ORGINAL ARTICLE

MOLECULAR CHARACTERIZATION OF MYCOBACTERIA CAUSING PULMONARY TUBERCULOSIS IN GONDAR, NORTH-WEST ETHIOPIA

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ABSTRACT

Background: *In human infections, molecular epidemiological studies have suggested that certain Mycobacterium tuberculosis types can be especially prone to drug resistance acquisition or to global dissemination. Some related types of Mycobacterium tuberculosis also appear to be strongly associated with specific geographic locations. Other Mycobacterium tuberculosis strains, such as the Beijing family are reported more pathogenic. Therefore, this study determined the dominant Mycobacterium tuberculosis spoligotype causing pulmonary tuberculosis in Gondar*

Objectives: *the aims of this study was to provide an initial and base-line data on the genetic biodiversity of Mycobacterium tuberculosis strains causing pulmonary tuberculosis in Gondar.*

Methods: *Sputum samples were collected from 116 smear positive tuberculosis patients and decontaminated with N-acetyl-L-cysteine (NALC)/sodium hydroxide. DNA was purified at an elution volume of 100 µl using the QIAamp DNA Mini cube. DNA samples were subjected to 16S rDNA/RD750 real time PCR, RD9 multiplex PCR and spoligotype. For 16S rDNA/ RD750 assay, PCRs were carried out in 25 µl volumes containing 20 µl of PCR Master Mix and DNA standard was diluted to 10^7, 10^6, 10^5, 10^4, 10^3 and 10^2 M. tuberculosis genomic DNA genomes/µl. For RD9 PCR assay, eighteen µl of the PCR mix were aliquot into a PCR tubes and 2 µl of the DNA template added to the respective tubes and PCR run with the corresponding PCR program in the thermo cycler. The preparations were subjected to gel electrophoresis and image taken using a 12 bit imaging camera. Spoligotype was run by adding 5 µl of DNA and 20µl of the PCR mix followed by Hybridization in water bath at 42°c and 60°C.The autorad was developed by subjecting films to infrared light and striped using 1% SDS at 80°c. The orientation of the autorad was read first using controls and the SIT number of each Mycobacterium tuberculosis complex determined from the SITVIT database.*

Results: *The 16S rDNA assay result showed that 22 samples were positive for Mycobacterium tuberculosis complex and the RD750 real-time PCR assay showed that tubercle bacilli in 5 sputum samples were with intact RD750. Ninety-four of the one hundred sixteen samples were RD9 intact and the other 22 RD9 deleted. Seventy-two of the 94 Mycobacterium DNA extracts with RD9 intact showed Mycobacterium tuberculosis with different Shared International type numbers (SITs) of which, Mycobacterium tuberculosis SIT 25, Central Asian Strain variant 1 (CAS-1 variant), accounted for 30.6% (n=22). In this study, 15 new Mycobacterium tuberculosis spoligotypes all with intact RD9 (MTb SIT NEW) were also observed.*

Conclusion: *This study showed diversity of Mycobacterium tuberculosis spoligotypes causing pulmonary tuberculosis in Gondar. The new Mycobacterium tuberculosis spoligotypes observed in the study area was different from the recently reported Woldiya lineage that suggests the existence of new Mycobacterium tuberculosis strains circulating in the locality. Therefore, we suggest that large scale community based studies are required to determine the prevalence of this new Mycobacterium tuberculosis spoligotypes around Gondar and the neighboring areas. The disease causing ability or virulence of these new Mycobacterium tuberculosis spoligotypes need to be determined by future works.*

Key words: *Mycobacterium tuberculosis, RD9, Spoligotype, Central Asian Strain variant 1*

INTRODUCTION

The establishment of the genome sequence of *M. tuberculosis* H37 Rv in 1998 paved the way for a major break through in understanding the biology of tubercle bacilli in particular and mycobacteria in general (1). The *Mycobacterium tuberculosis* complex (MTBC) comprises so-called *M. tuberculosis*, *M. bovis*, *M. microti*, *M. africanum*, *M. pinnipedii*, and *M. caprae* species (2). The genome of MTBC is highly conserved than other pathogenic bacteria (3). Nevertheless, genotyping tools have recently identi-

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fied several polymorphisms in the MTBC genome that have provided insight into its evolution. An association between geographic region and *Mycobacterium tuberculosis* families, defined by specific polymorphisms, has been demonstrated. The geographic structuring genetically, and perhaps phenotypically, distinct MTBC populations may contribute to the difference in clinical features, such as severity of disease (4). Of particular note are the recent publications suggesting that strains belonging to the *Mycobacterium tuberculosis* W/Beijing lineage possess unique attributes that confer an increased ability to cause disease and to be transmitted within certain geographic settings (5).

