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Molecular identification of mutations associated with anti-tuberculosis drug resistance among strains of *Mycobacterium tuberculosis*

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Summary
Background: Understanding the etiologic organism, antimicrobial resistance mechanisms, and transmission of multidrug-resistant tuberculosis (MDR-TB) can be of great value in optimizing strategies to control and prevent its development and transmission.
Methods: One hundred and fifty-five *Mycobacterium tuberculosis* complex isolates from patients with pulmonary tuberculosis (TB) in Cairo, Egypt were studied. In vitro drug susceptibility testing against rifampin (RIF), isoniazid (INH), streptomycin (SM), ethambutol (EMB), and pyrazinamide (PZA) was performed. Resistance was studied by the standard agar proportion method. Single strand conformation polymorphism (SSCP) and DNA sequence analysis were used to detect mutations in the genes that encode resistance to *rpoB*, *katG*, *rpsL*, and *embB*.
Results: Among 155 consecutive *M. tuberculosis* isolates, 25 (16.1%) were MDR-TB; 13 of these were from newly diagnosed untreated cases, 12 were from re-treated cases, and none of the MDR-TB isolates had matching IS6110 fingerprints. Among the MDR-TB isolates, *rpoB* mutations were found in 76% of RIF-resistant isolates, *katG* mutations were found in 47.1% of INH-resistant isolates, *rpsL* mutations were found in 55.6% of SM-resistant isolates, and *embB* mutations were found in 36.4% of EMB-resistant isolates.
Conclusions: No major differences were found in the frequencies of mutations or types of amino acid substitution between newly diagnosed untreated cases and re-treated cases. The high prevalence of MDR-TB at this hospital underscores the need for continuous monitoring of strains and antimicrobial resistance.

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Introduction

Tuberculosis (TB), especially multidrug-resistant TB (MDR-TB), is a global problem, and a prevalence of MDR-TB as high as 26.8% has recently been reported.[1–3] MDR-TB strains are generally defined as resistant to at least isoniazid (INH) and rifampin (RIF). These strains have been described worldwide, and their existence poses a serious threat to TB control programs in many countries. Understanding the etiologic organism, antimicrobial resistance mechanisms, and transmission of MDR-TB can be of great value in optimizing strategies to control and prevent its development and transmission.[4]

The frequency of resistance to multiple drugs varies geographically, and acquired (secondary) resistance is more common than primary resistance. High rates of acquired MDR-TB have been reported in Nepal (48.0%), India (33.8%), and in New York City (approximately 30%).[5] Although studies have shown an overall decrease in the number of MDR-TB cases reported in New York City and throughout the USA, the number of states reporting these organisms has actually increased substantially since the early 1990s.[5] The treatment of patients with MDR-TB is substantially more difficult than the treatment of individuals infected with drug-susceptible strains.

El-Gazzar et al. found that among 400 newly diagnosed patients with active pulmonary TB in urban and rural areas of Qualiobia governorate, Egypt, the prevalence of primary resistance to streptomycin (SM) was 32.3%, to INH was 25%, to ethambutol (EMB) was 17.5%, to RIF was 12.5%, and to pyrazinamide (PZA) was 7.5%.[6] The prevalence of primary resistance to one drug was 40%, to two drugs was 20%, and to three drugs was 5%, but no resistance was found to four or five drugs.[6] Abbadi et al. studied 25 *Mycobacterium tuberculosis* isolates from Assiut region in Egypt, and found 11 (44%) MDR isolates, but these patients had undergone at least one year of prior TB therapy.[7] Theoretically, MDR-TB strains could arise as a consequence of sequential accumulation of mutations conferring resistance to single therapeutic agents, by a single-step process such as acquisition of an MDR genetic element, or through a mutation that alters cell wall structure affecting drug uptake.[8] A well-documented example of how MDR-TB strains arise has been provided by the analysis of the evolution of two closely related subclones in New York City, arbitrarily designated as W and W1.[9] Automated DNA sequencing of representative organisms defined the series of distinct mutations conferring resistance to RIF, INH, SM, EMB, and PZA. For example, a His526Tyr amino acid substitution was responsible for conferring RIF resistance and a Ser315Thr substitution was responsible for INH resistance.[10] These MDR isolates arise because random mutations in genes that encode targets for the individual antimicrobial agents are selected by subtherapeutic drug levels that can occur due to processes such as treatment errors, poor adherence to treatment protocols, or other factors.[11]

