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Molecular Analysis of Rifampicin Resistance Conferring Mutations in *Mycobacterium tuberculosis*

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Abstract:

Mycobacterium tuberculosis resistance to rifampicin is mainly mediated through mutations in the *rpoB* gene. The effects of *rpoB* mutations are relieved by secondary mutations in *rpoA* or *rpoC* genes. This study aims to identify mutations in *rpoB*, *rpoA*, and *rpoC* genes of *Mycobacterium tuberculosis* isolates and clarify their contribution to rifampicin resistance. Seventy isolates were identified by acid-fast bacilli smear, Genexpert assay, and growth on Lowenstein Jensen medium. Drug susceptibility, testing was performed by the proportional method. DNA extraction, PCR, and sequencing were accomplished for the entire *rpoA*, *rpoB*, and *rpoC* genes. Twenty-three isolates (32.85%) showed resistance to rifampicin by either proportion method or Genexpert assay. Sequence analysis of the rpoB gene revealed fourteen different mutation patterns. Inside the rifampicin resistance determining region (RRDR), codons: S531L, D516V were highly mutated with frequencies of (21.73%, 17.39%) respectively. Outside the RRDR, there were nine different types of mutations, and M479L was the most prevalent one. Out of 23 RIF resistant isolates, seven isolates (30.43%) carried mutations in the *rpoA* gene, and twelve isolates (52.17%) harbored a mutation in *rpoC*. Most of the mutations were identified for the first time in this study. The current study demonstrated that mutations in *rpoB*, *rpoA*, and *rpoC* contributed to RIF resistance in *Mycobacterium tuberculosis* and this new finding may be relevant to realize how compensatory mutations in the *rpoA* and *rpoC* genes restore the fitness cost caused by rifampin resistance-conferring mutations in *rpoB*.

Key words: Pulmonary tuberculosis, *rpoA*, *rpoB*, *rpoC*, RRDR, Tuberculosis.

Introduction:

Mycobacterium tuberculosis (*M. tuberculosis*) causes tuberculosis (TB), which is the leading cause of death by infectious diseases worldwide, with an estimated 10.4 million new TB cases in 2016(1).

Iraq occupies 108th rank globally and the 7th position among the Eastern Mediterranean Region countries with high TB burden. About 3% of the total cases of TB exist in Iraq. In 2018, WHO estimated that there were 16,000 TB patients in Iraq and deaths due to TB exceeds 4000 annually. The estimated TB incidence rate was 42/100,000. According to the survey of 2014, levels of drug resistance in Iraq was low (1.1%) (WHO). In overall, Iraq has a higher incidence rate of TB than all neighboring countries i.e. Syria, Jordan, Iran, Saudi Arabia and Turke (2).

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This may be attributed to the instability, low socio-economic status and unsatisfactory treatment. The DOTS strategy has been adopted in Iraq since 1998, except for the three Iraqi Kurdistan provinces (Duhok, Erbil and Sulaimani), which has been implemented DOTS since 2001 (3).

Rifampicin, also called rifampicin an effective agent against different pathogens including mycobacteria and considers as an antituberculosis agent against susceptible strains as well as strains resistant to isoniazid or streptomycin (2). The antibacterial activity of rifampicin is due to its inhibition of transcription, by making specific contacts that involve the β-subunit of RNA polymerase (*rpoB gene*) and blocks growth of an RNA chain past 2 or 3 nucleotides (3). Elongating RNA polymerase is insensitive to rifampicin inhibition because RNA in the exit channel blocks rifampicin binding. Thus, runoff transcription continues after the addition of rifampicin (4).

Mutations in the *rpoB* gene, which encodes the β-subunit of RNA polymerase, lead to high-level resistance to rifampicin, three clusters site

mutation of *rpoB* gene in rifampicin-resistant *Escherichia coli* strains were identified by Jin and Gross (5). Recently, several researchers have investigated nucleotide sequences of the cluster I and II regions of the *rpoB* gene and exhibited mutations from rifampicin-resistant *Mycobacterium tuberculosis* strains (6) and (7). Moreover, several distinct regions in the genes *rpoA* and *rpoC* are potentially important regions in the compensatory evolution of *rpoB* (8).