Understanding the structure and dynamics of pathogen populations gives unique insights into crucial public health issues, such as the emergence of resistance to antibiotics (6). In Gondar, although the *Mycobacterium tuberculosis* strains causing pulmonary tuberculosis were not previously characterized, a 15.8% resistance to at least one first-line drug and a 5.0% multi-drug resistance were recently reported (7). The most effective means of assigning strains into a small number of unambiguous lineages is the method based on the detection of a large sequence of polymorphisms (LSPs) or regions of difference (RDs) that represent a series of well-characterized unique-event polymorphisms (deletions) (8). Differential hybridization arrays identified 18 regions of difference (RD1-RD18), ranging in size from 0.3bp to 12.7 kilo-base coding for 120 genes that are present in *M. tuberculosis* H37Rv but absent from *M. bovis* BCG strain (9).

The RD9 region was found in all *M. tuberculosis* and *M. canetti* strains but absent from *M. africanum*, and *M. bovis* (10). Recently, a two-tube combined TaqMan/SYBR Green assay targeting the mycobacterial 16S rDNA and the global lineage-defining region of difference (RD750) polymorphism in separate reactions within a single PCR run was reported as a method to differentiate MTBC strains (11). Spoligotyping is a new method for simultaneous detection and typing of *Mycobacterium tuberculoses* complex bacteria. Spoligotyping is based on Polymerase Chain reaction (PCR) amplification of a highly polymorphic direct repeat locus in the *Mycobacterium tuberculoses* genome (12). The genome of *Mycobacterium tuberculoses* complex carries a single region on the chromosome called the direct repeat locus and DNA polymorphism in this locus allows for strain typing.

Analysis of the genetic structure and evolution of population of pathogenic microbes is essential for understanding the mechanisms responsible for the ability to escape host immune disease (13). Although *Mycobacterium tuberculosis* exhibits very little genomic sequence diversity, there are evidence reports that documented phenotypic diversity among clinical isolates (14). The presence of significant sequence diversity in *Mycobacterium tuberculosis* would provide a basis for understanding pathogenesis, immune mechanisms, and bacterial evolution as there are increasing evidences that the inter strain variation that exists is biologically significant (15). Therefore, the major aim of this study was to provide an initial and base-line data on the genetic biodiversity of *Mycobacterium tuberculosis* strains and to determine the dominant *Mycobacterium tuberculosis* spoligotype causing pulmonary tuberculosis in Gondar.

METHODS

This cross-sectional study was conducted at the University of Gondar Teaching Hospital, Gondar Polyclinic and Gondar Army Hospital, northwest Ethiopia. A total of 116 smear positive tuberculosis patients were recruited within six months at the Outpatient Department after informed consent. Demographic data was collected using a standard questionnaire. Sputum samples were collected using dry, clean, leak proof and screwed plastic containers. The samples were decontaminated and homogenized through treatment with N-acetyl-L-cysteine-Sodium hydroxide (NALC/NAOH) solution. N-acetyl-L-cysteine was freshly prepared prior to use as it is readily inactivated by oxidation. An equal volume of digestent was added to the sputum and gently mixed for no longer than 30 seconds. The mixture was allowed standing for 15 minutes with occasional agitation followed by neutralizing by adding 67 mM phosphate buffer to within 1 cm of the top of the tube. Bacterial pellet was harvested after centrifugation at 2500 rpm for 20 minutes. Pellet was re-suspended with 500 µl Phosphate buffer saline (PBS).

Purification of DNA: One-hundred fifty µl of pellet suspended with 500 µl PBS was used for the DNA assay. Pellet was re-centrifuged at 13,000 rpm for 2 minutes, supernatant removed and the pellet re-suspended in 90µl sterile distilled water. This was boiled at 100^{o}C for 30 minutes to inactivate *Mycobacterium tuberculosis* and stored at -20^{o}c. Lysozyme stock was prepared by dissolving 100 mg lysozyme in 1 ml Tris buffer (20 mM Tris-HCl PH 8.0, 2 mM EDTA and 1.2% Triton). Nine hundred sixty µl of the stock was re-diluted with 1440 µl of

Qiagen protease to prepare 20 mg/ml lysozyme working solution. To each 90 µl of sample, 90 µl of lysozyme working solution was deposited in a 1.5 ml tube and vortex mixed. The preparation was transferred to 2 ml safe-lock micro-centrifuge tube. QIAamp Mini spin column and 1.5 ml collection tube were positioned on a rotor adapter. Buffers AW1 and AL (containing guanidine hydrochloride), 100% ethanol, and RNase free water were placed in a reagent bottle rack. Two hundred seventy-nine µl of proteinase K solution was deposited in a 1.5 ml tube to run 12 samples. Samples and reagents were placed into QIAamp DNA Mini cube for automated DNA purification, and DNA was purified at an elution volume of 100 µl. Sample preparation using the QIAamp DNA Mini cube followed 4 steps: lysis, bind, wash, and elute at a preferred centrifugation of 8000 rpm. After isolation, DNA samples were stored at -20^{o}C.

