Drug Resistance in *Mycobacterium tuberculosis* Isolates from Northeastern Sudan

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Authors’ contributions

This work was carried out in collaboration with all authors. SOH designed the study and wrote the first draft of the manuscript. MTM supervised the study and corrected the final draft. HME, AMSE and NSS participated in editing of the manuscript, data search and analysis. All authors have read and approved the final manuscript.

ABSTRACT

The present study intended to determine the drug resistance patterns of *Mycobacterium tuberculosis* isolates among human new tuberculosis cases from northeastern Sudan using conventional and molecular techniques. Of 100 mycobacterial isolates tested by proportion method, 6% and 2% were identified, respectively, as multi drug resistant-tuberculosis (MDR-TB) and non-multi drug resistant-rifampicin (non-MDR-RIF). A total of 8% was found resistant to rifampicin (RIF), 13% to isoniazid (INH), 34% to streptomycin (STM) and 12% to ethambutol (EMB). Mono-resistant were 0% for RIF, 1% INH, 18% STM and 3% ETH. The remaining 61% isolates were identified as sensitive. Resistance to pyrazinamide was evaluated conventionally for the first time in this country and was found as low as 1%.

Molecularly, mutations of MDR-TB, RIF, INH, EMB and STM resistant isolates were detected in 66.7%, 87.5%, 76.9%, 66.7% and 17.6% of corresponding conventional findings, respectively. The genetic mutations were related to the following codons: *rpoB* 531 (71.4%) and *rpoB* 526 (28.6%) for RIF, *katG* 315 (90%) and *mabA-inhA* 15 (10%) for INH, *embB* 306 (66.7%) for EMB and *rpsL* (17.6%) for STM.

The study showed that drug resistant tuberculosis increased steadily and provided...
potentially valuable information on resistant genes circulating in the community. The rapid solving of this problem can benefit both public health and patient management.

Keywords: Drug; resistance; Mycobacterium; tuberculosis; susceptibility; Sudan.

1. INTRODUCTION

Tuberculosis (TB) and multidrug resistant tuberculosis (MDR-TB) are still a major public health problem worldwide (Ormerod, 2005; WHO, 2007). In 2005, million new TB cases were estimated globally, of which 7.4 million were in Asia and sub-Saharan Africa (WHO, 2007). A point of great concern is that the human immunodeficiency virus exacerbated the burden of MDR-TB and threatened prevention and treatment of TB (Barnes et al., 2002; WHO, 2006).

The two major drugs used to treat tuberculosis are isoniazid (INH) and rifampicin (RIF). The other first-line drugs are pyrazinamide (PZA), ethambutol (EMB) and streptomycin (STM). The second line-drugs are more toxic or less effective, and they should be used in therapy only under extenuating circumstances because they result in treatment failure and multiple drug resistance. Second-line drugs include kanamycin, capreomycin, ethionamide, cycloserine, ofloxacin and ciprofloxacin. The WHO recommended standard treatment for new tuberculosis cases includes, in the initial phase, INH, RIF, PZA, and STM or EMB.

Mutations in genomic regions of Mycobacterium tuberculosis are involved in the occurrence of resistance to various anti-tuberculosis drugs. According to Ramaswamy and Musser (1998), the most common mutations of RIF resistant strains occur at codons ser531 and His526 in the central region of the RNA polymerase β-subunit rpoB gene, while INH-resistant organisms have mutation in the katG, inhA, kasA, and ahpC and ndh genes. Resistance in STM is mainly due to mutation in the rrs and rpsL genes encoding ribosomal protein S12. Approximately 70% of EMB-resistant strains have mutation due to amino acid replacement at position 306 of an arabinosyl transferase encoded by the embB gene. Resistance to PZA in the great majority of organisms is caused by mutation in the gene pncA encoding pyrazinamidase that result in diminished enzyme activity.

