First Insight into the Fluoroquinolone and Aminoglycoside Resistance of Multidrug-Resistant *Mycobacterium tuberculosis* in Saudi Arabia

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Abstract. In Saudi Arabia, there were no nationwide screening studies conducted so far to determine the aminoglycoside and fluoroquinolone resistance among multidrug-resistant *tuberculosis* (MDR-TB) isolates. Therefore, as the first attempt in the country, a retrospective analysis has been conducted on a nationwide collection of 2,956 *M. tuberculosis* clinical isolates screened with phenotypic drug susceptibility testing to define MDR-TB. Enrolled MDR-TB isolates were subjected to second-line drug susceptibility testing, detection of mutations conferring resistance to aminoglycosides and fluoroquinolone, followed by 24-loci mycobacterial interspersed repetitive unit-variable number of tandem repeat typing and spoligotyping. Overall, 83 isolates were identified as MDR-TB, and 13 (15.7%) isolates showed resistance to second-line drugs. Moxifloxacin (low level) showed higher resistant rates (10.8%) followed by ofloxacin (7.2%), capreomycin (3.6%), kanamycin (3.6%), and amikacin (2.4%). Overall fluoroquinolone resistance was 12%, whereas aminoglycoside resistance was 7.2%. Predominant mutations conferring resistance to fluoroquinolone were found in *gyrA* A90V and D94G, whereas aminoglycoside resistance was observed only with *rrs* gene A1401G mutation. The corresponding strain lineages predominated with Indo-Oceanic and East-African Indian origin. Interestingly, none of the isolates with second-line drug resistance was defined as extensively drug-resistant TB (XDR-TB). Surprisingly, many isolates (50.6%) were panresistant to first-line drugs. Saudi Arabia faces considerable burden of fluoroquinolone- and aminoglycoside-resistant MDR-TB. Higher incidence of panresistant MDR-TB reveals a threat for the emergence of XDR-TB strains in the near future.

INTRODUCTION

Tuberculosis (TB) caused by *Mycobacterium tuberculosis*, reemerges as a major threat to the global public health, sustained by increasing resistance to most effective drugs and significant association of human immunodeficiency virus (HIV) with active TB disease.^{1,2} Mutations in genes encoding drug targets and other mechanisms such as enzymes activating the drug and overexpression of the targets, lead to the evolution of drug resistance particularly in cases with incomplete or inadequate treatment.³ Sequential accumulations of mutations in target genes generally lead to resistance of multiple drugs. Resistance to isoniazid (INH) and rifampicin (RIF) were defined as multidrug-resistant TB (MDR-TB) which often challenges the efforts of global TB control programs.⁴ In addition, the emergence of extensively drug-resistant TB (XDR-TB) became a global reality and threatens the TB control management of any country. XDR-TB is defined as MDR-TB isolates with additional resistance to a fluoroquinolone and one or more of the following injectable drugs: kanamycin, amikacin, and capreomycin. When the first-line drugs no longer work, the second-line drugs fluoroquinolone, kanamycin, amikacin, and capreomycin are used in treatment.⁵ An average of 3.6% total TB cases in the world were reported as MDR-TB and 46 countries were reported with XDR-TB.⁶ XDR-TB is reported with highest mortality (98%) among HIV-coinfected patients in Africa and 33% mortality was reported among HIV-negative patients.^{7–9}

The new-generation fluoroquinolones (moxifloxacin, gatifloxacin), injectable aminoglycosides (kanamycin, amikacin),

and cyclic peptides (capreomycin) were considered as crucial drugs for the treatment of MDR-TB. Even though intrinsic drug resistance is possible in *M. tuberculosis*, the majority is acquired resistance caused by spontaneous mutations in chromosomal genes, mainly because of suboptimal therapy.¹⁰ For instance, the key drugs to define MDR-TB, RIF showed > 95% resistance due to the mutation in *rpoB* gene, whereas INH has a complex process of mutation in several genes, predominantly in *katG* and *inhA* followed by *ahpC*, *kasA*, *oxyR*, and *ndh*.^{11–13} Mutations in the hypervariable regions of *gyrA* and *gyrB* genes conferred fluoroquinolone resistance and accounted for majority (up to 94%) of phenotypic resistant isolates.^{14,15} On the other hand, kanamycin, amikacin, and capreomycin resistance is caused by the major mutation in 16S rRNA gene (*rrs*) in positions 1401, 1402, and 1484, respectively.^{16,17} In addition, kanamycin and capreomycin resistance were conferred by *eis* and *tlyA* gene mutations, respectively.^{18,19}