The Real-time PCR 16S rDNA / RD750 assay: For 16S rDNA assay, PCRs were carried out in 25 µl volumes containing 20 µl of PCR Master Mix (12.5 µl of 2xSYBR green, 1 µl of MYCO16SF, 1 µl of MYCO16SR, 1µl of MYCO16S probe, 4.5 µl of RNase free water) and 5µl of DNA lysate. For the RD750 assay, PCRs were also carried out in similar total volumes (20 µl Master Mix with 5 µl of DNA lysate) used for the16S rDNA assay. PCR Master Mix was prepared from 2xSYBR Green, 2 µM of RD750FP1, 2 µM of RD750RP2, 2 µM of RD750R3, 2 µM of RD750probe and 3.5 µl of RNase free water. DNA standard was diluted to 10^7, 10^6, 10^5, 10^4, 10^3 and 10^2 *M. tuberculosis* genomic DNA genomes/µl equivalent to 16S rDNA copies per µl. *Mycobacterium tuberculosis* H37Rv and RNase free water were used as positive and negative controls, respectively. All PCR assays were performed in duplicate. PCRs were performed in a Rotor-Gene^{TM} machine (Corbett Life Science, QLD, Australia) as follows: 95^{o}C for 15 minutes to activate *Taq* polymerase, followed by 40 cycles of 95^{c}C for 15 seconds, 60^{o}C for 60 seconds, 72^{o}C for 20 seconds, and 84^{o}c for 20 seconds. ROX and Cy5 signals were acquired at 60^{o}C while SYBR Green signals were acquired at 84^{o}C.

RD9 Multiplex PCR assay: Deletion typing was designed as a multiplex PCR using three different primers: RD9-Flank FW (forward), RD9-Internal Rev (reverse) and RD9-Flank Rev (reverse). DNA samples were lyophilized at the University of Leicester in a vacuum dryer at 55^{o}C for 1 hour to enhance preservation during transportation to Armauer Hansen Research Institute (AHRI), Ethiopia. The lyophilized DNA samples were investigated at AHRI for RD9 deletion typing and Spoligotype. Lyophilized DNA samples were re-constituted with RNase free water, vortex mixed and subjected to PCR. Multiplex PCR master mix for RD9 deletion typing was prepared on the day of use by mixing water, HotStat Master Mix, and primers in a sterile eppindroff tube following the AHRI standard operational procedures (SOPs). Eighteen µl of the PCR master mix was aliquot into PCR tubes and 2 µl of the DNA template added to the respective tubes. The tubes were placed in the PCR thermocycler (Multiplex PCR T3000 thermocycler Biometra, Thistle Scientific) and run at 95^{o}C for 15 minutes, 95^{o}C for 1 minute, 55^{o}C for 0.5 minute (35 cycles), 72^{o}C for 1 minute; 72^{o}C for 10 minutes, and 4^{o}C for 10 minutes.

Agarose gel was prepared by weighing out the corresponding amount of agarose to make 1.5% gel. Using an Erlenmeyer flask 1.5 gram agarose was mixed with 100 ml of 1% Tris-Chloride buffer (TAE). To prepare 50% Tris-Chloride buffer, the TAE stock was prepared by dissolving 242 gram Tris base to 750 ml de-ionized water. Carefully, 57.1 ml of glacial acetic acid was added and then 100 ml of 0.5M EDTA. Finally, the volume was adjusted to 1 litter. One percent of TAE buffer (running buffer) was prepared by dissolving 1 ml of 50% TAE in 49 ml of distilled water.

The agarose was heated in a microwave oven for 1 minute and 3µl ethidium- bromide was added to make the final volume of ethidium-bromide concentration 0.3µg/ml. The agarose was allowed to cool 50 -60^{o}C before casting the gel. After assembling the appropriate comb in the gel electrophoresis apparatus, the gel was allowed to polymerize for 30 minutes. The gel was placed in the electrophoresis tank and covered with 1% TAE buffer. To each PCR product 5 µl loading dye was added and mixed thoroughly. Eight micro litter of DNA ladder was deposited to the first well of the gel, followed by adding 10 µl loading dye-PCR mix to other wells, respectively. The preparation was electrophorized at 110 volt for 40 minutes. The gel was removed from the tank and placed in EPI Chemi II Dark room, UVP laboratory products and image were taken using a 12 bit colour imaging camera and saved on a Microsoft power point.

Spoligotyping of Mycobacterial isolates from sputum: The water, primers direct repeat (DRa) and DRb, and the Quiagen master mix were mixed in a sterile eppindroff following the AHRI SOPs. Twenty micro-litter of the PCR mix was dispensed into each

well of a 0.2 ml thin walled Plate. Samples were vortex mixed shortly and spinned for 30 seconds. Five micro-litters of each sample and the controls (*M. tuberculosis* H37 Rv, Distilled water and *M. bovis* AF21222/97) were transferred to the respective wells and placed on the thermocycler. PCR was run at 95°C for 15 minutes, 95°C for 1 minute, 55°C for 0.5 minute (35 cycles), 72°C for 1 minute; 72°C for 10 minutes, and 4°C soak.

