Original Article

Molecular Study of the (*rpoB*) Gene as a Diagnostic Tool in *Acinetobacter baumannii* and the Identification of the Mutations in this Gene Led to Rifampicin Resistance

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Abstract

Background: Rifampicins are bactericidal antibiotics that prevent the synthesis of RNA by interacting with the *rpoB*-gene encoded bacterial RNA polymerase beta subunit. The *rpoB* gene has emerged as a pivotal candidate gene for bacterial diagnostics and phylogenetic investigations. In recent years, the *Acinetobacter baumannii* acquired a prominent interesting as a result of their high resistance to broad spectrum of antibiotics. **Objective:** The current study was designed to identify the sequence of *rpoB* gene, the mutation led to rifampicin resistance and compared them to standard isolates in the NCBI and sensitive isolates in Iraq (local control). **Materials and Methods:** Three hundred samples in total were gathered from patients with various infections in Medical City Hospital during the period from July 1, 2022 to January 30, 2023. The Vitek 2 system was used to identify bacterial isolates, cultural media (blood agar, macconkey agar, chromagar™) and the partial sequence of the *rpoB* gene whereas Kirby-Bauer method was used to test antibiotic susceptibility. **Results:** Total genomic DNA was extracted from the most resistant (MDR) thirty bacterial isolates in order to genetically identified by PCR and sent for sequencing. PCR and DNA sequence analysis of *rpoB* revealed that the RIF-resistance determining region (RRDR) of the *rpoB* gene had been modified in at least nine of the isolates. And the 21 of the isolates with no mutation, six mutations were discovered (CAA, ATG, GGA, ACT, GCA, GAT), four of them are novel mutation G114G, T249T, A348A, and D444D (Silent mutation) identified for the first time in Iraq when compared with sensitive isolates (local control). Most *A. baumannii* isolates with *rpoB* gene mutations exhibited the rifampicin resistance in 8 isolates (88.8%), only one isolate with *rpoB* gene mutation was sensitive to rifampicin. **Conclusion:** At the end, the molecular analysis showed that *rpoB* gene mutations in some isolates play a significant role in rifampicin resistance, particularly if the mutation at the *rpoB*-binding site to rifampicin.

Keywords: *A. baumannii* mutations, *A. baumannii* resistance, rifampicin, *rpoB* gene

Introduction

The World Health Organization (WHO) recently ranked antibiotic resistance as one of the top three threats to human health. The most prevalent and harmful MDR pathogens are referred to by the abbreviation ESKAPE, which stands for "*Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumonia, Acinetobacter baumannii, Pseudomonas aeruginosa*, and *Enterobacter* spp."[1] During wars in "Lebanon, Afghanistan, and Iraq." One of the most common pathogens, *A. baumannii*, is in charge of numerous MDR infection outbreaks in combat casualties.[2] In particular, when examining closely related isolates, the RNA polymerase b-subunit-encoding "*rpoB*" gene has become a core gene candidate for phylogenetic studies and bacterial identification. Along with the 16S rRNA gene, *rpoB* has enhanced the analysis of bacterial communities, assisted in the discovery of new bacterial species, and assisted in the tracking of mutations that

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result in rifampicin resistance.[3] Alternating conserved and variable regions can be found in the *rpoB* gene.[4] For preserved regions with an internal variable region, broad-spectrum primers can be created.[5] It has been possible to examine bacteria from the same group or closely related groups using various *rpoB* gene fragments. The *rpoB* gene fragment used for identification determines the cut-off for bacterial identification using the *rpoB* gene sequence: correct species identification depends on a *rpoB* gene fragment sequence similarity of at least "94%–95% " for 300–600" bp.[6] A *rpoB* gene-fragment sequence similarity of at least 96%–97% is required for "600–825" bp.[7] The bacterial RNA polymerase subunit encoded by the *rpoB* gene interacts with rifampicins, which are bactericidal antibiotics that prevent the synthesis of RNA. Examples of rifampicins include rifampicin, rifabutin, rifapentine, and rifaximin. Rifampicin is the most frequently prescribed first-line treatment for many bacterial diseases among these. Rifampin resistance is a growing problem in both produced and third-world nations.[8] Patients with immune system disorders face a more challenging situation when dealing with this issue.[9] So, for the effective management of bacterial diseases, antibiotic resistance detection at an early stage is of the highest priority. Some studies have revealed the genetic causes of bacterial isolates' resistance to anti-bacterial antibiotics.[10]

The *rpoB* gene, which encodes the RNA polymerase beta subunit, is the most likely site of point mutations, small insertions, and deletions that result in rifampin resistance.[11]

Aim of the study

The aim of this study was to investigate the use of the *rpoB* gene as a diagnostic tool in *A. bauumannii* and the identification of the mutation in this gene led to rifampicin resistance.

Materials and Methods

Samples collection

Three hundred samples in total were gathered from patients with various infections (burns, wound, sputum and urine samples) in Medical City Hospital during the period from (July 1, 2022) to (January 30, 2023). All samples were collected by sterile cotton swaps from both genders aged 16–60 years and children aged 1–12 years, and then streaked on MacConkey agar and blood agar incubated for 24 h at 37ºC.

Identification of bacterial isolates

All clinical samples were cultured for 24 h on chromagar, MacConkey agar, and blood agar at 37°C. Each bacterial isolate was identified using morphological, microscopic, and biochemical tests such as "oxidase, catalase, triple sugar iron agar (TSI), indole production, urease production, and citrate utilization." With a 98% confidence level, the Vitek-2 system was used to accurately identify each bacterial isolate. Finally, the isolates were identified using the *rpoB* gene for molecular identification.