Saudi Arabia is the third biggest Arab country in the world with a moderate annual TB burden (22 cases/100,000 populations).² A moderate level of MDR-TB prevalence (4%) was recently reported with an increasing level of overall resistance (23.6%) to first-line drugs.²⁰ Furthermore, like many other countries, routine testing of second-line drug susceptibility is not conducted as part of the national TB control program in the country. Consequently, the availability of data on resistance to aminoglycosides or fluoroquinolone is very much limited. Therefore a study was designed to screen a nationwide collection of MDR-TB isolates with a notion to find XDR-TB. Phenotypic drug susceptibility testing, line probe assays to detect mutations, and phylogenetic analysis were applied on each isolate.

MATERIALS AND METHODS

Study population. During 2013–2014, 2,956 nonrepetitive *M. tuberculosis* clinical isolates were collected with the

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respective epidemiological data from different provincial hospitals in the country. Demographical and clinical data of the patients were collected from the laboratory records using standard data collection forms. All the data collected for the study were fully anonymized and no patient identifiers were used throughout the data collection and analysis period.

Phenotypic drug susceptibility testing. All the collected isolates were subjected to primary susceptibility testing to streptomycin (1 µg/mL), INH (0.1 µg/mL), RIF (1 µg/mL), and ethambutol (EMB 5 µg/mL) by using the MGIT SIRE kit (Becton Dickinson, Franklin Lakes, NJ). Isolates resistant to both INH and RIF were defined as MDR-TB and enrolled. All the enrolled MDR-TB isolates were subjected to drug susceptibility testing to amikacin, capreomycin, kanamycin, moxifloxacin, and ofloxacin by using commercially available drug formulations (Becton Dickinson). The tested critical concentrations of the drugs were 1.0 µg/mL, 2.5 µg/mL, 2.5 µg/mL, 0.5 µg/mL, and 2.0 µg/mL, respectively.

DNA extraction and mutation detection. Genomic DNA was extracted from the heat-killed isolates using QIAamp DNA mini kit (Qiagen, Hilden, Germany). All isolates confirmed as MDR-TB by the phenotypic susceptibility testing were evaluated to determine INH and RIF resistance conferring mutations by using the commercial line probe assay Genotype MTBDR_{plus} (Hain Lifescience, Nehren, Germany) according to the manufacturer's instructions. Mutations conferring resistance to fluoroquinolones and aminoglycosides were tested by using the Genotype MTBDR_s/ kit version-01 (Hain Lifescience, Nehren, Germany). The test strips after hybridization were scanned and interpreted by using the automated scanning system Genoscan and software package Blotrix (Hain Lifescience). The MTBDR_{plus} assay identifies mutations in *rpoB* gene responsible for resistance to RIF and mutations in *KatG* gene and *inhA* promoter region for low-level and high-level INH resistance, respectively. The MTBDR_s/ assay permits the identification of five most common mutations and one rare mutation of *gyrA* gene conferring resistance to fluoroquinolone. Any isolate which demonstrated the presence of a mutation band corresponding to any of the six mutations was declared as resistant to fluoroquinolone. There were two probes represented mutation in *rrs* gene. Isolates observed with those two mutation bands were defined as resistant to aminoglycosides or cyclic peptides.

Genotyping of MDR-TB isolates and data analysis.

