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Genotypic detection of rifampicin-resistant *M. tuberculosis* strains in Syrian and Lebanese patients

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Summary
Setting: The incidence of multi- and extensively drug-resistant TB cases is increasing in many countries. Resistance to rifampicin is widely considered a surrogate marker for multiple drug resistant TB. No efforts have been made to identify and quantify the drug-resistant genotypes in the Syrian and Lebanese communities.
Objective: The genotypic characterization of rpo B mutations in the rifampicin drug-resistance region (RRDR) of resistant *Mycobacterium tuberculosis* isolates in Syrian and Lebanese patients.
Design: The pyrosequencing technique was applied to DNA derived from the *M. tuberculosis* isolates of 56 patients.
Results: RRDR sequencing identified 97 modified codons representing 35 different mutations; 31 (34%) of the 97 modifications were novel and have not been previously reported. The changes were mostly within codons 531 (37/97: 38%), 533 (28/97: 29%) and 526 (9/97: 9%). Additionally, 30 (54%) isolates had multiple codon changes.
Conclusion: This study indicates the importance of the RRDR hotspot region for the detection of rifampicin resistance in MTB clinical isolates from Syrian and Lebanese patients. However, new mutations and mutations in other locations within the RRDR were also observed. The vast majority (95%) of the studied isolates from this pool of patients contained mutations in codons 531 and/or 533.

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Introduction

Approximately 95% of all of tuberculosis cases occur in developing countries, where the disease has typically remained endemic [1]. In recent years, a dramatic increase in the number of cases of drug-resistant infections has occurred. The number of multi- and extensively drug-resistant cases (MDR, XDR) was estimated to be approximately 440,000 in 2008, with 150,000 deaths [2]. MDR TB is thought to emerge in patients either through exogenous infection by resistant strains or through the endogenous emergence of mutations due to suboptimal treatment [3,4]. The treatment of resistant TB is medically difficult, economically expensive and has adverse health effects for patients [5,6]. Despite extensive treatment measures, levels of mortality are still high. However, mortality has decreased significantly [7] in recent years following the introduction of several measures, including the application of molecular diagnostic techniques [8], strain identification [9] and the investigation of transmission [10,11].

The combination of rifampicin and isoniazid is the backbone of first-line and short-course chemotherapy. Rifampicin, a macrocyclic antibiotic, targets mycobacterial DNA-dependent RNA polymerase, a complex oligomer composed of four different subunits (α, β, β' and σ, which are encoded by rpo A, rpo B, rpo C and rpo D, respectively). Rifampicin binds specifically to the rpo B-expressed subunit and suppresses the initiation step of transcription [12]. Resistance to rifampicin results from spontaneous mutations, which occur at a rate of 10^8. These mutations have been widely shown to localize to the rpo B region, primarily in codons 507—533. This 81-bp region is called the RIF resistance-determining region (RRDR). Resistance to rifampicin is largely considered a surrogate marker for MDR TB due to its association with other drug resistance phenotypes [13].

Pyrosequencing technology has recently been used to characterize the genotypes of resistant tuberculosis strains [14—16]. Pyrosequencing chemistry differs in several aspects from standard sequencing. For example, fluorochromes and radioactivity are not used, and no postreaction step is required when using this technique [17]. The technology enables the rapid prediction of mutations and is suitable for the simultaneous screening of short sequences in large numbers of samples. It is therefore a proven, reliable and high-throughput assay for the rapid and specific analysis of rifampicin-resistant *M. tuberculosis* strains [18].

The presence of drug-resistant tuberculosis in Syria and Lebanon is known [19]. However, no efforts have been made to identify and quantify the drug-resistant genotypes in this community. In this study, pyrosequencing was used to fully characterize the RRDR mutations prevalent in *M. tuberculosis* isolates obtained from Syrian and Lebanese patients for the first time.

