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Sequencing of the entire *rpob* gene and characterization of mutations in isolates of *Mycobacterium tuberculosis* circulating in an endemic tuberculosis setting

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ABSTRACT

Objective: To evaluate the use of a sequencing procedure for the entire *rpoB* gene of *Mycobacterium tuberculosis* to identify mutations pre-rifampicin resistance determining region (RRDR), within RRDR, and post-RRDR in isolates circulating in a region affected by tuberculosis (TB).
Methods: Five primers were designed, with which five DNA fragments of *rpoB* were obtained, sequenced by Sanger, and analysed in silico in order to identify mutations over the entire *rpoB* gene in rifampicin-sensitive and rifampicin-resistant TB.
Results: It was possible to analyse the entire *rpoB* gene in five rifampicin-sensitive and 15 rifampicin-resistant isolates. Thirty-six mutations were identified. Two mutations were found pre-RRDR, nine within-RRDR and 25 post-RRDR. The most frequent mutations within RRDR were S531L (53%), followed by S512T (20%), all of which were found in rifampicin-resistant isolates. Of the 25 mutations found post-RRDR, 14 were only in resistant isolates, and the most frequent was D853N, which was present in 85% of isolates. Mutations E818K, D836N and T882P were observed in 80% of the rifampicin-resistant and rifampicin-sensitive isolates.
Conclusions: The proposed sequencing method allowed identification of mutations in the entire *rpoB* gene. This procedure represents a useful tool for diagnosing rifampicin resistance. The number of mutations that were found raises new questions about the diversity of mutations in the *rpoB* gene and their role in rifampicin resistance in regions where TB is endemic.

1. Introduction

According to the global report on tuberculosis (TB), of the 10.5 million cases reported in 2017, 600 000 were rifampicin-resistant (RR), 490 000 were multidrug-resistant (combined resistance to rifampicin and isoniazid, MDR-TB) and 25 000 showed extensive drug resistance [1]. This increase in the incidence of drug-resistant TB is more significant in populations with a high prevalence of the disease, which are predominantly found in developing countries. Such is the impact of this increase that it has been suggested that this problem could threaten the attempts to eliminate TB as a global public health problem by 2030 [1].

The *rpoB* gene encodes for the β subunit of *Mycobacterium tuberculosis* (*M. tuberculosis*) RNA polymerase, and mutations in this gene have been described as the main reason for rifampicin resistance [2,3]. Of the 3519 bp that comprise this gene, 80–90% of the RR isolates have mutations within an 81 bp portion known as the rifampicin resistance determining region (RRDR) [4,5]. All of the genotypic tests for RR diagnosis focus on analysis and identification of mutations within the RRDR [6–9]. However, there is an increasing number of reports describing mutations associated with RR outside the RRDR and, as a consequence, the effectiveness of these genotypic diagnostic tests is decreasing [10–13].

Due to the importance of detecting new polymorphisms that could cause RR, the need to identify new mutations that increase the efficiency of genotypic testing, and the fact that RR has been

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established as a surrogate marker for MDR-TB, this paper aimed to develop a procedure with which to sequence the entire *rpoB* gene and characterise the polymorphisms.

2. Methods

2.1. Collection of clinical samples, Mycobacterium tuberculosis isolation, and drug susceptibility testing

Sputum samples were collected from different patients from the state of Veracruz, and confirmed for TB. Collection of the 20 isolates included in this study was conducted by the Mycobacteriology Department of the Public Health Laboratory of the State of Veracruz, Mexico, during January–December 2017. Sputum decontamination was performed using N-acetyl-L-Cysteine-NaOH and mycobacterial isolation was performed using Lowenstein-Jensen medium. Susceptibility testing followed the fluorometric method (BACTEC, MGIT 960 Becton-Dickinson) and considered the critical concentration for streptomycin (S) of 1.0 μg/mL, isoniazid (H) 0.1 μg/mL, rifampin (R) 1.0 μg/mL, pyrazinamide (Z) 100 μg/mL, and ethambutol (E) 5.0 μg/mL.

