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Tuberculosis drug resistance isolates from pulmonary tuberculosis patients, Kassala State, Sudan

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A B S T R A C T

Background: This study was conducted in Kassala Teaching Hospital, Kassala State, Sudan (January 2006–June 2008) to determine the rate of mycobacterium drug resistance to anti-tuberculous treatment and to explore the genotype of Mycobacterium tuberculosis resistant isolates using rpoB gene.

Methods: 53 isolates of mycobacterium isolated from pulmonary tuberculosis (PTB) patients from Kassala State were subjected to drug susceptibility testing (DST) to anti-tuberculous drugs; 10 M. tuberculosis complex (MTBC) resistant isolates were subjected to polymerase chain reaction (PCR), and commercially the amplified DNA was sequenced.

Results: DST detected resistance in 23/53 (43.39%) isolates, among which rifampicin had a high number of resistant isolates (13/23), followed by streptomycin (11/23), and multi-drug resistance was detected in 5 isolates.

DNA sequence analysis of 10 MTBC-resistant isolates detected variations within and outside the rifampicin resistant determining region (RRDR). Variation within RRDR was detected at positions 512 (AGC/ATC, Ser/Ile), and 528 (CGC/CTC, Arg/Leu). Outside the RRDR region variations were detected at positions 498 (GTG/GGG, Val/gly), 488 (ACA/ACC, Thr/Thr), which is a silent mutation. Insertions were observed at positions 484, 496 (GTG/GTG, CCG/CAGG, respectively). Deletion was observed at position 487 (ATC/_TC).

Discussion and conclusion: This study revealed that high resistance to rifampicin was associated with various point mutations in and out of the RRDR of the rpoB gene. Molecular methods are needed for early detection of TB disease and drug resistance.

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Introduction

Tuberculosis (TB) is a curable disease, though it still remains a major public health problem worldwide, especially in developing countries. Globally, it ranks as the second leading cause of death from an infectious disease. In 2012, the estimated new cases were 9.0 million and 1.5 million TB deaths [1]. The situation has become alarming due to dual infection with HIV and the development of drug resistance. The emergence of resistance to drugs used for TB treatment, and particularly
multidrug-resistant TB (MDR-TB), has become a significant public health problem in a number of countries and an obstacle to effective global TB control [2]. Globally, 3.6% of new TB cases and 20.2% of previously treated cases are estimated to have MDR-TB [3]. Both Rifampin (RIF) and Isoniazid (INH) resistance are reliable markers of MDR-TB [4]. Resistance to RIF is caused by mutations in the β subunit of RNA polymerase, a target of RIF, which is encoded by the rpoB gene. More than 95% of the resistant strains harbor mutations within an 81-bp hot-spot region (codons 507–533) of rpoB, named RIF resistant determining region (RDRR) [5]. In contrast, INH resistance is due to a mutation at one of two main sites, in either katG or inhA genes [6]. 

High incidence ranks Sudan among the high prevalence countries for TB in the Eastern Mediterranean region and accounts for 14.6% of the total TB burden [7]. No clear data on MDR-TB was reported in Sudan. A few studies have reported the detection of drug resistance in some States [8]. This study was initiated to determine the rate of mycobacterium drug resistance to anti-tuberculous treatment and to explore the genotype of MTB-resistant isolates using rpoB gene.

Methods

Study design and sample collection

This is a descriptive cross-sectional study. It was conducted at the Chest Department, Kassala Teaching Hospital, Kassala State, Sudan during the period from 2007 to 2009. 113 of the pulmonary TB cases that were subjected to treatment based on the laboratory diagnosis and/or clinical symptoms and X-ray findings were enrolled in the present study. Each patient was requested to give an adequate sputum sample in a specific container for culture and DST.