Antibiotic susceptibility test

Kirby-Bauer method was used to conduct an antibiotic susceptibility test in accordance with CLSI (2021) guidelines. Eighty eight *A. baumannii* isolates previously identified by morphological traits and the VITEK-2 system participated in this test were cultured in 5 mL of nutrient broth and incubated at 37°C for 24 h. The bacterial broth was centrifuged at 5000 rpm for 5 min; then the supernatant was removed and the bacterial precipitate was diluted until it reached 0.5 McFarland turbidity standard, which contains approximately $1–2 \times 10^8$ CFU/mL. Following that, a sterile cotton swab was dipped in a bacterial suspension and any excess liquid was removed by pressing on the side of the tube before streaking on Muller-Hinton agar medium in various directions. Each antibiotic disk was then gently pressed on the agar surface with the help of "sterile forceps" after the inoculated plates had been left at room temperature for 10 min to allow excess moisture to be absorbed. At 37°C, the plates were incubated for 24 h. After incubation, a ruler was used to measure the diameter of the inhibition zone, which was then compared to the standard value for each drug as published by "CLSI" (2021). The inhibition zone is defined as the area around the disk that has no obvious visible growth.

DNA extraction

The genomic DNA of rifampicin resistant *A. baumannii* (30) clinical isolates was extracted using a commercial purification system (Promega, USA). Using a spectrophotometer (NanoDrop) the DNA's purity and quantity were determined by measuring optical density (OD). The extracted DNA was then stored for future use at –20°C.

PCR reaction mixture

The primers, nuclease-free water, and master mix were all contained in the 0.2 mL Eppendorf tube. The total volume of PCR mixture was 25 µL as shown in Table 1.

Table 1: Reaction mixture of PCR

Components	Reaction volume (µL)	Final concentration
Master mix (2×)	12.5	1×
Forward primer (10 µM)	1	0.4 µM
Reverse primer (10 µM)	1	0.4 µM
DNA template	2	< 250 ng
Nuclease free water	8.5	–
Final volume	25	–

Table 2: PCR amplification program of *rpoB* for *A. baumannii*

Steps	Time (min)	Temperature (°C)	No. of cycles
Initial denaturation	5	95	1
Denaturation	2	95	30
Annealing	0.5	60	
Extension for rpoB	0.75	72	
Final extension	5	72	1

Table 3: Primers and their sequences

Primer	Sequence (5'–3')	Primer size (bp)	*Tm*	GC%	Product size (bp)	References
rpoB-F	GAGCGTGCTGTTAAAGAGCG	20	65	55	750	Current study
rpoB-R	GCCACGGTTTGCGATTACAC	20	65	55		Current study

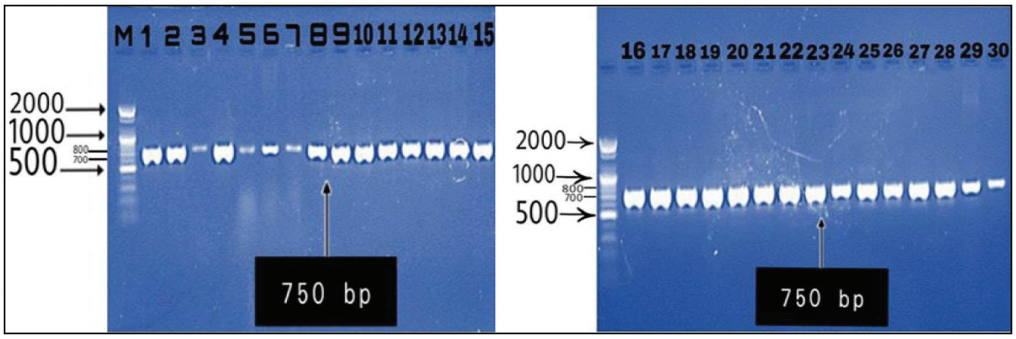

Figure 1: Amplified *rpoB* gene of thirty bacterial isolates of *A. baumannii* using specific primers (rpoB-F and rpoB-R). Electrophoresis was done using 1% agarose for 1 h at 70 V

The thermal cycler was filled with the PCR tubes to begin amplification reaction according to the specific program for each primer pair as in Table 2. In this study, the primers were applied as shown in Table 3.

Sequencing

The isolates were delivered to the Macrogen Company for sanger method sequencing; then an alignment of nucleotides sequencing of *rpoB* gene was compared with sensitive isolates and standard isolate in NCBI center and the phylogenetic tree were done by CLC WORKBENCH bioinformatics software according to the partial *rpoB* sequence.

Ethical approval

The study was conducted in accordance with the ethical principles that have their origin in the Declaration of Helsinki. It was carried out with patients verbal and analytical approval before sample was taken. The study protocol and the subject information and consent form were reviewed and approved by a local ethics committee according to the document number 12 (including the number and the date in June 4, 2022) to get this approval.

Results

Among the total of (300) samples, the findings revealed that 103 isolates (34.3%) were gram-positive and 109 isolates (36.3%) were gram-negative. Only 88 isolates (29.3%) shared the same morphological and biochemical characteristics as *A. baumannii*. Only 12 isolates were sensitive to rifampin, and the 30 most rifampin-resistant isolates were selected for molecular study and sequencing.

The detection of *rpoB* gene was done by using "*rpoB*-F and *rpoB* R" primers, and the size of the amplified fragment was 750 bp as shown in Figure 1.

Lane M: 100 bpDNA ladder

Variations in rpoB gene of Acinetobacter baumannii

Six mutations were discovered (CAA, ATG, GGA, ACT, GCA, and GAT). Table 4 describes the types of mutations, their positions, and their effects on gene expression. The *rpoB* gene's (RRDR) substitution mutations changed one base to another and resulted in either no change in the protein that was produced, known as silent mutations (sense mutation), or a change that resulted in an exchange of the produced protein (missense mutations).