2.2. DNA purification and PCR-amplification of rpoB

Extraction of DNA from the clinical isolates with resistance against rifampicin was conducted with one loop of cultured mycobacteria, according to Van Soolingen et al. [14]. The DNA was re-suspended in nuclease-free water, and its concentration determined by spectrophotometry in a Nanodrop 1000 (Thermo-Scientific, USA). The resulting DNA solution was then stored at –20 °C until subsequent processing.

For sequencing, the *rpoB* gene was divided into five fragments. Five forward and two reverse primers were used to amplify the fragments that covered the entire gene. Amplicons were designed to overlap so they could subsequently be assembled with no loss of nucleotide sequence (Table 1 and Fig. 1).

The PCR reaction for each of the fragments was standardised and consisted of: 2.5 μL of Buffer DreamTaq 10X (Thermo Scientific) that included 20 mM $MgCl_2$, 0.4 μL of dNTPMix, 10 mM (Thermo Scientific), 10 pmol of forward and reverse primers (PF and PR), specific to each fragment, 1.25 U DreamTaq DNA polymerase (5 U/μL, Thermo Scientific), 1.7 uL of 7% DMSO (J.T. Baker) and 60 ng of DNA template, then topped up with nuclease-free water to a final volume of 25 μL. Amplification of each fragment was performed in a T100™ Thermal Cycler (Bio-Rad) under the same conditions: 95 °C for 5 min, 30 cycles of 95 °C for 60 s, 60 °C for 60 s, and 72 °C for 4 min, with a final extension at 72 °C for 3 min.

Amplicons were electrophoretically analysed in 1.5% agarose gel, and the DNA concentration determined by Mass Ruler low range DNA Ladder (Thermo Scientific). Finally, the products were purified by using Exo-Sap according to provider conditions (Thermo Scientific).

Table 1
Forward and reverse primers used to amplify the *rpoB* gene of *Mycobacterium tuberculosis*.

Primer	Sequence 5′-3	Position (bp)	TM	Amplicon size
rpoB1 F	GAGCAAAACAGCCGCTAGTC	18	60.19	1552
rpoB2 F	GGCGCTGTTGGACATCTACC	798	62.93	772
rpoB3 F	GACCAGAACAACCCGCTGTC	1303	63.03	2216
rpoB4 F	TACCTACCGGATGCGCAAGT	1986	62.83	1533
rpoB5 F	GATCTCCGACGGTGACAAGC	2616	63.15	903
R 1	ACACGATCTCGTCGCTAACC	1570	60.29	–
R 2	TTACGCAAGATCCTCGACAC	3519	58.88	–

2.3. rpoB fragment Sanger sequencing and assembling

The sequencing reactions were performed in forward directions using 6 μL of Big Dye Terminator Cycle Sequencing Kit V3.1 (Applied Biosystems), 3.2 pM of forward primer, and 20 ng of purified PCR product in a final volume of 20 μL. The amplification conditions were 25 cycles of 95 °C for 30 s, 50 °C for 15 s, and 60 °C for 4 min. Amplicons were purified using the ZR DNA sequencing clean-up kit (Zymo Research), re-suspended in Hi-Di formamide (Applied Biosystems), heated to 95 °C for 5 min, cooled on ice, and finally loaded onto a 96-well MicroAmp reaction plate (Applied Biosystems). The DNA products were sequenced by capillary electrophoresis in a Genetic Analyzer 3500 (Applied Biosystems).

Quality analysis of the fluorescence spectra from the sequenced fragments was performed with the software Data Collection V1.01 (Applied Biosystems). Further assembly of the *rpoB* fragments from each isolate was conducted using the software CodonCode aligner (CodonCode Corporation) and further confirmed by manual revision. The obtained *rpoB* sequence was stored in FASTA format and the software BioEdit Sequence Aligner Editor V.7.2 (http://www.mbio.ncsu.edu/BioEdit/bioedit.html) was used to compare the sequences and identify polymorphisms, considering the *rpoB* from *M. tuberculosis* H37Rv as the wild-type reference sequence.