Sudan was found ranked the second in relation to the incidence of TB in the East Mediterranean region and accounted for 14.6% of total TB burden (WHO, 2007). Information on drug sensitivity tests of M. tuberculosis in the Sudan was scarce and limited to the central part of the country. The documented data on the resistance of M. tuberculosis isolates to anti-tuberculous drugs in the 1960s by Cavanagh (1965) and Grande (1967) were not followed by continuous surveys until Ali (2002) and Sharaf-Eldin et al. (2002) carried out studies in the early twenty-first century and both reported approximately 2% for MDR-TB among new cases. However, more recently, Sudan had begun a nationwide survey on resistance to anti-tuberculous drugs (WHO, 2008). The reliable prevalence studies and surveillance of resistant TB in Sudan is strongly recommended to evaluate the true extent of occurrence of MDR-TB. The present study aimed to determine the drug resistance patterns and genetic mutations causing resistance of M. tuberculosis strains among human new TB cases from northeastern Sudan.
2. MATERIALS AND METHODS

2.1 Mycobacterial Isolates

A total of one hundred sputa were collected from newly untreated and epidemiologically unrelated patients admitted to Port Sudan General Hospital, Red Sea state, implementing DOTS strategy from March 2006 to March 2007. All patients were living in Port Sudan city, most of them were male (68%), adults (≥18 years), belonging to 25 different Sudanese tribes, HIV/AIDS-negative and exhibiting clinical symptoms suggestive of TB. Acid fast bacilli from all patients were detected on smears stained with Ziehl-Neelsen stain and were recovered successfully upon culture on Löwenstein-Jensen (LJ) medium. The isolates were identified phenotypically as *M. tuberculosis* and confirmed molecularly as described previously (Grange et al., 1996; Kim et al., 2004; Bakshi et al., 2005, 2007).

2.2 Drug Sensitivity Tests

Drug sensitivity tests were performed using the proportion method as gold standard (WHO, 1998). Slopes of the LJ medium containing RIF (40 µg/ml), INH (0.2 µg/ml), STM (8 µg/ml), and EMB (2 µg/ml) were prepared each separately. Reference strain of *M. tuberculosis* H₃₇ Rv and well characterized local laboratory resistant isolates for each drug were included as controls. The same procedure described for the detection of pyrazinamidase activity (Grange et al., 1996) was used to determine susceptibility to pyrazinamide (PZA), since resistant strains are pyrazinamidase negative (McClatchy et al., 1981).

2.3 Molecular Analysis of Drugs Resistance

Each mycobacterial isolate, in addition to the sensitive and resistant control strains, was inactivated at 100°C for five minutes (Melchior and Drugeon, 1999). DNA was extracted and purified using AccuPrep® Genomic DNA Extraction Kit (Bioneer corporation, South Korea, Cat No.: k-3032). Molecular detection of drug resistance was performed by multiplex allele specific polymerase chain reaction (MAS-PCR) assay as described previously (Yang et al., 2005). Genetic mutation for STM was detected separately as described formerly (Jordaan and Victor, 1999) due to restriction enzyme digestion requirement. The most frequently found mutation; a substitution in codon 43 of the gene *rpsL*, in STM resistant *M. tuberculosis* was amplified firstly. The mutation was detected by restriction fragment length polymorphism. Briefly, ten microliters of each PCR product was added to 1 µl (2 units) of restriction endonuclease Mbo II (vivantis Product No. RE1290) and 2 µl of 10X enzyme buffer. Nuclease free sterile double distilled water was added to a final volume of 20 µl. The mixture was incubated at 37°C for 2 hours and inactivated at 72°C for 10 minutes. The digested products were run on 1.5% agarose gel and visualized after staining with ethidium bromide. Digestion of PCR products was indicative of STM sensitive, whereas STM resistance remained undigested. Primers used for identification of *M. tuberculosis* and detection of drugs resistance, in this study, were given in Table 1 and 2.
Table 1. Primers used for identification of *Mycobacterium tuberculosis*

| Primer name | Primer sequence | Product size | Reference |
|-------------|-----------------|--------------|-----------|
| Tbc1        | 5'-CGT ACG GTC GGC GAG CTG ATC CAA-3' | 235 bp | (Kim et al., 2004) |
| TbcR5       | 5'-C CAC CAG TCG GCG CTG GTG GGT CAA-3' | 136 bp | |
| M5          | 5'-G GAG CGG ATG ACC ACC CAG GAC GTC-3' | 136 bp | |
| RM3         | 5'-CAG CGG GTT GTT CTG GTC CAT GAA C-3' | 168 bp | (Bakshi et al., 2005, 2007) |
| CSB1        | 5’-TTCCGAATCCCTTGATGA-3' | 262 bp | |
| CSB2        | 5’-GGAGAGCGCTCTGATGTA-3' | 270 bp | |
| CSB3        | 5’-AGTCCGCTGGCCTTTCC-3' | 270 bp | |

