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Molecular characterization of *Mycobacterium tuberculosis complex* in Gambella region, Southwest Ethiopia

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The information on the genetic diversity of *Mycobacterium tuberculosis* in certain geographic region is needed for a better understanding of epidemiology of TB and could have implications for development of new diagnostics, drugs, and vaccines. The aim of this study was to provide information on the strains of *M. tuberculosis* circulating in Gambella region. Cross-sectional study was conducted for six months (November 2012 and April, 2013) in order to generate a primary data on the genetic diversity of *M. tuberculosis.* Clinical examination, Ziehl Neelsen staining, Mycobacterium culturing and molecular typing were used. *M. tuberculosis* isolates were characterized using region of difference 9 (RD9) and spoligotyping. The patterns of strains identified by spoligotyping were compared with the patterns registered in the SpoIDB4 database of the Pasteur Institute. Culture positivity was confirmed in 53.49 % (46/86) of the smear positive cases. Upon molecular characterization, 86.95% (40/46) of culture positive samples were *M. tuberculosis.* The spoligotyping of the 40 isolates lead to the identification of 24 spoligotype patterns. Seven of the patterns were clustered and consisted of 2-3 isolates while the remaining 17 patterns were non-clustered consisting of a single isolate. The most prevalent strain was Spoligotyping International Typing (SIT) 289 with a proportion of 20.0% (8/40). Grouping of the isolates into lineages showed that 32.50% (13/40) were Euro-American, 17.50% (7/40) Indo-Oceanic, and amazingly the remaining 50.0% (20/40) of the isolates could not be grouped into the already known lineages and hence they were new. Findings from this study show that most of strains of *M. tuberculosis* circulating in the region are different from those reported from the other areas of the country. Hence, TB control program should give attention to the control of TB in Gambella region. In addition, characterizing the strains of *M. tuberculosis* circulating in the region would play a great role towards the mapping of molecular epidemiology of TB in Ethiopia.

Key words: Mycobacterium tuberculosis, molecular typing, strain, lineage, Gambella region.

INTRODUCTION

Tuberculosis (TB) is a chronic infectious disease mainly caused by *Mycobacterium tuberculosis Complex (MTBC).*

The main source of infection is untreated smear-positive pulmonary TB (PTB) patient discharging the bacilli. It mainly spreads by an airborne route when the infectious patient expels droplets containing viable bacilli. It is alsotransmitted by consumption of raw milk containing *Mycobacterium bovis (M. bovis)* (Harries and Dye, 2006; Palomino et al., 2007).

According to a recent WHO report the case detection rate of TB trend in Ethiopia was in increasing manner which was 11% in 1995, and increased to 72% in 2011 (WHO, 2012). A ten year TB trend epidemiological analysis of TB conducted in Ethiopia, by Nigatu and Abraha (2010), indicated that the incidence rate of TB is increasing in at a rate of 5 new TB cases per 100,000 populations per year. Urban agro-ecological zones have been more affected by the disease throughout the ten-year period. Extra-pulmonary rate and smear-negativity has shown a modest increment and males were disproportionately affected by TB within the ten-years. On the other hand case detection rate and treatment success rate are found to be increasing at a rate of 0.5% per year. Federal Ministry of Health of Ethiopia also indicated the rising of detection rate of TB was seen since 2004 to 2009 (FMoH, 2011). WHO (2009) report that the status of TB in Gambella Region was the highest from all the Ethiopian Regions, with the notification rate (new and relapse) 261-421/100, 000 population (WHO, 2009). In high TB incidence settings, like Ethiopia, determination of distinct transmission patterns is often hard to define, but may be greatly enhanced by the use of both molecular and conventional epidemiological tools.

Using genotyping methods and markers for molecular characterization of TB have been applied extensively in the epidemiological study worldwide in the last decades (Candia et al., 2007; Sharma et al., 2008; Valcheva et al., 2008).