The aims of this study to determine the epidemiological relevance of synonymous and nonsynonymous mutations in *rpoB, rpoA,* and *rpoC*.

Materials and Methods:

Bacterial Isolates (*Mycobacterium tuberculosis*)

Four hundred and twenty-five sputum sample were received from patients at Chest and Respiratory Diseases Centre in Iraq-Erbil province from April 2016 to April 2017. Sputum smears were stained by ZN stain Kit (Atom Scientific, UK) (9), and further investigations performed by the growth rate, pigment production, colony morphology, niacin test, and nitrate reduction test. The sputum samples were digested by Petroff's Method and cultured on LJ slant (10).

Drug Susceptibility Testing

Rifampicin susceptibility tests were carried out by Genexpert assay and proportion method. Genexpert assay was performed on sputum samples then isolates were cultured from sputum underwent susceptibility testing using the L-J proportion method according to the standard instructions Lowenstein Jensen medium, 0.25µg/ml rifampicin, and 40µg/ml isoniazid, the slants were incubated at 37°C and results were read at 28 days and up to 42 days, depending on control growth (11).

Genomic DNA Extraction

Chromosomal DNA was extracted by the lysozyme-proteinase K cetyl-trimethyl ammonium bromide (CTAB) method and stored at 4^{o}C (12). The purity of DNA was checked with the NanoDropND-1000 spectrophotometer (NanoDrop Technologies).

Polymerase Chain Reaction (PCR) and Primers

Three primer sets were designed for target genes (*rpoB*, *rpoA* and *rpoC*) as described in (Table 1) by NCBI primer designing tool (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) and Clone Manager software was used for checking primers specificity.

Table 1. List of primers.

Target Gene	Primer pair	Primer sequence (5'->3')	Product Size (bp)
rpoA	1	F: TGTTCGGTTTCGGCGTAGTC R: ATGCTGATCTCACAGCGCC	1037
rpoB	1	F: TTGGCAGATTCCCGCCAGAG R: GTGGTCGATGTCGTCGGTTTC	1098
	2	F: GACGACATCGACCACTTC R: CATCTCGCCGTCGTCAGTACAG	1038
	3	F: TGTACTGACGACGGCGAGATG R: CATCGGACTTGATGGTCAACAG	1165
rpoC	1	F: CTCCGCATCGGTCTTGCTAC R: GCGGTACAGGTCGTTCAAGT	1008
	2	F: CTGTTCAAGCCGTTCGTGATG R: CACCAGGCCCTTCATACCG	1122
	3	F: GATCCGGAGATTGACGCTCTG R: TGTAGCCGTAGTCGTCCAGC	1102

Target genes of *M. tuberculosis* were amplified by PCR using TC-412 thermal cycler (Techne-Japan) and amplicon sizes confirmed. 50 µL reaction mixture consisted of 25 µL of master mix (GeneDirex/UK) 2 µL of each primer set, 20 µL of ddH2O and 3µl µL (30 ng) of bacterial genomic DNA.

For rpoB, the PCR Amplification conditions included an initial denaturation step at 94°C for 2 minutes, followed by 35 cycles of 94°C for 1 minute, annealing at 65°C for 1 minute, and 72°C for 1 minute with a final extension at 72°C for 10 minutes. For both *rpoA* and *rpoC* genes, PCR Amplification conditions comprised an initial denaturation step at 94°C for 1 minutes, followed by 35 cycles of 94°C for 1 minute, annealing at 58°C (*rpoA)* and 59°C (*rpoC)* for 1 minute, and 72°C for 45 seconds with a final extension at 72°C

for 10 minutes. Each PCR products analysed by Agarose Gel Electrophoresis (13).

