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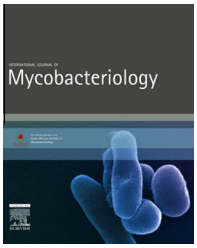

Tuberculosis drug resistance isolates from pulmonary tuberculosis patients, Kassala State, Sudan

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ARTICLE INFO

Article history:
Received 24 November 2014
Accepted 30 November 2014
Available online 29 January 2015

Keywords:
Mycobacterium tuberculosis
Drug resistance
Sudan

ABSTRACT

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/GTGA, CGG/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.

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

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Peer review under responsibility of Asian African Society for Mycobacteriology.
http://dx.doi.org/10.1016/j.ijmyco.2014.11.064

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 (RRDR) [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 [ETH]) 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:

tbc1 5′-CGT ACG GTC GGC GAG CTG ATC CAA-3.
tbcR 5-CCG ACA GTC GGC 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//ncbiblast).

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 RRDR.

Single change was demonstrated in resistant isolates at 512 (AGC/ATC, Ser/Ile), and 475 (GTC/GGC, 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 devel-

Table 1 – Sensitivity test of the MTB isolates to first line anti-tuberculous drugs.

Drug susceptibility	No. of isolates	%
Sensitive	30	56.6
Resistance to one drug	12	22.64
Resistance to two drugs	7	13.2
Resistance to three drugs	4	7.5
Total	53	100