Several methods such as direct sequencing of PCR products, single-strand conformation polymorphism (SSCP) analysis, the base pair-mismatch assay, the reverse hybridization-based line probe assay, and other strategies, are designed to exploit the observation that specific mutations that are found in resistant strains are absent in susceptible organisms. The fact that natural populations of drug-susceptible *M. tuberculosis* complex isolates recovered globally have remarkably few polymorphisms in structural genes, greatly simplifies interpretation of these assays. The restricted allelic variation in structural genes means that virtually all susceptible organisms will have the same wild-type allele of the target gene. Hence, one generally needs to differentiate between a single wild-type sequence and mutant sequences. Among the many techniques used to identify drug resistance-associated mutations, automated DNA sequencing of PCR products has been the most widely applied. One important advantage of sequence-based approaches is that the resulting data are virtually unambiguous because a resistance-associated mutation is either present or absent. Telenti et al. similarly demonstrated good performance of SSCP-based interrogation of target sequences. Missense mutations and short deletions in one region of the gene that encodes the RNA polymerase beta subunit (*rpoB*) cause RIF resistance in *Escherichia coli*.[12] This insight led to the characterization of *rpoB* in *M. tuberculosis*,[13] and to the identification of a wide variety of mutations conferring RIF resistance in this species as well.[12]

All genotypic drug resistance strategies suffer from the fact that the molecular mechanisms explaining the resistance of anti-tuberculosis agents are not fully understood.[14] Hence identification of resistance-associated mutations is clinically informative, whereas lack of a mutation in the target sequence must be interpreted with considerable caution. In Egypt, there is no database for MDR-TB. We investigated mutations associated with anti-tuberculosis drug resistance among strains of *M. tuberculosis* isolated from patients at the Abbasia Chest Hospital, Cairo, Egypt, a major reference hospital with 900 beds and the largest case load of TB patients in Egypt. This hospital is considered to be a reference hospital for pulmonary diseases throughout the Middle East.

Materials and methods

Bacterial isolates and patient analysis

One hundred and fifty-five *M. tuberculosis* isolates were included in this study. They were isolated from 371 successive sputum specimens collected from symptomatic patients with pulmonary tuberculosis at Abbasia Chest Hospital, Cairo, Egypt. Two hundred and fifty-three isolates were from acute untreated cases and 118 were from chronic previously treated cases.

A PCR restriction fragment length polymorphism (RFLP) assay to identify a polymorphism in *oxyR* was performed to distinguish *Mycobacterium bovis* and *M. bovis* BCG from other members of the *M. tuberculosis* complex.[15]

Anti-tuberculosis drug susceptibility testing

Drug susceptibility testing was performed by a modified proportion method as described by Kent and Kubica using Middlebrook and Cohn 7H10 agar plates containing the following concentrations of each of five drugs: RIF (1.0 μg/ml), INH (0.2, 1.0, and 5.0 μg/ml), SM (2.0 and 10.0 μg/ml), EMB (5.0 μg/ml), and PZA (25.0 μg/ml).[16]

Strain typing

Strain typing was performed at the Mycobacteriology Laboratory Branch, Centers for Disease Control and Prevention, Atlanta, Georgia, USA, using a standardized RFLP method. A chemiluminescence-labeled amplification product of the insertion element IS6110 was used as a genomic probe.[17]

PCR-based single-strand conformational polymorphism (SSCP) analysis

Crude lysates containing genomic DNA for use as templates for PCR were prepared from Middlebrook 7H9 broth cultures by disruption of cells with siliconized glass beads as previously described.[18] Regions of *rpoB, katG, rpsL,* and *embB* were PCR-amplified using previously described conditions and oligonucleotide primers (Table 1).[7,19,20]