Agarose Gel Electrophoresis

Agarose gel electrophoresis was carried out using 1.5% agarose gels to analyze DNA according to its molecular weight mobility (13). 6 µL from each PCR products were mixed with 2 µL of loading dye and 4 µL of SYBR® Safe™ DNA Gel Stain (Life Technologies, UK) per 100 mL of agarose and run using 1× TAE buffer at 45 V for 15 min and then 90 V for 35-45 min. The size of DNA fragments in the tested samples was evaluated using the GeneRuler 1 kb DNA ladder (Quick-Load®,UK). The bands were visualized using a UV lamp at 365 nm, and images then captured by a Canon D100 (Canon Co., Japan).

Amplified PCR products were purified from the gel using (Gel Extraction Kit/Geneaid/Korea) then sent to Bioneer Corporation (South Korea), for sequencing using the Sanger sequencing method by ABI Genetic Analyzer 3130 (Applied Biosystems). Preliminary analysis of sequences and sequences alignment was performed using BioEdit. The sequences were compared with to wild type *Mycobacterium tuberculosis* sequence available at NCBI.

Nucleotide Sequence Accession Numbers

The nucleotide sequences of target genes mutation were deposited in the GeneBank database under the following accession numbers: MK874753- MK874779, and MK887216-MK887231.

Quality Control

H37RV strain of *Mycobacterium tuberculosis* was selected as a control.

Ethical Approval

Ethical approval for this study is obtained from the ethical board of Chest and Respiratory Diseases Centre in Iraq-Erbil province.

Results:

Diagnosis and susceptibility of *M. tuberculosis* isolates to anti-TB drugs

Overall, four-hundred and twenty-five sputum samples were collected from suspected pulmonary TB patients, seventy isolates gave positive result by AFB smear microscopy, Genexpert assay, and grew on LJ medium as in (Table 2). The identity of the isolates was confirmed by cultural, morphological, and biochemical characteristics. All of the *M. tuberculosis* isolates were positive for niacin, nitrate reduction, nicotinamidase, pyrazinamide, and binding of neutral red tests. They were catalase negative at 25°C and 68°C (10).

Table 2. Two-by-two table comparing Genexpert MTB/RIF assay and sputum acid-fast bacilli smear.

GeneXpert	AFB positive	AFB negative	Total
MTB/Rif			
MTB detected	70	80	150
MTB not	1	274	275
Detected			
Total	71	354	425

Fifteen isolates were MDR-TB by Genexpert assay. The proportion method detected twenty-three (28.57%) isolates resistant to rifampicin, all of which were isoniazid resistant, hence considered as MDR. Among MDR isolates by proportion method, twelve isolates (70.5%) showed resistance to rifampicin by Genexpert assay and (3/47) rifampicin sensitive isolates by the proportion method considered as resistant by Genexpert assay as in Table 3.

Table 3. Two-by-two table comparing DST by Genexpert MTB/RIF assay and the proportion method on LJ medium.

GeneXpert MTB/RIF	Rif resist on LJ	Rif sensitive on LJ	Total
Rif resist. detected	12	3	15
Rif resist. not detected	11	47	55
Total	23	50	70

Mutations in *rpoB*

Amplified PCR products of *rpoB* are shown in Fig. 1. Among 23 rifampicin resistant isolates, pre-RRDR contained four different mutation patterns, the most common one was M479L (6/23,26.08%). Five mutation patterns were detected inside RRDR of (15/23, 65.21%) isolates. Five isolates (5/23, 21.73%) carried the S531L mutation along with M479L mutation in four isolates. The M479L mutation was found to coexist with the V475D mutation in one isolate. Four isolates (17.29%) harbored the D516V mutation. Moreover, two isolates (8.69%) possessed the S522L mutation associated with the T481I in the pre-RRDR of one isolate. Two isolates owned the H526R mutation. Isolates number (1,13,17) were regarded as sensitive by Genexpert assay while they carried more than one mutation outside the RRDR. Among 47 RIF sensitive isolates, nine (19.14%) harbored mutations outside the RRDR. No synonymous mutations exist within RRDR while

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one synonymous mutation was present in the pre-RRDR (P99P).