Materials and methods

Bacterial strains

A total of 56 clinical rifampicin-resistant *Mycobacterium tuberculosis* isolates (resistant) were selected. These clinical isolates were provided by the Medical Biotechnology Section of the National Commission for Biotechnology in Syria and the Azm Center for Research in Biotechnology and Its Applications at Lebanese University. The isolates were derived from 45 Syrian, 7 Lebanese and 4 Iraqi (living in Syria) patient samples collected between July 2003 and October 2005 from all Syrian and Lebanese provinces (muhafazat) [20,21]. The drug resistance pattern of the Syrian samples was previously established according to the recommendations of the National Committee for Clinical Laboratory Standards [21] and that of the Lebanese samples was also previously established [20]. All isolates were stored at −80 °C. The reference strain H37Rv (ATCC 25177) was used as a control for the wild-type sequence. The research was approved by the responsible institutional ethics committee.

DNA extraction

DNA extraction was performed with maximum precautions under a biosafety class two hood according to [20]. The isolates were incubated in a water bath at 80 °C for approximately 30 min to kill the bacteria and then centrifuged for 10 min at 8000 rpm. TE buffer containing 1% Triton X-100, 0.5% Tween 20, 10 mM Tris—HCl pH 8.0 and 1 mM EDTA was added to the pellet. The rest of the procedure was performed according to the instructions provided with the Qiagen DNA Blood Mini Kit (Qiagen, Germany) with one minor modification: the incubation period at 37 °C was 2 h instead of 90 min.

PCR

The primers used to amplify and sequence the rifampicin resistance-determining region (RRDR) were synthesized according to [22] by Thermo

Scientific, USA. One set of forward and reverse primers was used to amplify the target region. The size of the PCR product was 297 bp. The PCR reaction mixture consisted of the following: 1× PCR buffer, 2 mmol/L $MgCl_2$, 0.125 μmol/L of each nucleotide (dATP, dTTP, dCTP, and dGTP), and 1.5 U Taq polymerase (Sigma, Germany) in a total volume of 50 μL. DNA amplification was performed using a MyCycler Bio-Rad 96-well thermocycler (Bio-Rad, Germany), using the following cycling program: initial denaturation and enzyme activation 95 °C for 5 min; 40 cycles of three steps of 95 °C for 30 s, 63 °C for 30 s, and 72 °C for 30 s; and final extension at 72 °C for 5 min. PCR products (5 μL) were visualized on a 2% agarose gel stained with ethidium bromide under ultraviolet light using the ChemiDoc program (Bio-Rad, Hercules, CA, USA).

Pyrosequencing

Pyrosequencing was performed on all 56 clinical isolates and the ATCC25177 reference strain. PCR products were immobilized on streptavidin-coated Sepharose beads (GE, USA) to provide single-stranded DNA templates. The beads containing the immobilized templates were captured on a filter by vacuum filtration and were washed with 70% ethanol for 5 s. DNA was denatured by applying 0.2 M NaOH for 10 s and then washed for 5 s with 10 mM Tris-acetate, pH 7.6. The beads were subsequently transferred to a 96-well plate containing an annealing solution (38.4 μL) and the two sequencing primers (1.6 μL) (R1, R2) [22]. Two separate sequencing primers (R1, R2) (Thermo Scientific, USA) were used to sequence the relatively long sequence (81 bp) of interest within the amplified (297 bp) product. Pyrosequencing was performed using a PyroMark ID96 instrument, which is an automated PSQ 96 ID system (Qiagen, Germany), using the PSQ Gold 96 SQA reagent kit containing the enzyme. The reaction cascade consisted primarily of the incorporation of nucleotides into the growing DNA chain, culminating in the production of light. The pattern of light emitted in relation to the nucleotide dispensation order and the number of nucleotides incorporated was subsequently illustrated on a pyrogram. The mutations were detected based on a sequence comparison with the reference strain ATCC 25177. An internal control was also used to validate the results. The BLAST database was used to search for the obtained sequences, and a 90% minimal similarity match with the *M. tuberculosis* genome was obtained.