The PCR products were analyzed for mutations using non-radioactive SSCP electrophoresis. Briefly, SSCP was performed by heating a mixture consisting of 5 μl (approximately 50 ng) of PCR product and 15 μl of deionized formamide at 95 °C for 4 min, followed by electrophoresis in 4—20% gradient acrylamide gels (Invitrogen Corp., Carlsbad, CA, USA) at 300 V for 1.75 h in Tris-borate-EDTA (TBE) buffer maintained at 13 °C.[20]

DNA sequencing and DNA sequence analysis

Sequencing of both strands of the PCR product was performed on an ABI373 sequencing instrument according to the protocol supplied by the manufacturer using the Big Dye™ Terminator Cycle Sequencing Ready Reaction kit (PE Applied Biosystems, Foster City, CA, USA).

Results

Anti-tuberculosis drug susceptibility results

Among the 155 cases, 121 (78.1%) were untreated newly diagnosed cases and 34 (21.9%) were re-treated cases. Drug susceptibility testing using the standard proportion method revealed that 79 (51.0%) isolates were susceptible in vitro to all anti-tuberculosis drugs tested; 62 of these isolates were from untreated patients and 17 isolates were from previously treated patients. Thirty-four (21.9%) isolates were mono-resistant to RIF, INH, SM, EMB, or PZA, and 33 of these isolates were from untreated patients. Twenty-one (13.6%) were mono-resistant to SM, three (1.9%) to RIF, six (3.9%) to INH, and two each (1.3%) to EMB and PZA. Twenty-five (16.1%) isolates were MDR (at least to RIF and INH), and 12 of these were from previously treated patients. These 12 MDR isolates accounted for 35.3% of the 34 isolates from previously treated patients as compared to 10.7% (13 MDR isolates among 121) isolates from previously untreated patients (Table 2). Seventeen isolates that were resistant to multiple drugs but not to both INH and RIF, included five isolates (3.2%) that were resistant to SM and RIF, two (1.3%) to INH and EMB, seven (4.5%) to INH and SM, one (0.7%) to SM, RIF, and EMB, one (0.7%) to PZA, SM, RIF, and EMB, and one (0.7%) to PZA and SM. Thirteen of these isolates were from previously untreated patients. All INH-resistant isolates were resistant to either 0.2 μg/ml or 1.0 μg/ml, and none was resistant to 5 μg/ml, INH. The six PZA-resistant isolates were tested by *oxyR* PCR-RFLP and none was identified as *M. bovis*.

Strain typing

There were no strain clusters among the 25 MDR-TB isolates as determined using IS6110 RFLP analysis. Twenty-five unique banding patterns with from 5 to16 copies of IS6110 were observed.

Mutations

The gene regions shown in Table 1 were examined using DNA sequence analysis and SSCP electrophoresis, and 41 mutations were found among the 25 MDR-TB strains (Table 3). The *rpoB* resistance determinant region (RRDR) was examined in 25 RIF-resistant MDR isolates, *katG* in 17 INH-resistant MDR isolates, *rpsL* in 18 SM-resistant MDR isolates, and *embB* in 11 EMB-resistant MDR isolates (Table 3). Amino acid substitutions in *rpoB* were found in 19 isolates (76%) with the most common being Ser531Leu, which was found in 13 of the 19 *rpoB* mutants. Amino acid substitutions in *katG* were identified in eight of 17 isolates examined (47.1%) all of which involved the *katG*315 codon. All of the 17 isolates examined were resistant to 1 μg/ml INH, but were susceptible to 5 μg/ml. Four Lys43Arg and six Lys88Arg substitutions were identified in 10 of 18 (55.6%) SM-resistant isolates, all of which were resistant to 10 μg/ml SM. No *rpsL* mutations were found among eight isolates with low-level SM resistance (2 μg/ml $<$ minimum inhibitory concentration (MIC) $<$ 10 μg/ml). Mutations in *embB* that cause substitutions at codon 306 were found in four of 11 EMB-resistant isolates (36.4%). The SSCP band patterns of the nine mutations shown in Table 3 were different compared with wild-type SSCP patterns (Figure 1).