The sequence of *rpoB* gene was aligned with control isolates (sensitive isolates 29,30) and with NCBI (standard isolate).

PCR and DNA sequence analysis of *rpoB* showed that nine of the isolates carried at least one mutation at (RRDR) of the *rpoB* gene and the 21 of the isolates with no mutation. The most common variation (Silent mutation), found in nine isolates (30%), was the substitution of ACA to ACT at position 249. The rest of the silent mutations were as follows: substitution of GCT to GCA found in eight isolates (26.6%) at position 348, substitution of GGC to GGA found in five isolates (16.6%) at position 114, and a less prevalent mutation was a substitution of GAC to GAT found in two isolates (6.6%) at position 444. Also there are two missense mutations. Substitution of histidine with glutamine at position 60 was detected in four isolates (13.3%); this mutation had previously appeared in a study in Italy by,[15] and the substitution of isoleucine with methionine at position 198 was detected in five isolates (16.6%). Most *A. baumannii* isolates with *rpoB* gene mutations exhibited the rifampicin resistance in eight isolates (88.8%), only one isolate with *rpoB* gene mutation was sensitive to rifampicin, and this isolate was the only one that did not contain mutations in the drug-binding site (No. 10). In addition, another isolate (No. 35) was resistant to rifampicin, but it contained two silent mutations outside the binding sites.

rpoB partial sequence analysis and alignment with standard isolate in NCBI of four novel *rpoB* gene mutations in the target region:

ACT

The sequencing outcome showed that SNP A → T [Table 4]. The noticed SNP was a silent mutation; it was substitution variation, "the Codon ACA was changed into codon ACT." The altered codon continued to encode the identical amino acid (threonine), so this point mutation had no impact on the expression of the gene; this variation was found in nine (30%) isolates (2, 3, 6, 8, 11, 12, 25, 26, 27). The partial sequencing profile of *rpoB* gene who shows this variation in resistant isolates when compared to the sensitive isolates (local control) as shown in Figures 2 and 3, respectively.

Then an alignment of nucleotides sequencing of 750 pb gene who shows no variation in resistant isolates when compared with standard isolate in NCBI center using automated sequencer and analyzed by BLAST data, as shown in Figure 4.

GCA

The sequencing outcome showed that SNP T → A [Table 4]. The noticed SNP was a silent mutation; it was substitution variation, "the codon GCT was changed into

Table 4: Variations in *rpoB* gene in *Acinetobacter baumannii* isolates

No.	Mutation	Type	Position	Wild type codon	Mutated codon	Change of amino acid*	Effect on translation	Kind of mutation	No. of isolates
1	T → A	Substitution	60	CAT	CAA	H → Q	Missense mutation	Point mutation	4
2	C → G	Substitution	198	ATC	ATG	I → M	Missense mutation	Point mutation	5
3	C → A	Substitution	114	GGC	GGA	G → G	Silent mutation	Point mutation	5
4	A → T	Substitution	249	ACA	ACT	T → T	Silent mutation	Point mutation	9
5	T → A	Substitution	348	GCT	GCA	A → A	Silent mutation	Point mutation	8
6	C → T	Substitution	444	GAC	GAT	D → D	Silent mutation	Point mutation	2

H: histidine, Q: glutamine, I: isoleucine, M: methionine, G: glycine, T: threonine, A: alanine, D: aspartate

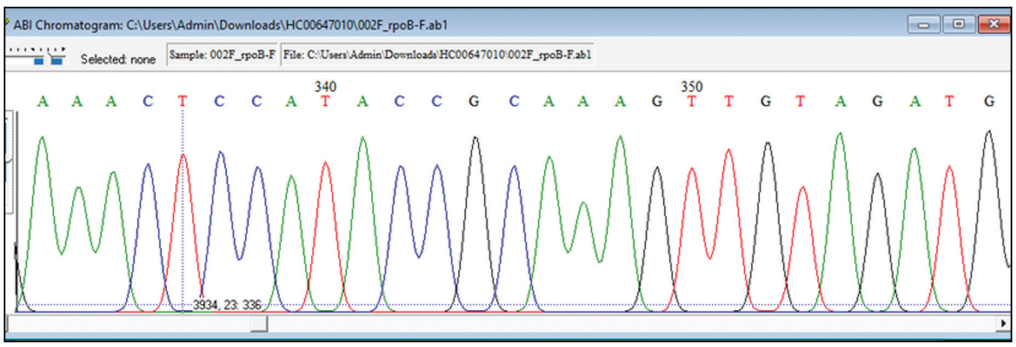

Figure 2: Nucleotide sequencing profile of *rpoB* gene for resistant (test) *Acinetobacter baumannii* isolates

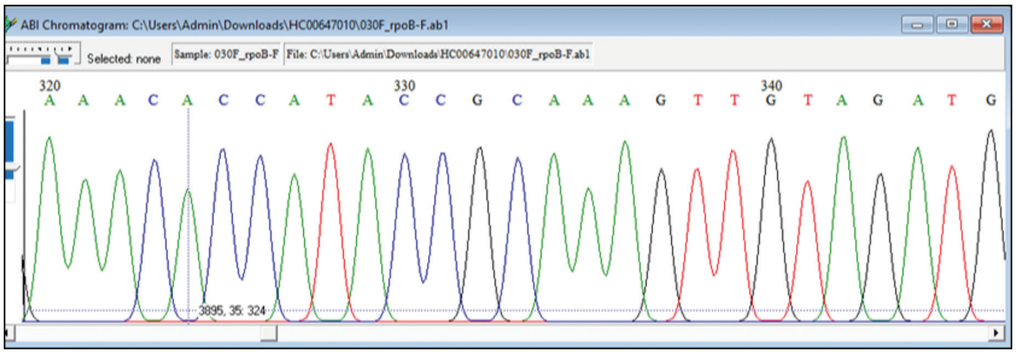

Figure 3: Nucleotide sequencing profile of *rpoB* gene for sensitive (control 29, 30) *Acinetobacter baumannii* isolates

