Asian; absence 33–36 for Euro-American; absence of 29–32, 34 spacers, and presence of 33 spacer for Indo-Oceanic; and absence of 4–7 and 23–34 spacers for East-African Indian). In addition, lineage assignment on the basis of spoligotype alone required that ≥1 spacer from spacers 29–36 be present [15,12]. In this database, two or more patient isolates sharing identical spoligotype patterns with the previous existing ones in the data base are defined as SIT (spoligotype international type) whilst single spoligo patterns or the ones which were not reported in the data base previously are defined as “orphan” isolates.

3. RESULTS

3.1 Culture Result

Sputum sample from smear positive TB patients (n=53 subjects) were collected, transported to Aklilu Lemma Institute of Pathobiology and cultured. Out of 53 smears positive samples cultured, 64.15%of samples were culture positive and 35.85% of samples were culture negative as shown by Fig. 2.

Among culture positive individuals, 52.94% of the individuals were males and 47.06% of them were females. The proportion of culture positive result was highest in 18-30 age category, accounting 70.59% of the total within age categories (Table 1).

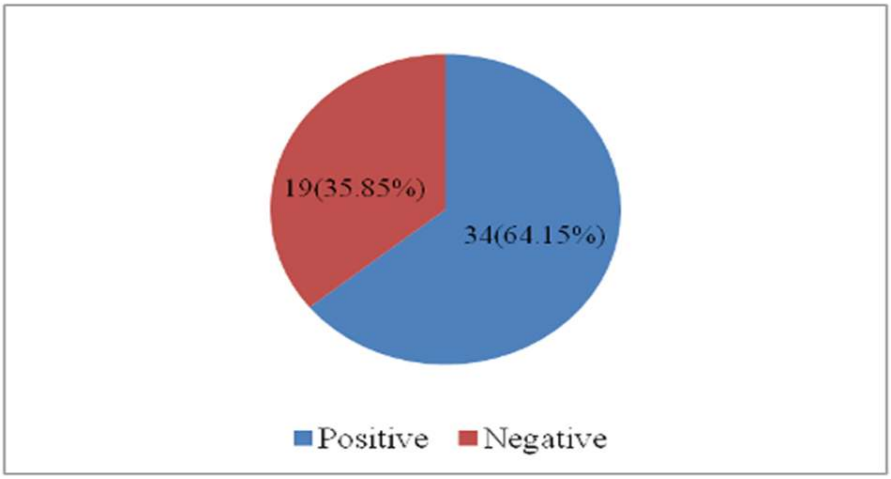


Fig. 2. The proportion of culture results among smear positive sputum samples. The figure shows the percentage of culture positive and culture negative samples among all smear positive samples cultured

Table 1. Culture result by institution, sex and age of the patients

	Negative N (%)	Positive N (%)	Total N (%)
Sex			
Male	13 (68.42)	18 (52.94)	31 (58.49)
Female	6 (31.58)	16 (47.06)	22 (41.51)
Age			
18-30	14 (73.68)	24 (70.59)	38 (71.70)
31-40	3 (15.79)	4 (11.76)	7 (13.21)
41-50	1 (5.26)	4 (11.76)	5 (9.43)
≥ 51	1 (5.26)	2 (5.88)	3 (5.66)
Institution			
AH	5 (26.32)	17 (50.00)	22 (41.51)
AHC	0 (0.00)	1 (2.94)	1 (1.89)
BHC	2 (10.53)	0 (0.00)	2 (3.77)
MHC1	1 (5.26)	1 (2.94)	2 (3.77)
PH	11 (57.89)	15 (44.12)	26 (49.06)
Total	19 (100.00)	34 (100.00)	53 (100.00)

AH= Asossa Hospital, AHC= Asossa Health Centre, BHC= Bambasi Health Centre, MHC1= Mambuk Health Centre, PH= Pawe Hospital

3.2 RD9 Deletion Typing Result

The colonies from culture positive samples were collected and mycobacterial genomic DNA was extracted by heat killing at 85ºC. DNAs from 34 isolates were subjected to multiplex PCR RD9 deletion for species identification. Region of difference analysis for RD9 (RD9 typing) of all the isolates indicated that 33 out of 34 isolates were with intact RD9 and therefore *M. tuberculosis* species. Only one isolate did not give a signal in response to RD9 typing.

