Characterization of *rpoB* Mutations in Rifampin-Resistant Clinical Isolates of *M. tuberculosis*.

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ABSTRACT

Drug resistant strains of *M. tuberculosis* pose serious public health problem in Egypt. Inadequate treatment regimens and inefficient laboratory diagnosis of drug-resistant strains are likely major contributors to these problems. Mutations of the *rpoB* gene associated with rifampin resistance were studied in 25 rifampin resistant clinical strains of *Mycobacterium tuberculosis* isolated in Egypt. Of the 25 resistant isolates, 17 had a mutation in the 81 bp region of the *rpoB* gene by single strand conformation polymorphism (SSCP) and DNA sequencing. Only two resistance patterns could be detected by SSCP analysis. Sequence analysis revealed that Ser_{531}→Leu arose most frequently missense mutation (76%) followed by Asp_{516}→ Val (24%). The sensitivity and specificity of the SSCP result were 100% compared with that of the DNA sequencing. These results suggest that SSCP is an efficient method for predicting rifampin resistance which would reduce the time required for susceptibility testing from 4-8 weeks to few days. It is useful for rapid screening of rifampin resistance in isolates of *M. tuberculosis*.

INTRODUCTION

The management of tuberculosis depends not only upon accurate diagnosis but also upon appropriate therapeutic decision. Conventional methods such as BACTEC and agar proportion may provide susceptibility results in 4 to 21 days starting with pure cultures of *M. tuberculosis*. Mutations in specific bacterial genes have been associated recently with clinical resistance to anti-tuberculosis drugs. The identification of these mutations may be useful in the development of rapid screens for drug resistance, providing results in 1-2 days [1].

The frequency of resistance to multiple drugs varies geographically, and acquired (secondary) resistance is more common than primary resistance. 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%, 25% for INH, 17.5% for ethambutol (EMB) and 12.5% for RIF [2]. Abbadi *et al.* studied 25 *M. tuberculosis* isolates the from Assuit region in Egypt and found 11 (44%) MDR isolates, but these patients had undergone at least one year of prior TB therapy [3]. MDR-TB strains could arise as a consequence of sequential accumulation of mutations conferring resistance to single therapeutic agent, by a single-step process such as acquisition of an MDR element, or mutation that alters cell wall structure affecting drug uptake [4]. Several methods such as direct sequencing of PCR products, SSCP analysis, base pair-mismatch assay, a reverse hybridization-based line probe assay, and other strategies, are designed to exploit the observation that specific polymorphisms found in resistant strains are absent in susceptible organisms. All genotypic drug resistance strategies suffer from the fact that the molecular mechanisms explaining the resistances of antituberculosis agents are not fully understood [5]. Hence identification of resistance-associated mutations is clinically informative whereas lack of a mutation in the target sequence must be interpreted with considerable caution.

Rifampin (RIF) was proved to be effective anti-tuberculosis agent against susceptible strains as well as strains resistant to INH or SM. RIF binds to the beta subunit of DNA-dependent RNA polymerase and leads to abortive inhibition of transcription. Mutations in an 81 bp region of the *rpoB* gene (encoding the beta subunit of DNA-dependent RNA polymerase) have been found in ~95% of RIF-resistant *M. tuberculosis* [6].

This study aimed at detecting MDR-MTB by the conventional agar proportion method and a DNA-based method, which is the Single Strand Conformation Polymorphism (SSCP) and to characterizing mutations associated with drug resistance by DNA sequencing.

MATERIALS & METHODS

Strains: *M. tuberculosis strains:* Twenty five RIF^r *M. tuberculosis* isolates from patients with pulmonary TB, attending Abbasia Chest Hospital, Cairo.

Drug susceptibility testing: Drug susceptibility testing was performed by a modified proportion method as described by Kent and Kubica [7] using Middlebrook and Cohn 7H10 agar plates containing rifampin (RIF) (1.0 µg/ml). Additional drug susceptibility were tested for the following drugs: isoniazid (INH) (0.2, 1.0 and 5.0 µg/ml), streptomycin (SM) (2.0 and 10.0 µg/ml), ethambutol (EMB) (5.0 µg/ml), pyrazinamide

(PZA) (25.0 µg/ml), and rifabutin (RBT)(2.0 µg/ml).