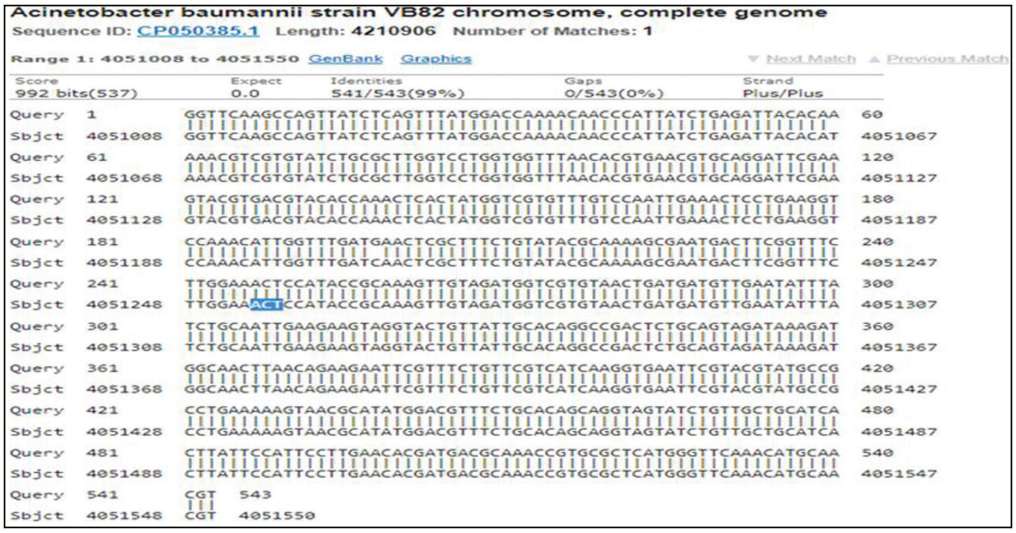

Figure 4: Using BLAST, the alignment of the *A. baumannii* variation's *rpoB* gene sequence at position (249) was examined. The query number denotes the current results, while the subject denotes the reference sequence

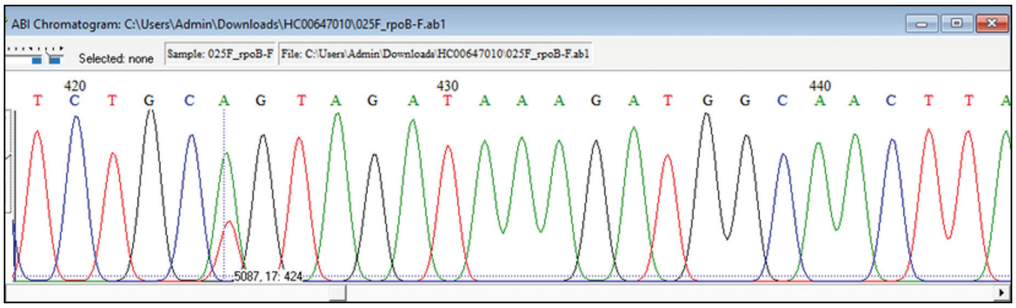

Figure 5: Nucleotide sequencing profile of *rpoB* gene for resistant (test) *Acinetobacter baumannii* isolates

codon GCA." The altered codon continued to encode the identical amino acid (Alanine), so this point mutation had no impact on the expression of the gene; this variation was found in 8 (26.6%) isolates (2, 3, 8, 11, 12, 27, 26, 25). The partial sequencing profile of *rpoB* gene who shows this variation in resistant isolates when compared to the sensitive isolates (local control) as shown in Figures 5 and 6, respectively.

Then an alignment of nucleotides sequencing of *rpoB* gene who shows no variation in resistant isolates when compared with standard isolate in NCBI center using automated sequencer and analyzed by BLAST data, as shown in Figure 7.

GGA

The sequencing outcome showed that SNP C → A [Table 4]. The noticed SNP was a silent mutation; it was substitution mutation. "The codon GGC was changed into GGA." The altered codon continued to encode the identical amino acid (Glycine), so this point mutation had

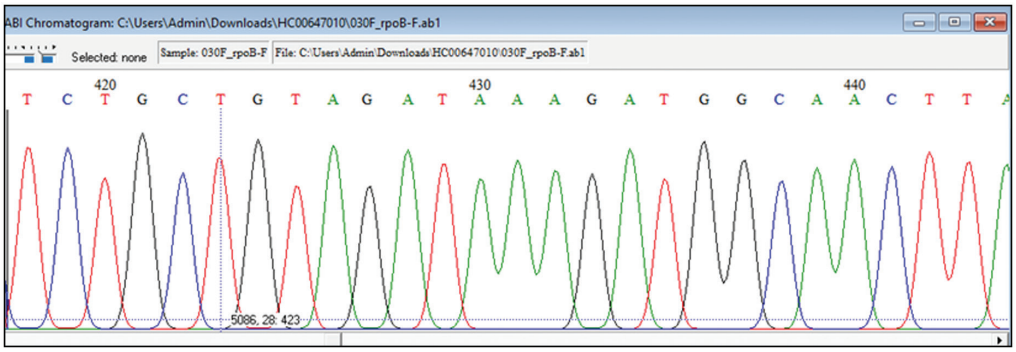

Figure 6: Nucleotide sequencing profile of *rpoB* gene for sensitive (control 29, 39) *Acinetobacter baumannii* isolates