3. Results

3.1. Recovered clinical isolates and rpoB amplification

Of the 20 collected isolates, 25% (n = 5) were sensitive to rifampicin, 75% (n = 15) were simultaneously resistant to R and H (MDR-TB), 50% (n = 10) were resistant to S, 35% (n = 7) to Z, 30% (n = 6) to E, and 20% (n = 4) showed resistance to all first-line drugs (R + H + Z + E + S). By using the primers and established PCR protocol, it was possible to successfully amplify the five fragments of the *rpoB* gene. The expected theoretical and observed weights of the products were similar (Fig. 1). Sequencing of these products produced fluorescence spectra with a signal of sufficient quality to allow identification of the nucleotides and the subsequent splicing of fragments without loss of nucleotides.

3.2. Mutations identified in rpoB

The characteristics and locations of the 36 non-synonymous polymorphisms found in the *rpoB* gene of the 20 analysed isolates are detailed in Table 2. To facilitate analysis, these mutations were organised into three regions: pre-RRDR, within-RRDR, and post-RRDR. In the pre-RRDR region, two mutations were identified: V227G was observed in three resistant isolates, while the E411K mutation was observed in five rifampicin-sensitive and seven rifampicin-resistant isolates. In the within-RRDR region, nine mutations were identified in the RR isolates only. The most abundant was S531L, which was present in eight isolates (53%), followed by S512T in three isolates (20%), and H526D and R529Q in two isolates (13%) each. One RR isolate (1159), as well as the five rifampicin-sensitive isolates, presented no polymorphisms (Table 2). In the post-RRDR region, 25 mutations were identified. The most frequent was D853N, which was present in 17 (85%) rifampicin-sensitive and rifampicin-resistant isolates, followed by the mutations E818L, D836N and T882P, presented in 16 (80%) isolates. Fourteen mutations were seen exclusively in RR isolates and eight were observed only in sensitive isolates (Table 2).

3.3. Presence of double and triple mutations in the analysed isolates

The most frequent double combination of mutations was found between codons 897 + 1075 observed in 47% (7 of 15) isolates,