Table 2. Primers used for detection of drugs resistance of *Mycobacterium tuberculosis*

| Target       | Drug | Primer name | Primer sequence | Product size | Reference |
|--------------|------|-------------|-----------------|--------------|-----------|
| embB         | EMB  | embB306     | 5’- GGCTACATCCCTGGGCATG -3’ | 335 bp | (Yang et al., 2005) |
|              |      | embBR2      | 5’- GAGCCGAGCGGTGATGAT -3’ | 335 bp | |
| katG315      | INH  | katG5R<sup>a</sup> | 5’- ATACGACCTCGATGCGC-3’ | 292 bp | (Yang et al., 2005) |
|              |      | katGOF<sup>a</sup> | 5’- GCAGATGGGCTGATCTACG-3’ | 292 bp | |
| mabA-inhA-15 | INH  | InhAP-15    | 5’- GCGCGCTGCTCCATTCCACA-3’ | 270 bp | (Yang et al., 2005) |
|              |      | inhAPF2     | 5’- CACCCCGCAACCTATCAC-3’ | 270 bp | |
| rpoB516      | RIF  | rpoB516     | 5’- CAGCTGACCAATCTGGA-3’ | 218 bp | (Yang et al., 2005) |
| rpoB526      | RIF  | rpoB526     | 5’- TTGACCCGCGCTACAC-3’ | 218 bp | |
| rpoB531      | RIF  | rpoB531     | 5’- TACAGCGCGCGACTGTC-3’ | 170 bp | (Yang et al., 2005) |
| rpsL         | STM  | STR52       | 5’-GTGAAGACCGCGCGCTGAA-3’ | 272 bp | (Jordaan and Victor, 1999) |
|              |      | STR34       | 5’-TTTCTTGACACCGCTGATC-3’ | 272 bp | |
3. RESULTS

Phenotypically, 6% and 2% of the 100 mycobacterial isolates were identified as MDR-TB and non-MDR-RIF resistance, respectively. Of the isolates, 8% were resistant to RIF, 13% to INH, 34% to STM and 12% to EMB. Resistance to PZA was found as low as 1%, and the isolate was multidrug resistant to all the other four drugs. Mono-resistant to the four first line anti-tuberculosis drug were 0% for RIF, 1% INH, 18% STM and 3% ETH. The remaining 61% isolates were identified as sensitive.

The MAS-PCR findings are given in Fig. 1. Mutations of RIF were detected in 87.5% (7/8) of phenotypically resistant isolates, of which, 71.4% and 28.6% had mutations at rpoB 531 and rpoB 526 codons, respectively.

Out of 13 isolates tested resistant to INH conventionally, 76.9% proved molecularly as mutants. Of these, 90% and 10% were found, respectively, having mutations at katG 315 and mabA-inhA-15 codons.

The MDR-TB was found molecularly harbored mutations at rpoB 531 and katG 315 codons in four out of six (66.7%) conventionally resistant isolates.

The conventional findings of the 12 EMB-resistant isolates revealed genetic mutations in 66.7% at codon embB 306.
A total of 17.6% of the isolates culturally resistant to STM were undigested and proved as STM-resistant carrying mutation at this position (Fig. 2). Comparison between conventional and molecular findings was demonstrated in Table 3.

