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Original Article

Analysis of Nucleotide Sequences Similarity and Protein Prediction of Some Resistance Genes in *Escherichia coli* Isolated from Iraqi Patients with Urinary Tract Infections

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Abstract

Antibiotic resistance leads to a dramatic increase in the morbidity and mortality caused by infectious diseases. Even though estimates vary widely, the economic cost of antimicrobial-resistant bacteria is on a rise. The current aimed to identify the antimicrobial resistance of *Escherichia coli* (*E. coli*). In fact, this study focused on the recent deep-learning methods (sequencing) to investigate *E. coli* antibiotic resistance and their protein sequences. To evaluate antibiotic resistance, the sequencing method could be considered the method of choice. The *E. coli* was identified by either specific biochemical tests or polymerase chain reaction (PCR) using the 16S rRNA gene. The results of *aadA1* gene sequences demonstrated 10 nucleic acid substitutions throughout, as compared to the reference NCBI database (MG385063). Out of the 10 nucleic acid substitutions, 9 missense effects were observed. While the *dfr*A1 gene sequences illustrated 20 nucleic acid substitutions throughout, compared to the reference NCBI database (KY706080), out of the 20 nucleic acid substitutions, 8 missense effects were observed. Furthermore, the *sul1* gene sequences displayed 20 nucleic acid substitutions throughout, in comparison with the reference NCBI database (CP069561), and out of the 20 nucleic acid substitutions, 12 missense effects were detected. The *cat1* gene sequences showed 14 nucleic acid substitutions throughout, compared to the reference NCBI database (NC017660), and out of the 14 nucleic acid substitutions, 8 missense effects were observed. The precise point (Missense) mutation in four genes (aadA1, dfrA1, sul1, and cat1) in the expected sequence is interpreted to be the target site of a site-specific recombination mechanism that led to antibiotics resistance in *E. coli* isolates.

Keywords: *aadA1, cat1, dfrA1, Escherichia coli,* NCBI, Resistance genes, Sequencing, *sul1*

1. Introduction

Antimicrobials are small molecules that can either destroy or repress most bacteria, except for multidrug-resistant bacteria which limit treatment options and increase morbidity and mortality in clinical settings (1, 2). In recent decades, several methods and tools have been used to detect antibiotic resistance. Organizing sequencing data is an important pre-processing step before the analysis of antimicrobial resistance genes. Short-read sequencing generated by such technologies as Illumina can be processed using assembly-based methods, in which sequencing reads are first assembled into contiguous fragments (contigs) and then annotated by making a comparison with custom or public reference databases. Moreover, they can be directly analyzed using read-based methods in which resistance determinants are predicted by mapping reads directly to a reference database.

Owing to the published data from whole-genome sequencing (WGS) and whole-metagenome sequencing (WMS) for detecting genetic determinants of antimicrobial resistance (1), the researchers discovered

the data with more than 130 resistance genes (3). They contain genes for aminoglycoside adenylyltransferase (*aadA1*), dihydrofolate reductase (*dfrA1*), sulphonamide resistant (*sul1*), and chloramphenicol acetyltransferase (*cat1*), which confer resistance to streptomycin, trimethoprim, sulfonamides, and chloramphenicol, respectively (4, 5). Due to the significance and risk of *Escherichia coli (E. coli)*, the increased incidence of infection, the possibility of an infection epidemic, and the lack of treatment, the *E. coli* infection and the antibiotic resistance have led to the global focus of research on the improvement of efficient molecular detection using advanced technologies, as well as the reduction of time and effort.

The best diagnostic methods, such as polymerase chain reaction (PCR) and sequencing techniques, are characterized by the technique of specificity and high speed in the detection of the *E. coli* genes. Advancements in sequencing technology have increased the availability of bacterial sequence data, and continuously reducing costs have made sequencing a viable antimicrobial resistance surveillance tool. In light of the aforementioned issues, the present study aimed to investigate *E. coli* antibiotic resistance by a sequencing-based method and their protein prediction.