Results

Of the 56 rifampicin-resistant clinical isolates analyzed, 45 were from Syrian patients, 7 were from Lebanese (living in Lebanon) patients, and four were from Iraqi citizens (living in Syria). The pyrograms of the two sequenced rpoB regions (507—520 and 521—533) indicated the presence of 97 modified codons (Table 1) representing 35 different codon changes (Table 2). All resistant strains contained at least one non-synonymous codon change relative to the ATCC reference strain. One codon change was a consequence of a single base pair deletion. Five codon changes resulted in silent mutations through nucleotide substitutions, and the rest resulted in missense mutations. All silent mutations were accompanied by non-silent mutations. Codon changes occurred primarily at codons 531 (37/97: 38%), 533 (28/97: 29%) and 526 (9/97: 9%); only one, two or three codon changes were detected in each of the remaining codons. The 97 codon changes were distributed in the 56 tested isolates as indicated in Table 2. Isolate 56(S) had the greatest number of codon changes (5 codon changes). Eight isolates had three codon changes (8/56: 14%), and twenty-one isolates had two codon changes (21/56: 38%) (Table 1). The isolates with multiple codon changes generally included changes at codon 533 (26/30: 87%). The remaining isolates (26) had only one codon change (26/56: 46%), most commonly at codon 531 (22/26: 85%) (Table 1). Changes to codons 531 and 533 occurred in 53 patients (53/56: 95%). The mutation S531 L (TCG/TTG) was by far the most frequent (35 patients: 35/56: 63%), followed by L533R (CTG/CGT) (12 patients: 12/56: 21%), L533P (CTG/CCG) (7 patients: 7/56: 13%), L533R (CTG/CGG) (5 patients: 5/56: 9%) and H526D (CAC/GAC) (4 patients: 4/56: 7%) (Table 2).

Based on the information provided by the TB drug resistance mutation database [23], which lists all published mutations that have been associated with rifampicin resistance, 15 of the 30 different missense codon changes obtained (excluding silent codon changes) represent novel codon changes (50%). Most of the novel changes were located in codon 533. Novel codon changes represent 31 of the total 92 codon changes (34%) and were identified in 30 of the 56 patients (54%) (Table 2). The new codon changes at positions 529 and 532 indicate mutations at new locations.

The codon changes included (43) different base pair changes (nucleotide position or base) resulting in a total of 134 bp changes (63 transitions and 71 transversions). Of the 97 codon changes, 68 (70%) included a single base pair change, 22

Table 1 The patients location and resistance profile of *M. tuberculosis* strains, in relation to obtained rpoB RRDR codon changes.