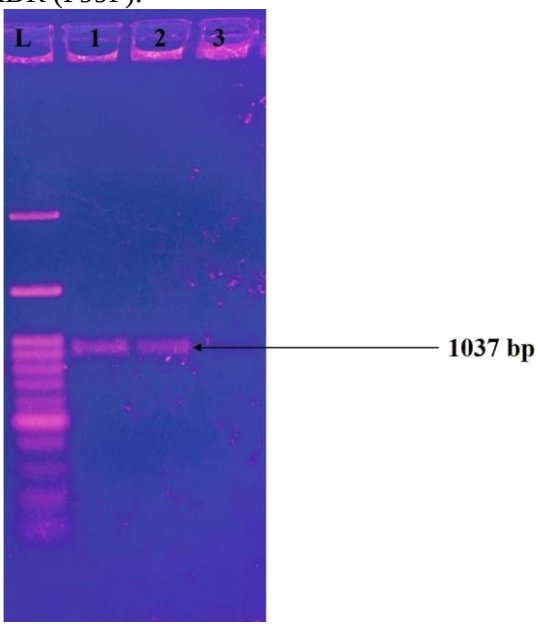

Figure 1. *rpoA* gene. Lane 1: Ladder, Lane 1: Positive control, Lane 2: Positive sample, Lane 3: Negative sample.

Mutations in *rpoA* and *rpoC*

Out of 23 RIF resistant isolates, seven isolates (30.43%) carried nonsynonymous mutations in the *rpoA* gene as in Table 4. The mutations in the *rpoA* included Y59S, S87L, T187A, V183G, R182L, T332S, and the most prevalent one was R182L. One isolate with the L194P mutation in *rpoA* gene has no mutation in *rpoB* and *rpoC*.

Seven different types of mutations were identified in *rpoC*. Six of these mutations at codons (I281V, S260L, Q600E, G941A, D1120A, and A1201V) are reported for the first time in this study. The most common mutations were Q600E and S260L. No nonsynonymous mutations were detected in *rpoA or rpoC*.

Data Availability Statement

The [DNA Sequence data] used to support the findings of this study have been deposited in the [GenBank] under the following accession numbers: MK874753- MK874779, and MK887216-MK887231.

Table 4. Frequency of mutations identified by sequencing in the *rpoA, rpoB, rpoC* genes of *Mycobacterium tuberculosis* isolates.

RIF sensitive isolate no.		RIF resistant isolate no.		*rpoB*				*rpoA*		*rpoC*	
Xpert	LJ	Xpert	LJ	RRDR		Outside RRDR		Codon change	Amino acid change	Codon change	Amino acid change
				Codon change	Amino acid change	Codon change	Amino acid change				
1			1	none	none	ATG > TTG	M479L	none	none	none	none
		2	2	TCG > TTG	S531L	ATG > TTG	M479L	TAC > TCC	Y59S	none	none
		3	3	CAC > CGC	H526R	none	None	TCG > TTG	S87L	ATC>GTC	I281V
4			4	none	none	none	None	none	none	TCG>TTG	S260L
		5	5	CAC > CGC	H526R	none	None	GTC > GGC	V183G	TTC>CTC	F452L
		6	6	CTG > CCG	L533P	GTG > GCG	V927A	none	none	CAG>GAG	Q600E
7			7	none	none	none	None	CTG> CCG	L194P	none	none
		8	8	TCG > TTG	S531L	GTC > GAC	V475D	none	none	GGT>GTT	G941A
		9	9	GAC > GTC	D516V	none	None	CGG > CTG	R182L	GAG>GCG	D1120A
		10	10	TCG > TTG	S522L	ACC > ATC	T481I	none	none	GCG>GTG	A1201V
		11	11	CTG > CCG	L533P	none	None	none	none	CAG>GAG	Q600E
		12	12	TCG > TTG	S531L	TTC > TTG ATG > TTG	F584L M479L	none	none	none	none
13			13	none	none	ATG > TTG	M479L	none	none	none	none
		14	14	GAC >	D516V	GTG >	V597A	CGG>CTG	R182L	none	none