Fig. 2. Results of PCR showing mutations at rpsL codon 43 of *M. tuberculosis*-STM-resistant strains. lane 1, 100 bp DNA ladder; lane 2, *M. tuberculosis* H37 Rv; lanes 3 to 8, strains had mutation at rpsL codon 43; lanes, 9-15 sensitive isolates; lane 16, negative control

Table 3. Comparison of conventional and molecular assays of the 100 mycobacterial isolates for drug sensitivity tests of the 100 mycobacterial isolates from northeastern Sudan

| Drug | Conventional sensitivity tests | Molecular assay | Mutation |
|------|-------------------------------|-----------------|----------|
|      | No. of resistant isolates | No. of sensitivity isolates | No. of resistant isolates | |
| RIF  | 8 | 92 | 7 | rpoB 531 and rpoB 526 |
| INH  | 13 | 87 | 10 | katG 315 and mabA-inhA-15 |
| EMB  | 12 | 88 | 8 | embB 306 |
| STM  | 34 | 66 | 6 | rpsL |

4. DISCUSSION

The most important measure of TB drug resistance is the number of new cases that are MDR-TB (Dye et al., 2002). Studies in the Sudan have shown that the frequency of MDR-TB among new TB cases was 1.9% - 2.1% (Ali, 2002; WHO, 2008). Accordingly, the 6% MDR-TB among the new cases, presented in this study, could be considered at alarming level. This increment of MDR-TB was expected as a result of increase in the INH and RIF resistance.

The present study also evidently indicated that most of the resistance in RIF and INH were due mainly to genetic mutations of rpoB 531, rpoB 526, katG 315 and to a lesser extends to
mabA-inhA-15 genes. Similar findings were reported by several workers (Ramaswamy and Musser, 1998; Telenti et al., 1997; Lee et al., 1999; Gonzalez et al., 1999; Kiepiela et al., 2000), a result which means the majority of MDR-TB in the Sudan can be detected molecularly depending on selection of these genes.

The low proportion (2%) of non-MDR rifampicin resistance that detected conventionally is consistent with the previous report of the WHO (2008) which indicated that rifampicin resistance unaccompanied by isoniazid resistance is rare.

It is needless to state that the higher frequency of resistance to STM was not surprising because the drug was in use for treatment of many infectious diseases including brucellosis and tuberculosis for many years. Moreover, same result was reported previously in new cases (Ali, 2002), but the genetic mutation harbored rpsL 43 gene in 17.6% of the total resistant strains was unexpected, since this mutation was known to be highly predictive for STM resistances (Mieskes et al., 2000). However, this unpredicted mutation might have been due to the adoption of current study to detect resistance in STM at rpsL 43 gene solely. This appeared clearly from the results obtained by Sharaf-Eldin et al. (2002) who found that genetic mutations of STM were associated with rpsL 43 and rrs 513 genes at equal frequency (4% each) among new TB cases. Consequently, it could be suggested that approximately 35% of the STM genetic mutations might have been detected if resistance to rrs 513 had included. It is worth mentioning that the molecular mechanism of resistance of about 33% STM-resistant isolates was unknown (Cole et al., 1995; Morris et al., 1995) and the prevalence of mutations may vary by geographical areas (Dobner et al., 1997; Yang et al., 2005).

Sensitivity to PZA was achieved for the first time in this country and the low level of resistance was in line with the fact that mono-resistance to PZA is uncommon.

The results of resistance of EMB was incompatible with others who had reported 100% sensitivity to EMB in new TB cases either by radiometric method (Ali, 2002) or dot blot strategy (Sharaf-Eldin et al., 2002). Problems encountered with the conventional sensitivity tests of M. tuberculosis to EMB were explained elsewhere (Heifets, 1986; Gangadharam et al., 1990; Madison et al., 2002), whereas molecular techniques are effective only for drug-resistant isolates for which the genetic mechanisms have been identified. This means sensitivity and specificity of molecular techniques are not 100%, and false-sensitive or negative result do occur. However, conventional and molecular methods and the relatively large sample size, in this study, gave the reasons for confirmation of resistance to EMB among new TB cases.

An interesting finding emerged from this report that sensitivity of acid-fast microscopy was 100%. This is contrary to the general opinion that smear microscopy is less sensitive. It is well obvious that application of standardized methods by qualified individual provides superior result.

4. CONCLUSION

The study shows a reflection to the drugs resistant status of M. tuberculosis from northeastern part of Sudan, and provided potentially valuable information on resistant genes circulating in the community. This report may be an eye opening to those involved in control programme of human TB to solve this problem.
CONSENT

The proposed study was approved by the Research Council of Sudan Academy of Science and informed consent was obtained from all patients.

ETHICAL APPROVAL

All authors hereby declare that all experiments have been examined and approved by the Research Council of Sudan Academy of Science and have therefore been performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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