PCR number	Location	RIf	INH	Strp	EMB	Mutation
ATCC 25177	U.S.A	S	S	S	S	No mutation
68(S)	Aleppo	R	R	R	R	531 TCG(S)→TTG(L) 533 CTG(L)→GGC(G)
57(S)	Aleppo	R	R	R	R	531 TCG(S)→TTG(L)
58(S)	Aleppo	R	R	R	R	522 TCG(S)→TTG(L)
39(S)	Aleppo	R	R	R	R	531 TCG(S)→TTG(L) 533 CTG(L)→CGT(R)
53(S)	Aleppo	R	R	R	R	526 CAC(H)→CAG(Q) 533 CTG(L)→CCG(P)
50(S)	Aleppo	R	R	R	R	525 ACC(T)→ATC(I) 531 TCG(S)→TTG(L)
28(S)	Aleppo	R	R	R	R	526 CAC(H)→CGC(R) 528 CGC(R)→ACG(T) 533 CTG(L)→CCG(P)
29(S)	Aleppo	R	R	S	R	G deletion Position 15 533 CTG(L)→CCG(P)
31(S)	Aleppo	R	R	R	R	531 TCG(S)→TTG(L) 533 CTG(L)→CGT(R)
32(S)	Aleppo	R	R	R	R	531 TCG(S)→TTG(L) 533 CTG(L)→CGT(R)
13(S)	Aleppo	R	R	S	S	531 TCG(S)→TTG(L)
77(S)	Aleppo	R	R	S	R	531 TCG(S)→TTG(L)
14(S)	Aleppo	R	R	R	R	526 CAC(H)→TGC(C) 533 CTG(L)→CGC(R)
83(S)	Aleppo	R	S	S	S	526 CAC(H)→GAC(D) 533 CTG(L)→CGT(R)
108(S)	Aleppo	R	R	R	R	531 TCG(S)→TTG(L)
104(S)	Aleppo	R	R	R	R	531 TCG(S)→TTG(L) 533 CTG(L)→CGG(R)
111(S)	Aleppo	R	R	R	R	531 TCG(S)→TTG(L)
118(S)	Aleppo	R	R	R	R	531 TCG(S)→ATT(I) 533 CTG(L)→CCG(P)
11(S)	Latakia	R	R	R	R	531 TCG(S)→TTG(L)
25(S)	Latakia	R	R	R	S	526 CAC(H)→CGC(R) 528 CGC(R)→ACG(T) 533 CTG(L)→CCG(P)
110(S)	Latakia	R	R	R	R	531 TCG(S)→TTG(L)
16(S)	Deir ez Zor	R	R	S	R	531 TCG(S)→TTG(L)
19(S)	Deir ez Zor	R	R	R	R	531 TCG(S)→TTG(L)
38(S)	Deir ez Zor	R	R	R	S	526 CAC(H)→CGC(R) 529 CGA(R)→CAA(Q) 533 CTG(L)→CGT(R)
20(S)	Homs	R	R	S	R	517 CAG(Q)→CCA(P) 531 TCG(S)→TTG(L) 533 CTG(L)→CGG(R)
66(S)	Homs	R	S	R	S	531 TCG(S)→TTG(L)
56(S)	Homs	R	R	R	R	512 AGC(S)→CTC(L) 513 CAA(Q)→CCA(P) 524 TTG(L)→CTC(L) 529 CGA(R)→CGC(R) 533CTG(L)→CGT(R)