				GTC		GCG					
		15	15	TCG > TTG	S531L	ATG > TTG	M479L	none	none	none	none
		16	16	TCG > TTG	S531L	ATG > TTG	M479L	none	none	none	none
17			17	none	none	CTG > CCG	L943P	ACC > GCC ACC> AGC	T187A T332S	none	none
	18	18		GAC > GTC	D516V	CGT > CAT	R945H	none	none	none	none
	19	19		GAC > GTC	D516V	GTG > GCG	V691A	none	none	none	none
	20	20		TCG > TTG	S522L	none	None	none	none	TCG > TTG TTC > CTC	S260L F452L
21			21	none	none	none	None	none	none	CAG> GAG	Q600E
22			22	none	none	none	None	none	none	GCG > GTG	A1201V
23			23	none	none	none	None	none	none	GGT > GTT	G941A

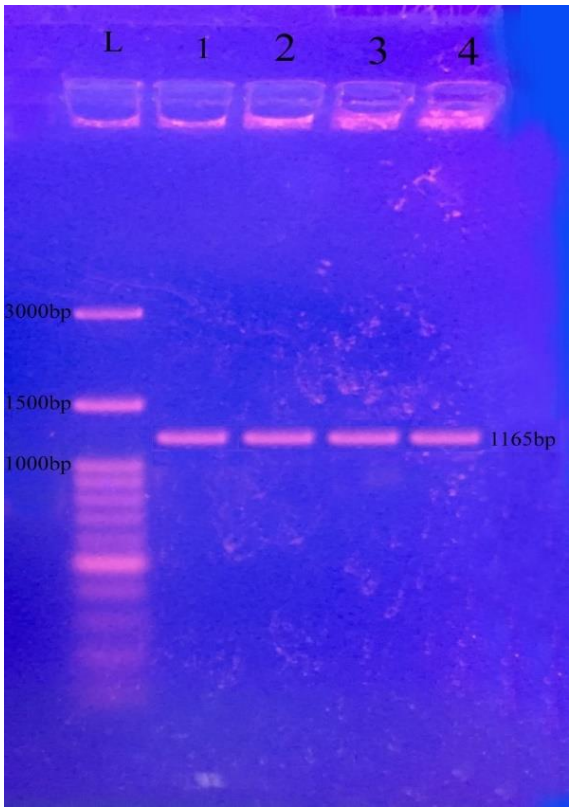

Figure 2. Agarose gel electrophoresis of amplified PCR products of the *rpoB* gene of *M. tuberculosis*.
Lane L: 100 bp DNA ladder
Lane 2-4: Amplified PCR products

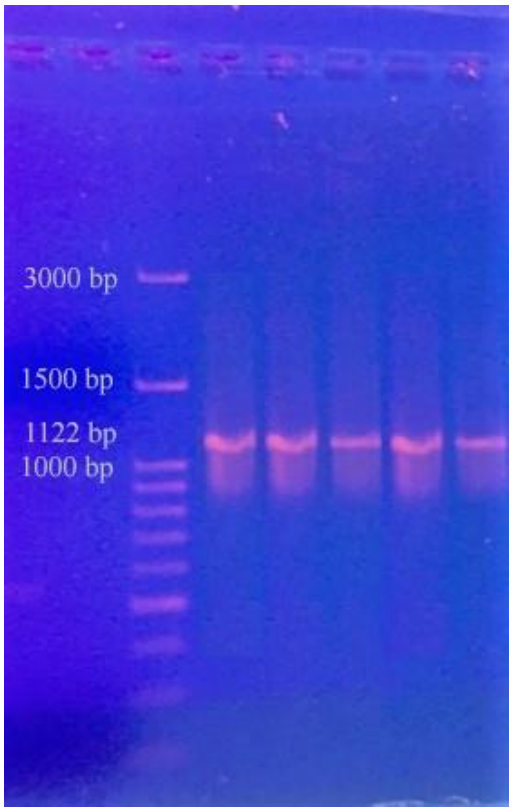

Figure 3. Agarose gel electrophoresis of amplified PCR products of the *rpoC* gene of *M. tuberculosis*.
Lane L: 100 bp DNA ladder
Lane 2-6: Amplified PCR products

