**Original article**

# *Mycobacterium tuberculosis* genotypes associated with drug resistance in Isolates from Sudan

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## Abstract

Tuberculosis (TB) is still one of the deadliest infectious diseases worldwide, it is one of the leading disease causing morbidity and mortality in Sudan. However, little scientific information is available on the drug sensitivity profiles of the strains of Mycobacterium tuberculosis in Sudan. This study was conducted to detect the relation between drug resistance and specific genotyping. A total of 74 clinical isolates of M. tuberculosis from patients with pulmonary TB were used. The phenotypic drug susceptibility test was done for isolates in LJ media contain anti-tuberculosis drugs (Rifampicin, Isoniazid, Streptomycin and Ethambutol), Spoligotyping to detect 43 known spacers in the direct repeat locus was performed with a commercially available kit, according to the instructions supplied by the manufacturer (Isogen Bioscience B.V., Maarsen, Netherlands). Both sensitive and MDR isolates were spoligotyped. Results were compared with the SITVIT Database4 (spolDB4.0 - Institut Pasteur, Guadeloupe). Phenotypically, 12.2% of the 74 tested isolates were drugs sensitive while 87.8% of them were multi-drug resistant (MDR).The spoligotype patterns of (90.5%) of isolates had patterns that matched those in the database while (9.5%) of isolates were defined as orphans. 15 different patterns were identified. The predominant were spoligotype international type (SIT) 54 and SIT25, each consisting of 43 and 7 isolates, respectively. The most of MDR M.tb isolates belong to SIT54 spoligotype pattern (58.8%). In conclusion, the SIT54 genotype was prevalent in this study and results indicate that a higher percentage of drug-resistant isolates are belonging to this genotype.

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## Introduction

The number of people newly diagnosed with TB and reported is dropped globally as impact of COVID19 pandemic, this fell from 7.1 million in 2019 to 5.8 million in 2020, an 18% decline back to the level of 2012 and far short of the approximately 10 million people who developed TB in 2020. Reduced access to TB diagnosis and treatment has resulted in an increase in TB deaths. Best estimates for 2020 are 1.3 million TB deaths among HIV-negative people (up from 1.2 million in 2019) and an additional 214000 among HIV-positive people (up from 209 000 in 2019), with the combined total back to the level of 2017. Other impacts include reductions between 2019 and 2020 in the number of people provided with treatment for drug-resistant TB (-15%, from 177 100 to 150 359, about 1 in 3 of those in need) and TB preventive treatment (21%, from 3.6 million to 2.8 million) (WHO, 2021).

The prevalence of TB infection is probably very different in different regions of Sudan (WHO, 2003). In the Fourth Global Report on anti-tuberculosis drug resistance the MDR rate amongst new TB cases in Sudan is estimated at 1.9% while the rate among previously treated cases is estimated at 9.8% (WHO, 2008). Total number of confirmed MDR TB cases by culture and DST is 90 (Sudan Federal Ministry, 2012). According to WHO report, (2016) TB incidence rate in Sudan was 25-99 new TB cases per 100, 000 population per year, in 2017 the incidence rate was increased to 53-105 new cases per 100, 000 population per year (WHO, 2018a), the incidence rate was 50-99 new TB cases per 100, 000 population per year in 2020 (WHO, 2021).