For hybridization, the primary, secondary, and striping buffers were prepared and aliquot in flasks and allowed to equilibrate to correct temperatures in water bath (42°C and 60°C). The striping buffer was placed at 60°c. Thirty millilitre of primary buffer was kept in a diluting tray at room temperature. 2xSSPE and 20mM EDTA were also kept at room temperature. One hundred fifty µl of the primary buffer was dispensed in to a new 96-well plate. To this, 20 µl of PCR product was added. The plate was tightly closed and placed on a thermocycler for 10 minutes at 96°C. The membrane which was placed in 20mM EDTA was washed in 250 ml pre-wormed primary buffer for 10 minutes on the shaking platform in the 60°C hybridization oven. The denatured PCR samples were removed from the thermocycler and placed on ice.

Using a P200 pipette, 150 µl of primary buffer were added to lanes 1 and 45 of the mini-blotter. One hundred fifty µl of the denatured samples were added to the other lanes of the mini-blotter, in the order they appear in the 96-well plate. This was hybridized for 60 minutes at 60°C on a horizontal surface with no shaking. The samples were removed from the mini-blotter by aspiration and then the mini-blotter was disassembled, followed by transferring the membrane to a thermo-resistant container (~20x20cm). The membrane was washed twice with 250 ml secondary buffer at 60°C for 10 minutes on a shaking platform. The mini-blotter was cleaned with a detergent and stored at room temperature in a water container until next usage. The membrane was rolled and transferred to a rolling bottle containing distilled water and allowed cooling.

The water was discarded and assured that the membrane was nicely rolled along the glass in the rolling bottle. 2.5 µl of streptavidin-peroxidase conjugate (500 unit/ml) was added to 10 millilitre of secondary buffer. The streptavidin-peroxidase solution was tipped into the rolling bottle and the lid was placed securely, incubated at 42°c for 60 minutes in the hybridization oven. The membrane was removed from the rolling bottle and placed in a plastic container, washed twice with 250 ml of secondary buffer for 10 minutes at 42°C on the shaking platform in the oven. The membrane was rinsed twice with 250 ml of 2xSSPE for 5 minute at room temperature. The ECL detection fluid was prepared and the membrane was immersed two times to ensure that the membrane is completely covered in the solution. In the dark room, the membrane was placed on a covered autorad and wrapped in a clean cling film. The wrapped membrane was placed facing up in an autorad "hypercassette blue' and a new ECL film was positioned on top. The cassette was closed, wrapped in a black sack, and left in a drawer for 20 minutes.

To develop an autorad, the temperature of the developer was fixed at 22°C and 100 millilitres of the film developer, water, and fixer was poured into three separate developing trays (20x25cm). The infrared safety light was turned on; the autorad was removed from the cassette, and placed in the developer ensuring that it is entirely submerged. The autorad was tipped gently until an image of black squares was visible-approximately for 2 minutes. The autorad was rinsed in distilled water for 10 seconds and transferred to the tray containing fixer, agitated gently while incubating for 2 minutes. The autorad was rinsed with water for 2 minutes and allowed to dry. Once satisfied with the autorad, the PCR product of the membrane was striped by setting an oven at 80°C. The membrane was unwrapped and placed in a thermo-resistant plastic container and washed with 250 ml of warm stripping buffer (1%SDS) for 30 minutes at 80°C while shaking. After the second wash, it was left at room temperature for 5 minute to cool before discarding the liquid, and then washed for another 5 minutes in 250 ml of 20mM EDTA. The liquid was discarded and another 250ml of 20mM EDTA added and stored in a fridge until the membrane is needed.

Ethical considerations: All the necessary ethical issues were considered and informed verbal and written consent was taken. Ethical clearance and permission was obtained from the University of Gondar Research and Publications Office (RPO) with Ref.NO:- RPO/55/138/2001 E.C. The sputum specimens obtained from the study subjects for a routine examination purpose was used for this study. The demographic data was collected only after informed consent was obtained from the study subjects. Volunteers were given adequate and clear information about the study purpose, conduct, and expected outcome. No attempt was made to get consent from a patient whose sensorial was disturbed or was in acute pain, and all data were kept confidentially.

RESULTS

One hundred sixty-three patients were initially reported positive for acid–fast bacilli (AFB), but only 116 patients were included in this study. The socio-demographic characteristics of TB patients showed that 68 patients were male and 48 female. Forty-seven patients were married, 44 single, 15 widowed and 10 divorced. The educational status showed that 57 patients were illiterate, 28 completed primary school, 25 completed secondary school and only 6 patients had diploma or degree. The majority of the TB patients (41.4%) were farmers by occupation, followed by students (16.4%) and daily labourers (13.8%).

All the 116 patients were reported positive for AFB by the three laboratories after conducting three smears for each patient (spot-morning-spot sputum sample) and staining the smears with Zeihl-Neelsen stain by neither decontaminating the sputum nor concentrating the organism by centrifugation. Each sputum sample AFB score was re-examined after the sputum was decontaminated by N-acetyl-L-Cysteine/ NaOH (NALC/NaOH) decontamination method. One hundred three sputum samples were positive for AFB and the other 13 were negative when re-examined. The AFB score of each sputum sample was determined based on CDC classification (16). Accordingly, 33 sputum samples were 4+, 20 (3+), 26 (2+), 24 (1+) and the other 13 negative after NALC/NaOH treatment.