Some of the more popular MTBC typing methods being used include IS*6110*-based restriction fragment length polymorphism (RFLP) (van Embden et al., 1993) and PCR based methods like spoligotyping (Kamerbeek et al., 1997), and others like mycobacterial interspersed repetitive units - variable number of tandem repeats (MIRU-VNTR), single-nucleotide polymorphisms and large-sequence polymorphism analysis (Frothingham et al., 1998; Le Fleche et al., 2002; Savine et al., 2002). In this study, we set out an objective to investigate the circulating MTBC isolates, and use spoligotyping typing to distinguish MTBC isolates from Gambella regional hospital, located southwest Ethiopia. The present study has been based on the assumption that patients with genotypically clustered strains are epidemiologically linked and represent recent transmissions. In contrast, patients infected with different types of strains are not considered indicative of recent transmission.

MATERIALS AND METHODS

Study area

The study was conducted in Gambella Peoples National Regional State, Gambella town. The region is located in the South Western part of Ethiopia and borders two other regions - Oromia to the North and east and the Southern Nations, Nationalities and Peoples" Regional State to the south and South Sudan to the west. The Gambella is a name for both the region and the city, which is located about 753 km West of Addis Ababa perched at an elevation of 526 m above sea level. The town is founded on the banks of the Baro River, Ethiopia's widest and the only navigable river. Geographically, the portion of Gambella Region is situated in what is known as the western lowland has coordinates 60 28'38" to 80 34' North Latitude and 330 to 350 11'11" East Longitude. Gambella town is located inside the boundary of the Gambella Zuriya woreda, which again this is bordered on the south by the Abol Woreda, on the North and East by Oromia Regional state, on the West by Itang woreda (Figure 1). Based on the 2007 Ethiopian Statistical projections estimate the total population estimated was 390, 593 by the end of 2012 (CSA, 2012). All the people of the region estimated at 390, 593 assumed to be the catchment of the hospital, while around 44, 269 people visit in the hospital and gets service annually (Gambella Regional Health Bureau, 2012).

Study design

An institutional based cross-sectional study was conducted for six month (November 2012 to April, 2013) to see the period prevalence of TB and molecularly characterize *M. tuberculosis* complex species along with strain diversity and lineages. Besides, TB positive individuals were requested to take HIV testing during sample collection period.

Study population

The study populations were those who were attending Gambella Regional Hospital TB clinic from November 2012 to 10 April 2013.

Sample collection and processing

Three times (Spot-morning-spot) sputum samples were collected from each study participant and transported to laboratory for immediate process.

Laboratory investigations

Microscopic examination

Smeared on clean slides in the hospital laboratory independently, by the ZN staining technique and positive samples were transported to Aklilu Lemma Institute of Pathobiology for further diagnosis by putting under the ice box at 4°C.