Drug-resistance in tuberculosis originates from two possible sources; either initial or acquired drug resistance. Initial resistance refers to the spread of already resistant strains giving rise to primary resistance in a patient. Acquired resistance on the other hand originates from the selection for resistance due to inadequate treatment. This can be caused by several of reasons where interrupted drug supply and compliance to treatment are amongst a few. Another Plausible scenario for acquired resistance is treatment with drugs that the strain already are resistant to; this enhances the risk of creating resistance to even more drugs. Inappropriate treatment regimens and poor patient compliance have led to the appearance of multi drug resistant (MDR) and extensively drug resistant (XDR) *M. tuberculosis* strains which make the treatment of TB costly, lengthy and difficult (Singha *et al.,* 2012). By definition, Multidrug resistant *Mycobacterium tuberculosis* (MDR-TB) is resistant to at least isoniazid and rifampin. The treatment of infections caused by MDR-TB is a major concern for TB control programs worldwide because it requires prolonged use of multiple second-line anti-TB drugs which are more expensive and toxic than the first-line drugs. TB death toll is very high although it could be prevented with a timely diagnosis and correct treatments (WHO, 2013). The emergence of drug resistant tuberculosis is a serious threat to global public health security, substantially increasing the burden of global tuberculosis control. In the treatment of drug-sensitive TB, the abuse of anti- TB drugs, irrational treatment program, and inadequate drug administration are the most common reasons that cause MDR-TB (Liang *et al.,* 2012). Therefore, the key to the control of MDR-TB is early diagnosis. Diagnostic methods for MDRTB include phenotypic and molecular diagnostic techniques. Drug susceptibility testing (DST) is the golden standard for MDR-TB diagnosis, and it is also an important basis for the formulation of MDR-TB treatment programs (Kim, 2005). However, the DST method is time-consuming, and the detection rate is low; thus, it is not suitable for early diagnosis and treatment. Microscopic-observation drug susceptibility (MODS) testing shortens the time of tuberculosis culture to 1-2 weeks (Moore *et al.,* 2004); however, false positives are likely. Results using the fluorophage method are rapidly obtained and matched 100% with the results of DST of first-line anti-TB drugs but the technique is expensive (Jain *et al.,* 2012). The detection of second-line anti-TB drugs requires further investigation. The polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method only detects drug resistance-related gene mutations (Ahmad *et al.,* 2007; Pulimood *et al.,* 2008), yet mutation of the targeted gene does not fully explain all mechanisms of MDRTB (Pule *et al.,* 2016).

*Mycobacterium tuberculosis* Genotyping and determination of phylogenetic lineages are becoming important for Tuberculosis (TB) outbreaks surveillance, transmission patterns interpreting, and infection control plans (Kato-Maeda *et al.,* 2011). Spoligotyping (Spacer oligonucleotide typing) was the second most widely used method for *M.tb* complex genotype after IS6110-based fingerprinting and gained increased international acceptance as a rapid, first line and discriminatory test (Brudey *et al.,* 2006). It depend on polymerase chain reaction (PCR) amplification of a single direct repeat (DR) locus which consists of alternating identical DRs and variable spacers harboring 36 base pairs (bp) direct repeats interspersed with unique 34–41 bp spacer sequences.

The entire DR locus is amplified by PCR, using two inversely oriented primers complementary to the sequence of short DRs. The PCR products are hybridized to a membrane containing 43 oligonucleotides corresponding to the spacers from MTB H37Rv and *M. bovis* BCG. The presence or absence of each of those 43 spacers in the DR region of the analyzed isolate will be represented as the pattern of positive or negative hybridization signals (Kamerbeek *et al.,* 1997). The hybridization signals are detected by chemiluminescence through biotin labeling of the PCR products (one of the primers is biotinylated) and a streptavidin-peroxidase conjugate system that could be visualized by either autoradiography or using the Luminex® technology. In this latter, the synthetic spacer oligonucleotide probes are immobilized on microspheres by means of covalent coupling. The detection is then achieved via fluorochromes attached to the beads and to the hybridized PCR product. The Luminex® platform provides greater robustness and reproducibility since it eliminates the membrane steps with its subjective visual data interpretation, replaced by mathematical cut-offs (Zhang *et al.,* 2010). International databases, such as the World Spoligotyping Database, SpolDB4.0 and SITVIT2, have shown the clonal structure of *M.tb* isolates in different geographical settings. The SITVIT2 updated database includes classification for spoligotypes and description of the genetic families of MTB for 111,635 clinical isolates from 169 countries of patient origin (131 countries of isolation, representing 1032 cities); these isolates contain more than 7,100 patterns of spoligotyping that are grouped into 3882 SIT (Shared International Type) codes (Couvin *et al.,* 2018). The evolution of direct repeat (DR), renamed CRISPR (Clustered-Regularly-Interspersed-Short-Palindromic-Repeats) has enabled the analysis of *M.tb* population structure, and the classification of *M.tb* in seven lineages based on Whole-Genome-Sequencing (WGS) was shown to be compatible to spoligotyping-based classification in most cases (Comas and Gagneux, 2009). The WGS-based approach revealed major lineages, designated as L1 to L7 (Coll *et al.,* 2014). Some lineages are made-up of many sublineages; for L4, some known sublineages are found in Africa, e.g. Uganda (4.6.1), Cameroon (4.6.2), South Africa (4.4), in Asia (L2/Beijing and L3/Central Asia), in America and Europe: Latin American-Mediterranean/LAM (4.3), X (4.1.1), Ghana (4.1) and Haarlem (4.1.). L1 is found in South-East Asia and East Africa. The L1-L4 lineages affect humans, whereas other lineages (L5-L6) such as the *M. africanum*, *M. bovis*, *M. caprae and M. microti* families primarily affect animals (Wirth *et al.,* 2008).