36(S)	Hama	R	R	R	S	516 GAC(D)→CTC(L) 517 CAG(Q)→CAA(Q) 533 CTG(L)→CGG(R)
64(S)	Hama	R	R	R	R	531 TCG(S)→TTG(L) 53 3 CTG(L)→CGT(R)
33(S)	Damascus	R	R	R	S	531 TCG(S)→TTG(L) 533 CTG(L)→GCG(A)
49(S)	Damascus	R	R	R	R	531 TCG(S)→TTG(L)
115(S)	Damascus	R	R	R	R	531 TCG(S)→TTG(L)
82(S)	Damascus	R	R	R	R	516 GAC(D)→GTC(V)
96(S)	Damascus	R	R	R	R	526 CAC(H)→GAC(D 532 GCG(A)→GGC(G) 533 CTG(L)→CGG(R)
61(S)	Damuscus	R	R	R	S	531 TCG(S)→TTG(L)
65(S)	Damascus	R	R	R	R	531 TCG(S)→TTG(L)
69(S)	Damascus	R	S	R	S	511 CTG(L)→CCG(P) 533 CTG(L)→CGT(R)
51(S)	Al Hasakah	R	R	R	R	CAG(Q)→AGA(R) 531 TCG(S)→TTG(L)
62(S)	Al Hasakah	R	R	S	S	531 TCG(S)→TTG(L)
79(S)	Qamishli	R	R	R	R	TCG(S)→TTG(L) 532 GCG A)→CGC(R) 533 CTG(L)→TGG(W)
106(S)	AL Qamishli	R	R	R	R	513 CAA(Q)→CAG(Q) 533 CTG(L)→CCG(P)
70(S)	Quneitra	R	R	R	R	526 CAC(H)→GAC(D)
9(S)	Abu Kamal	R	R	S	S	531 TCG(S)→TGC(C)
60(S)	Rif Dimashq	R	R	R	R	512 AGC(S)→ACC(T) 531 TCG(S)→TTG(L)
42(S)	Adra Prison	R	R	S	S	533 CTG(L)→CGT(R)
40(I)	Iraq	R	R	R	R	531 TCG(S)→TTG(L)
43(I)	Iraq	R	R	R	R	531 TCG(S)→TTG(L) 533 CTG(L)→CGT(R)
97(I)	Iraq	R	R	R	R	531 TCG(S)→TTG(L)
44(I)	Iraq	R	R	S	R	531 TCG(S)→TTG(L)
6(L)	Dekweneh	R	R	S	S	512 AGC (S)→ TCG(S) 513 CAA (Q)→ CCA(P) 533 CTG(L) →CCG(P)
22(L)	ALhamam Al Askari	R	R	S	S	526 CAC(H) →GAC(D) 533 CTG(L)→CGG(R)
26(L)	MajdelAnjar	R	R	S	S	531 TCG(S) →TTG(L)
50(L)	HaretHreik	R	S	R	S	531 TCG(S) → TTG(L)
58(L)	Kafaat	R	R	R	S	522 TCG(S)→ TTG(L) 533 CTG(L)→ CGT(R)
60(L)	Sayfeh	R	R	S	S	531 TCG(S) → TTG(L)
61(L)	Mazraa	R	R	R	S	531 TCG(S) → TTG(L) 533 CTG(L)→ CGT(R)