All the isolates reported with resistance to either aminoglycosides or fluoroquinolone were subjected to spoligotyping (Ocimum Biosolutions, Hyderabad, India) and 24-loci-based

mycobacterial interspersed repetitive unit-variable number of tandem repeat (MIRU-VNTR) typing (Genoscreen, Cedex, France) by using the commercially available kits and method described elsewhere.^{21,22} The assignment of MIRU-VNTR alleles were initially performed with the help of Gene Mapper version 4.0 software package (Applied Biosystems, Woburn, MA) and further alignment was carried out in MIRU-VNTR Data Manager Version 1.0 (Genoscreen, Lille, France). The spoligo-signatures were converted into octal codes. The spoligo and MIRU-VNTR patterns were analyzed together to assign the strain lineages by using the MIRU-VNTR Plus online database (www.miru-vntrplus.org).

The statistical analysis of the data was carried out by using SPSS, version-19.0 (IBM Corporation, New York, NY). Patients without history of anti-TB drug therapy or with a history of less than 1 month were defined as "New" cases, whereas patients who received more than 1-month anti-tuberculosis therapy were considered as "previously treated" according to the World Health Organization guidelines.²³

RESULTS

Of the total 2,956 isolates screened, 83 were confirmed with resistance to INH and RIF and further defined as MDR-TB. Phenotypically, among the 83, 18 (21.7%) were resistant only to INH and RIF, and 42 (50.6%) were panresistant. There was no large difference in drug resistance pattern among patients with a history of anti-TB drug treatment (Table 1). Phenotypical second-line drug susceptibility testing showed 15.7% resistant cases. Of the total 83, 10.8% were resistant to moxifloxacin and 7.2% to ofloxacin, respectively. Overall fluoroquinolone resistance was 12%, whereas aminoglycoside resistance was 7.2% (Table 2).

Of the 83 RIF-resistant isolates, 64 (77.1%) were mutated in codon 531 of *rpoB* gene. On the other hand, 69 (83.1%) INH-resistant isolates showed mutation in *katG* gene codon 315 and 14 (16.9%) in *inhA* promoter region 15. Analysis of mutations conferring resistance to fluoroquinolone and aminoglycosides showed that 13 of the 83 MDR-TB cases have mutations in either *gyrA* or *rrs* genes, whereas none of them showed mutation in both genes. Among the *gyrA* mutated isolates, five (38.5%) showed the D→G mutation in codon 94, whereas four (30.7%) cases showed A→V mutation in codon 90. However, *rrs* gene mutation was observed only among three (23.1%) isolates within the nucleic acid position 1401 showing an A→G mutation. Mutations conferring RIF and INH resistance among these 13 isolates showed a domination of *rpoB* codon 531 and

TABLE 1
First-line phenotypic and genotypic drug resistance pattern of 83 multidrug-resistant *Mycobacterium tuberculosis* isolates

Drug resistance pattern*	Total (no/%)	Treatment history†		Type of mutations conferring resistance‡				
		New (N/%)	Previously treated (N/%)	S531L + S315T1	S531L + C15T	H526Y + S315T1	D516V + S315T1	H526D + C15T
H + R	18 (21.7)	8 (44.4)	10 (55.6)	12	1	3	1	1
S + H + R	17 (20.5)	8 (47)	9 (53)	8	4	3	2	—
H + R + E	6 (7.2)	4 (66.7)	2 (33.3)	2	1	1	2	—
S + H + R + E	42 (50.6)	19 (45.2)	23 (54.8)	30	6	4	1	1
Total	83	39 (47)	44 (53)	52 (62.7)	12 (14.5)	11 (13.2)	6 (7.2)	2 (2.4)

*E = ethambutol; H = isoniazid; R = rifampicin; S = streptomycin.

†New: < 1 month of treatment history or newly diagnosed; previously treated: > 1 month treatment history.

‡The mutations detected by the Genotype MTBDR_{plus} assay.

TABLE 2
Second-line phenotypic and genotypic drug resistance pattern of multidrug-resistant *Mycobacterium tuberculosis* isolates

Drug resistance pattern*	Total (no/%)	Type of mutations conferring resistance†			
		<i>gyrA</i> A90V	<i>gyrA</i> D94N/D94Y	<i>gyrA</i> D94G	<i>rrs</i> A1401G
M + O	5	3	1	1	–
M	4	1	–	3	–
O	1	–	–	1	–
A + K + C	2	–	–	–	2
K + C	1	–	–	–	1
Total	13 (15.7)	4 (4.8%)	1 (1.2%)	5 (6%)	3 (3.6%)

*A = amikacin; C = capreomycin; K = kanamycin; M = moxifloxacin; O = ofloxacin. The table shows the corresponding mutations in phenotypic resistant isolates.