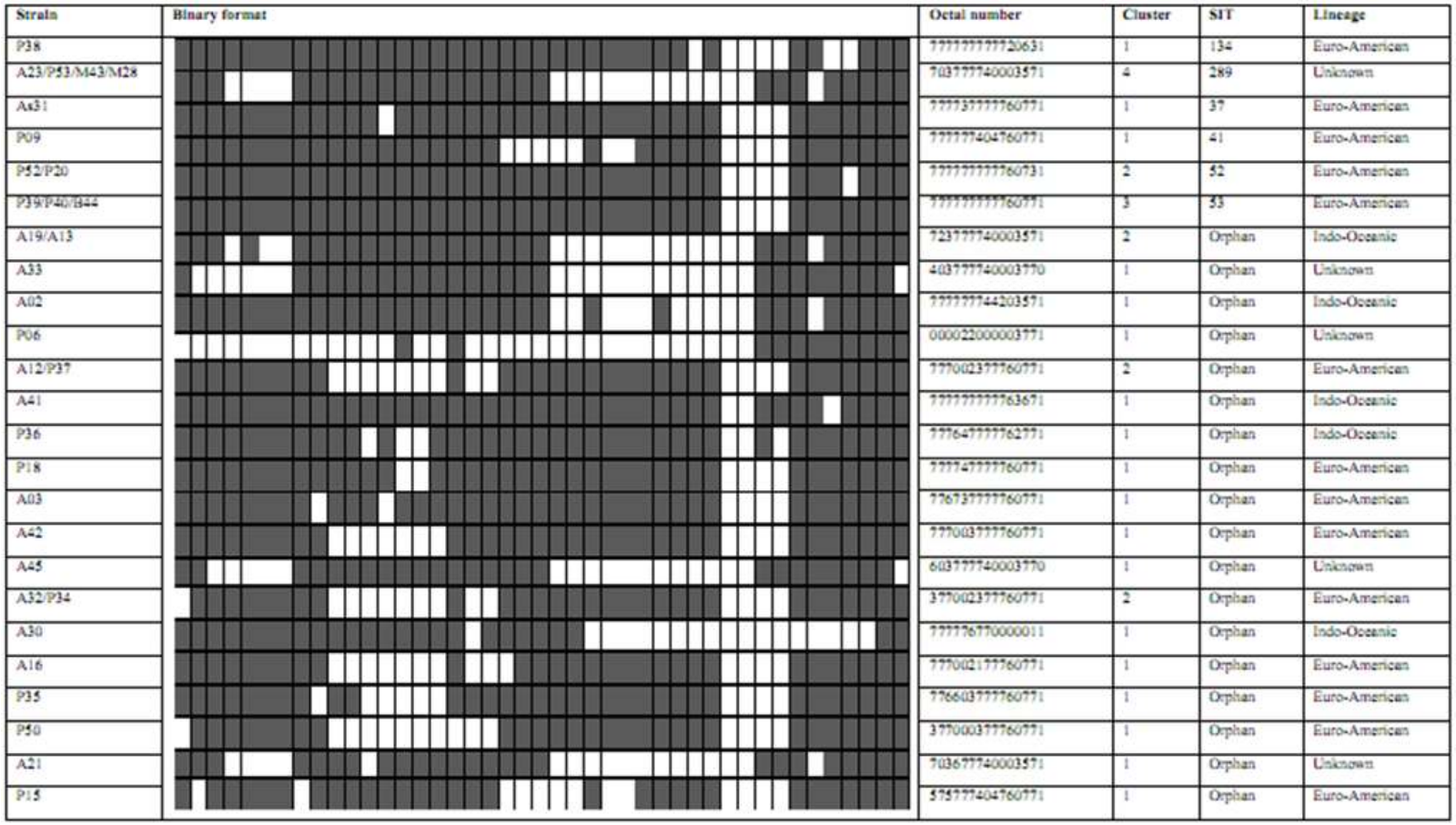
3.3 Spoligotyping Result

After deletion typing all the isolates were subjected to spoligotyping (Fig. 3). A 43-spacers spoligotyping result for 33 DNA samples was obtained. A total of 24 individual patterns were found, among which, 18 patterns were unique whereas 15 clinical isolates were distributed in six clusters containing two to four isolates (Fig. 3). Eighteen patterns were not previously reported in the SpolDB4 international database nine of which were presenting the typical signature of the Euro-American lineage (absence of spacers 33–36), six of which presenting the typical signature of Indo-Oceanic (absence of 29–32, 34, and presence of 33 spacer) and four of which presenting the typical signature of Unknown lineage.

In the current study, classification of the 33 isolates into the lineages showed that 57.58% of the isolates belonged to Euro-American, 18.18% to Indo-Oceanic while the remaining 24.24% could not be categorized to the presently known lineages (Fig. 4).

4. DISCUSSION

Out of 53 smears positive samples cultured, only 34 (64.15%) were culture positive. This result is in line with the findings reported in Nigeria (67.2%) by [16]. The percentages for positivity with AFB microscopy compared with culture obtained in this study may also be compared with those from various parts of the world as reported by [17]. Cases with numerous AFB on microscopy did not show an increased likelihood for yielding positive results on culture than those with scanty AFB. This is due to the fact that the recovery of AFB from microscopy is significantly affected by several factors including the adequacy of the specimen, the processes of collection, transport and decontamination of the specimen, the temperature of