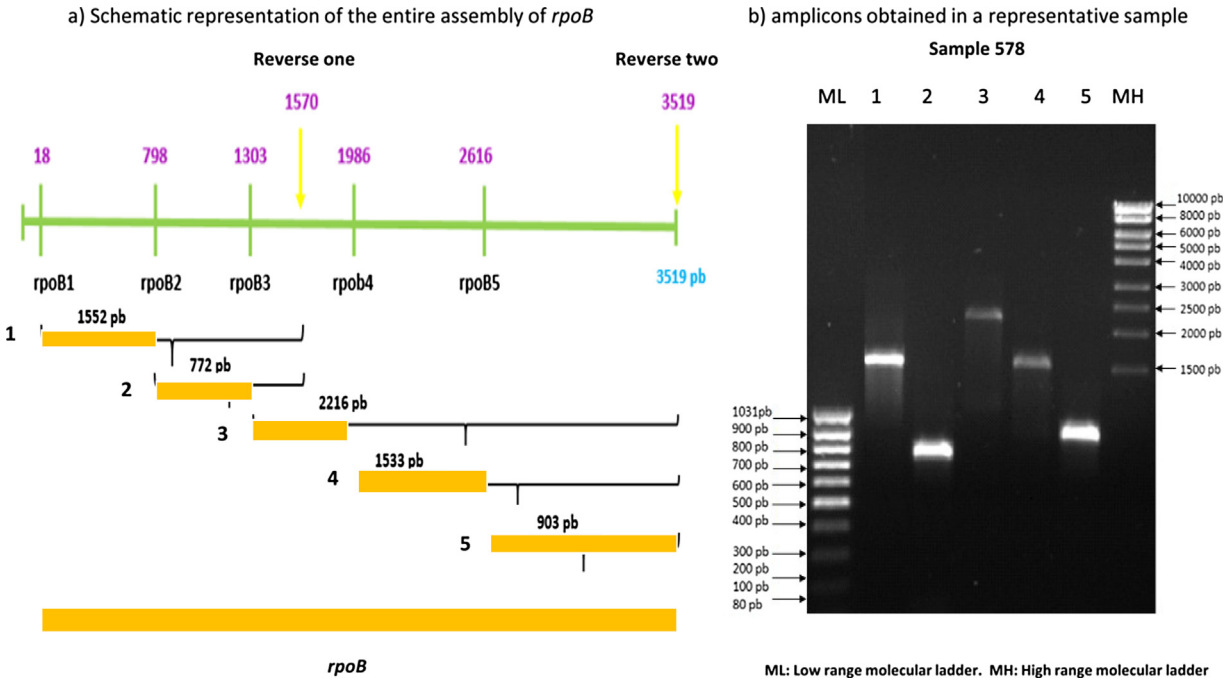

Fig. 1. Location of primers, fragments and construction of the entire *rpoB* gene.

followed by the combination in codons 531 + 1045 and 1045 + 1075, each present in 40% (6 of 15) of isolates. Five combinations, with triple mutations were identified; the most frequent were at codons 897 + 1045 + 1075, observed in 40% (6 of 15) of the isolates, followed by mutations at codons 846 + 897 + 1045 and codons 531 + 897 + 104.

3.4. Analysis of mutations in the rpoB *gene from whole genomic sequences of tuberculosis isolates*

In order to identify mutations in the entire *rpoB* gene obtained from 15 whole genome sequences of *M. tuberculosis* isolates with RR (GenBank codes: CP002992, CP002885, CM000789, CP001658, CM001044, CP009100, CP009100, CP009101, CP012506, CM000788, CP010968, JLCQ0100003, JANH010000011, CP010873, and NZ_JAQI01000016), the same in silico protocol of analysis was followed. The results showed seven mutations, which were distributed as follows: pre-RRDR, two mutations were found, D382Y and A384G, each in one isolate. Inside-RRDR, three mutations were found: S531L observed in 50% (7 of 15) of isolates, D516Y/G/V found in 33% (5 of 15) and L533P in 20% (3 of 15) of isolates. Finally, post-RRDR presented only two mutations, L812P, observed in 20% (3 of 15) of the isolates and I1187T in 17% (2 of 15) of the isolates.

4. Discussion

The amplification and subsequent assembly of the five *rpoB* fragments conducted in this study enabled complete sequencing of the entire gene in *M. tuberculosis* isolates. Despite the limited number of analysed samples, it was possible to identify mutations within the RRDR in 14 (93%) of the 15 isolates with RR. This coincides with several reports indicating that between 80–90% of the RR strains contain mutations within the RRDR [4,15–21]. Isolate 1159 showed no mutations within the RRDR; however, analysis of the entire gene allowed identification of sixteen mutations post-RRDR, therefore it will be necessary to evaluate the potential participation of each one of these mutations.

The mutation S531L, which is commonly described in Mexico [22], was the most frequent mutation observed within the RRDR. However, it occurred in 53.3% of isolates, which is a low percentage that coincides with reports from China and Kuwait [18,23], even in the same setting [9,13,24]. The second most frequent mutation was S512T, which was observed in three isolates (20%); this is a rare mutation associated with a low level of RR [25] and its location in second place contrasts with other reports where the second most frequent mutation was located at codon 526 [17,18,26,27].

