



Initial second-line drug resistance of *Mycobacterium tuberculosis* isolates from Sudanese retreatment-patients

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ABSTRACT

Setting: Multidrug-resistant tuberculosis (MDR-TB) and extensively drug-resistant tuberculosis (XDR-TB) are a major public health threat.

Objective: This study aimed to determine resistance patterns to second line anti-TB drugs (SLDs), and to determine the frequency of extensively drug resistant *Mycobacterium tuberculosis* (XDR-TB).

Design: During the period from July 2009 to July 2010; sputum specimens were collected from TB retreatment patients; isolates were tested for sensitivity to first line anti-TB drugs by the 1% proportion method; MDR strains were tested for second line anti-TB drugs sensitivity by 1% proportion method and by version 1. Hain GenoType MTBDRsl Assay.

Results: One hundred and forty three mycobacterial isolates were successfully recovered from a total of 239 specimens (143/239; 59.8%). Fifty six strains were rifampicin resistant (RR); of these 54 were multi-drug resistant (MDR); two were RIF/INH-resistant mycobacterium other than tuberculosis (MOTT). Five of MDR (5/50; 10%) showed resistance to at least one second line drug and one isolate (1/50; 2%) was XDR. The XDR strain was concordantly detected by the two methods.

Conclusion: Initial resistance to second line anti-TB drugs among MDR-TB patients is at 10% levels and XDR-TB is prevalent at low levels (2%). Nevertheless; without great efforts from national tuberculosis control program (NTP) this figure can fuel the TB epidemics in Sudan.

1. Introduction

Drug resistant tuberculosis has emerged as a significant global public health problem and a great challenge for TB control. In 2014 WHO estimate 480 000 multi drug resistant (MDR) cases; Most of these cases are in India, China, Russian Federation and South Africa. Extensively drug-resistant TB (XDR-TB) had been reported by 105 countries by 2015. The magnitude of XDR-TB is not small (9.7% of MDR-TB) in addition XDR-TB is an important killer of TB patients [1,2].

Multi-drug resistant tuberculosis (MDR-TB) is defined as *Mycobacterium tuberculosis* strain that resistant to at least isoniazid (INH) and rifampicin [3]. While extensively drug-resistant tuberculosis (XDR-TB) is MDR-TB with additional resistance to any fluoroquinolone and to at least one of three injectable drugs used for TB treatment: capreomycin, kanamycin, or amikacin [4]. The term Pre-XDR-TB means MDR-TB with resistance to either a fluoroquinolone or a second-line injectable agent but not both [5].

When first line drugs fail, second line drugs are used to treat MDR-TB, unfortunately, some second line drugs are toxic and can lead to negative side effects. These drugs must be taken for up to two years. Moreover they are expensive and less effective, with poor treatment outcomes and high death rates [2,6,7].

Prescription of second line anti-TB drugs for MDR-TB patients started in 2008 in the Sudan. The program was run based on presumptive diagnosis and managed cases empirically. In 2010, Sudan signed the Green Light Committee (GLC) Initiative Document to combat MDR-TB by improving access to affordable second-line anti-TB drugs. To our knowledge; In Sudan second line drug resistance and XDR has never been studied before, Therefore, in the present study, we investigated the initial second line drug resistance.

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2. Methods

2.1. Study specimens' origin

During the period from July 2009 to July 2010; smear positive sputum specimens were collected from TB retreatment cases; treatment failures, relapsed and return after default from National referral centers for tuberculosis diagnosis and management in Abu Anja Specialized TB Hospital, Academic Hospital and the National Public Health Laboratory; Greater Khartoum, Sudan.

2.2. Isolation and identification of mycobacteria

Lewenstein-Jensen (L.J.) medium containing glycerol and Lewenstein-Jensen supplemented with pyruvate were used for isolation of mycobacteria from sputum specimens. Petroff method for decontamination (4% NaOH for 20 min) was used to homogenize and decontaminate sputum specimens; alkaline was neutralized to the end point with 1 N HCl containing 0.1% phenol red indicator. Aliquots were directly inoculated onto the culture media without centrifugation. Isolates were firstly identified phenotypically according to their reaction with ZN staining method, growth rate, colonial morphology and pigment production and by their susceptibility to Para nitro benzoic acid (PNB). Then MDR isolates were confirmed genetically by hybridization of DNA Amplicons with TUB probe incorporated on validity zone of MTBDRsl strips.