There are little data about the distribution of *M.tb* strains in Sudan, Manu2/SIT54, CAS1 Delhi/SIT25, CAS1 Kili/SIT21, H3/SIT50, TI/SIT53, CAS/SIT142, H3/SIT316 patterns were identified by Sharaf eldein *et al.,* (2011) and SIT (29/U, 40/T4, 240/U (like LAM), 289/CAS1 Delhi, 294/H3, 1690/H3, 1787/CAS1 Delhi, 1634/Manu2), were identified by Eldirdery *et al.,* (2015), there was no data about the association between the *M.tb* strains and drug resistance, therefore, more studies are needed to give a clear picture about the distribution of *M.tb* strains and to help in starting the base of strain library. **The aim of the study** is to detect the different genotypes of the *M. tb* isolates using spoligotyping technique and their association with the drug resistance PTB.

**Materials and methods:**

**The study population**:

The study population was TB patients receiving treatment at Omdurman Educational Hospital for chest diseases (Abu anja). The study was conducted with the authorization from the Human Ethical Committee of Tropical Medicine Research Institute (TMRI). Written informed consent was obtained from all participants. For each of the PTB patients included, the consent form and questionnaire were filled, followed by sputum sampling National Tuberculosis Reference Laboratory, National Public Health Laboratory; Khartoum, Sudan.

**Sample collection and examinations:**

Sputum samples were collected from 100 PTB patients in the

National Tuberculosis Reference Laboratory and consecutively screened for acid fast bacilli (AFB) using Ziehl-Neelsen (ZN). Sputum specimens submitted for culture and DST was processed using 4% sodium hydroxide (NaOH) decontamination because the specimens susceptible to contamination by more rapidly growing normal flora, these would rapidly overgrow the entire surface of the medium and digest it before the tubercle bacilli start to grow, so the specimens was being subjected to digestion and decontamination procedures that liquefies the organic debris and eliminates the unwanted flora. Sputum sample was unloaded in falcon tube 15 ml, equal volume of 4% sodium hydroxide was added and then mixed well and left at room temperature for 20 minute, then was mixed three times after each 5 min for homogenization. Then 15ml of distilled water was added and centrifuged at 3000xg for 15 minute. The supernatant was discarded and also 13ml of distilled water was added and centrifuged again for five minutes. The supernatant was poured off and the sediment was inoculated in the LJ media, (WHO, 1998). Two or three drops of the decontaminated sample were inoculated in the LJ medium and were incubated at 37C°, the bottles were tightening loosely for 24 hours to allow evaporation and then well tighten. Contamination was examined for 72 hours and then culture was examined weekly for the growth of *Mycobacterium tuberculosis* for eight weeks. The contaminated bottles were discharged (WHO, 1998).