Discussion

Patients in this study were all from Abbasia Chest Hospital, which serves patients from all regions of Cairo as well as

Table 1 Genome regions examined for mutations.

Drug	Gene	Nucleotide	Codons	Size (bp)	Reference
RIF	*rpoB*	2336—2463	500—541	128	Miller et al. (13); Cooksey et al. (20)
INH	*katG*	725—1047	243—349	321	Abbadi et al. (7)
EMB	*embB*	7771—8047	270—362	276	Sreevatsan et al. (32)
SM	*rpsL*	4—310	2—103	306	Honore and Cole (37)

RIF, rifampin; INH, isoniazid; EMB, ethambutol; SM, streptomycin.

Table 2 Antimicrobial resistance patterns among 155 *Mycobacterium tuberculosis* isolates from Abbasia Chest Hospital, Cairo, Egypt.

	Resistance patterns					Previous treatment					
						No		Yes		Total	
	PZA	SM	RIF	INH	EMB	*n*	%	*n*	%	*n*	%
Fully susceptible (*n* = 79)	–	–	–	–	–	62	51.24	17	50	79	50.97
SDR (*n* = 34)	–	+	–	–	–	20	16.53	1	2.94	21	13.55
	–	–	+	–	–	3	2.48	0	0	3	1.94
	–	–	–	+	–	6	4.96	0	0	6	3.87
	–	–	–	–	+	2	1.65	0	0	2	1.29
	+	–	–	–	–	2	1.65	0	0	2	1.29
MDR (*n* = 25)	–	–	+	+	–	4	3.31	1	2.94	5	3.23
	–	+	+	+	–	1	0.83	2	5.88	3	1.94
	+	+	+	+	–	0	0	1	2.94	1	0.65
	–	–	+	+	+	1	0.83	1	2.94	2	1.29
	–	+	+	+	+	6	4.96	7	20.59	13	8.39
	+	+	+	+	+	1	0.83	0	0	1	0.65
ODR (*n* = 17)	–	+	+	–	–	3	2.48	2	5.88	5	3.23
	–	–	–	+	+	1	0.83	1	2.94	2	1.29
	–	+	–	+	–	6	4.96	1	2.94	7	4.52
	–	+	+	–	+	1	0.83	0	0	1	0.65
	+	+	+	–	+	1	0.83	0	0	1	0.65
	+	+	–	–	–	1	0.83	0	0	1	0.65
		Total				121	100	34	100	155	100

+, resistant; –, susceptible; PZA, pyrazinamide; SM, streptomycin; RIF, rifampin; INH, isoniazid; EMB, ethambutol; SDR, single drug resistance; MDR, multidrug resistance; ODR, other drug resistance.

other nearby governorates in Lower Egypt. This makes our data a likely representation of the genetic makeup of strains throughout this region of Egypt. All of the 155 cases in our study were directly observed therapy (DOT) patients. This and other treatment practices at Abbasia that could influence antimicrobial resistance do not differ from other regions in Egypt.

The patients included 121 who had never received anti-tuberculosis therapy and for whom primary resistance is more likely, as well as 34 who had received anti-tuberculosis therapy and for whom secondary resistance is more likely. Multidrug resistance (resistance to at least RIF and INH) was found in 25 isolates (16.1%), and additional resistance to SM, EMB, and PZA was found in 18, 16, and two isolates, respectively. Thirteen MDR isolates (10.7%) were from newly diagnosed untreated cases and 12 (35.3%) were from re-treated cases. We found single drug resistance in 34 of 155 isolates (21.9%), which is less than in a previous study of TB in Egypt in which resistance to a single drug was found in 18 of 50 isolates (36%).[21] We also observed lower percentages of isolates that were resistant to two drugs or three drugs than in the previous study in which 13 isolates (26%) were resistant to two drugs and two isolates (4%) were resistant to three drugs. However, none of the 50 isolates in the previous study was

Table 3 Mutations among 25 multidrug-resistant *Mycobacterium tuberculosis* strains.

