Abstract

Background: The aim of this study was to characterize the drug resistance profile, and the specific lineages of Mycobacterium tuberculosis (MTB) strains isolated from patients with pulmonary TB in the state of Khartoum in Sudan. Methods: Consecutive sputum samples and clinical data were collected from 406 smear-positive TB patients with pulmonary TB in 2007–2009. The samples were cultured, and drug susceptibility testing (DST) was performed using the proportion method (PM) on solid Löwenstein–Jensen medium, and species were identified using biochemical methods at the National Reference Laboratory (NRL) in Khartoum. Extracted deoxyribonucleic acid from a total of 120, 60 suspected multidrug-resistant isolates (MDR), and 60 non-MDR isolates were subsequently sent to the WHO supranational reference laboratory (SRL) in Stockholm at the Public Health Agency of Sweden, for confirmation of the drug resistance profile, examinations by line probe assay (LPA), and molecular epidemiology analysis with Spoligotyping. Results: LPA results correlated 100% for non-MDR and 62% for the suspected MDR strains when compared to the DST results obtained by PM at the NRL. Two strains were initially using the PM identified as MDR-TB but later shown by Hain GenoType Mycobacterium CM/AS to belong Mycobacterium avium complex (Mycobacterium intracellulare). These two strains were excluded from the study material for further analysis. The remaining 58 MDR strains were analyzed using LPA, and 36 strains were confirmed as MDR, 10 as rifampicin monoresistant, and eight as isoniazid-monoresistant. Spoligotyping for all the 118 MTB isolates revealed a total of 115 patterns in which four patterns represented major clusters with a total of 108 (91%) of the strains. The CAS1_Delhi/family was the predominant type and detected in 62 isolates (52%), of which 26 were MDR and 36 were susceptible. It was followed by H3/family with 19 (16%) strains, and 11 Latin American Mediterranean3/family, 16 T2/T1, and two strains each of the Beijing and S lineage. Conclusion: Comparison of DST results obtained using PM and LPA showed 100% agreement for the non-MDR strains but only 62% for the MDR strains. Taking in consideration the time, risk of contamination and the cost of labour to identify MDR TB, the LPA have clear advantages in early detection of MDRTB than the PM. Additionally in this study material Spoligotyping revealed the CAS1 Delhi as the most predominant family. We could not see no major difference in lineages between MDR and non-MDR strains.

Keywords: Genotype lineages, line probe assay, pulmonary tuberculosis, spoligotyping, Sudan

INTRODUCTION

The Stockholm WHO Supranational Reference Laboratory (SRL) for tuberculosis (TB) at the Public Health Agency of Sweden is one of the 31 WHO-SRLs located in different regions globally. The reference laboratory in Stockholm provides technical and external quality assurance (EQA) support to ten national reference laboratory (NRL) located in WHO-EURO and Eastern Mediterranean region (EMR). Similar support is also offered to the five clinical Swedish TB laboratories.

The Sudanese NRL is one of the laboratories in the Stockholm SRL network, and the SRL provides training and technical support to the Sudanese NRL including yearly DST proficiency testing (PT) of for EQA purposes.

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At the time of this study, the NRL, located in the capital city of Khartoum, was the only laboratory in the country with a population over 40 million, with the capacity to perform DST. In recent years, a significant reduction in TB-related mortality and a decline in incidence and prevalence in all forms of TB have been observed.\(^1,2\)

HIV positivity rate among TB patients in Sudan is highest (7.7%) in the EMR, necessitating an expanded HIV testing in all the TB Management unit (TBMUs). In 2016, only 3659 (17%) of all notified TB cases were screened for HIV. Almost all 279 (99%) patients who were also HIV positive started antiretroviral therapy.

Today, the NRL has a molecular laboratory and in addition to routinely performed DST with proportion method on solid media (PM) also examine drug resistance with line probe assay (LPA) and GeneXpert. To upgrade the capacity to control TB countrywide, the national TB laboratory network comprises four regional TB culture laboratories equipped with GeneXpert and in addition 13 states laboratories with GeneXpert.

**Methods**

A total of 406 consecutive sputum samples were collected from smear-positive TB patients and cultured on Löwenstein–Jensen (LJ) medium in 2007–2009 at the Sudanese NRL in Khartoum. Biochemical species identification of the strains was done using nitrate and catalase tests.

DST of isoniazid (INH) (0.2 µg/ml), rifampicin (RIF) (40 µg/ml), streptomycin (SM) (4 µg/ml), and ethambutol (EMB) (2 µg/ml) was performed at the NRL with the PM on LJ media.\(^2,3\)

Deoxyribonucleic acid (DNA) from 60 suspected MDR (two later found to be NTM and excluded) and 60 non-MDR TB strains were selected for EQA and molecular epidemiology purpose, coded and shipped to SRL Stockholm.