Nine mutations were identified within the RRDR. According to previous reports, all of these are associated with RR [28]. This diversity of mutations observed in the isolates from the setting is similar to that reported in previous studies conducted in Mexico [24,29]. However, there are differences between these findings and those reported from other countries, supporting the hypothesis that the geographic and socio-demographic characteristics of the region affected by TB play an important role in the selection of polymorphisms involved in the development of RR [26,30].

Most of the mutations observed outside the RRDR showed a frequency of occurrence close to 30–70%, eliminating the possibility that they could be derived from errors in the sequencing, assembly or subsequent analysis of the gene sequenced. Of the mutations found pre RRDR, V227G was found in three RR isolates and E411K was observed in five rifampicin-sensitive and seven RR isolates. In recent years, there has been an increased number of reports that describe the presence of mutations in this region of the *rpoB* gene at codons A381V, A184A, H481Y, I491F, V498G, A500V, I502V and F505S, and with participation in RR [10,16,26,31–33]. Additional studies are required to evaluate the mutations' (found in the analysed isolates) participation in RR.

In post-RRDR, 14 mutations were observed only in resistant isolates, none of which had previously been described. Various reports have confirmed the presence of mutations related to RR in this region such as L538F, I569L, S574L and D626E [10,26,34]; however, the resolution limit of the capillary sequencing is <900 pb, so the unusual number of mutations identified post-RRDR should be carefully considered. Work is currently underway

Table 2
Mutations identified in the *rpoB* gene from rifampicin-sensitive and rifampicin-resistant *Mycobacterium tuberculosis* isolates.

Region	Codon	Amino acid	Isolates (n = 20)	Rifampicin-sensitive (n = 5)	Rifampicin-resistant (n = 15)	Frequency	
						n	%
Pre-RRDR	227[a]	V→G	478, 417, 533	0	3	3	15
	411[a]	E→K	393, 578, 482, 379, 387, 426, 364, 649, 533, 478, 507, 417	5	7	12	60
RRDR (codons 507-533)	509	S→T	533	0	1	1	5
	512	S→T	533, 478, 417	0	3	3	15
	513	Q→H	417	0	1	1	5
	515	M→L	417	0	1	1	5
	526	H→D	413, 364	0	2	2	10
	527	K→N	C-171	0	1	1	5
	529	R→Q	507, C-171	0	2	2	10
	531	S→L	421, 559, 649, 1131, MLC 120, 423,426, 417	0	8	8	40
	533	L→P	507	0	1	1	5
Post-RRDR	602	E→K	393, 578, 482, 379, 387, 426, 421, 533, 478, 559, 1139	5	6	11	55
	676	D→N	393, 482, 379, 387, 423, C171, MLC 120, 533, 478, 507, 559, 1131	4	8	12	60
	664	V→G	393, 578, 379, 387	4	0	4	20
	721	E→K	393, 578, 482, 379, 649, 423, 1159, MLC120, 421, 507, 559, 1131	4	8	12	60
	785	D→N	578, 482, 379, 387, 649, 413, 1159, C171, 421, 533, 507, 559, 1131, 417	4	10	14	70
	818	E→K	393, 578, 482, 379, 387, 649, 413, 423, 1159, C171, MLC120, 533, 507, 559, 1131, 417	5	11	16	80
	827	I→F	393, 578, 482, 379, 387	5	0		25
	836	D→N	393, 578, 482, 379, 387, 413, 423, 1159, c171, MLC120, 421, 533, 507, 559, 1131, 417	5	11	16	80
	853	D→N	393, 578, 482, 379, 387, 649, 413, 423, 1159, c171, MLC120, 421, 533, 507, 559, 1131, 417	5	12	17	85
	882	T→P	393, 578, 482, 379, 387, 649, 413, 423, 1159, MLC120, 421, 533, 507, 559, 1131, 417	5	11	16	85
	846[a]	R→P	649, 423, MLC 120, 533, 507, 1131	0	6	6	30
	893[a]	E→D	1159, MLC 120, 533, 507, 1131	0	6	6	30
	897[a]	R→L	649, 413, 423, 159, c171, MLC120, 421, 507, 559, 1131	0	10	10	50
	944	V→G	393, 379, 387	3	0	3	15
	984[a]	D→N	413, C171, MLC120, 507, 1131	0	5	5	25
	1045[a]	A→P	426, 649, 423, 1159, MLC120, 478, 507, 559, 1131, 417	0	10	10	50
	1074[a]	D→N	426, 649, 1159, 533, 559, 417	0	7	7	35
	1075[a]	V→L	649, 413, 423, 1159, 421, 507, 559, 1131	0	8	8	40
	1167[a]	K→N	649, 423, C171, MLC120, 559, 1131, 417	0	7	7	35
	1221[a]	G→K	364, 413, 423, 1159, C171, MLC120, 507, 559, 1131	0	9	9	45
	1234[a]	A→P	413, 423, 1159, C171, MLC120, 507	0	6	6	30
	1243[a]	R→P	649, 413, 423, 1159, C171, MLC120, 507, 559	0	8	8	40
	1245[a]	E→K	649, 413, 423, 1159, C171, MLC421, 507, 559, 1131	0	10	10	50
	1246[a]	S→P	413, 423, 1159, C171, MLC120, 507, 559	0	7	7	35
	1247[a]	A→P	413, 423, 1159, C171, 507, 559, 1131	0	7	7	35