2. Materials and Methods

2.1. Patients and the Bacterial Isolates

Approximately 100 samples were randomly collected from patients with urinary tract infections (UTIs) admitted to various hospitals between January and March of 2021. The bacterial isolates were identified using specific *E. coli* biochemical tests. Thereafter, the previous bacterial identification findings were verified by using the 16s rRNA gene (molecular identification).

2.2. Antimicrobial Resistance Genes Analysis

2.2.1. Polymerase Chain Reaction Analysis

All isolates were subjected to PCR amplification in order to identify resistance-encoding genes. These genes were as follows: *aadA1* (which confers streptomycin antibiotic resistance), *dfrA1* (which confers trimethoprim antibiotic resistance), *sul1* (which confers sulfonamide antibiotic resistance), and *cat1* (conferring resistance to Chloramphenicol antibiotic). The primers listed in table 1 were used to perform PCR amplification of the resistance genes.

Table 1. Identification of the genes by polymerase chain reaction with specific primers

Genes	Primers	Sequence of the nucleotides (5´ to 3´)	Polymerase chain reaction product (bp)	Annealing temperature (°C)
16S rRNA	F:16S rRNA R:16S rRNA	GGAAGAAGCTTGCTTCTTTGCTG GAGCCCGGGGATTTCACAT	546	55
dfrA1	F: dfrA1 R: dfrA1	GGAGTGCCAAAGGTGAACAGC GAGGCGAAGTCTTGGGTAAAAAC	367	55
sul1	F: sul1 R: sul1	TTCGGCATTCTGAATCTCAC ATGATCTAACCCTCGGTCTC	822	55
cat1	F: cat1 R: cat1	AGTTGCTCAATGTACCTATAACC TTGTAATTCATTAAGCATTCTGCC	547	55
aadA1	F: aadA1 R: aadA1	TGATTTGCTGGTTACGGTGAC CGCTATGTTCTCTTGCTTTTG	284	55

2.2.2. DNA Sequencing

All PCR amplicons were sequenced using the Sanger method with gene-specific forward primers (Table 1), according to the sequencing company's instructions (Macrogen Inc. Seoul, South Korea). Using BioEdit suit, the sequencing results of the PCR products were edited, aligned, and analyzed in the reference database with the corresponding sequences. The observed variations in PCR amplicons in each sequenced sample and their specified location within the referred genome were counted.

2.2.2.1. The Analysis of Single-Nucleotide Polymorphisms

Each query sequence was aligned with the reference sequence using the alignment software BioEdit

(http://mummer.sourceforge.net/). To detect potential single-nucleotide polymorphisms (SNP) sites, the variation sites between the query and reference sequences were identified and preliminarily filtered. Filtering out SNPs found in repeat regions yielded credible SNPs.

2.2.2.2. Analysis of Insertion/ Deletion

The reference sequence and query sequence were aligned, by the BioEdit software (http://www.bx.psu.edu/miller lab/dist/README.lastz-1.02.00/). The NCBI (http://bio-bwa.sourceforge.net/) and samtools (http://samtools.sourceforge.net/) were used to verify the alignment results.

2.2.3. Protein Sequencing

The amino acid sequences of the target proteins were obtained from the protein data bank (http://www.ncbi.nlm.nih.gov). This protocol describes a community-wide web-based method for protein tertiary structure modeling with RaptorX (http://raptorx.uchicago.edu/) and visualizing the 3D structure models with the molecular graphics software PyMOL (http://www.pymol.org/).

3. Results

3.1. Bacterial Isolates and Identification of the Resistance Genes

Out of the 100 samples, only 80 (80%) cases tested positive for growth. The distribution of bacterial isolates based on typical morphological characteristics and PCR detection by 16S rRNA identification of *E. coli* gene 50/80 (62.5%) isolates were more prevalent in patients, as compared to other bacterial isolates. The current study aimed to determine the resistance properties of 50 *E. coli* isolates and the prevalence of detected resistance genes (*aadA1*, *dfrA1*, *sul1*, and *cat1*) in all isolates.