Drug	Gene (number tested)	Number of strains	Amino acid change	Nucleotide change
Rifampin	*rpoB* (25)	4	Asp516Val	GAC→GTC
		13	Ser531Leu	TCG→TTG
		2	His526Cys	CAC→TGC
		6	wt	wt
Isoniazid	*katG* (17)	7	Ser315Thr	AGC→ACC
		1	Ser315Arg	AGC→AGA
		9	wt	wt
Streptomycin	*rpsL* (18)	4	Lys43Arg	AAG→AGG
		6	Lys88Arg	AAG→AGG
		8	wt	wt
Ethambutol	*embB* (11)	2	Met306Ile	ATG→ATA
		2	Met306Val	ATG→GTG
		7	wt	wt

wt, wild type.

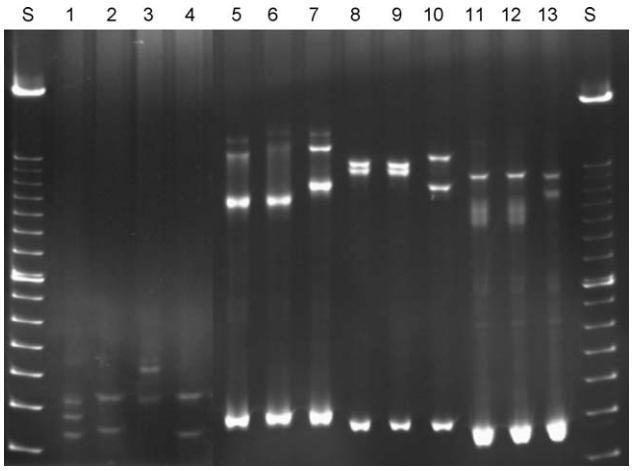

Figure 1 Single strand conformation polymorphism patterns of multidrug-resistant *Mycobacterium tuberculosis* isolates from Abbasia Hospital, Cairo, Egypt. PCR products (Table 1) were denatured and electrophoresed in 4—20% gradient acrylamide gels for 1.75 h, 300 V, 13 °C. Lane 1, *rpoB* mutant (Asp516Val); lane 2, *rpoB* mutant (Ser531Leu); lane 3, *rpoB* mutant (His526-Tyr); lane 4, *rpoB*wt; lane 5, *katG* mutant (Ser315Thr); lane 6, *katG* mutant (Ser315Arg); lane 7, *katG*wt; lane 8, *rpsL* mutant (Lys43Arg); lane 9, *rpsL* mutant (Lys88Arg); lane 10, *rpsL*wt; lane 11, *embB* mutant (Met306Ile); lane 12, *embB* mutant (Met306-Val); lane 13, *embB*wt; lane S, 100-bp size standard.

resistant to four drugs, even though primary resistance to SM was reported in 22 isolates (44%), to INH in 16 isolates (32%), and to RIF in nine isolates (18%).[21]

The overall prevalence of resistance among our isolates was lower than that reported in 1996 by the Egyptian National Tuberculosis Program (ENTP) for pulmonary isolates.[22] Among 250 isolates tested in the ENTP study, 55.2% were resistant, compared to 49.0% of our isolates. Resistance to INH, however, was more common among our isolates (25.8%) than among the ENTP isolates (6.4%).