PCR-based single-strand conformational polymorphism (PCR-SSCP) analysis: Crude lysate containing genomic DNA was prepared for use as templates for PCR. Cells grown in Middlebrook 7H9 broth cultures were lysed by disruption with siliconized glass beads as previously described [8]. Regions of the *rpoB* gene from nucleotide 2335 to 2463 encompassing the 81 bp region associated with RIF resistance in *M. tuberculosis* were amplified by PCR using oligonucleotide primer BC 55 (CGC CGC GAT CAA GGA GTT CT) and primer BC 56R (5` GCT CAC GTG ACA GAC CGC CG). These primers amplify a 128 bp nucleotide sequence on *rpoB* gene (2336-2463). Amplification reaction mixture (50 µl) contained 5µl of 10X *Taq* polymerase buffer, 2.5 unit *Taq* polymerase, 0.2mM of each dNTP's, 1 µl of DNA template and 3 µl of each primer [9].

SSCP Electrophoresis: PCR products used for the polyacrylamide gel electrophoresis-SSCP analysis were denatured with methyl mercury hydroxide in 20µl volume (5µl PCR product, 0.4 µl 1M methylmercury hydroxide, 1 µl loading buffer, and 13.6µl 1XTBE buffer), electrophoresed through Novex 4-20% vertical gradient acrylamide gel (Novex Corp., San Diego, CA, USA) at 300 V for 45 min at 13°C [9].

DNA sequencing and DNA sequence analysis: Sequencing of both strands of the PCR product was performed on an ABI373 sequencing apparatus according to the protocol supplied by the manufacturer using the Big DyeTM Terminator Cycle Sequencing Ready Reaction Kit (PE Applied Biosystems, Foster City, CA).

Cycle sequencing of PCR products: was done using ABI PRISM BigDye Terminator Cycle Sequencing ready reaction kit (PE Applied Biosystems) which contained: Ampli-*Taq* polymerase, $MgCl_2$, Tris-HCl pH 9, dNTP's, and terminator dye containing A,C,G, and T–terminator labeled with d-Rhodamine acceptor dyes. A master mix was prepared from 3.2 ul (1 uM) of each primer, 4 ul terminator mix, 2 ul 5X sequencing buffer, 1 ul PCR product and 9.8ul distilled water in a final volume of 20 ul. Thermocycling started by initial denaturation at 96°C for 5 min, followed by 25 cycles each included denaturation at 96°C for 10 sec, annealing at 50°C for 5 sec, and extension at 60°C for 4 min. PCR products were purified from the unincorporated dyedeoxy-terminators by a spin column kit as instructed by the manufacturer (Princeton Separations Inc., Adelphia, NJ). The purified samples were dried in a speed vacuum and stored at 4°C.

Polyacrylamide gel electrophoresis: The polyacrylamide solution was freshly prepared by adding 40g urea, 12 ml 40% acrylamide (BioRad), 27 ml water and 1 gm mixed bed ion-exchange resin (BioRad). The solution was filtered and 80 ml 10x TBE buffer were added (0.89 M Tris, 0.89 M Boric acid, 0.02M EDTA, pH 8.3) followed by 400 ul ammonium persulfate (Boehringer-Mannheim) and 45 ul TEMED (N,N,N`,N`-tetramethyl ethylenediamine, BioRad) to polymerize the gel.

DNA sequencing: sequencing of both strands of the PCR product was performed with an ABI373 sequencing apparatus according to the protocol supplied by the manufacturer and with the Big Dye Terminator Cycle Sequencing Ready Reaction kit (PE Applied Biosystems). The gel and buffer chambers were installed in the sequencer. To each sample, 4.5 ul of resuspension buffer were added (5:1 mixture of deionized formamide and 25 mM EDTA containing 50 mg/ml Blue Dextran). Samples were heated to 90°C for 2 min followed by sudden chilling on ice. The gel was loaded (2 ul/sample) and allowed to run for 16 h, visually inspected to assess the overall quality of the sequencing run. Mutations were detected using Applied Biosystems Sequence Navigator software package. An asterisk appeared at any base that differed from the wild type sequence, locating the site of a mutation.

RESULTS

Drug susceptibility testing using the standard proportion method revealed that among the RIF^r isolates, there were 7 patterns of susceptibility to 5 additional drugs tested (INH, SM, EMB, PZA, and RBT). All isolates were considered MDR since they were resistant to INH and RIF (Table 1).

Only two SSCP patterns were being identified from all RIF^r isolates (Figure 1). SSCP results divided the 25 RIF^r isolates into 2 main groups: Group I: 8 isolates (32%) with SSCP pattern identical to the wild type (wt) strain H37Rv, Group II: 17 isolates (68%) with a SSCP pattern different from the wt. The *rpoB* resistance determinant region sequence (RRDR) was examined in the 25 RIF^r MDR isolates. DNA sequencing revealed amino acid substitutions in *rpoB* gene of 17 isolates (68%) with the most common being Ser_{531} Leu, detected in 13 of the 17 *rpoB* mutants, while Asp_{516} Val mutation was found in 4 isolates (Table 2 and Figure 1)

Table (1): Resistance pattern to different drug combination among *M. tuberculosis* strains.