2.3. Drug susceptibility testing (DST)

2.3.1. 1% proportion method

Based upon laboratory standard operating procedures (SOPs) of media, reagents and solutions preparation, drug dilutions and bacillary suspensions preparation, instrument validation and administration; tests quality control was performed. As resistant strains was not available at that time and due to biosafety consideration second line quality control was performed only by H37Rv strain. Drug susceptibility testing (DST) was performed following the standard 1% proportion method. First line Anti-TB drugs were added to reach critical concentrations as following: Rifampicin (R), 40.0 µg/ml; Isoniazid (H), 0.2 µg/ml; Streptomycin (S), 4.0 µg/ml; and Ethambutol (E), 2.0 µg/ml. Second line Anti-TB drugs were added to reach different critical concentrations as following: Kanamycin (KM), 30 µg/ml; Capreomycin (CM), 40 µg/ml; Ofloxacin (Ofx), 2.0 µg/ml [8] and Amikacin (AM) 40 µg/ml [9]. Quality assurance was performed by designing an internal audit system; panel of ten isolates (five sensitive to all SLDs and all five of any resistance strains) was retested by another colleague; and results showed 100% concordance.

2.3.2. Hain genotype MTBDRsl assay

Hain GenoType MTBDRsl is nucleic acid amplification assay based on reverse hybridization with specific oligonucleotide probes on nitrocellulose strips. MTBDRsl identifies *M. tuberculosis* complex and detects resistance to fluoroquinolone, second line injectable drugs and first line ethambutol evident as mutations of *gyrA*, *rrs* and *embB* genes respectively. DNA extraction, amplification, Hybridization and band detection, reading and interpretation of strips patterns were performed according to version 1. Hain GenoType MTBDRsl commercial kits [10].

2.3.3. Data handling

Data were analyzed by using statistical package for social sciences (IBM SPSS statistics 20). Descriptive in addition to the association between dependent and independent variables were applied.

3. Results

3.1. Sputa collection sites, isolate types and growth behavior

A total of 239 smear positive sputa were collected from retreatment TB patients with mean age 37.2 and SD 1.9; range (13 to 75 years). The number of male was 175 and the number of female was 64 patients. Majority of samples came from the National Public Health Laboratory (45.2%, 108/239). Comparable numbers came from Abu Anja (29.3%, 70/239) and The Academic Hospital (25.5%, 61/239). Samples collected from those patients classified into 55 as failure cases, 63 as relapse, and 121 as return after default. More than half (59.8%, 143/239) of the isolates were successfully recovered, while 40.2% (96/239) failed to grow. Two strains (2/143; 1.4%) were identified as RIF/INH-resistant MOTT, and the rest (141/143; 98.6%) was *Mycobacterium tuberculosis* complex strains. Contamination rate was 3.9%. Fifty four strains (54/141; 38.3%) were MDR. Of these four isolates (4/54; 7.4%) died during subsequent subculture.

3.2. 1% proportion method results

Forty five males (out of 129), and nine females (out of 54) were reacted positive for MDR-TB with *p. value* 0.041. The majority of the MDR isolates were insignificantly found among failure patients (27 out of 54) with *p. value* of 0.115.

Fifty four MDR isolates were collected from patients had mean age of 34.7 ± 11.2. Among those MDR patients, there were 44 males and 10 females. The XDR case is a female aged 20 years old. Five of the MDR isolates (5/50; 10%) showed resistance to at least one second line drug and one isolate (1/50; 2%) was XDR, the breakdown of results was shown in Table 1. Amikacin and Capreomycin shows no resistance except within XDR strain.

3.3. Hain genotype MTBDRsl assay results

XDR, Capreomycin and Amikacin resistant and MOTT isolates were concordantly detected by gold standard 1% proportion method and by Hain GenoType MTBDRsl line probe Assay. Kanamycin-resistant isolate showed discordant result between the two techniques. Ofloxacin showed one false positive and one false negative result in the Hain Genotype assay.