Discussion:

To the best of our knowledge, this is the first study in Iraq which deals with rifampicin resistance and its associated genes using sequencing of the entire *rpoB, rpoA,* and *rpoC* genes. This study was undertaken on sputum samples collected from presumptive TB patients. Among four hundred and twenty-five presumptive TB patients, seventy were positive by AFB, LJ culture, and Genexpert assay. One of the currently available molecular techniques that simultaneously detects MTB and RIF resistance-conferring mutations within RRDR of the rpoB gene is Genexpert assay (14). In our study, this technique detected MTB in (150/425, 35.29%) sputum samples. Among these, (53.33%, 80/150) were AFB negative. One AFB positive isolate was negative by Genexpert assay, which is attributed to the inability of this method to detect *Mycobacterium bovis*. This low level of detection with AFB is due to the presence of less than105/ml bacteria in the sample (15).

Multidrug resistance tuberculosis (MDR-TB) denotes *M. tuberculosis* which is resistant to at least rifampicin and isoniazid (16). Annually, about half a million cases of MDR-TB recorded resulting in almost 0.25 million deaths per year. According to the survey of 2014, levels of drug resistance in Iraq was low (1.1%) (17). Out of the 150 samples detected by Genexpert assay, rifampicin resistance was detected in (15/150,10%). This result is higher than the studies of (18) and (19) which were (4.1%), and (6.9%) of their isolates that showed resistance to rifampicin. According to our results, there is a relatively high MDR cases (23/70, 32.85%). Improper treatment is an important reason for the increasing of MDR-TB.

Resistance to rifampicin is greatly associated with RRDR (codon 507-533) of the gene encoding RNA polymerase *B*, and several studies indicated the existence of novel mutations in *rpoB* gene (20) and (16). Several studies have described the presence of common mutations in this region of *rpoB* gene. In this study, we reported the presence of common and novel *rpoB* gene mutations in clinical *M. tuberculosis* isolates.

All culture positive isolates were investigated for mutations in *the rpoB* gene. Among 23 RIF resistant isolates, the entire rpoB gene sequence contains different mutation patterns. In our study, (15/23, 65.21%) isolates showed resistance to rifampicin and had a mutation in 81 bp RRDR of rpoB gene. There were five mutation patterns within the RRDR, the most common of which are S531L (5/23, 21.73%), and D516V (4/23, 17.29%). The less common mutations were L533P, S522L, H526R which have similar prevalence (8.69%). This result is concordant with the study of (21) who reported common mutations at codons 531, 526 and 516 in Kuwait.

The most common mutation outside the RRDR was M479L found in (6/23, 26.08%) of the isolates. Although most of the studies revealed single mutation in *rpoB* gene, we found double mutation in (9/23, 39.13%) isolates and triple mutation in one isolate. The current results are similar to the studies of (22, 23)and even triple mutation as reported by (22).

In addition to common mutations within RRDRD, new non-synonymous mutations were identified in our study including Met479 Leu, Val475Asp, Thr481Ile, Phe584Leu, Val 597Ala, Val691Ala, Val927Ala, Leu943Pro, and one synonymous mutation Pro99Pro. In addition to the previously reported mutations, there are newly identified mutations in the current study. The increase in mutations probably due to the ability of *M. tuberculosis* to fit itself with anti-tubercular drugs through more than one mutations in RIF resistance-related genes.

Brandis *et al.*, (2012) (24) declared that compensatory mutations in the genes *rpoA* and *rpoC* relieve the fitness cost caused by rifampicin resistance-conferring mutations in *rpoB.* Another study (Comas et al.,2011) (25) proposed that secondary mutations in the *rpoA* or *rpoC* gene could alleviate the fitness cost incurred by *rpoB* mutations, especially those in the RRDR. We investigated the *rpoC* mutation patterns in drug-resistant and susceptible *M. tuberculosis* isolates. Seven different types of mutations were identified, six of which are reported for the first time in this study. Mutation at codons 600 and 260 were the most common mutations (7/24, 29.2%).