The 16S rDNA/RD750 real-time PCR assay on Mycobacterial DNA extracts from sputum: A total of 48 DNA extracts obtained from smear positive sputum samples with AFB score of 4+ (24 samples), 3+ (16 samples), and 2+ (8 samples) were subjected to 16S rDNA/RD750 real-time PCR assay. The 16S rDNA assay result showed that 22 samples were positive for *Mycobacterium tuberculosis* complex (Taqman probe positive 'T^{+}' and Sybergreen positive 'S^{+}'); 22 samples were Taqman probe negative but Sybergreen positive (T^{-} and S^{+}) suggestive of non-tuberculosis mycobacteria or not Mycobacteria and 4 samples negative (No Taqman or Sybergreen signal; T^{-} and S^{-}) which means not Mycobacteria. The RD750 real-time PCR assay result showed that tubercle bacilli in 5 sputum samples were with intact, 7 deleted RD750 and the other 36 negative (No Taqman or Sybergreen signal).

RD9 deletion typing on mycobacterial DNA extracted from sputum: All the 116 lyopilized tubercle bacilli DNA samples were subjected to RD9 deletion typing after they were re-constituted with sterile distilled water. The result showed that 94 samples were with intact RD9 and the other 22 RD9 deleted. The RD9 deletion types of some of the *Mycobacteria* are shown in Figure 1. Previously, when the result of the acid-fast stain (AFS), Ziehl-Neelsen (ZN) stain, delivered by the hospital laboratory in Gondar was compared with the result of another AFS after the sputum samples were decontaminated by NALC/ NaOH, 13 were negative for AFB.. The result of RD9 PCR assay on these samples showed that 10 of them were RD9 intact and the other 3 an RD9 deleted (no signal).

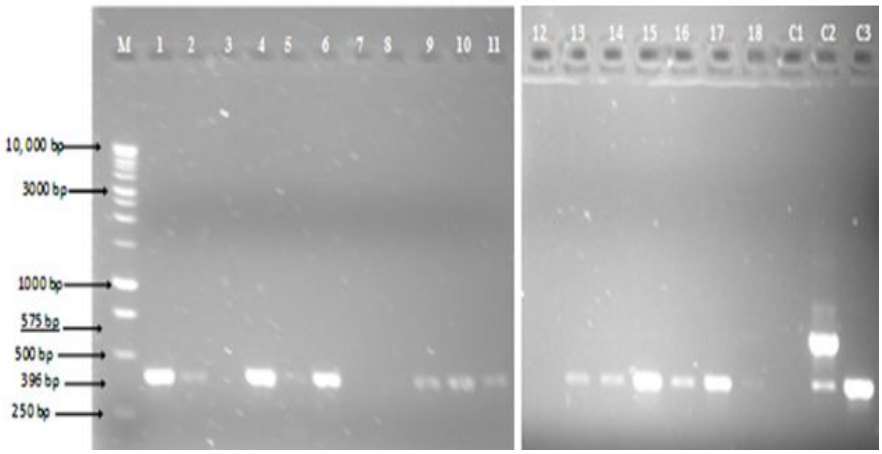

Figure 1: *RD9 deletion typing of some of the Mtb DNA extracts*
Expected PCR Product: *396 bp= Mycobacterium tuberculosis; 575 bp=MTBC other than M. tuberculosis (e.g. M.bovis, M. africanum, M.pinnipedi, M. caprae).*
Result: *Lane M, 1Kb DNA ladder; Lanes 1, 2, 4, 6, 9, 10, 11, 13-17= M .tuberculosis; C1= QH_2O; C2= M.bovis; C3= M. tuberculosis H37Rv;* ***Primers:*** *RD9-F (forward), RD9-Flank & RD9-R (reverse).*

Spoligotype on DNA extracts from mycobacteria obtained directly from sputum: Once the spoligotype pattern of each DNA extract was recorded on an autorad, the orientation of the autorad was first read using the control samples. The spacer deletion pattern was also recorded on a spread sheet to be compared with the International database (SITVIT database). Of the 94 *Mycobacterium* DNA extracts with RD9 intact PCR result, 72 showed *Mycobacterium tuberculosis* with different Shared International type numbers (SITs) (figure 2) of which, MTb SIT 25 accounted for 30.6% (n=22), MTb SIT 53 for 12.5% (n=9), MTb SIT 149 for 12.5% (n=9). On the other hand, MTb SIT 523 (n=3), MTb SIT118 (n=3), MTB SIT 26 (n=3), MTB SIT 50 (n=2), MTB SIT 291 (n=2), and MTB SIT 21 (n=2) accounted for 20.8% of the Mycobacterial isolates causing pulmonary tuberculosis in Gondar. Others with Shared International numbers (SITs) of MTb SIT 159, MTb SIT 37, MTb SIT 522 , MTb SIT 41, MTb SIT 316, MTb SIT 910, MTb SIT 343, MTb SIT 1688, MTb SIT 244, MTb SIT1539, MTb SIT 35, MTb SIT 75, MTb SIT 952, MTb SIT 1198, MTb SIT 815, and MTb SIT 245 collectively accounted for 23.6%.