RRDR, rifampicin resistance determining region.
[a] Mutation reported for the first time.

with the aims of analysing smaller fragments of the post-RRDR of *rpoB*, further confirming the presence of the changes found, and subsequently evaluate the real participation through in silico or experimental directed mutagenesis [35,36].

Twelve isolates showed the same patterns of double mutations and five isolates had triple mutations. Five RR isolates with double mutations showed the same combination of rpoB531 + rpoB1045 and four strains presented the pattern rpoB531 + rpoB1045 + rpoB897. Combinations of double mutations in *rpoB* have been described in isolates from regions with endemic TB and significant levels of RR, such as Sri Lanka [26], Vietnam [37] and China [27]. Two possible explanations for these patterns are: 1) they could be related to specific mycobacterial clusters/lineages, and 2) they function as compensatory mutations that give rise to a coordinated action that maintains or increases the level of resistance against rifampicin.

Analysis of the *rpoB* sequences derived from whole genome sequencing showed the occurrence of commonly described mutations within the RRDR: 516, 531 and 533 [8,25,28]. Moreover, four mutations were found outside this region. These results contrast with the number of mutations observed using Sanger sequencing of *rpoB* in isolates from different regions [11,16,26,34,38,39]. This could demonstrate the advantages of Sanger sequencing over whole genome sequencing in terms of identifying new mutations that could participate in resistance against rifampicin in TB, supporting the use of this procedure for diagnostics.

There were two major limitations in this study: the relatively small number of samples included in the analysis and difficulty in determining the type of lineage present in the studied isolates. Nevertheless, the generated information raises important questions about the frequency and diversity of mutations in the *rpoB* gene and the need for more in-depth studies in order to evaluate their participation in RR. Three major inferences arose from the findings: 1) additional studies are needed in order to confirm the

participation of mutations in pre-RRDR and post-RRDR in the analysed isolates; 2) it is necessary to analyse the implications of these mutations in terms of the function of RNA polymerase and their potential participation as neutral or compensatory mutations; and 3) it is also necessary to determine how this information could impact the implementation of a molecular diagnostic test for resistance against rifampicin in regions where TB is endemic and where cases of RR are increasing in number.

In conclusion, the *rpoB* sequencing procedure described in this study enabled the identification of an important number of polymorphisms. In addition, comparison of the sequence analysis with the drug sensibility profile determined by MGIT revealed a sensitivity and specificity value >90%, and it is therefore considered that this molecular analysis could be a useful tool for diagnosing resistance against rifampicin in TB isolates circulating in endemic regions.

Competing interests

None.

Ethical approval

Not required.

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