centrifugation and the amount of centrifugal force applied [18]. The revised definition of a new sputum smear-positive PTB case is based on the presence of at least one acid fast bacillus (AFB+) in at least one sputum sample in countries with a well functioning external quality assurance (EQA) system [19]. Hence, the small proportion of culture positive result in this study may be due to these mentioned reasons.



Strain	Binary format	Octal number	Cluster	SIT	Lineage
P38		777777777720631	1	134	Euro-American
A23/P53/M43/M28		703777740003571	4	289	Unknown
As31		777737777760771	1	37	Euro-American
P09		777777404760771	1	41	Euro-American
P52/P20		777777777760731	2	52	Euro-American
P39/P40/B44		777777777760771	3	53	Euro-American
A19/A13		723777740003571	2	Orphan	Indo-Oceanic
A33		403777740003770	1	Orphan	Unknown
A02		777777744203571	1	Orphan	Indo-Oceanic
P06		000022000003771	1	Orphan	Unknown
A12/P37		777002377760771	2	Orphan	Euro-American
A41		777777777763671	1	Orphan	Indo-Oceanic
P36		777647777762771	1	Orphan	Indo-Oceanic
P18		777747777760771	1	Orphan	Euro-American
A03		776737777760771	1	Orphan	Euro-American
A42		777003777760771	1	Orphan	Euro-American
A45		603777740003770	1	Orphan	Unknown
A32/P34		377002377760771	2	Orphan	Euro-American
A30		777776770000011	1	Orphan	Indo-Oceanic
A16		777002177760771	1	Orphan	Euro-American
P35		776603777760771	1	Orphan	Euro-American
P50		377000377760771	1	Orphan	Euro-American
A21		703677740003571	1	Orphan	Unknown
P15		575777404760771	1	Orphan	Euro-American

Fig. 3. Spoligotyping result revealing the Octal numbers, clusters, SIT and lineages of strains that were observed in *M. tuberculosis* isolates from patients with tuberculosis in two hospitals (Asossa and Pawe) and two health centers (Bambasi and Mambuk) in Benishangul Gumuz Region