Drug sensitivity test was done for isolates in LJ media containing drugs; Rifampicin, Isoniazid, Streptomycin and Ethambutol with the different concentrations (0.2µg/ml for INH, 40 µg/ml for RIF, 4 µg/ml for SM and 2.0 µg/ml for ETH) for drug susceptibility test of *Mycobacterium tuberculosis* strains. Plain LJ medium was used as control (WHO, 2018b). The strain was considered as resistant if the ratio is greater or equal to 1% (Sethi *et al*., 2004). The growth was preserved in Glycerol.

**Spoligotyping of *Mycobacterium tuberculosis*:**

Spoligotyping was used to analyze a variation in the DR locus (i.e., the absence or presence of 43 different spacers) as described by (kamerbeek *et al* 1997); oligonucleotides derived from the known spacers in the DR cluster are covalently linked to an activated membrane in parallel lines. PCR products are hybridized perpendicular to the oligo lines. After hybridization the membrane is incubated in streptavidin peroxidase, which binds to the biotin label on the PCR products. Detection of hybridization signals is optimized by the enhanced chemiluminescence (ECL) detection system. The peroxidase present on the streptavidine catalyzes a reaction resulting in the emission of light which can be detected by autoradiography of the membrane.

**DNA Extraction:**

An automated extraction method using the GenoXtract instrument (Hain Lifescience, Germany) in combination with the GXT DNA/RNA extraction kit (Hain Lifescience, Germany) were used to extract the Deoxy-ribonucleic acid (DNA) from cultivated samples preserved in distilled water. The automated DNA/RNA extraction device GenoXtract is based on magnetic beads and handles up to 12 samples in parallel using prefilled cartridges. DNA extraction with GenoXtract and the GTX DNA/RNA extraction kits were performed according to their instructions for use. Briefly, after the loading of the GenoXtract instrument with the prefilled cartridges and disposable pump units, using graduate disposable transfer pipette, samples in distilled water was mixed by pipetting up and down, 700µl of the sample were transferred to 2ml screw cap tube and entered to GenoXtract instrument for DNA extraction, and then the eluate was directly used for PCR.

**Amplification of DNA by PCR:**

PCR reaction was carried for 25 µL volume include: 5 µL of DNA, 2 µL of each primer (DRa: 5'-GGT TTT GGG TCT GAC GAC-3', DRb: 5'-CCG AGA GGG GAC GGA AAC-3'), 12.5 µL of Q master mix and 3.5 µL of $QH_2O$. The tubes were placed in a PCR-apparatus for amplification. Denaturation was carried out at $96^0$C for 15 min, followed by 30 cycles of $96^0$C for 1 min, $55^0$C for 1min, $72^0$C for 30 sec,

and 72°C for 10 min and soaked in $4^0$C. (kamerbeek *et al.*, 1997).

15 min 96°C
1 min 96°C
1 min 55°C } 30x
30 sec 72°C
10 min 72°C

**Hybridization:**

Hybridization of the biotin-labeled PCR products to the immobilized spacer-oligos that represent spacers of known sequence. The presence of spacers is visualized on film as black squares after incubation with streptavidin-peroxidase and ECL-detection. Briefly 20 µL of the amplified PCR product was diluted in 150 µl of 2X SSPE–0.1% SDS and denatured by heat in 99°C for 10 miutes and cooled in ice immediately. The membrane and a support cushion were placed into the miniblotter in such a way that the slots are perpendicular to the line pattern of the applied oligonucleotides then the residual fluid from the slots of the miniblotter was removed by aspiration. The diluted samples were pipetted into the parallel channels in the miniblotter apparatus. Hybridization was done for one hour at $60^0$C. After hybridization, the membrane was washed twice in 250 ml of 2X SSPE/0.5% SDS for 10 min each time at $60^0$C and then incubated in 1:14000-diluted streptavidin-peroxidase conjugate for 60 min at $42^0$C. The membrane was washed twice for 10 min each time in 250 ml of 2X SSPE/0.5% SDS at $42^0$C and rinsed with 250 ml of 2X SSPE for 5 min at room temperature. Detection of hybridizing DNA was done by using chemiluminescent ECL (Amersham) detection liquid, followed by exposure to X-ray film (Hyperfilm ECL; Amersham) in accordance with the instructions of the manufacturer. For repeated use of membranes, the membranes were stripped by being washed two times for 30 min each time in 1% SDS at $80^0$C and then incubated for 15 min in 20 mM EDTA (pH 8) at room temperature. Membranes were sealed in plastic and stored at $4^0$C until further use. The spoligotype patterns were entered in an Excel spreadsheet and compared to the international database (SpoIDB4) and SITVIT2 (Institut Pasteur de Guadeloupe) to determine the specific MTB complex strain (Figure 1). Spoligotyping was done in (AHRI) institute in Ethiopia country.