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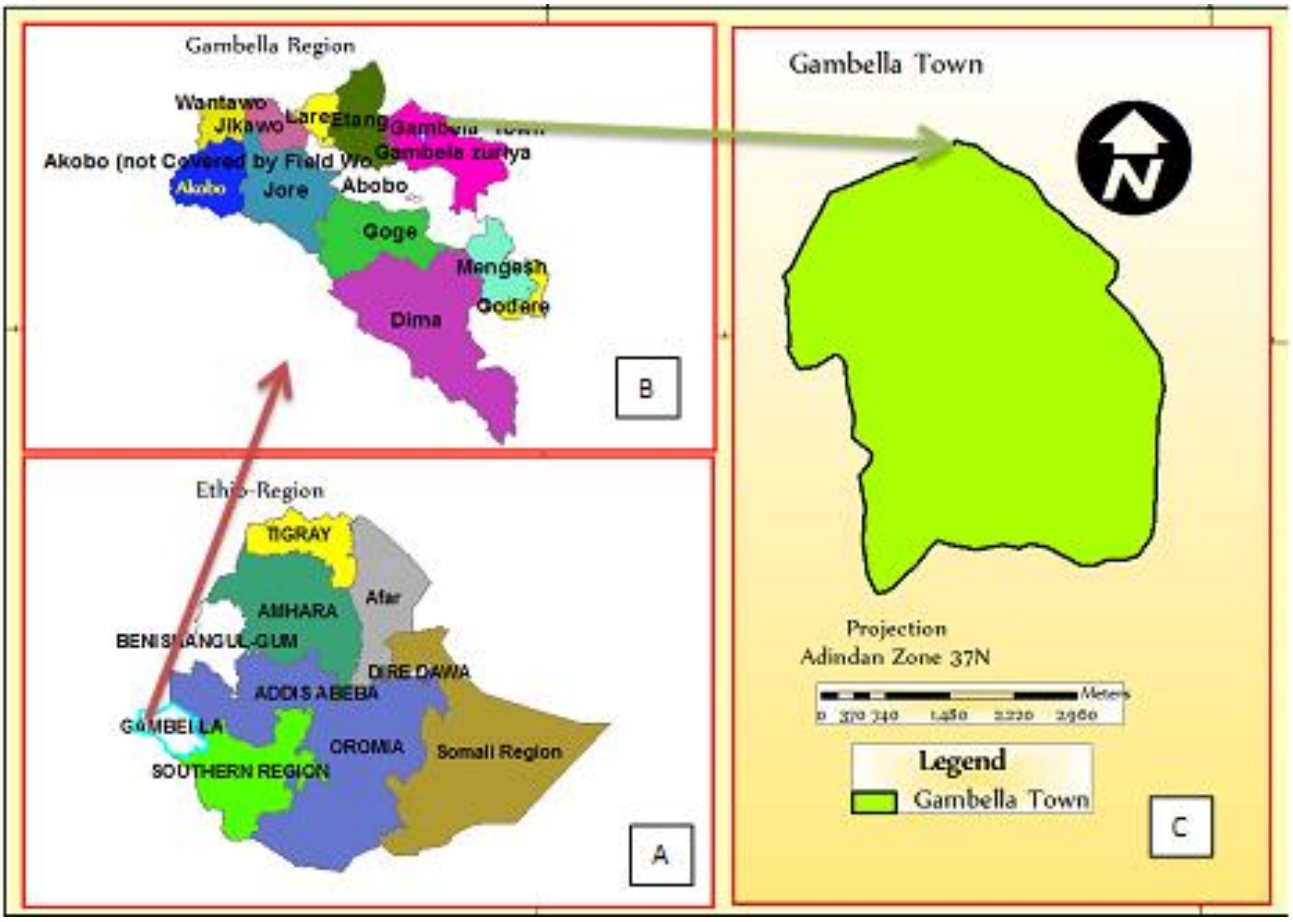

Figure 1. Map of study area.. A) Map of Ethiopia. B) Gambella Regional state. C) Gambella town located inside the Gambella Zuriya woreda. Source: Designed by ArcGIS arc map 10 software based on the CSA 2007 data of Ethiopia by region.

Control Quality control of the slides

During sputum sample examination positive and negative controls were run each day, when new reagents changed, in order to monitor the quality of reagents. Sputum samples collecting cups and slides were labeled carefully.

Culture

The three samples from each patient were pooled, diluted with normal saline and concentrated (Bruchfeld et al., 2000). Mycobacterial culturing was done on conventional Löwenstein-Jensen (LJ) egg medium containing 0.6% sodium pyruvate and 5% glycerol was performed with all sputum samples separately. Before culturing, the samples were digested and decontaminated of non-mycobacterial microorganisms by 4% NaOH (Groothuis and Yates, 1999). This is done by transferring the collected sputum into centrifuge tube and adding equal volume of 4% NaOH to centrifuge tube. Then it is mixed and centrifuged for 15 min at 3000 rpm at 4°C. Then the supernatant decanted and the sediment mixed very well. Following decontamination, we added a phenol red indicator and well-mixed sediment until became red, then 2N HCl added drop by drop until the color changed from purple to yellow. Following neutralization inoculation was conducted by adding one to three drop of neutralized sediment of sputum into two LJ media (pyruvate and glycerol containing), followed by incubation at 37□C with 10% CO_2 air at slant position for one week and upright for 8 weeks by monitoring every week for growth at the same temperature and air condition until complete growth.

Molecular typing

Heat killed cells were tested by PCR based RD9 deletion typing for the presence or absence of region of difference-9 (RD9) so as to identify *M. tuberculosis* from other species of *M. tuberculosis* complex species. For this deletion typing, a procedure described by Brosch et al. *(*2002) was used. In brief: reaction mixtures were made in a total volume of 20 µl consisting of 10 µl HotStarTaq Master Mix (Qiagen, UK), 7.1 µl distilled water, 0.3 µl of each of the three oligonucleotid primer (100 µM), and 2 µl DNA template samples or controls. *M. tuberculosis* H37Rv and *M. bovis* 2122/97, and water were used as positive and negative controls, respectively. The primers used for RD9 deletion typing were RD9flankF, 5'-GTG TAG GTC AGC CCC ATC C-3'; RD9intR, 5'-CTG GAC CTC GAT GAC CAC TC-3'; and RD9falnkR, 5'-GCC CAA CAG CTC GAC ATC-3'. The reaction mixture was then heated in Thermal Cycler using the following amplification program: 95°C for 10 min for enzyme activation; 95°C for 1 min for denaturation; 55°C for 0.5 min for annealing; 72°C for 2 min for extension, involving