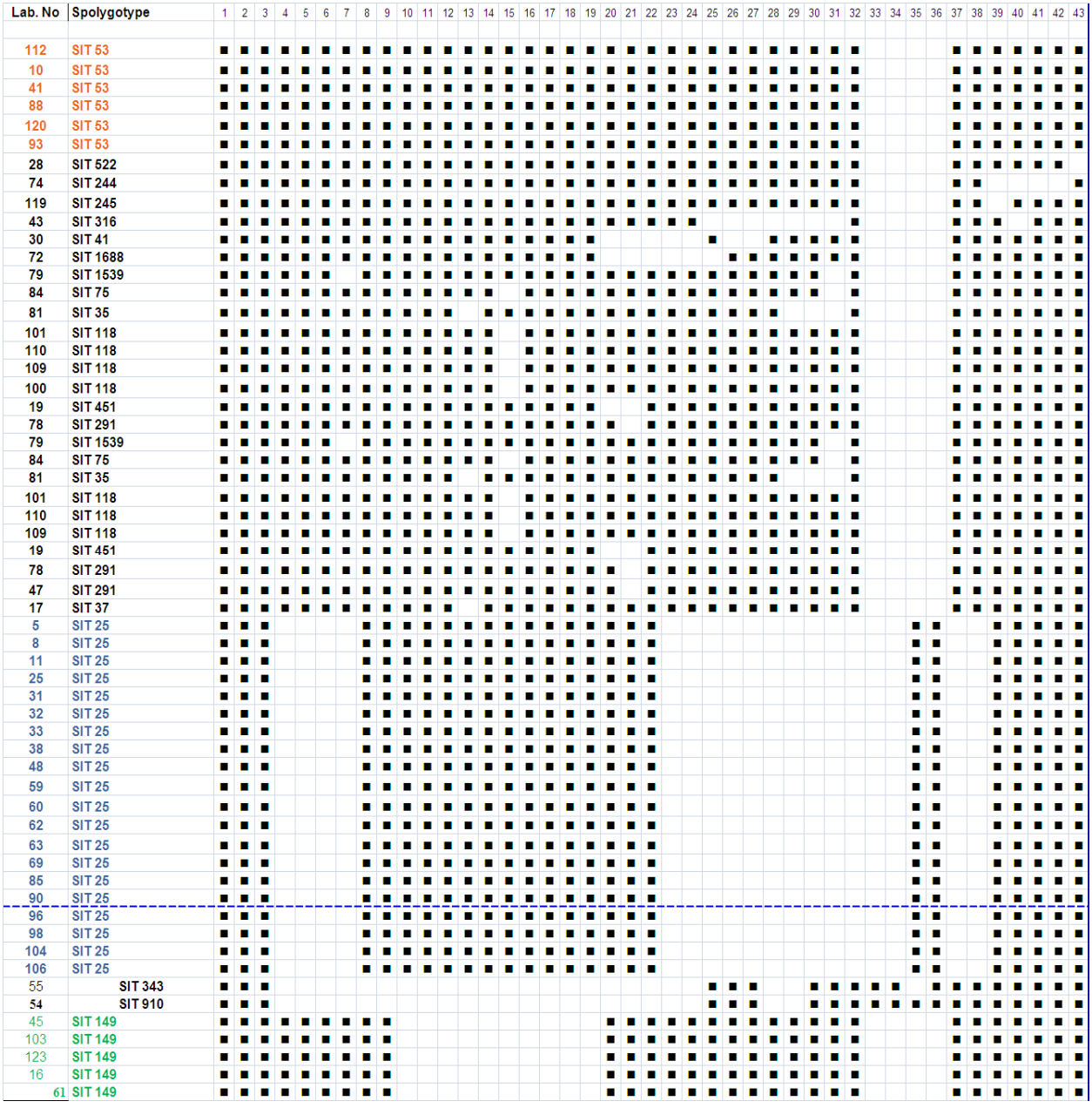

Figure 2: *The spoligotype patterns of Mycobacterial DNA extracts from pulmonary tuberculosis patients in Gondar, Ethiopia.*

In this study, 15 new *Mycobacterium tuberculosis* spoligotypes, tentatively given *Mycobacterium tberculosis* of the Gondar linage (MTb SIT NEW) (figure 3), which were not reported previously nor documented in the International database (SITVIT database) were observed. The spoligotype pattern of 5 *Mycobacterium* DNA extracts showed *Mycobacterium bovis* SBO 133. However, the RD9 deletion type result for all the five demonstrated an intact RD9 which is very unusual and spoligotype pattern for these particular cases might be unreliable.