Culture and DST

200 μL from each of the digested and decontaminated sputum samples was inoculated in Lowenstein Jensen (LJ) medium. A subculture was prepared for the successful growth samples followed by DST, which was done for 53 clinical isolates of mycobacterium using the proportion method described by Sethi et al. [9]. Briefly, the LJ medium with each drug incorporated in various concentrations (0.2 μg/mL for INH, 40 μg/mL for RIF, 4 μg/mL for streptomycin [SM] and 2.0 μg/mL for Ethambutol [ETB]) and a plain medium for control were prepared. The bacterial serial dilution suspensions (10^-2 and 10^-4) were inoculated in the prepared media, and then incubated at 37°C. The readings of the incubated samples were taken after 28 days, and the second one was taken after 42 days. The resistance was calculated as the ratio of the number of colonies on the drug-containing medium and those of the control media. The isolate was considered as resistant if the ratio was greater or equal to 1% [9].

DNA extraction, amplification and sequencing

DNA was extracted by boiling method as described by Khosravi [10]; 2-5 loops-full of mycobacterial colonies were harvested in 500 μL double-distilled water in sterile Eppendorf tube, boiled in a water bath at 100 °C for 10 min, then centrifuged at 13,000 rpm for 5 min, the supernatant was collected in a sterile Eppendorf tube and stored at −20 °C until used as a template for PCR. Amplification of the product was done as described by Kim [11] using the primer:

\[
\text{tbc1 5’-CGT ACG TGC GGC GAG CTG ATC CAA-3’}
\]

\[
\text{tbcR 5'CGG ACA GTC GCC GCT TGT GGG TCA-3’}
\]

The amplified DNA of 10 resistant isolates of MTBC was commercially sequenced (Macro GEN Company, Seoul, South Korea) to detect the change of DNA sequences. The result of MTBC-resistant isolate sequences were aligned with the rpoB gene sequence of MTB H37RV strain by using Blast (http://WWW/nchiblast).

Results

The DST showed that 30 (56.6%) isolates were sensitive to the minimum concentration of the drugs, while 23 (45.3%) were resistant to at least one anti-tuberculous drug. The resistance varied from single 12 (52.17%), double 6 (26.08%), to multidrug resistance 5 (21.74%) as shown in Table 1. RIF revealed the highest resistance pattern in combination with other drugs 13 (56.53%). Among the resistant isolates, 10 (43.47%) were sensitive to RIF.

DNA sequence analysis of 10 isolates showed no change in 5 (50%) isolates, while the others had different types of mutations. Mutations included substitution, deletion and insertion of nucleotides within and out of RDRR.

Single change was demonstrated in resistant isolates at 512 (AGC/ATC, Ser/Ile), and 475 (GTC/GCC, Val/Gly). Deletion at 487 (ATC_/TC) was observed in a susceptible isolate, but resistant to SM and ETH. Insertion was in site 496 (CGG/CAGG). In other isolates more than one change was observed at 528 (CGC/CTC, Arg/Leu), 498 (GTG/GGG, Val/Gly), and 488 (ACA/ACC, Thr/Thr) (Table 2).

From the above result, only two variations were demonstrated at RRDR, and the remaining variations were outside that region. No variation was detected in four resistant isolates, among which one was RIF resistant, two were resistant to INH and the fourth was SM resistant.

Discussion

Resistance to anti-tuberculous drugs is an emerging global health problem [12]. Spread of multidrug-resistant strains of MTB has become a major public health concern in both

<table>
<thead>
<tr>
<th>Drug susceptibility</th>
<th>No. of isolates</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sensitive</td>
<td>30</td>
<td>56.6</td>
</tr>
<tr>
<td>Resistance to one drug</td>
<td>12</td>
<td>22.64</td>
</tr>
<tr>
<td>Resistance to two drugs</td>
<td>7</td>
<td>13.2</td>
</tr>
<tr>
<td>Resistance to three drugs</td>
<td>4</td>
<td>7.5</td>
</tr>
<tr>
<td>Total</td>
<td>53</td>
<td>100</td>
</tr>
</tbody>
</table>
opposed and developing countries. This study documented resistance to first-line anti-tubercular drugs in 23 (45.3%) out of 53 tested isolates. This may be due to inadequate therapy or poor drug absorption [13]. Drug resistance was reported in Sudan [14].