35 cycles all in all; and final extension at 72^0C for 10 minutes. The product was electrophoresed by Agarose Gel Electrophoresis System (BIO RAD, UK) in 1.5% agarose gel in 1X TAE running buffer. Ethidium Bromide at a ratio of 1:1000, 100 bp DNA reference ladder and orange 6x loading dye were used in agarose gel electrophoresis. The gel was visualized using Multi Image Light Cabinet (Alpha Innotech Corporation, UK) and the photograph was taken. The results were interpreted as *M. tuberculosis* (RD9 present) when a band size of 396 bp was observed.

Finally, PCR products were electrophoresed in 1.5% agarose gel in 10x TAE running buffer with ethidium bromide at a ratio of 1:10. A 100 bp DNA (Promega Cooperation, USA) ladder and Orange 6x loading dye were also used for visual tracking of DNA migration during electrophoresis. The gel was visualized in a Multi- image TM light cabinet using Alpha Innotech version 1.2.0.1 (Alpha Innotech Corporation). The presence of RD9 (i.e. *M. tuberculosis*) gives a product size of 396bp (RD9 FlankF + RD9 Internal) and its absence (*M. africanum*, *M. bovis*) gives a product size of 575bp (RD9 FlankF+RD9 FlankR).

Spoligotyping

Spoligotyping was performed as previously described by Kamerbeek et al. (1997) and according to the spoligotype kit supplier's instructions (Ocimum Biosolutions Company, Ijsselstein, The Netherlands). The DR region was amplified by PCR using oligonucleotide primers (DRa: 5' GGT TTT GGG TCT GAC GAC 3' and DRb: 5' CCG AGA GGG GAC GGA AAC 3') derived from the DR sequence. The DRa is biotinylated at the 5'-end.

Data processing and analysis

The collected data was checked for any inconvenience and inconsistence and entered to excel spread sheet, coded, and transferred to stata version 11. A p-value less than 0.05 was considered statistically significant. The laboratory result of spoligotype was fed to web based international database of *M. tuberculosis* SITVITWEB version (Dermay et al., 2012) and lineage of mycobacteria, was obtained by SPOTCLUST online software (http://www.tbinsight.cs.rpi.edu/).

Ethical clearance

Ethical clearance was obtained from Addis Ababa University, Aklilu Lemma Institute of Pathobiology. A working permission was also obtained from Gambella Regional State Health bureau and Gambella town district health office. Finally written informed consent was signed by study participants.

RESULTS

Prevalence of TB cases among the study participants

During the cross-sectional study period, there were 651-suspected individuals registered as TB cases from November 2012 to April 2013 at the Gambella Regional Hospital. Table 1 indicates the proportion of sex, smear result, patient type and age category relative to TB status. The smear positive period prevalence was 13.21% (86/651) among the suspected cases.

RD9 deletion typing result

Majority (87%) of the culture positive samples gave positive signal to RD9 deletion typing as shown in Figure 2 and all the isolates were *M. tuberculosis*. The remaining did not show any band signal to RD9 typing implying that they are not members of the *M. tuberculosis* complex.

Strain characterization

Identification of the strains and assigning their respective lineages made for 40 *M. tuberculosis* isolates. Accordingly, 57.5% (23/40) of the isolates were found in seven clustered patterns while the remaining 42.50% (17/40) were non-clustered patterns (Table 2). Assigning the isolates to the lineages showed 32.50% (13/40) of the isolates belongs to Euro-American and 17.50% (7/40) Indo-Oceanic. Amazingly, 50.00% (20/40) of the isolates could not be assigned to the presently defined lineages (Table 2). The spolitotyping result shown in Figure 3, with controls and samples. The black squares indicate the presence of spacers while the spaces loss of spacers. The spoligotyping patterns of the isolated strains showed that there is no significant change in their distribution in sex, age residential area or HIV status of the patients (Table 3).