3.2. Resistance Genes Sequence

A direct sequencing strategy was conducted in the currently investigated isolates to resolve the pattern of genetic diversity in each analyzed isolate (used five isolates for each gene). The results of *aadA1* gene sequences of five streptomycin resistance *E. coli* isolates (Table 2, Figure 1) demonstrated 10 nucleic acid substitutions, compared to the reference NCBI database (MG385063). Out of the 10 nucleic acid substitutions, 9 missense effects (L23P, E27K, D35N, I36F, W39G, E51K, N55I, K63E, and K63R) were observed in 5 investigated isolates (EC39, EC44, EC47, EC48, and EC49).

The results of the current study on the novel *aadA1* gene sequence mutations in the NCBI database were deposited under accession numbers MN317260, MN317261, MN458556, MN458557, and MN458558. The results of *dfrA1* gene sequences of five trimethoprim resistance *E. coli* isolates (Table 3, Figure 2) illustrated 20 nucleic acid substitutions throughout, in comparison with the reference NCBI database (KY706080). Out of the 20 nucleic acid substitutions, 8 missense effects (C28G, T32P, I37F, I47F, E57K, W68G, D70N, and R112P) were observed in 5 investigated isolates (EC39, EC44, EC47, EC48, and EC49).

The results of the current study on the novel *dfrA1* gene sequence mutations in the NCBI database were deposited under accession numbers MW363921, MW363922, MW363923, MW363924, and MW363925. The results of *sul1* gene sequences of 5 sulfonamide resistance *E. coli* isolates (Table 4, Figure 3) showed 20 nucleic acid substitutions throughout, compared to the reference NCBI database (CP069561). Out of the 20 nucleic acid substitutions**,** 12 missense effects (E3D, S4N, D8Y, D25N, R48K, D55N, A56P, I100F, D129N, E133K, R239Q, and A249P) were observed in five investigated isolates (EC39, EC44, EC47, EC48, and EC49).

In this study, novel *sul1* gene sequence mutations in the NCBI database were deposited under accession numbers (MN527462, MN527463, MN527464, MN527465, and MN527466). The results of *cat1* gene sequences of five chloramphenicol resistance *E. coli* isolates (Table 5, Figure 4) indicated 14 nucleic acid substitutions throughout, compared to the reference NCBI database (NC017660). Out of the 20 nucleic acid

substitutions, 8 missense effects (F6L, L7F, Y18N, M29V, K40E, I46V, S114G, and M147V) were observed in 4 investigated isolates (EC39, EC44, EC47, EC48, and EC49). The results of the current study on novel *cat1* gene sequence mutations in the NCBI database were deposited under accession numbers MW357541, MW357542, MW357543, MW357544, and MW357545.

Table 2. Nucleic acid substitutions and effect on amino acid of the *aadA1* gene

NO.	Mutation isolates in *aadA1*	Nucleotide		Amino acid		Type of Mutations
		Position	Change codon	Position	Change	
1.	EC47, EC49	51	CCT-CCG	17	P-P	Silent
2.	EC47, EC48, EC49	68	CTC-CCC	23	L-P	Missense
3.	EC48, EC49	79	GAA-AAA	27	E-K	Missense
4.	EC44	103	GAC-AAC	35	D-N	Missense
5.	EC39, EC44, EC48	106	ATC-TTC	36	I-F	Missense
6.	EC39, EC44	115	TGG-GGG	39	W-G	Missense
7.	EC39	151	GAA-AAA	51	E-K	Missense
8.	EC39	164	AAT-ATT	55	N-I	Missense
9.	EC39, EC47, EC48, EC49	187	AAG-GAG	63	K-E	Missense
10.	EC44	188	AAG-AGG	63	K-R	Missense