A=Asossa, B =Bambasi, M=Mambuk, P=Pawe

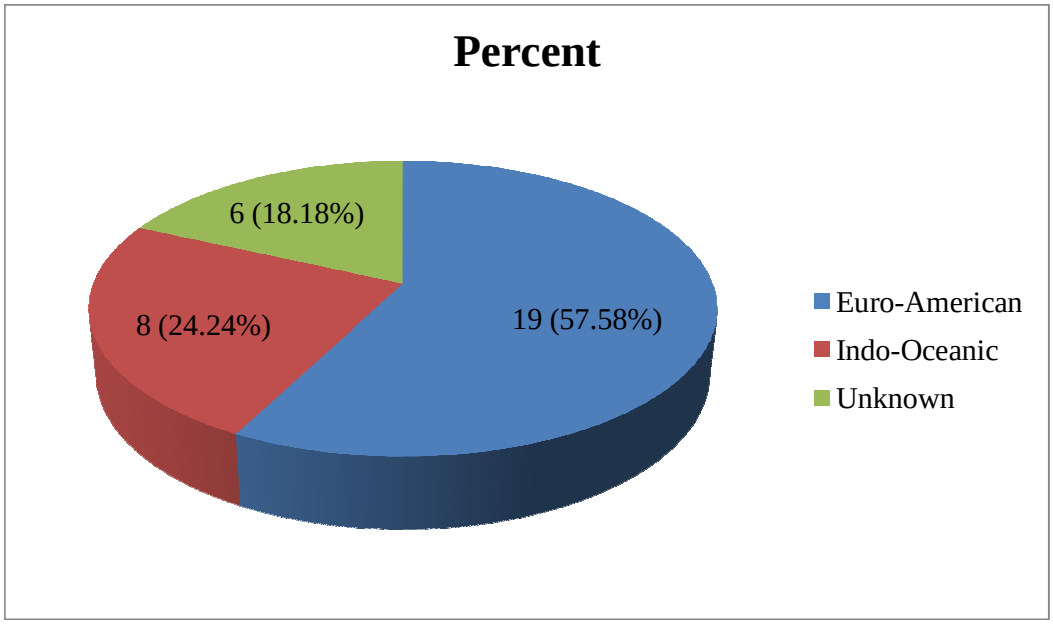


Fig. 4. Proportion of lineages identified by spoligotyping pattern (n=33) in the present study. The figure represents the two common lineages and one unknown lineage to which the identified *M. tuberculosis* strains were grouped

The PCR, result showed that 33 (97.06%) of infections were caused by *M. tuberculosis* among culture positives. This finding showed higher proportion of *M. tuberculosis* infection when compared with a study conducted in Nigeria which reported around 85% [20], but almost similar with the finding reported in Addis Ababa in which 98.37% [21] of TB infection was caused by *M. tuberculosis*.

The spoligotyping pattern of 33 DNA samples revealed 19 (57.6%) Euro-American (lineage-4), 6 (18.2%) Indo-Oceanic (lineage 1) and 8 (24.2%) unknown lineage based on the guidelines [13,14]. In the current study, lineage 4 was the most prevalent lineage. This finding is in line with the findings of [22] and [23] where they reported high prevalence of lineage 4, which was the most common lineage in Ethiopia. Among 33 samples 21 (63.64%) of them were orphans and new to Ethiopia. Some of the strains with SIT (37, 41, and 53) identified in the current study were similar to that of the strains identified elsewhere in Ethiopia by [22]. The proportion of clustered patterns is lower than the proportion of unique patterns in this study. This may be due to the fact that the Region being border setting of the country has a mixed population from different boarder countries and regions. The other possible reason may be due to fact that the Region is visited by investors, refugees and visitors from different regions of the country as well as from bordering countries. As it was indicated earlier, the mixing of populations from different regions leads to fewer clustering [24].

5. CONCLUSION

The study on preliminary characterization of the *M. tuberculosis* strains in the present study revealed the identification of new strains and lineages which could not match with the existing strains and lineages suggesting the localization of these strains and lineages in this Region. Hence, further research is required to identify and document the strains and lineages circulating in the Region.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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