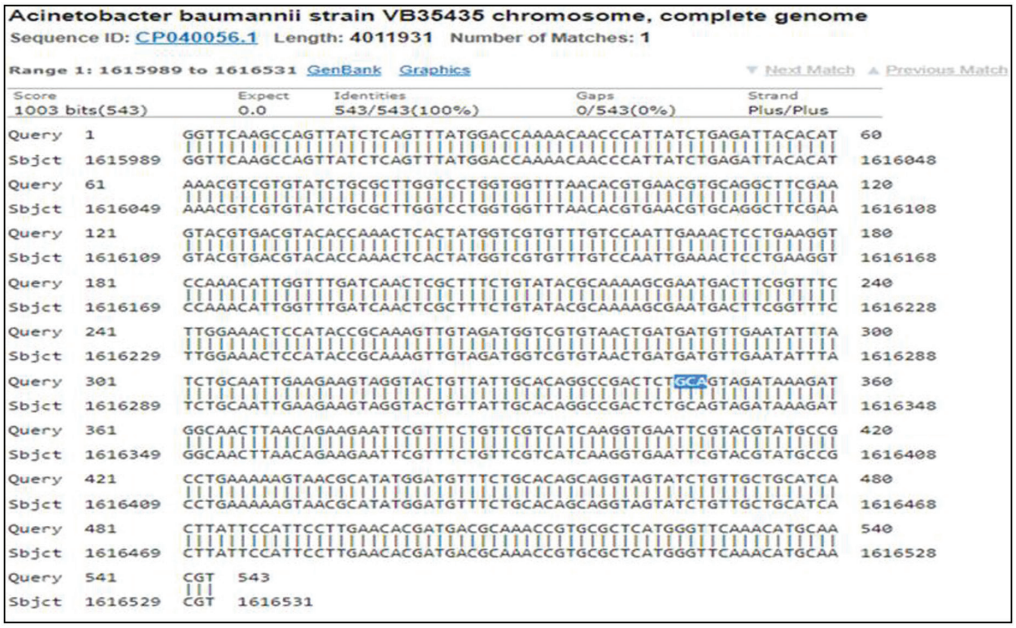

Figure 7: Using BLAST, the alignment of the *A.baumannii* variation's *rpoB* gene sequence at position (348) was examined. The query number denotes the current results, whereas the subject denotes the reference sequence

no impact on the expression of the gene. This variation was found in five (16.6%) isolates (2, 3, 8, 12, and 26). The partial sequencing profile of *rpoB* gene who shows this variation in resistant isolates when compared to the sensitive isolates (local control) as shown in Figures 8 and 9 respectively.

Then an alignment of nucleotides sequencing of *rpoB* gene who shows no variation in resistant isolates when compared with standard isolate in NCBI center using automated sequencer and analyzed by BLAST data, as shown in Figure 10.

GAT

The sequencing outcome showed that SNP C → T [Table 4]. The noticed SNP was a silent mutation; it was substitution mutations. The codon "GAC was changed into codon GAT." The altered codon continued to encode the identical amino acid (Aspartate), so this point mutation had no impact on the expression of the gene, these variation was found in 2 (6.6%) isolates (25, 27). The partial sequencing profile of *rpoB* gene who shows this variation in resistant isolates when compared to the sensitive isolates (local control) as shown in Figures 11 and 12, respectively.

Then an alignment of nucleotides sequencing of *rpoB* gene who shows no variation in resistant isolates when compared with standard isolate in NCBI center using automated sequencer and analyzed by BLAST data, as shown in Figure 13.

Phylogenetic tree of *Acinetobacter baumannii*

The *rpoB* partial sequences were delivered as.ab1 chromatogram files. The sequence files were viewed, trimmed, and examined using the BioEdit sequence alignment editor version 7. BioEdit was also used to check for and correct sequencing errors. Molecular Evolutionary Genetics Analysis (MEGA11) software was used for multiple sequence alignment with ClustalW and phylogenetic analyses.

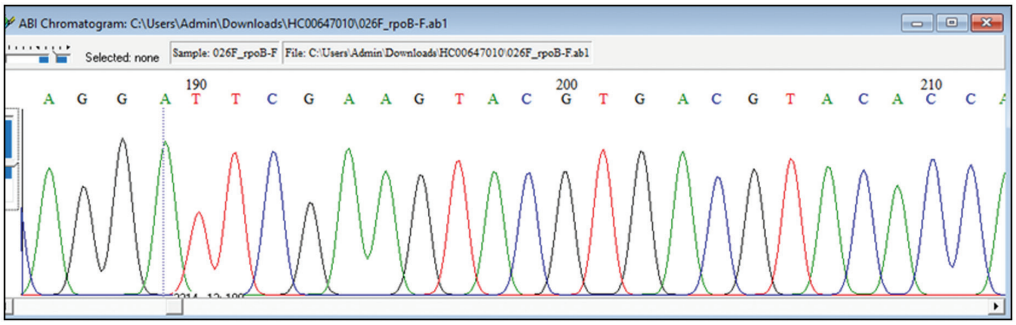

Figure 8: Nucleotide sequencing profile of *rpoB* gene for resistant (test) *Acinetobacter baumannii* isolates