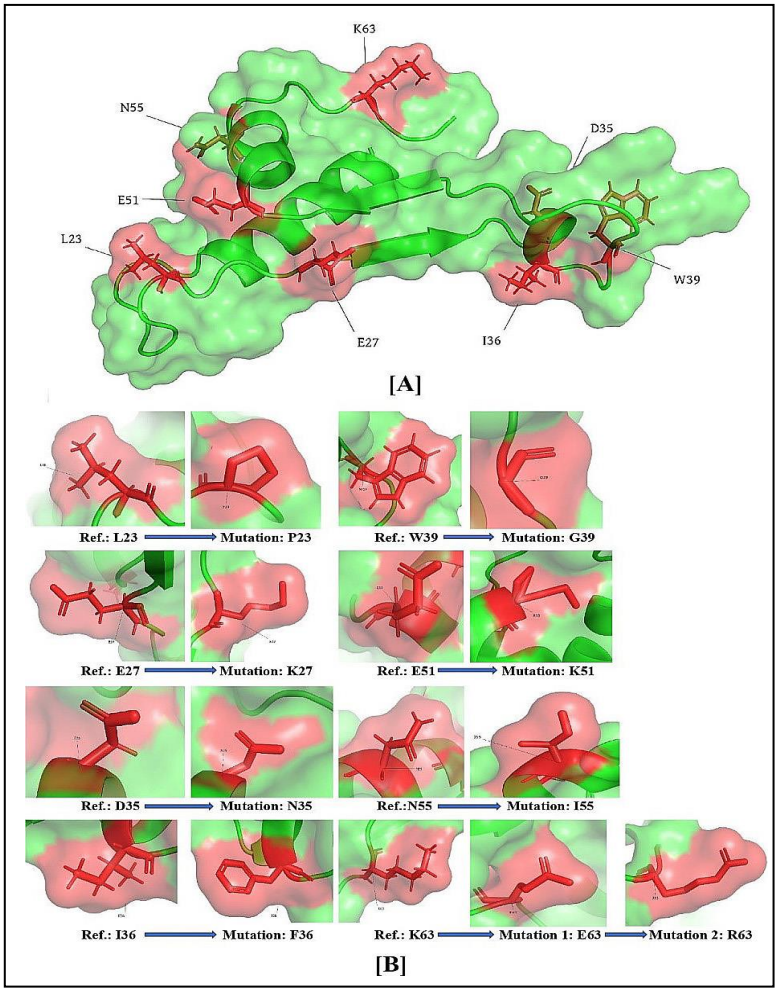

Figure 1. 3D structure models of the *aadA1* gene are produced with PyMOL (http://www.pymol.org/). **A:** for reference sequence, **B:** Missense substitutions

Table 3. Nucleic acid substitutions and effect on amino acid of the *dfrA1* gene

NO.	Mutation isolates in *dfrA1*	Nucleotide		Amino acid		Type of Mutations
		Position	Change codon	Position	Change	
1.	EC47	3	GAA-GAG	1	Q-Q	Silent
2.	EC44	13	CTA-TTA	5	L-L	Silent
3.	EC39	21	CCA-CCC	7	P-P	Silent
4.	EC47	30	GTT-GTG	10	V-V	Silent
5.	EC39, EC44	45	GAC-GAT	15	D-D	Silent
6.	EC44	75	CCG-CCG	25	P-P	Silent
7.	EC39	81	GTA-GTG	27	V-V	Silent
8.	EC44	82	TGC-TGG	28	C-G	Missense
9.	EC39	90	CGT-CGA	30	R-R	Silent
10.	EC48, EC49	94	ACG-CCT	32	T-P	Missense
11.	EC48, EC49	96	ACG-CCT	32	T-P	Missense
12.	EC44, EC47	109	ATC-TTC	37	I-F	Missense
13.	EC44, EC47	139	ATC-TTC	47	I-F	Missense
14.	EC47, EC48	169	GAA-AAA	57	E-K	Missense
15.	EC47, EC48	202	TGG-GGG	68	W-G	Missense
16.	EC44, EC47	208	GAT-AAT	70	D-N	Missense
17.	EC48, EC49	222	CCT-CCG	74	P-P	Silent
18.	EC48, EC49	264	CAT-CAC	88	H-H	Silent
19.	EC44, EC47	297	AAT-AAC	99	N-N	Silent
20.	EC44, EC47	335	CGC-CCC	112	R-P	Missense