The prevalent strain was SIT289 that comprised of 20.00% (8/40) of the total isolates, while 40% (16/40) did not match the international spoligotyping data base (Table 4).

This figure shows some of the isolates samples; briefly positive (M. tuberculosis H37Rv 2X at 1st and last position) control), negative (distilled H2O at the second position) control; positive (*M. bovis* 2X at 3rd and 21th position) control; 15 unique isolates and 6 clustered isolates

DISCUSSION

TB has long been recognized as a major public health problem since the 1950s worldwide. By considering the impact, WHO initiated implementation of DOTS strategy in the 1990s to minimize the problem. Advent of HIV / AIDS epidemic makes TB to, remain a major public health problem in endemic developing countries including Ethiopia (WHO, 2005).

In this study, a higher sputum smear positive (SSP) prevalence of TB 69 (14.50) was observed in the age group of 15-44 years while a lower percentage of 2 (12.50%) was seen in age groups of 65 years and above. This finding was similar to observation of Kolifarhood et al. (2012) who reported that 11.2 - 14.5% while, opposite in the age groups of 65≥ years which is 33.5% cases. Proportionally cases residing in Gambella

Table 1. Prevalence of TB in the Gambella Regional Hospital from November 2012 to April 2013.

Variable	Status of TB				Total (N=651)	χ^{2}/fisher Exact	p-value
	SSP (n=86)	SSN (n=119)	EPTB (n=32)	Non-TB (n=414)			
Sex							
Male	51 (13.49)	77 (20.37)	23 (6.08)	227 (60.05)	378 (100.00)	6.49	0.09
Female	35 (12.82)	42 (15.38)	9 (3.30)	187 (68.50)	273 (100.00)		
Total	86 (13.21)	119 (18.27)	32 (4.90)	414 (63.19)	651 (100.00)		
Smear result							
Positive	86 (100.00)	-	-	-	86 (100.00)		0.000
Negative	-	119 (21.06)	32 (5.66)	414 (73.27)	565 (100.00)		
Total	86 (13.21)	119 (18.27)	32 (4.90)	414 (63.19)	651 (100.00)		
Age category							
0-14years	-	24 (36.36)	7 (10.61)	35 (53.03)	66 (100.00)		0.000
15-44years	69 (14.50)	84 (17.65)	24 (5.04)	299 (62.82)	476 (100.00)		
45-64years	15 (16.13)	7 (7.53)	-	71 (76.34)	93 (100.00)		
>=65years	2 (12.50)	4 (25.00)	1 (6.25)	9 (56.25)	16 (100.00)		
Total	86 (13.21)	119 (18.27)	32 (4.90)	414 (63.19)	651 (100.00)		
Patient type							
New	86 (13.87)	97 (15.65)	25 (4.03)	412 (66.45)	620 (100.00)		0.000
Relapse	-	2 (50.0)	2 (50.0)	-	4 (100.0)		
Failure	-	3 (60.0)	1 (20.0)	1 (20.0)	5 (100.00)		
Defaulter	-	7 (70.0)	3 (30.0)	-	10 (100.00)		
Transfer in	-	10 (83.33)	1 (8.33)	1 (8.33)	12 (100.0)		
Total	86 (13.21)	119 (18.27)	32 (4.90)	414 (63.19)	651 (100.00)		

SSP= sputum smear positive, SSN= sputum smear negative, EPTB= extra pulmonary TB.