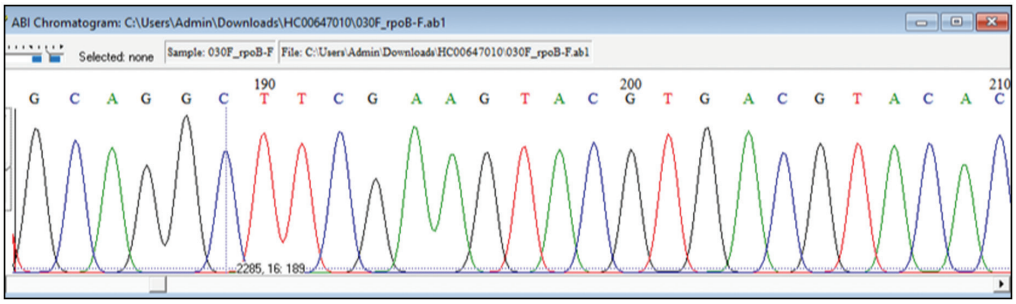

Figure 9: Nucleotide sequencing profile of *rpoB* gene for sensitive (control 29,30) *Acinetobacter baumannii* isolates

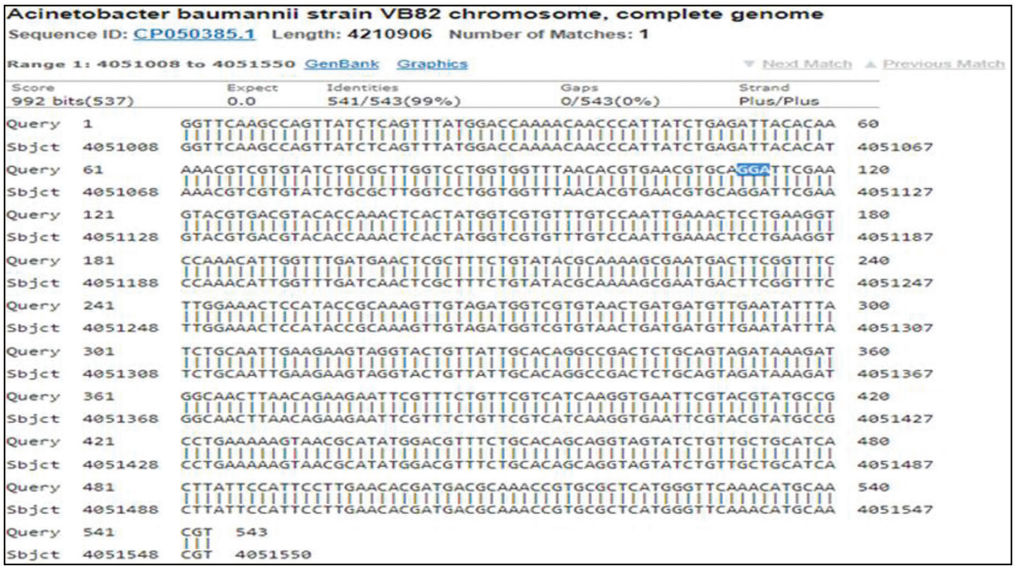

Figure 10: Using BLAST, the alignment of the *A. baumannii* variation's *rpoB* gene sequence at position (114) was examined. The query number denotes the current results, whereas the subject denotes the reference sequence

Figure 14 clarifies the phylogenetic tree of 30 *A. baumannii* isolates depending upon the partial *rpoB* sequences.

Discussion

Under normal and pathophysiological conditions, all cells in an organism express housekeeping genes, which are typically constitutive genes required for basic cellular function maintenance.[12,13] The results revealed that 30 out of 30 isolates (100%) have *rpoB* gene. A number of issues arise when using 16S sequencing, including primer bias, gene copy number, PCR or sequencing artifacts, and contamination. Metabarcoding approaches that target a taxonomically relevant marker, such as the *rpoB* gene, are a potential alternative, allowing at least some of these challenges to be overcome.[14] Rifampicin resistance is caused by mutations in the *rpoB* gene, and these modifications reduce the affinity of RNAP for rifampin.[16] When bacteria are exposed to environmental stresses, the bacteria get adapted by coordinated transcriptional changes; this is called bacterial

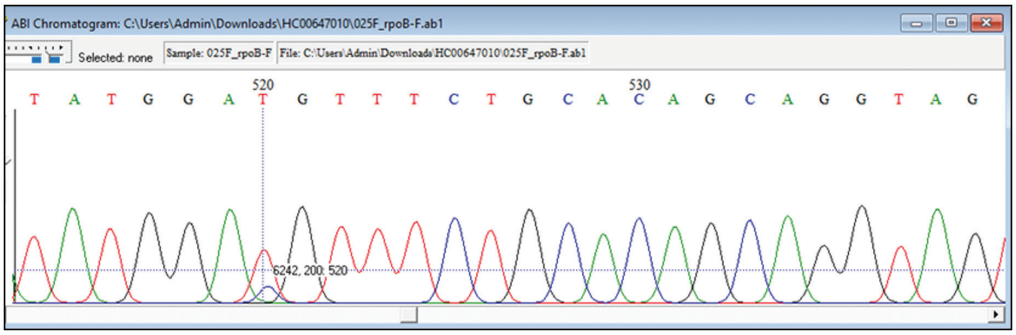

Figure 11: Nucleotide sequencing profile of *rpoB* gene for resistant (test) *A. baumannii* isolates