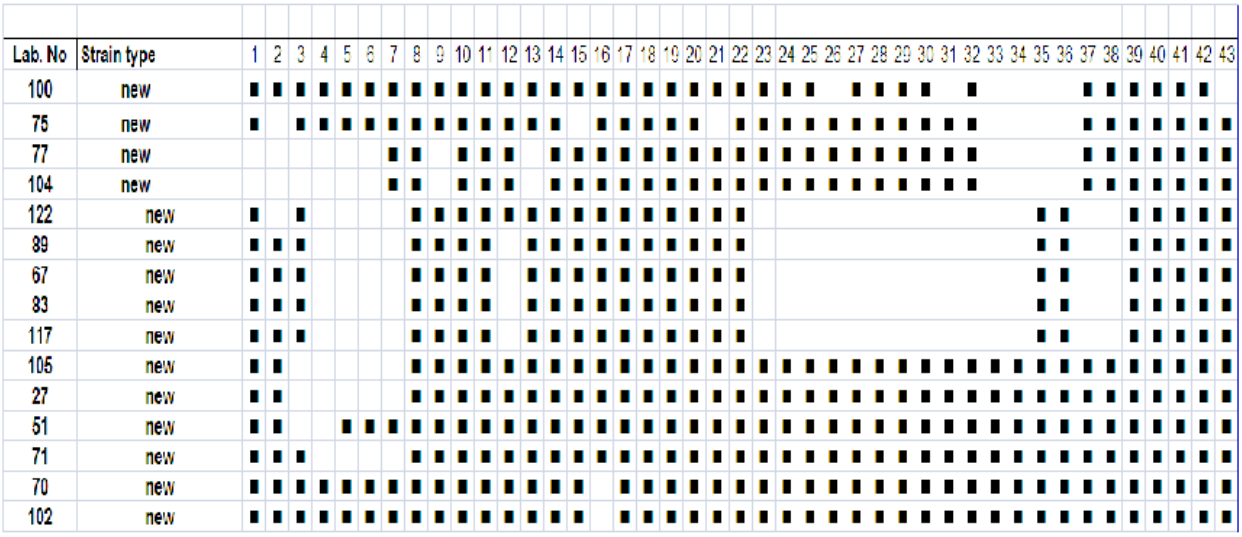

Figure 3: The new Mycobacterium tuberculosis spoligotype isolated from pulmonary tuberculosis patients in Gondar, Ethiopia

All samples with an RD9 deletion were also subjected to spoligotype. The spoligotype result on the RD9 deleted samples showed that 8 of the 22 DNA extracts demonstrated *Mycobacterium tuberculosis* spoligotype with shared International number of MTb SIT 25 (n=3), MTb SIT 815 (n=2), MTb SIT 26, MTb SIT 50 , and MTb SIT523. Seven RD9 intact *Mycobacterium tuberculosis* DNA extracts showed an anomalous spoligotype pattern. On the other hand, 14 samples with RD9 deleted demonstrated an anomalous spoligotype pattern, too. When the result of the16S rDNA real time PCR assay was compared to spoligotype, all the 22 16S rDNA positive samples also showed a positive result on spoligotype. In addition, fifteen 16S rDNA negative samples were positive for *Mycobacterium tuberculosis* strains by the spoligotype method.

DISCUSSION

One hundred sixty-three patients out of 1914 TB suspects (8.5%) were smear positive for acid-fast bacilli. The 2008 Ministry of Health, Ethiopia, report showed an incidence of TB of all forms and smear positive 341 and 152/ 100,000 with prevalence and mortality of 546 and 73 per 100,000 population, respectively (17). Ethiopia conducted a population-based national tuberculosis prevalence survey by the year 2010/2011. According to the Ethiopian Health and Nutrition Research Institute (EHNRI) recent report (2011, unpublished), the prevalence of smear positive and bacteriologically confirmed (smear and culture positive) pulmonary tuberculosis infection in the general population was 108/100,000 and 277/100,000, respectively.

Thirteen sputum samples were reported positive for AFB when the sputum samples were examined without decontamination but negative for AFB when samples were decontaminated with NALC/NaOH. Sodium hydroxide, also known as lye or caustic soda, is a very strong alkali that could be harsh to the survival of the tubercle bacilli during decontamination. However, Soumitesh et al reported a sensitivity and specificity of NALC/NaOH decontamination 80% and 97.7% compared to 68.6% and 92.6%, respectively, for the direct method to detect tubercle bacilli in sputum (18).