**Results:**

**Spoligotyping of *Mycobacterium tuberculosis* isolates:**

DNA from 74 *M. tuberculosis* isolates was extracted and genotyped by spoligotyping. Spoligotyping to detect 43 known spacers in the direct repeat locus was performed with a commercially available kit, according to the instructions supplied by the manufacturer (Isogen Bioscience B.V., Maarsen, Netherlands). Both sensitive and MDR isolates were spoligotyped. Results were double checked visually by an experienced operator to eliminate any systematic artifact caused by using commercial membranes. Results were compared with the SITVIT Database 4 (spolDB4.0 - Institut Pasteur, Guadeloupe).

**Table1: Frequency and percentage of spoligotyping patterns of *M.tb isolates***

| NO | Spoligotype cluster | Frequency | |
|---|---|---|---|
| | | MDR | Sensitive |
| 1 | Manu2/SIT54 | 38 | 5 |
| 2 | CAS1 Delhi /SIT25 | 7 | 0 |
| 3 | H3/ SIT50 | 1 | 1 |
| 4 | CAS1 Kili / SIT21 | 1 | 0 |
| 5 | H3/SIT53 | 1 | 0 |
| 6 | T4/SIT40 | 1 | 0 |
| 7 | U /SIT29 | 1 | 0 |
| 8 | CAS /SIT142 | 1 | 0 |
| 9 | U(LikeLAM)/SIT240 | 1 | 0 |
| 10 | T1/SIT294 | 1 | 0 |
| 11 | CAS1 Delhi /SIT289 | 0 | 1 |
| 12 | H3/SIT316 | 0 | 1 |
| 13 | Manu2/SIT1634 | 1 | 0 |
| 14 | H3/SIT1690 | 4 | 0 |
| 15 | CAS1Delhi /SIT1787 | 1 | 0 |
| 16 | Orphan | 6 | 1 |
| Total | | 65 | 9 |

In this study the spoligotyping of 74 mycobacterial isolates produced a total of 15 different spoligotype patterns, (90.5%) of isolates had patterns that matched those in the database while (9.5%) of isolates were represented by a unique pattern. Spoligotypes that did not match any existing pattern in the database were defined as orphans. The most commonly occurring clustered spoligotypes were SIT 54 and SIT25 consisting of 43 (58.1%) and 7(9.5%) isolates, respectively. The most of MDR *M.tb* isolates belong to Manu2/SIT54 spoligotype pattern; out of 65 MDR isolate 38(58.5%) were found belong to this pattern and most of orphan isolate also classified in this pattern, 6 (9.2%) (**Table 1).**