RIF is an effective anti-TB therapy and is the key component of the short course of multi-drug therapy. Resistance to RIF predicts resistance to INH and serves as a marker of multi-drug resistance. In this study a high number of resistant isolates to RIF was observed (56.5%), unlike the result reported by Chand et al. [15] who reported high resistant isolates to SM. MDR resistance was observed in 21.74% of cases raising a major concern about future management of TB patients in Sudan.

RIF resistance in MTB is associated with mutations in the \textit{rpoB} gene; it has been demonstrated in a genetic study that more than 95% of resistance is due to mutations in the \textit{rpoB} gene [16]. Highly conservative \textit{rpoB} genes play an essential role in the physiology of MTB. DNA sequence studies indicate that more than 96% of the RIF-resistant MTB had mutations within the 81-bp hot spot region of the \textit{rpoB} gene (RRDR) corresponding to codons 507–533 [17]. In this study DNA mutations were observed within and outside the RRDR region of the \textit{rpoB} gene in 5 isolates identified as resistant to anti-tubercular drugs. Within the RRDR region, DNA mutations were detected at 512 (AGC/ATG, Ser/Ile) and 528 (CGC/CTC, Arg/Leu). The mutations outside the RRDR of the \textit{rpoB} of this region were detected at different sites, including 498 (GTC/GGC, Val/Gly) 488 (ACA/ACC, Thr/Thr), which is a silent mutation. Ramasoota [18] reported the same result from the study conducted in Thailand, and also reported a silent mutation at the 488 codon. Deletions and insertions, which were observed in two clinical isolates, resulted in a frame shift and a formation of a truncated and non-functional protein [19]. No mutations were observed in 5 isolates, among which only 1 was RIF resistant and the others were resistant to other anti-tubercular drugs; these findings were in agreement with the results reported in the study conducted in the Russian Federation [20], in Turkey [21], and in China [22] and in Japan [17].

The mutation at 528 was reported in the study conducted in Pakistan and at 512 where a double mutation was reported [23].

### Table 2 – Detected DNA sequence variations in MTBC resistant isolates.

<table>
<thead>
<tr>
<th>Isolate number</th>
<th>Drug susceptibility</th>
<th>Codon number</th>
<th>Mutated bases</th>
<th>Type of mutation</th>
<th>Change in amino acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Resistant</td>
<td>512</td>
<td>AGC/ATG</td>
<td>Substitution</td>
<td>Serine/Isoleucine</td>
</tr>
<tr>
<td>2</td>
<td>Resistant</td>
<td>496</td>
<td>CGG/CAAGG</td>
<td>Insertion</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Resistant</td>
<td>475</td>
<td>GTC/GGCC</td>
<td>Substitution</td>
<td>Valine/Glycine</td>
</tr>
<tr>
<td>4</td>
<td>Resistant</td>
<td>528</td>
<td>CGC/CTC</td>
<td>Substitution</td>
<td>Arginine/Leucine</td>
</tr>
<tr>
<td></td>
<td></td>
<td>498</td>
<td>GTC/GGCC</td>
<td>Substitution</td>
<td>Valine/Glycine</td>
</tr>
<tr>
<td></td>
<td></td>
<td>488</td>
<td>ACA/ACC</td>
<td>Silent</td>
<td>Threonine/Threonine</td>
</tr>
<tr>
<td></td>
<td></td>
<td>487</td>
<td>ATC/TC</td>
<td>Deletion</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>Sensitive</td>
<td>No change</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>Resistant</td>
<td>No change</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>Sensitive</td>
<td>No change</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>Sensitive</td>
<td>No change</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>Sensitive</td>
<td>No change</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>Sensitive</td>
<td>No change</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

### Conclusion and recommendation

This study revealed that high resistance to RIF was associated with various point mutations inside and outside of the RRDR of the \textit{rpoB} gene. Molecular methods are needed for early detection of TB disease and drug resistance. The study of adequate sample size from different sites in Sudan is needed to reflect the clear picture of drug resistance in Sudan.

### Conflict of interest

None declared.

### Acknowledgements

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### REFERENCES