†The mutations detected by the Genotype MTBDRs/ version-01 assay.

katG gene codon 315 mutations in majority of the cases (Table 2).

Second-line drug resistance was relatively higher among non-Saudi (53.8%) patients compared with the Saudi (46.1%) population. Age of the patients showed a median of 40 years and a domination of male patients (69.2%). All cases with second-line drug resistance had pulmonary infection except one case with both pulmonary and extrapulmonary. Geographical origin of the non-Saudi patients showed three (23.1%) from south and southeast Asia and two (15.4%) each from Africa and Yemen. The treatment history of the 13 cases showed a majority (61.5%) of the cases as "New" and 38.5% cases as "previously treated." Most of the non-Saudi patients fell under the definition of "New" cases (46.1%). Genotyping of the 13 isolates showed mainly East African Indian (4, 30.8%), Delhi/Central Asian (3, 23.1%), and Cameroon (2, 15.4%). On the other hand, one case each of Beijing, S, Ghana, and Uganda-I strains were also observed (Table 3).

DISCUSSION

This is the first study of its type conducted in Saudi Arabia to analyze the second-line drug susceptibility of MDR-TB isolates with possible molecular characterization on a nationwide collection. Although a considerable number of second-line drug resistance cases were defined among the studied MDR-TB isolates, no XDR-TB phenotypes were observed. The phenotypic and genotypic susceptibility testing results showed a 100% concordance. The massive concordance may probably be due to the facts that existence

of mutations other than the common one covered in line probe assay is minimal in the country and second-line resistance cases were very few (less than eight case per drug) to make solid conclusion.^{24,25}

Genotypic or phenotypic susceptibility testing to second-line anti-TB drugs is not usually performed in the diagnostic laboratories in the country. Therefore, this study shed some light onto the possible second-line drug resistance pattern and mutations found in the two target genes *gyrA* and *rrs* which majorly confer resistance to fluoroquinolone and aminoglycosides.

In the current study, 53% of the patients had a history of previous treatment, whereas 47% were newly diagnosed cases. All drug-resistance patterns except combined resistance to INH, RIF, and EMB were found predominant (> 50%) among the isolates with a previous treatment history. This is in concordance with a previous survey which demonstrated that patients with prior history of TB treatment are at greater risk to acquire or develop MDR-TB, secondary to the emergence of resistant strains to multiple drugs in the country.²⁰ However, transmission of drug-resistant strains to a relatively higher level among the Saudi nationals and immigrants are also recently reported.^{24,26} In addition, Varghese and others recently demonstrated a new trend of TB transmission particularly among newly diagnosed immigrant patients, which shows the possibility of reactivation of remote infection immediately followed by a reinfection with drug-resistant strains including MDR-TB.²⁷ These recent findings clearly justify that the chances of drug-resistant TB in Saudi Arabia is not only limited to acquired resistance but also to recent transmissions. The

TABLE 3
Genetic and demographical diversity among second-line drug-resistant *Mycobacterium tuberculosis* isolates

Case	Country of origin	Age/sex	Infection type	Treatment history	Strain lineage*	Mutations in gene codon				
						<i>katG</i>	<i>inhA</i>	<i>rpoB</i>	<i>gyrA</i>	<i>rrs</i>
1	Indonesian	35/Female	PTB	New	Beijing	–	C15T	S531L	A90V	–
2	Saudi	31/Male	PTB	PT†	Delhi/CAS	S315T1	–	S531L	A90V	–
3	Pakistan	42/Male	PTB	New	EAI	–	C15T	S531L	A90V	–
4	Saudi	40/Male	PTB	PT	EAI	–	C15T	S531L	A90V	–
5	Saudi	21/Female	PTB	PT	Delhi/CAS	S315T1	–	S531L	D94N/D94Y	–
6	Ethiopian	30/Male	PTB and EPTB	New	Ghana	S315T1	–	S531L	D94G	–
7	Saudi	43/Male	PTB	PT	EAI	S315T1	–	S531L	D94G	–
8	Yemeni	20/Female	PTB	New	Uganda-I	S315T1	–	S531L	D94G	–
9	Saudi	41/Male	PTB	New	Delhi/CAS	S315T1	–	S531L	D94G	–
10	Saudi	72/Female	PTB	New	S	S315T1	–	S531L	D94G	–
11	Senegal	40/Male	PTB	New	Cameroon	S315T1	–	S531L	–	A1401G
12	Yemeni	23/Male	PTB	PT	Cameroon	–	C15T	S531L	–	A1401G
13	Filipino	43/Male	PTB	New	EAI	–	C15T	S531L	–	A1401G