Resistance to:	N°. of Isolates	%
RIF + INH	5	20%
RIF + INH+SM	4	16%
RIF + INH+SM + EMB	10	40%
RIF + INH+SM + EMB +RBT	2	8%
RIF + INH+SM + RBT+ PZA	1	4%
RIF + INH+ EMB	2	8%
RIF + INH+SM + PZA + EMB	1	4%
Total	25	100%

RIF: rifampin; INH: isoniazid, SM: streptomycin, PZA: pyrazinamide, EMB: ethambutol, RBT: rifabutin (RBT).

Table (2): Mutations in the *rpo*B gene detected in 25 RIF^r isolates

No of Isolates (%)	Codon	Amino acid changes	Nucleotide mutation	SSCP
4 (16)	516	Asp → Val	GAC→ GTC	M
13 (52)	531	Ser → Leu	TCG → TTG	M
8 (32)		No change		WT

Asp: aspartate; Val: valine; Ser: serine; Leu: leucine; M: mutant pattern; WT: wild-type pattern.

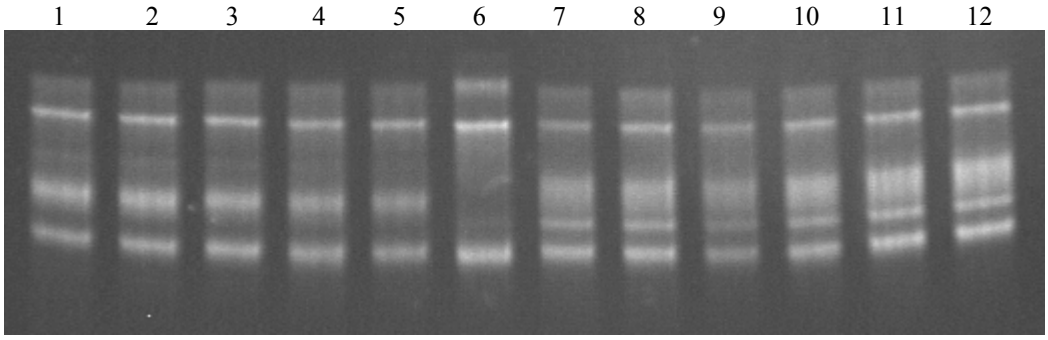

Figure (1): SSCP pattern of the rifampin-resistant *M. tuberculosis* isolates
Lane 1: wild type reference strain as a control; Lanes 2-5: wild type *M. tuberculosis* isolates; Lane 6: mutant RIF-resistant *M. tuberculosis* isolate (SSCP pattern A); Lanes 7-12: mutant RIF-resistant *M. tuberculosis* isolates (SSCP pattern B).

DISCUSSION

Drug resistant strains of *M. tuberculosis* pose serious public health problem in Egypt. Inadequate treatment regimens and inefficient laboratory diagnosis of drug-resistant strains are likely major contributors to these problems [3]. Early diagnosis of this disease and rapid identification of resistance to primary anti-TB agents are essential for efficient treatment and control of MDR strains.

Patients in this study were all from Abbasia Chest Hospital which serves patients from all regions of Cairo as well as other nearby governorates in upper Egypt. This makes our data a likely representation of the genetic makeup of strains throughout this region of Egypt. RIF is one of the most potent anti-TB drugs, therefore, resistance to RIF often results in high clinical relapse rates, particularly if RIF resistance is associated with resistance to other anti-TB drugs [10]. In this study, 25 RIF^r *M. tuberculosis* isolates characterized by the phenotypic drug susceptibility test were genetically identified by using SSCP and DNA sequencing techniques. Only 17 isolates were correctly identified as RIF^r by SSCP and DNA sequencing. The discrepancy between the results of SSCP and agar proportion method could be explained by the fact that SSCP only identifies single polymorphism in PCR products and is incapable of identifying resistance conferred by any alternative mechanism [4]. Conversely, the phenotypic conventional method detects drug

resistance regardless of the resistance mechanism. PCR-SSCP is a promising, inexpensive and easy to perform technique for monitoring emergence of drug resistance. Although molecular genetic testing can't as yet fully replace traditional phenotypic susceptibility testing, a molecular genetic test might be a good alternative for drug resistance screening test in the context of MDR DOTs-plus strategy. Although ~95% of RIF^r is due to *rpoB* mutations [11], mutations were only detected in 68% of the isolates in this study. This might be due to mutations present in another part of *rpoB* gene or the presence of other gene implicated in RIF resistance.