We identified *rpoB* mutations in 76% of the 25 RIF-resistant isolates, which is consistent with the results of Abbadi et al., who found mutations in 73% of RIF-resistant isolates.[7] However, previous studies have reported higher correlations of *rpoB* mutations with RIF resistance, and this inconsistency may warrant additional investigation.[8,23] We found three distinct *rpoB* mutations among 19 MDR isolates, all of which were reported previously among Egyptian isolates, and globally as well.[7,23,24]

The role of *katG* in mediating susceptibility to INH has been established.[25,26] Investigators have reported that many (approximately 50—60%) INH-resistant isolates have missense mutations or small deletions or insertions in *katG* that are not present among INH-susceptible control strains.[27] Amino acid substitutions in the *katG*315 codon are more abundant than other mutations among INH-resistant strains.[28] Among the 17 MDR-TB isolates we studied, eight (47.1%) had mutations at codon 315 in *katG*; seven (87.5%) had Ser315Thr (AGC→ACC) substitutions, one (12.5%) had a Ser315Arg (AGC→AGA) substitution, and nine had no detectable mutations in the region of *katG* we studied. Our results agree with the study by Abbadi et al. who found that 40% of the INH-resistant isolates had mutations in *katG* (all Ser315Thr).[7] Gomaa identified *katG* mutations in 77.8% of INH-resistant isolates.[23] Musser et al. studied INH-resistant strains from global sources, and identified a *katG*315 missense substitution in 58% of epidemiologically associated isolates.[28] Also, Haas et al. reported that 68% of INH-resistant strains from Africa had codon 315 missense changes (most often Ser315Thr substitutions), and Dobner et al. found that 26 of 27 (96%) INH-resistant isolates from Germany and Sierra Leone also had codon 315 mutations.[29,30] Statistically, the most common amino acid substitution is AGC (Ser)→ACC (Thr), but substitution of Ser with Arg, Asn, Ile, or Gly has also been reported.[26] It is likely, however, that mutations were present in other genes that we did not evaluate (e.g., *inhA*, and other *katG* regions).[31] In this study INH-resistant strains with MICs between 0.2 and 1 μg/ml had *katG*315 amino acid substitutions. Previous reports have failed to identify amino acid substitution strains with low level INH MICs (1—2 μg/ml).[31]

The 25 MDR-TB isolates included 18 SM-resistant isolates and two distinct mutations were identified in ten of these (55.6%). Four of these (40%) had mutations at codon 43 (Lys→Arg; AAG→AGG), and six (60%) had mutations at codon 88 (Lys→Arg; AAG→AGG). Our findings are in agreement with previous studies of SM-resistant strains by Sreevatsan et al. and by Abbadi et al. who identified that most missense mutations occur at either codon 43 or codon 88 of *rpsL*.[7,32]

Mutations in *rpsL* were detected in 10 isolates with high-level SM resistance (MIC $>$ 10 μg/ml), but not in eight isolates with low-level SM resistance (2.0 $<$ MIC $<$ 10 μg/ml). This finding agrees with Cooksey et al. who analyzed 45 SM-resistant isolates for phenotypic resistance and mutations, and found that all 24 high-level (MIC $>$ 500 μg/ml) resistant isolates had Lys43Arg changes in *rpsL* and that among the low level (MIC $<$ 10 μg/ml) SM-resistant isolates, one had a C→G substitution at position 903 of the 16S rRNA gene (*rrs*) and none had *rpsL* mutations.[33]

EMB resistance is most often found in association with mutations at codon 306 of *embB* that cause Met to Ile or Met to Val substitutions.[34] In our study, 16 of the 25 MDR-TB were EMB-resistant, and two distinct mutations in *embB* codon 306 were found in four isolates (36.4%). Two isolates each had Met to Ile or Met to Val amino acid substitutions. These mutations have previously been reported by Telenti et al., Sreevatsan et al., and by Abbadi et al.[7,32,35]

We evaluated the 155 study isolates for in vitro susceptibility to PZA even though this test is not routinely performed in Egyptian mycobacteriology laboratories and PZA is not routinely used in clinical practice in Egypt. Six isolates (3.9%) were PZA-resistant; two of these were resistant only to PZA, two were MDR-TB, and all six were determined to be *M. tuberculosis* complex species other than *M. bovis* using *oxyR* PCR-RFLP. Genotypic susceptibility testing for the six PZA-resistant isolates was not done since the array of mutations in *pncA* is so diverse and there are no convenient genotypic assays yet available for screening for PZA resistance.[36]

Conflict of interest: No conflict of interest to declare.

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