16S rDNA/RD750 assay: The 16S rDNA real time PCR assay result of the current study on Mycobacterial DNA extracts obtained directly from sputum was by far different from previous reports conducted on mycobacterial cultures obtained from the *Mycobacterium* Growth Indicator Tube (MGIT). In that experiment, a total of 70 mycobacterial cultures were subjected to 16S rDNA assay, but only two failed to give

Taqman signals (11). The negative results in 16S rDNA /RD750 assay on sputum samples could be explained as failed PCR assays. The PCR failures could be due to the presence of PCR inhibitors in sputum samples. Amicosante et al reported that PCR performance with clinical samples may be limited by the presence of PCR inhibitors, such as hemoglobin in sputum and other samples (up to 20% false negatives) (19). Forbes and Hicks detected interference of PCR in 52% of respiratory specimens. PCR inhibitors in sputum have been a knotty problem in the molecular diagnosis of pulmonary tuberculosis (20). Collectively, we suggest that the 16S rDNA/RD750 real- time PCR assay, although showed an excellent result on mycobacterial isolates from culture previously, may not deliver an accurate result on tubercle bacilli DNA extracted directly from sputum, as there are many PCR inhibitors in the sputum and this needs further study.

The AFB score of 13 sputum samples was negative when the sputum was decontaminated by NALC/ NaOH. Of these 13 samples, 10 showed RD9 positive and 3 RD9 deleted results. Yajko *et al* (21) reported the negative impact of NaOH decontamination on the survival of *Mycobacterium tuberculosis*. The mean (± standard deviation) number of *Mycobacterium tuberculosis* colony forming unit (CFU) recovered on Mitchison's agar medium after NALC/ NaOH treatment was 0.96 ± 0.76 $\log_{10}$ CFU less than the number recovered after treatment with NALC alone, corresponding to an average recovery rate of 11% for NaOH-treated samples.

RD9 deletion typing on mycobacterial DNA extracts from sputum samples: In this study, 81% (94/116) and19% (22/116) of the mycobacterial DNA extracts obtained directly from sputum showed an RD9 intact and an RD9 deleted PCR results, respectively. The result indicates that the dominant mycobacterium causing pulmonary tuberculosis in Gondar was *Mycobacterium tuberculosis*. RD9 deletion analysis discriminates *M. tuberculosis* from other *Mycobacterium tuberculosis* complex (22). The RD9 PCR product of *M. tuberculosis* reaches 396 bp long, but others such as *M. africanum*, *M.microti* and *M. bovis* each produce a PCR product of 575 bp long, making the primer "RD9-internal Rev" redundant in such PCR amplification (23). Successive loss of DNA, reflected by RD9 deletion was identified for an evolutionary lineage represented by *M. africanum*, *M.microti*, and *M. bovis* (24).

Mycobacterium strains causing pulmonary tuberculosis in Gondar: The fourth international spoligotyping database, SPolDB4, describes 1939 shared-types (STs) representative of a total of 39,295 strains from 122 countries which are tentatively classified into 62 clades/lineages using a mixed expert-based and bioinformatical approach (25). In this study, *Mycobacterium tuberculosis* MTb SIT 25 accounted for 30.6% (n=22), MTb SIT 53 for 12.5% (n=9), MTb SIT 149 for 12.5% (n=9). SIT 25 is a linage with spacer deletion at the 4 to 7, 23 to 34, 37 and 38 spacers which is the Central Asian Strain variant 1 (CAS-1 variant). Before this work, Firdessa *et al* (26) reported that the Euro-American and CAS lineages of *Mycobacterium tuberculosis* are predominant in Ethiopia. On the other hand, the SIT 53, SIT 149, and SIT 50 clades are the T1 (supper family T1), T3-Ethiopia, and H3 (Haarlem 3) *Mycobacterium tuberculosis* lineages. The SIT 149 (T3-Ethiopia) was previously shown to be frequent in Ethiopia and Denmark among Ethiopian immigrants (27).

Fifteen of the *Mycobacterium tuberculosis* spoligotypes were new, which means they were not registered in the international tuberculosis database and given a temporary lineage name as the *Mycobacterium tuberculosis* Gondar lineage. The spacer deletion pattern on these *Mycobacterium tuberculosis* spoligotypes varied from each other. Nine of the 15 new spoligotypes showed a spacer deletion at spacer 4 to 7, 2 of the others at spacers 1 to 7 and still 2 others at spacers 3 to 7. On the other hand, 4 of the 15 new spoligotypes and 5 of the others demonstrated spacer deletion of 33 to 36 and 23 to 32, respectively, in addition to other spacer deletions which are different from one spoligotype to another. These spacer deletions are quite different from the Firdessa et al (26) report of the Woldiya lineage. Deletion of spacer 4 to 24 was reported on the *Mycobacterium tuberculosis* isolates of the Woldiya lineage. The Woldiya lineage identified sits between the 'Ancient' and 'Modern' *Mycobacterium tuberculosis* branches.

The identification of new *Mycobacterium tuberculosis* spoligotypes in Gondar and Woldyia strengthens the suggestion documented by Ferdissa et al, which states that unidentified *Mycobacterium tuberculosis* lineage may be circulating in the Amhara National state in particular and in Ethiopia in General which may also hold true to Africa. Therefore, a large scale study is required to determine the exact prevalence of the new *Mycobacterium tuberculosis* spoligotypes. Moreover, we also recommend experimental studies to determine the disease causing ability or virulence of these new *Mycobacterium tuberculosis* spoligotypes.

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