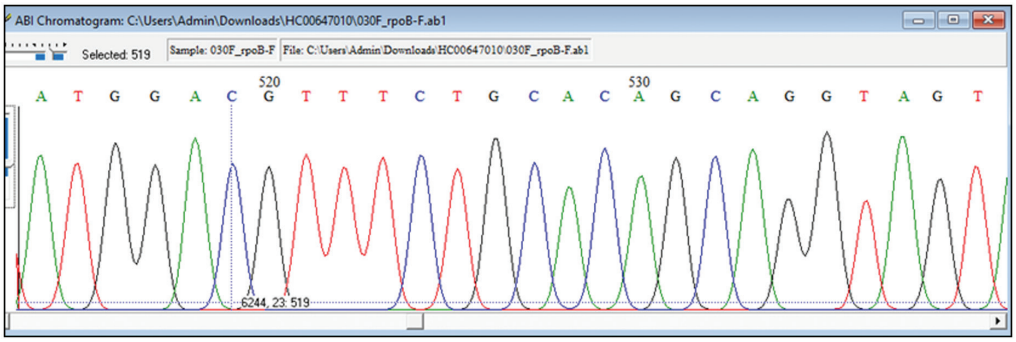

Figure 12: Nucleotide sequencing profile of *rpoB* gene for sensitive (control 29, 30) *A. baumannii* isolates

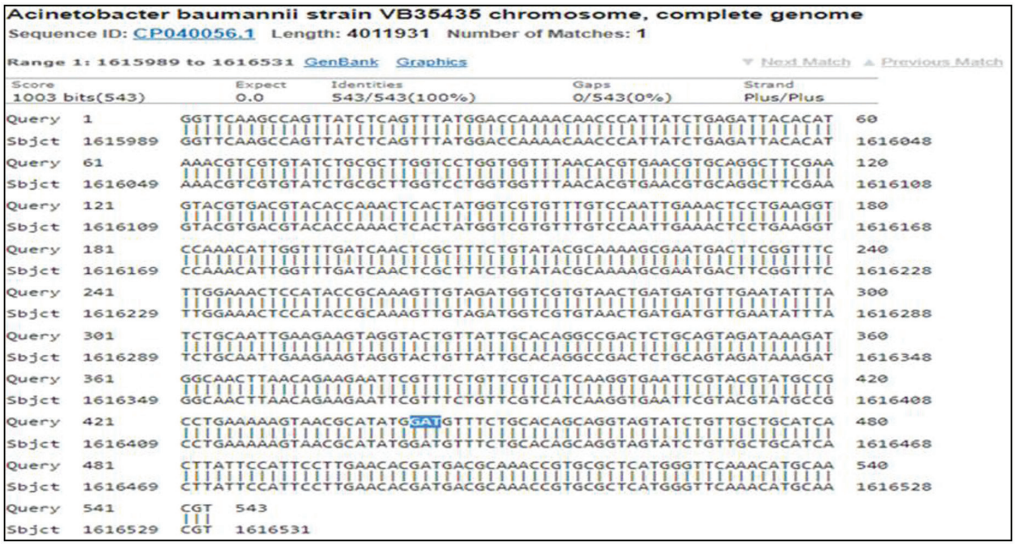

Figure 13: Using BLAST, the alignment of the *A.baumannii* variation's *rpoB* gene sequence at position (444) was examined. The query number denotes the current results, whereas the subject denotes the reference sequence

stress responses.[17] When these phenotypic processes are exhausted and the majority of cells are either killed or have their growth inhibited, there is a strong selective pressure for the emergence of adaptive mutations that confer resistance. Among these stressors are nutritional deficiencies, DNA damage, temperature changes, and antibiotic exposure. All of these universal stress responses have components that may lead to an increase in genetic variability. Multiple stress responses include the upregulation and activation of error-prone DNA polymerases, the downregulation of error-correcting enzymes, and the movement of mobile genetic elements.[18] Mutations in the *rpoB*/*rpoC* genes, which encode the β and β' subunits of (RNAP) core enzyme, these two subunits, which make up the enzyme's active site and account for 80% of its total mass, can help the enzyme adapt to a variety of environmental and antibiotic stresses.[19] The high rates of conservation of housekeeping genes like the RNAPC (*rpoB* and *rpoC*) have a proclivity to rapidly acquire adaptive mutations that alter their sequences in response to a variety of selective pressures. The bacterial RNA polymerase