Confirmation of the DST results and further characterization of the drug resistance profile were done at the SRL for the 58 MDR and the 60 susceptible strains using Genotype MTB DR\(^+\) assay (Hain Lifesciences, Nehren, Germany), according to the manufacturer’s recommendations.\(^4\)

Molecular characterization to identify clusters was performed with spoligotyping as described by Kamerbeek et al.\(^5\) The whole direct repeat region of TB genome was amplified with Dra and Drb primers. The amplified products were then hybridized to a set of 43 oligonucleotides, and the hybridized polymerase chain reaction products were detected using the enhanced chemiluminescence (Amersham) visualized by a charge-coupled device-camera (MF-ChemiBis 3.2) after incubation with streptavidin–peroxidase and detected using the enhanced chemiluminescence system.\(^5\)

Spoligotypes in binary and octal format were entered into an Excel spreadsheet and compared to an updated Spoligotypes International Type VNTR International Types (SITVIT) Database of the Pasteur Institute of Guadeloupe.\(^6,7\) The octal codes found for each strain were entered into the SITVIT2 database, which is the updated version of the previously released spoligotyping database. In this database, SIT designates spoligotypes shared by two or more isolates included in the database. The SITVITWEB incorporates multimarker data which gives a global vision of MTB complex genetic diversity. The database contains clinical isolates from >62,000 patients coming from 105 different countries. Major phylogenetic clades were assigned according to the signature provided in the database, which is defined in 62 genetic lineages/sublineages. The SITVITS contains around 3000 SITs with global genotyping information on about 54,000 clinical isolates from >153 countries of origin.\(^6,7\)

The patterns obtained were analyzed using visual examination and by sorting the results in Bionumerics software version 5.1 (applied Keistraat, Belgium).\(^8\) A spoligotype cluster was defined as two or more strains sharing identical patterns. If no matching spoligotypes were identified in the database, the isolate was defined as orphan or unique. Finally, a dendrogram was constructed using the Unweighted Pair Group Method with Arithmetic Mean and Jaccard’s distance method.

**Results**

**Drug susceptibility testing and external quality assurance results**

In total, 406 samples were cultured from patients with pulmonary TB and subjected to DST using PM at the NRL. The results show that 41.4% (123/297) of the strains isolated from patients with no previous history of TB exhibited resistance to at least one anti-TB drug. In the earlier-treated patients, this number was 60.6% (66/109). The initially estimated rate of MDR was 14.8% (60/406), after exclusion of the two misclassified *Mycobacterium intracellulare* strains, it was corrected to 58/404 (14.4%), and 28/297 (9.4%) in the newly diagnosed, and 30/109 (27.5%) in the patients with a relapse or treatment failure.

The GenoType MDR\(^+\) LPA assay was used on the 118-coded DNA extracts from the 404 MTB isolates. DST results by GenoType MTBDR\(^+\) assay and PM were compared head to head in all 118 MTB isolates.

GenoType MTBDR\(^+\) identified 64 samples as susceptible to both RIF and INH, 36 samples as MDR, 10 as RIF monoresistant, and eight as INH monoresistant. Among the RIF monoresistant isolates, we found two strains with missing bands, one with wide type (wt) 5 and 6, the other with wt 6 and 7 missing. This results according to the manufacturers can be mutations in codon 531 or 533 and could be a mutation in Leu 533 pro, a mutation that according to the literature can give INH resistance on LJ but not in MGIT liquid culture DST. The results are summarized in Table 1.
**Spoligotyping results**

Spoligotyping was performed on the 118 TB strains to examine lineages and clustering and showed 112 different patterns [Figure 1 and Table 2]. The Central-Asian 1 (CAS1) Delhi was the most predominant, detected in 49 isolates (43%), 20 of which were MDR, and 28 susceptible [Table 2 and Figure 2].

The second most common type was H3 (16 [20%]), followed by Latin American Mediterranean3 (LAM3) (four strains), LAM10_CAM (three strains), T2/T1 (eight strains), and Beijing type (two strains) as shown in Table 2.

**DISCUSSION**

Two strains previously misclassified as MDR-TB at the NRL were shown to be *M. intracellulare*. Illustrating the risk of overestimating the MDR-TB problem in a setting where reliable species identification tools are missing. In addition, a number of isolates initially classified as MDR by phenotypic DST were shown by LPA to be RIF or INH monoresistant isolates. If these initial results are trusted, it means that several patients who can be treated by RIF or INH would have for them suboptimal therapy with second-line drugs. With the molecular tests used at the NRL in Sudan, these mistakes would not have happened, which illustrate that molecular tests not only offer a more rapid detection of MDR-TB but also a more specific.