S: Syrian sample, L: Lebanese sample, I: Iraqi national; R: resistance, S: sensitivity; (amino acid one letter symbol).

(23%) included two, and 7 (7%) included three base pair changes. It appears that 18 codon changes involved 2 bp inversions (Table 1). Most of the missense codon changes represented non-conservative amino acid replacements. The most frequent codon changes at position 531 involve a switch from a polar to a hydrophobic residue (S/L, I), while the changes at position 533 resulted in a switch from a hydrophobic to a charged residue (L/R). Several of the codon changes involved mutations to proline, a known secondary structure disrupter (Table 2).

Discussion

The fact that all isolates with phenotypic resistance to rifampicin used in this study exhibited amino acid changes in the RRDR region demonstrates the importance of the RRDR hotspot region in the resistance of clinical TB isolates in Syria. Several studies have indicated that this region is responsible for 90—95% of RIF-resistance cases [24]. However, many new mutations were identified in this study, and some were found at new locations

Table 2 The distribution of various codon changes among isolates.

Codon	Codon change	Patients (S: Syrian, L: Lebanese, I: Iraqi)
511	# G deletion	
	Position 15	29(S)
511	CTG(L) → CCG(P)	69(S)
512 si	AGC(S) → TCG(S)	6(L)
512	**#AGC(S) → CTC(L)**	56(S)
512	AGC(S) → ACC(T)	60(S)
513	CAA (Q) → CCA(P)	6(L) 56(S)
513 si	CAA(Q) → CAG(Q)	106(S)
516	**#GAC(D) → CTC(L)**	36(S)
516	GAC(D) → GTC(V)	82(S)
517 si	CAG(Q) → CAA(Q)	36(S)
517	**#CAG(Q) → AGA(R)**	51(S)
517	CAG(Q) → CCA(P)	20(S)
522	TCG(S) → TTG(L)	58(L) 58(S)
524 si	TTG(L) → CTC(L)	56(S)
525	ACC(T) → ATC(I)	50(S)
526	CAC(H) → GAC(D)	70(S) 83 (S) 96 (S) 22(L)
526	CAC(H) → CGC(R)	25(S) 28(S) 38(S)
526	CAC(H) → CAG(Q)	53(S)
526	CAC(H) → TGC(C)	14(S)
528	**#CGC(R) → ACG(T)**	25(S) 28(S)
529	**#CGA(R) → CAA(Q)**	38(S)
529 si	CGA(R) → CGC(R)	56(S)
531	TCG(S) → TTG(L)	26)L) 60(L) 61(L) 11(S) 13(S) 16(S) 19(S) 20(S) 31(S) 32(S) 33(S) 39(S) 40(I) 43(I) 44(I) 49(S) 50(S) 51(S) 57(S) 60(S) 61(S) 62(S) 64(S) 65(S) 66(S) 68(S) 77(S) 79(S) 97(I) 104(S) 108(S) 110(S) 111(S) 115(S) 50(L)
531	**#TCG(S) → TGC(C)**	9 (S)
531	**#TCG(S) → ATT(I)**	118(S)
532	**#GCG)A) → CGC(R)**	79(S)
532	**#GCG(A) → GGC(G)**	96(S)
533	**#CTG(L) → CGC(R)**	14(S)
533	**#CTG(L) → CGG(R)**	22(L) 20(S) 36(S) 96(S) 104(S)
533	**#CTG(L) → CGT(R)**	58(L) 61(L) 31(S) 32(S) 38(S) 39(S) 42(S) 43(I) 56(S) 64(S) 69(S) 83(S)
533	CTG(L) → CCG(P)	6(L) 25(S) 28(S) 29(S) 53(S) 106(S) 118(S)
533	**#CTG(L) → GCG(A)**	33(S)
533	**#CTG(L) → GGC(G)**	68(S)
533	**#CTG(L) → TGG(W)**	79(S)
Total different codon changes: 35	Total codon changes: 97	

Si: silent mutation; #: new mutation.
The bold signifies novel mutations as the sign # does.

within the RRDR. Notably, the vast majority of patients (95%) had mutations in codons 531 and/or 533. This could greatly reduce the expense and complexity of future early detection efforts in the local patient pool.

Earlier studies [24] have asserted the importance of codon positions 526 and 531 to the observed resistance. This is true also in neighboring countries, such as Turkey. A similar earlier study of the hotspot rpo B locus in isolates from Turkish patients primarily identified mutations at codons 531 (56.1%) and 526 (19.5%) [25]. Eight of the 14 different mutations observed in that study (57%) were present in our patient pool. The present study also emphasizes the frequency of codon changes at position 533. In clear contrast to previous reports [26,27], the majority of isolates in this study exhibited more than one codon change (2—5).

Many codon changes involved more than one base pair change. A significant portion appeared to involve a two-base pair inversion, while others were likely to involve multiple base pair

substitutions through point mutations. The high GC/AT ratio may contribute mechanistically to the mutability of this hot spot region.

Noticeably, codon changes at 533 were accompanied by other codon changes in almost all of the isolates (with one exception). Changes at this position are reported to result in variable resistance; therefore, additive resistance could be a significant resistance mechanism in these strains.

Some rpoB codon changes have been shown to cause cross-resistance to antibiotics other than rifampicin in *M. tuberculosis* isolates. Codon changes at 513, 526, and 531 are associated with high-level resistance to rifampicin and rifabutin. Codon changes at 514, 515, 516, 522, and 533 have been reported to cause rifampicin resistance concomitant with susceptibility or low resistance to rifabutin [28]. Thus, depending on the genotype, the use and disuse of other antibiotics (e.g., in second-line Tb drug treatment) can be suggested [28]. However, this conclusion depends on the assessment of the novel codon changes and the additive effects of multiple codon changes.

Despite the dominance of isolates with the genotype S531 L, the diversity of the isolate genotypes is striking. With respect to the 18 isolates obtained from Aleppo, 6 had the S531 L genotype, while the rest (12) had 9 different genotypes. This diversity is consistent with the lower exogenous transmission of resistant strains in Syria, which was suggested by a previous strain genotyping study [21].

One drawback of this study is the small number of Lebanese samples, which cannot be considered representative of the rpo B pool of mutations in Lebanon. Future comparisons with other neighboring countries await more extensive local studies of the rpoB sequence.

Conflict of interest

The authors have no competing interests to declare.

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