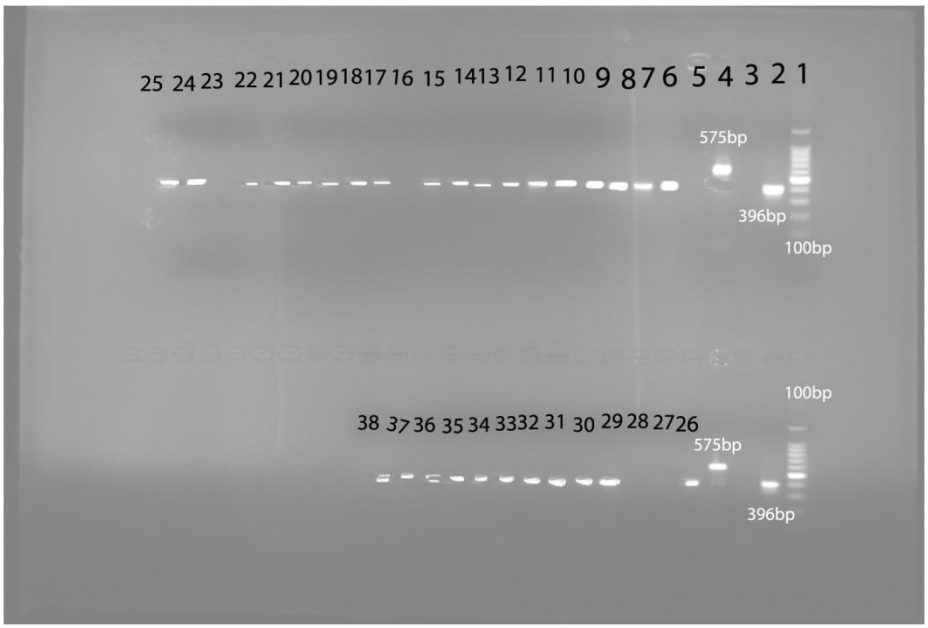

Figure 2. Gel electrophoresis separation of PCR products of RD9 deletion typing on mycobacteria isolates from humans. Lane 1, 100bp DNA Ladder; Lane 2, M. tuberculosis H37Rv (positive control 396 base pair band size); Lane 3, Qiagen H2O (Negative Control); Lane 4, M. bovis (positive control 575 base pair band size); Lanes 5-38) sample isolates from humans. Lane 5; 16; 23; 27 and 28 did not give RD9 signal implying there negative. (Study area is figure 1).

Table 2. Proportion of TB lineage and genotyping pattern.

Characteristics	Proportion n (%)
TB lineage	
Euro-American	13 (32.50)
Indo-Oceanic	7 (17.50)
Unknown	20 (50.00)
Spoligotyping pattern	
Non-clustered genotype	17 (42.50)
Clustered genotype	23 (57.50)
Strain type	
Orphan	16(40.00)
Known	24(60.00)