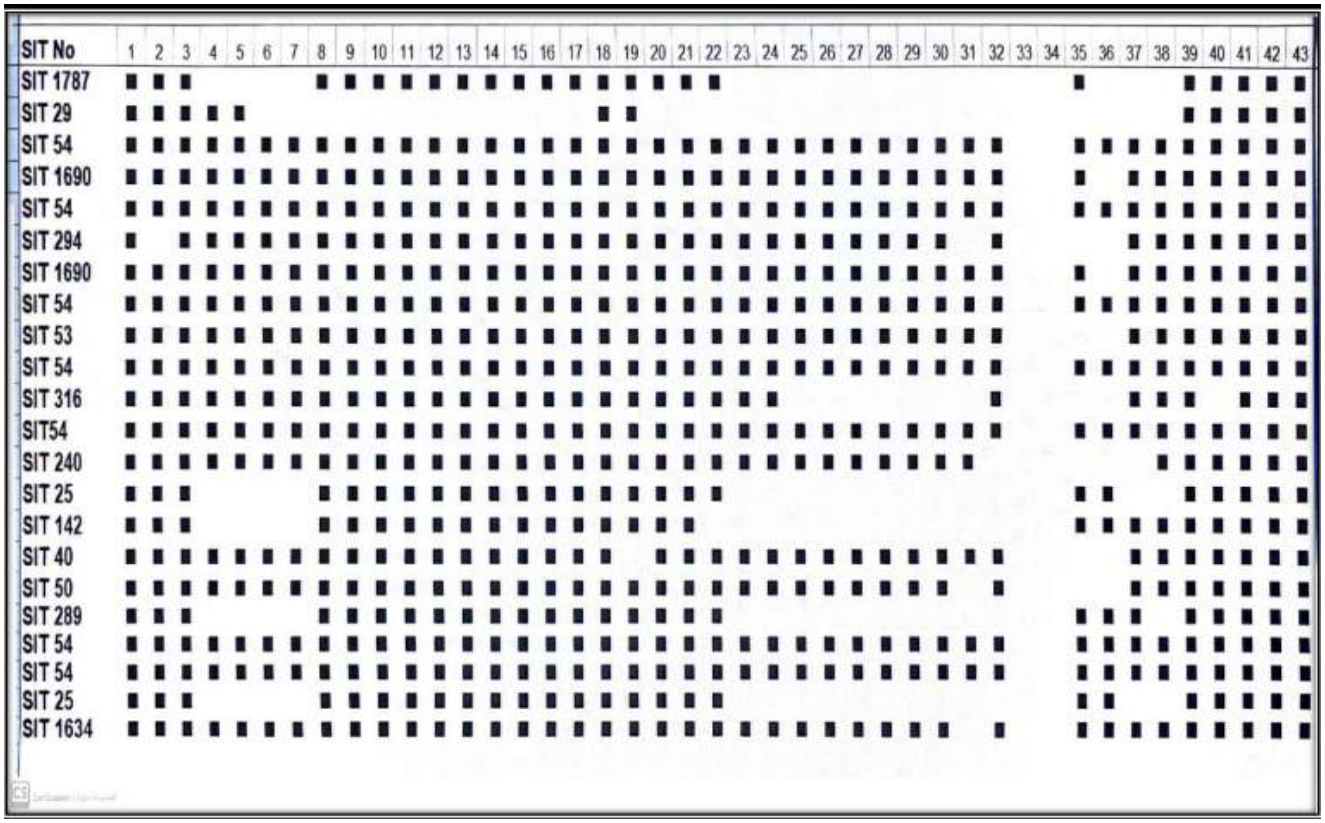


**Figure1: *M.tb* clusters detected by Spoligotyping, a black box represent the presence of a specific spacer at that location.**

Cluster analysis revealed that Manu2 clade is the dominant clade includes SIT54, SIT1634 and SIT1690, followed by Casi Dalhi clade and it include SIT25, SIT289 and SIT1787, comparing to previous study was done in Sudan; this finding is similar to study was done by Elderdirey *et al*., 2015 which found that the MANU2\SIT54 is the most common gynotyping clade but differ from what was recorded by Sharafeldien *et al.,* (2011) which found that Casi Dalhi was dominant spoligotypes pattern, this difference may be due to the time difference between the studies where in this period the tuberculosis was spread and we expect that existing strains spread more broadly also the predominance of Manu2 spoligtypes rather than other genotypes in this study suggest that it has been present in the study area for a sometime. The orphan MTB genotypes detected in the study could be attributed to the use of spoligotyping but the coupling spoligotyping to other techniques, such as Variable Number of Tandem Repeat (VNTR) analysis and WGS is needed to

improve taxonomical assignments and possibly to reveal anti-tuberculosis drug resistance profile.

**Conclusion**:

The MANU2\SIT54 genotype was prevalent in this study population, and results indicate that a higher percentage of drug-resistant isolates are belonging to this genotype.

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