Among 23 RIF resistant isolates analyzed for mutations in the *rpoA* gene, (6/23, 26.08%) harbored nonsynonymous mutations. The most prevalent one is R182L. There is no nonsynonymous mutation in *rpoA.*

Conclusions:

This study provided the first investigation that mutations in *rpoB, rpoA*, and *rpoC* contribute to RIF resistance in *M. tuberculosis*. Also, there are mutations outside the RRDR of *rpoB* which provided resistance to rifampicin. Five mutation patterns are detected inside the RRDR of *rpoB*; the most common is S531L mutation. Although there are no synonymous mutations within the RRDR, there is one synonymous mutation (P99P) outside the RRDR.

The number of mutation sites in both *rpoA* and *rpoC* is equal. There are seven nonsynonymous mutations in the *rpoA* gene, and the most prevalent one is R182L. The most common mutations in *rpoC* are Q600E and S260L. No synonymous mutations are detected in *rpoA or rpoC.* These new findings may be relevant to realize how compensatory mutations in the *rpoA* and *rpoC* genes restore the fitness cost caused by rifampin resistance-conferring mutations in *rpoB.*

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Authors' declaration:

- Conflicts of Interest: None.

- We hereby confirm that all the Figures and Tables in the manuscript are mine ours. Besides, the Figures and images, which are not mine ours, have been given the permission for re-publication attached with the manuscript.
- Ethical Clearance: The project was approved by the local ethical committee in Salahaddin University.

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التحليل الجزيئي لمقاومة الريفامبيسين يمنح طفرات في مرض السل المتفطرات

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الخلاصة:

ان مقاومة بكتريا (*Mycobacterium tuberculosis*) للمضاد الحيوى Rifampicin يكون عن الطريق طفرة الجين . *rpoB* ان تاثيرات طفرات *rpoB* يمكن ان ينزل بواسطة طفرات ثانوية في الجين *rpoA* و *rpoC*. هذه الدراسة هدفها كشف و تحديد الطفرات فى الجينات *rpoB* و *rpoA* و *rpoC* في *M. tuberculosis* المعزولة و مساهمتها في مقاومة.سبعون عينة معزولة تم تحديدها بطريقة مسمة Acid-Fast Bacilli Smear ونمو في وسط Lowenstein Jensen . تم الفحص استجابة البكتريا للادوية بالطرق المتناسبة. استخلاص ال DNA التسلسل المنجز للجينات *rpoB* و *rpoA* و *rpoC* . ثلاثة و عشرون معزولة 23% اظهرت مقاومة للرفامبسين اما بطريقة المتناسب او فحص Genexpert. اظهر تحليل التسلسل للجين اربعة عشرة نمط طفرة. في داخل المنطقة المحددة RRDR لمقاومة الريفامبسين هناك هناك طفرات كبيرة في codon S531L,D516V, مع تكرار (٢١،٧٣٪ و١٧،٣٩٪) بالتوالي. في خارج المنطقة المحددة RRDR هناك تسعة طفرات مختلفة و M479L الأكثر تواجداً.سبعة من اصل ثلاثة وعشرون عزلة مقاومةتحمل طفرات في *rpoA* وإثناعشرة عزلة تحمل طفرة في *rpoC*. الأكثر الطفرات مشخصة لأول مرةفي هذه الدراسة. الدراسة الحالية تظهر بأن الطفرات في *rpoB*، *rpoA*، *rpoC* تعزى الى مقاومة الريفامپسين في البكتريا. هذه الطفرات المشخصة لأول مرة من الممكن ان تكون لها العلاقة ذات الصلة لإدراك كيف تقوم الطفرات التعويضية في جينات *rpoA* و *rpoC* باستعادة تكلفة اللياقة الناتجة عن طفرات ريفامبين المقاومة للمقاومة في *rpoB*.

الكلمات المفتاحية: السل الرئوي، جين *rpoA*، جين *rpoB*، جين *rpoC*، منطقة RRDR ، السل.