Our results revealed two main kinds of point mutations in the RIF^r *M. tuberculosis* Egyptian isolates by DNA sequencing. SSCP results were 100% in agreement with DNA sequencing results in this study. Mutations were identified only in 68% of 25 RIF^r isolates. These findings are consistent with the results of Abbadi *et al*. [3] who found mutations in 73% of RIF^r Egyptian isolates. Meanwhile, our results are inconsistent with the results of Ramaswamy & Musser [4] and Gomaa [12] who reported that the percentage of *rpoB* mutations among RIF^r isolates were >90% and 86.7%, respectively. Two different mutations were identified among 17 RIF^r isolates. Four isolates (16%) had substitution at codon 516 (GAC→ GTC; $Asp_{516}Val$) and 13 isolates (52%) had substitutions at codon 531 (TCG→TTG; Ser_{531} Leu). In a previous study in Shandong, China, 57% of RIF^r isolates contained codon 531 mutation, while 7% showed mutation in 516 codon while 36% of isolates showed mutations in six other codons [13]. In Taiwan, codons 531 and 516 mutants were detected in 49% and 8.6% of 162 RIF^r isolates, among 11 other codons [14]. In Brazil, seven different kinds of nucleotide substitution were revealed in four codons of the *rpoB* gene. The mutations were located in codon 531 (58%) and in codon 526 (26%). Mutations in codons 522 (5%) and 533 (5%) have also been detected [15].

All *rpoB* gene mutations appeared in this work were reported previously among Egyptian isolates [3,12] and globally as well [13, 16]. Such similarity in gene mutations among Egyptian RIF^r isolates may necessitate continuous gene-mutation monitoring, as well as isolates subtyping to detect -and hence controlling- possible endemic foci throughout the country.

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دراسة الطفرات في الجين "اربى او بى" للسلالات الإكلينيكية من بكتيريا الدرن المقاومة لعقار الريفامبيسين

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يعتبر مرض الدرن احد اهم المشكلات المتعلقة بالصحة فى مصر بعد مرض البلهارسيا ويعتبر وجود سلالات مقاومة لعقار الريفامبين من اهم الدلائل على وجود سلالات مقاومة للعديد من المضادات الحيوية الاخرى. هذه الدراسة قد اجريت على ٢٥ سلالة مصرية تم تعريفها بأنها سلالات مقاومة لعقار الريفامبين عن طريق اختبار الاجار التناسبى. واوضحت النتائج انه عند اجراء اختبار التعدد الشكلى لشريط الحمض النووي د.ن. أ الفردى " اس اس سى بى" وجود عدد ١٧ سلالة (٦٨%) مقاومة لعقار الريفامبين موجودين فى نمطين مختلفين من بين الخمس وعشرين سلالة المختبرة سابقا بواسطة اختبار الحساسية.

وعند مقارنة النتائج السابقة بطريقة التتابع الجينى القياسية والتى تعتبر ادق الطرق الجينية للتعرف على الطفرات، لوحظ وجود ١٧ سلالة بها `طفرات فى جين " أربى أو بى". هذا وقد بينت النتائج وجود ١٣ سلالة (٧٦%) بها طفرات فى الكودون ٥٣١ وادت الى تغيير الحمض الامينى سيرين الى الحمض الامينى ليوسين وكذلك وجود عدد ٤ سلالات (٢٤%) بها طفرات فى الكودون ٥١٦ حيث ادت الى تغيير الحمض الامينى اسبراجين الى فالين.

وق وجد ان النمطين الموجودين فى اختيار التعدد الشكلى لشريط ال د. ن. أ. الفردى " أس. أس. سى. بى" على علاقة وثيقة بالطفرات المكتشفة بواسطة اختبار التتابع الجينى حيث وجد ان النمط (أ) مماثل لما تم تحديده بوجود طفرة فى الكودون ٥٣١ (سيرين--> ليوسين) اما النمط (ب) فمماثل لما تم تحديده بوجود طفرة فى الكودون ٥١٦ (اسبراحين --> فالين).

ومما سبق يمكننا استنتاج ان طريقة التعدد الشكلى قد اثبتت فاعليتها فى اكتشاف مقاومة ميكروب الدرن المرتبطة بالطفرات الجينية. وعلى ذلك فهى طريقة سريعة وسهلة ويمكننا الاعتماد عليها فى التعرف على مقاومة ميكروب الدرن للعديد من العينات فى خلال يومين بدلا من طريقة حساسية الدرن للادوية التى تستغرق من ٤-٦ اسابيع.