4. Discussion

Extensively drug-resistant TB (XDR-TB) had been reported by 105 countries by 2015, similar to estimates for previous years (9.6% in 2012, 9.0% in 2013 and 9.7% in 2014), 9.7% of people with MDR-TB have XDR-TB [1]. The low frequency of XDR-TB (2%) within the study cohort is within the reported frequency that was reported from many countries (0.8–15.2%) but is also constrained by the limited study sample [11].

As far as we are aware this is the first report of resistance to second line anti TB in Sudan and it the first time testing second line drug resistance and XDR TB; regarding the fact that like drug-sensitive and other forms of drug resistant TB; XDR-TB can spread from person to

Table 1
Breakdown of second line drug resistance results.

		XDR	Pre-XDR KM	Pre-XDR Ofx	Sensitive to all SLDs	Died	Total
MDR patterns	RHSE	1	1	2	12	2	18
	RHS	0	0	1	11	2	14
	RHE	0	0	0	14	0	14
	RH	0	0	0	8	0	8
Total		1	1	3	45	4	54

person [12] it is to be concluded that such high XDR-TB figure is alarming. Retrospective report from Norway reveal that XDR was in existence since 1995 and started with one patient, after ten years IS6110 RFLP and spoligotyping DNA studies revealed that most of patients diagnosed were carried the same patterns [13]. Moreover most studies from different parts of the world suggested genetic similarities of the XDR strains that belonged to 1 of 2 epidemiological clusters, either a single-family cluster or a cluster of close contacts [14,15].

Over time, age groups and genders affected by TB did not change much as is shown by our findings [16,17]. Higher XDR-TB among women is more than in males, which may reflect the treatment accessibility and partially to the fact that ladies are more stigmatized than males [11]. Recovery of mycobacteria can be reduced due to many reasons alluded by Frieden, 2004 [18]. In this study low recovery rate (59.8%) may explain by the use of without centrifugation pretreatment method and culture contamination.

Injectable second line drugs [Kanamycin, Amikacin, and Capreomycin] exhibited similarly low frequencies of resistance (2%, 0% and 0%) respectively. This indicates that second line injectable drugs have no superiority over each other. The lower cost of Kanamycin makes it preferable by some. In addition amikacin had a greater risk of developing the more severe forms of hearing loss, compared to the use of kanamycin for the same indication (75% versus 56%). The reported low frequencies of resistance to injectable anti-TB drugs are similar to that reported by different parts of the world [19,20]. Different from other settings; aminoglycosides cross resistance was not detected except within the XDR isolate [21]. Moreover amikacin pre-XDR pattern was not observed in this study partially this reflect the use of critical concentration of 40 µg/ml, lately; WHO recommended concentration of 30 µg/ml [9,22]. Discordant results of kanamycin reflect the missing mutations at the level of the *eis* promoter region in the, MTBDRsl v1.0 assay.

Fluoroquinolones Resistance is common, in eastern European countries [23]. Although Ofloxacin was not widely used in Sudan as ciprofloxacin; resistance to Ofloxacin [fluoroquinolone] has been detected (3/50 6%) this figure is less than that reported by WHO, 2015 and that reported in India and of Dalton and colleagues in 2012 [1,3,11]. this may reflect the full or near complete cross resistance between Ofloxacin and Ciprofloxacin and/or other fluoroquinolone [24]. Discrepancies Of ofloxacin results between 1% proportion method and line probe assay might be because of mutations conferring fluoroquinolones resistance in genomic regions not incorporated in MTBDRsl.

5. Conclusion

It conclude that initial resistance to second line anti-TB drugs among Sudanese MDR-TB patients is at 10% levels, XDR-TB is prevalent at low levels (2%), nevertheless it is alarming figure and need strong effort from NTP to prevent fueling the TB epidemics.

Authors' contributions

MAMA and HMHA: acquisition, analysis and interpretation of data.
MAMA: Conception and design of study and drafting the article.
EAGK: critically revising and approved the final manuscript version.

Ethics and consent

The research proposal has been approved by the Ethics Committee of Institute of Endemic Diseases, University of Khartoum.

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