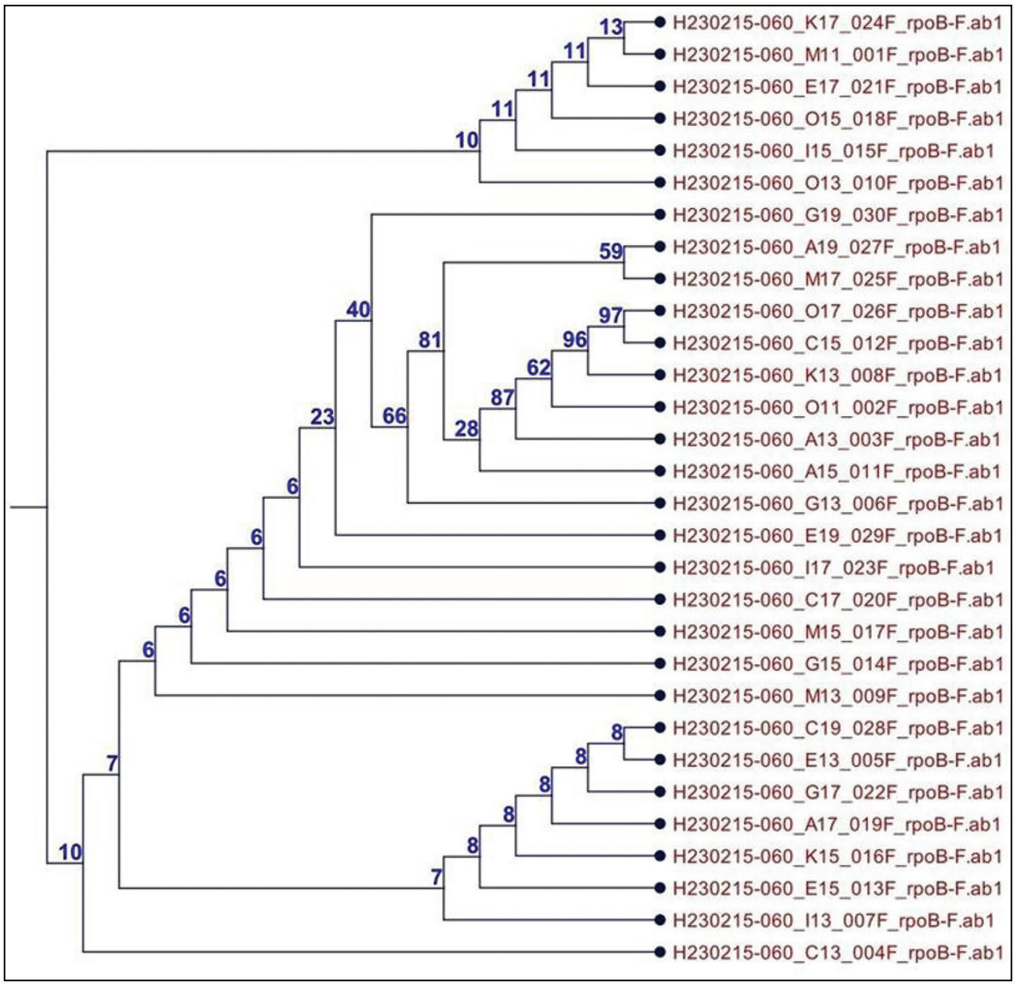

Figure 14: Phylogenetic tree of the 8 resistant local isolates of *A. baumannii* compared to sensitive control (two local isolate) based on the partial sequence of *rpoB* gene Done by CLC WORKBENCH bioinformatics software

can be used as a model to comprehend fundamental ideas at play in all cellular RNA polymerases because of this extensive conservation.[20] These mutations caused modifications to the RNA polymerase active site in the amino acids surrounding the RIF-binding pocket.[21] Mutations are more common in the amino acid residues of the RNAP -subunit active site, such as aspartic acid and histidine, which are involved in drug binding.[22] The most effective mutations for promoting adaptive responses are those that replace the glutamine (Q) and histidine (H) residues in the cluster I region of *rpoB*. They cause the bacteria to behave differently on a global scale, causing them to use alternative substrates, engage in secondary metabolism, produce spores or germinate, or develop antibiotic hetero-resistance.[23] In our study, both mutations occurred such as H60Q (missense mutation) and D 444 D (Silent mutation), in addition to other mutations that occurs outside of those regions I198M (missense mutation), A348A, G114G, T249T, (Silent mutation), and the silent mutations are a novel mutation in local isolates identified for the first time in Iraq when compared with sensitive isolates (local control) as shown in Figures 2 and 3. RIF's effectiveness, like that of other antibacterial agents, can be determined by the degree to which various *Acinetobacter* isolates are sensitive to this compound and this might be because of the microbial isolate's unique resistance mechanisms, which could involve altering the antibiotic's permeability, modifying the target's enzymatic structure, and chromosomal changes to the (RNAP) -subunit *rpoB* target gene.[22] However, silent mutation behaves differently than standard antibiotic resistance mutations. Usually they involve modifying the genetic code so that a different chain of amino acids with distinct functions is produced when the genetic material is "read" by ribosomes and translated into a protein. The same amino acid chain is produced by this mutation, but the structure of the crucial mRNA intermediate is altered, making ribosomes unable to read the protein's code and produce it.[24] Some bacteria develop *rpoB* mutations, and the majority of the mutated residues are in hotspots that are kept across species and are referred to as the (RRDR). The majority of mutations are point mutations, even though there have been some small insertions "1–2 codons" and deletions "2–3 codons" found.[25] The phylogenetic tree done by CLC WORKBENCH bioinformatics software, a branching diagram or tree illustrating the relationships

between various biological species or other entities based on their genetic or physical similarities and differences.[26] The genetic divergence between the isolates was determined according to a value of bootstrapping. Numerous studies have shown the value of using the RNA polymerase (*rpoB*) subunit gene for phylogenetic analysis and *Brucella* strain identification, especially in highly homologous isolates. ADditionally, novel bacterial species can be found and the bacterial community can be studied thanks to *rpoB*-based genotyping.[27] The bacterial "*rpoB* and *rpoC*" sequences are sufficiently kept to allow for the use of those genes as slowly evolving phylogenetic markers.[28] With hardly any overlap between the various conditions, the adaptation to various conditions involves a specific set of RNAPC positions. ADditionally, positions involved in adaptation tend to be even more conserved in bacteria than other RNAPC positions when combined across conditions, and they also tend to be located closer to the protein complex's active site. Finally, we show that when resources are scarce, adaptations within non-RNAPC housekeeping genes occur frequently within the more conserved areas of these proteins.[19]

Conclusion

The molecular study revealed that mutations of *rpoB* genes in some isolates play a major role in rifampicin resistant, especially if the mutation at the binding site of *rpoB* to rifampicin. Four novel mutations such as G114G, T249T, A348A, and D444D (silent mutation) are identified for the first time in Iraq when compared with the sensitive isolates (local control). The *rpoB* gene can be used as a diagnostic tool at the species and subspecies level.

Financial support and sponsorship

Nil.

Conflicts of interest

There are no conflicts of interest.

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