Since global reports indicate that MDR and extensively drug-resistant TB are on the rise, early identification of resistant strains, and prompt initiation of correct treatment have a great impact in the control of TB.[9] Today, we have molecular tools which can be easily integrated in TB microscopy centers located in remote areas. Strengthening the microscopy centers and having a strong central NRL, which can give training and support, can improve early detection of TB patients. These new rapid molecular tools in combination with the WHO recommended algorithms can strengthen the laboratory capacity in detecting resistant strains faster. Indirectly, this will improve the cure rate of TB patients and limit transmission of resistant strains.[10] Several studies have shown that molecular techniques, such as GenoType MTBDRplus, have clear advantages compared to using the time-consuming and laborious technique such as the PM.[11,12]

In this study, we used spoligotyping to identify the most dominant clones of MTB circulating in Sudan. Since half of the strains were MDR and half of them susceptible, we aimed to have an insight of the ongoing transmission among MDR and non-MDR TB cases [Figures 2-4].

A spoligotype study was done in Sudan in 2002, reported a dominance of a single genotype where more than half of the strains studied (29/49) shared the same spoligotype.[13] Another study done by the same author in 2011 found 35% belonged to the spoligotype CAS1_Delhi/family and 14% belonged to the same lineage but with different SIT numbers.[14] Using spoligotyping on TB isolates from the Sudanese patients show that highly diverse clades are circulating in the country. The CAS1_Delhi/family was the predominant shared type detected in 48 isolates followed by H3/family with 16 isolates, and four isolates with LAM3/family, eight isolates with T2/T1/family type, and two isolates each with Beijing type. We found 36 of the isolates were not available in the SpolDB database and were defined as orphans. In previously published paper from Saudi Arabia and Ethiopia, the CAS1_Delhi/family was found as the major family circulating in the region.[15]

Visually inspecting the spoligo patterns of Figure 3 (MDR) and Figure 4 (non-MDR) suggest that there are significant similarities within each group. This can indirectly indicate that there might be some active transmission within both groups. There is a need to further explore transmission rates among MDR and non-MDR patients by more in-depth studies.

The main goal of this study was to compare the phenotypic results obtained at the NRL and with that of molecular techniques. In addition, spoligotyping was used to see which patterns circulated in Sudan and to investigate possible differences between the MDR and non-MDR groups and increase the knowledge of ongoing transmission in these two groups.

Since the study material was on limited number of patients, we cannot give a concrete picture of the transmission rate. To give a clearer idea about transmission rates and which clones are dominant therefore a broader study material is needed. Such study designs need to have information of all TB

### Table 1: Drug susceptibility testing and line probe assay results for 118 the mycobacterial isolates

| DST result          | LJ PM | MTBDRplus |
|---------------------|-------|-----------|
| Susceptible         | 60    | 64        |
| RIF - monoresistant | 0     | 10        |
| INH - monoresistant | 0     | 8         |
| NTM                 | 0     | 2         |
| MDR                 | 60    | 36        |
| Total               | 120   | 120       |

RIF: Rifampicin, DST: Drug susceptibility testing, LJ PM: Löwenstein–Jensen proportion method, NTM: Nontuberculosis mycobacteria, MDR: Multidrug-resistant, INH: Isoniazid

### Table 2: Lineages of multidrug-resistant and susceptible tuberculosis isolates from Sudan

| Lineage     | MDR strains | Susceptible strains |
|-------------|-------------|---------------------|
| CAS1 Delhi  | 20          | 28                  |
| H3          | 8           | 8                   |
| LAM 3       | 1           | 3                   |
| LAM10_CAM   | 2           | 1                   |
| T2/T1       | 6           | 2                   |
| Beijing     | 1           | 1                   |
| Orphans     | 20          | 17                  |
| Total       | 58          | 60                  |

MDR: Multidrug resistant
Figure 1: Tree of spoligotype patterns Mycobacterium tuberculosis isolates ($n = 118$ profiles)
cases detected including names of frequent contacts, sites of residence, healthcare, work and social activities. In this study material, we found only two Beijing strains, one MDR, and the other non-MDR. This explains that the Beijing type is not yet, so widely spread in the country.

**Conclusion**

Using spoligotyping on TB isolates from the Sudanese patients show that highly diverse clades are circulating in the country. The CAS1_Delhi/family was the predominant shared type detected in 48 isolates followed by H3/family with 16 isolates, and four isolates with LAM3/family, eight isolates with T2/T1/family type, and two isolates each with Beijing type. We found 36 of the isolates were not available in the SpolDB database and were defined as orphans [Figure 4]. In previously published paper from Saudi Arabia and Ethiopia, the CAS1_Delhi/family was found as the major family circulating in the region.[15]

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**Conflicts of interest**

There are no conflicts of interest.

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