EAI = East African Indian; EPTB = extrapulmonary tuberculosis; PTB = pulmonary tuberculosis.

*Defined using www.miru-vntrplus.org database.

†Previously treated (> 1 month of treatment received).

drug resistance pattern showed a higher rate of pan-resistant isolates (50.6%) which is very high compared with previous large-scale studies and an indicative factor of XDR-TB emergence.^{28–30}

Mutation analysis of RIF resistance showed a domination of *rpoB* codon 531 (77.1%) followed by codon 526 (13.2%). This finding distinctly reflects the conclusions of one of our recent nationwide study and other reports which showed high rate of mutation in codon 531 as the main cause of RIF resistance in Saudi Arabia.^{24,31} On the other hand, INH resistance was dominated with mutations to *katG* gene codon 315 and mutation in the position 15 of the *inhA* promoter region, which is in concordance with our previous reports and other international studies.^{24,32,33}

Fluoroquinolone resistance was 12% among the studied MDR-TB isolates with the mutation majorly in *gyrA* gene on codons 90 and 94. The main mutations observed were D94G and A90V. On the other hand, all the isolates with aminoglycoside resistance were found with a conferring mutation to the *rrs* gene in position 1401. Prevalence of these mutations among the MDR-TB and XDR-TB isolates were reported in other countries also.^{34–37}

We analyzed the demographical background of the patients along with the phylogenetic profile of the isolates and corresponding mutations. Among the 13 patients with second-line drug resistance, 53.8% were immigrants. Supportively, Saudi Arabia is unique in the population structure with an approximate 9.3 million (31.8%) of immigrants within the 29.2 million of its total population.³⁸ Recent reports showed a very high rate of annual TB notification among immigrants (26.7 cases/100,000 populations) compared with Saudi nationals (11.5 cases/100,000 populations).³⁹ While the overall MDR-TB burden among the newly diagnosed cases in the country stays as only 4%, a higher rate was reported among the non-Saudi population (5%) compared with the local population (3%).²⁰ Hence, the overall higher rate of drug resistance including second-line drugs is as expected among immigrants. Majority of the non-Saudi patients were reported as newly diagnosed TB cases without any previous treatment history. This is an interesting finding, which is mostly supported by the facts; reactivation of remote TB infection is high among immigrant and majority of them belonged to the high-TB burden countries from Asia and Africa. This fact has been evidenced in many immigrant-receiving countries.

The phylogenetic origin of the isolates showed the predominance (61.5%) of PGG-I (Indo-Oceanic, East-African Indian) lineages. This is in concordance with previous studies that showed higher representation rate of Beijing, Delhi/CAS, and EAI clades among MDR isolates particularly XDR-TB.^{26,40}

This study has few limitations: genome sequencing techniques were not considered to reconfirm the detected mutations or to find unknown or new mutations; the elevated low resistance of moxifloxacin was not tested with higher concentrations; and due to the small size of the study group, phylogenetic analysis on transmission dynamics could not be performed.

CONCLUSION

In conclusion, as a first attempt in the country to screen the scope of second-line drug resistance among the MDR-TB

isolates, a considerable volume of resistance was observed. Interestingly, none of the isolates conformed to the definition of XDR-TB. However, the large presence of panresistance to first-line drugs is a key indication of emergence of XDR-TB in the near future. A close surveillance must be kept on implementing the TB control strategies around the country on MDR-TB cases.

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