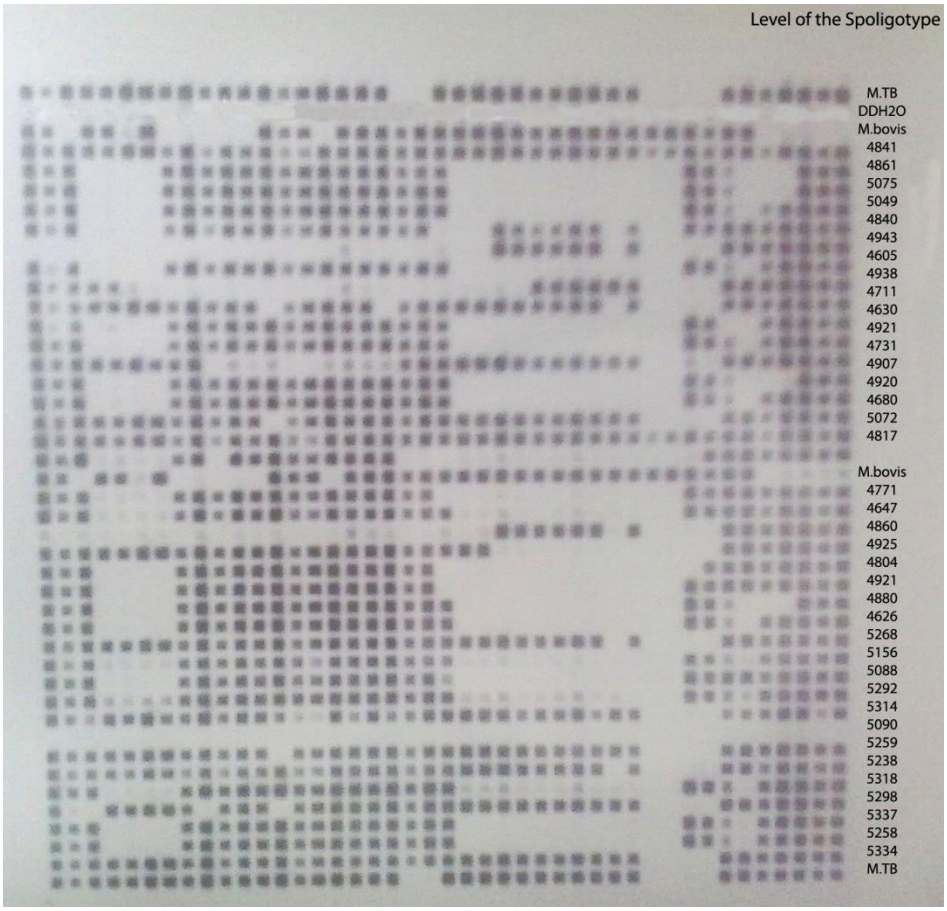

Figure 3. Spoligotype patterns of isolates obtained from Gambella.

town were higher as compared to those residing outside the city; this showed that urban dwellers are more prone to infection by TB. This could be due to increasing population density and high contact and social interaction, crowdedness in rapidly growing cities (Palomino et al., 2007; Rahman, 2010).

Based on the earlier established method by Shabbeer et al. *(*2012), 50% of the isolates were classified into the existing lineages while the remaining 50% of the isolates could not be grouped into the existing lineages and thus were new/unknown lineages. Further exploration is required to identify the lineages of these isolates and

Table 3. Proportion of spoligotyping pattern of the strains with different variables.

Characteristics	Genotype patterns of the strains n (%)			
	Unique	Clustered	χ^2/fisher exact	*P-value*
Sex				
Male	12 (48.00)	13 (52.00)	0.82	0.36
Female	5 (33.33)	10 (66.67)		
Age category				
15-44years	13 (40.63)	19 (59.38)		0.49
44-64years	4 (57.14)	3 (42.86)		
≥65years	-	1 (100.00)		
Residential area				
Outside Gambella	5 (35.71)	9 (64.29)	0.40	0.52
Gambella Town	12 (46.15)	14 (53.85)		
HIV status				
Positive	4 (40.00)	6 (60.00)	0.10	0.95
Negative	9 (45.00)	11 (55.00)		
Refused	4 (40.00)	6 (60.00)		

Table 4. Distribution of spoligotypes and their report status from Ethiopia.

SIT number	Proportion	Report from Ethiopia
523	2 (5.00)	No
289	8 (20.00)	No
25	1 (2.50)	Yes
37	3 (7.50)	No
21	1 (2.50)	No
142	2 (5.00)	No
26	2 (5.00)	No
602	1 (2.50)	No
50	2 (5.00)	No
118	1 (2.50)	No
53	1 (2.50)	Yes
Orphans	16 (40.00)	Not reported from any country
Total	40 (100.00)	

further study is required to know if these belong to a new lineage. The predominance of Euro-American lineage finding (also known as linage 4) in this study also similar with the reports of previous studies, reported as the dominant lineage in different regions of Ethiopia (Diriba et al., 2013; Firdessa et al., 2013; Tessema et al., 2013). In a study conducted in Switzerland, the Euro-American and Indo-Oceaninc linage proportion were 72.7 and 8.3% which are higher and lower respectively compared to the present study result (Fenner et al., 2012). In another study conducted in USA, the Euro-American linage prevalence was lower than the present finding which was 13.8% while, the Indo-Oceanic prevalence in this study was lower as compared to 22.6% (Eleanor et al., 2012).

Clustered strains were less common in the present findings suggesting low frequency of transmission of TB in the area. However, there were significant numbers of unique strains in the area, which suggest reactivation of TB rather than new infection. The presence of orphans and unknown lineages in the area could suggest the localization of such strains and lineages in Gambella Region and warrants for further research. SIT289 is a common isolates from Saudi Arabia, India, Pakistan and Bangladesh (Filliol et al., 2002; Hasan et al., 2006).

In conclusion, our study shows the importance of the disease in the area that revealed the presence of unknown TB strains and lineages. Transmission is particularly well contained in the activation of previous infection rather than new infection. We identified *M. tuberculosis* was the only agent that was identified as the causative agent of human TB in Gambella Region. More attention in TB control program, strict follow up during treatment, and further study on the newly identified lineages and strains using more advanced tools is encouraged.

Conflict of interests

The authors did not declare any conflict of interest.

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