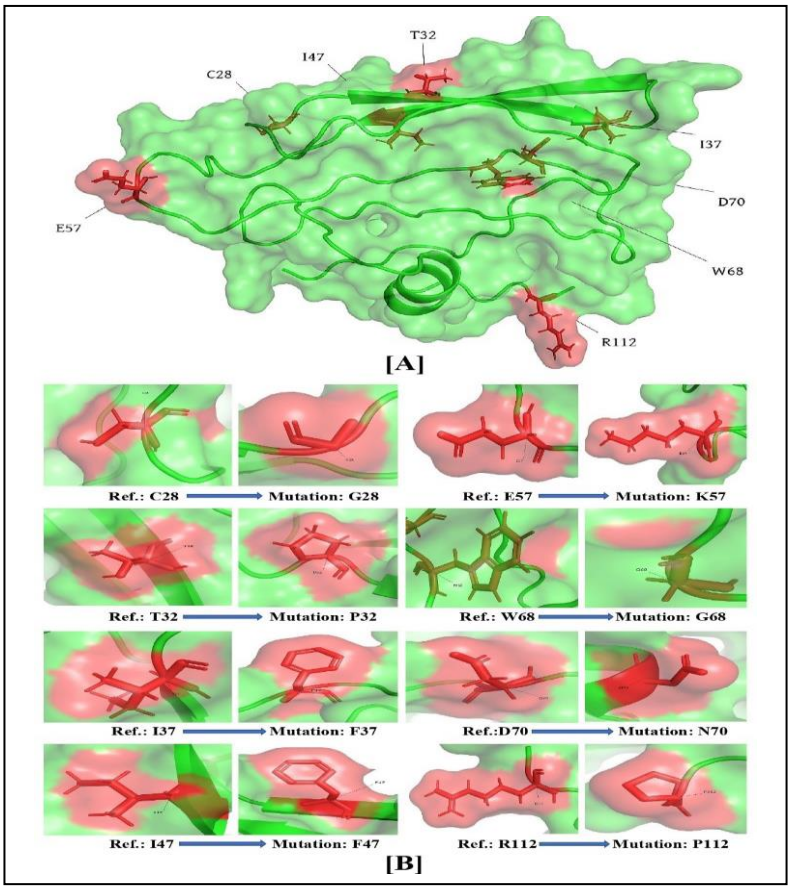

Figure 2. 3D structure models of the *dfrA1* gene are produced with PyMOL (http://www.pymol.org/). **A:** for reference sequence, **B:** Missense substitutions

Table 4. Nucleic acid substitutions and effect on amino acid of the *sul1* gene

NO.	Mutation isolates in *sul1*	Nucleotide		Amino acid		Type of Mutations
		Position	Change codon	Position	Change	
1.	EC49	3	TTC-TTT	1	F-F	Silent
2.	EC48	9	GAG-GAT	3	E-D	Missense
3.	EC39	11	AGC-AAC	4	S-N	Missense
4.	EC48	22	GAC-TAC	8	D-Y	Missense
5.	EC44, EC47, EC48, EC49	73	GAC-AAC	25	D-N	Missense
6.	EC47, EC48, EC49	143	AGA-AAA	48	R-K	Missense
7.	EC48, EC49	163	GAC-AAC	55	D-N	Missense
8.	EC49	166	GCC-CCC	56	A-P	Missense
9.	EC47, EC48, EC49	180	CAG-CAA	60	Q-Q	Silent
10.	EC48	222	CAG-CAA	74	Q-Q	Silent
11.	EC48	231	GCG-GCC	77	A-A	Silent
12.	EC49	298	ATT-TTT	100	I-F	Missense
13.	EC48	385	GAC-AAC	129	D-N	Missense
14.	EC48	397	GAG-AAA	133	E-K	Missense
15.	EC48	399	GAG-AAA	133	E-K	Missense
16.	EC47	531	TCG-TCA	177	S-S	Silent
17.	EC49	716	CGA-CAA	239	R-Q	Missense
18.	EC47	732	TTC-TTT	244	F-F	Silent
19.	EC47	745	GCG-CCG	249	A-P	Missense
20.	EC39	747	GCG-GCC	249	A-A	Silent

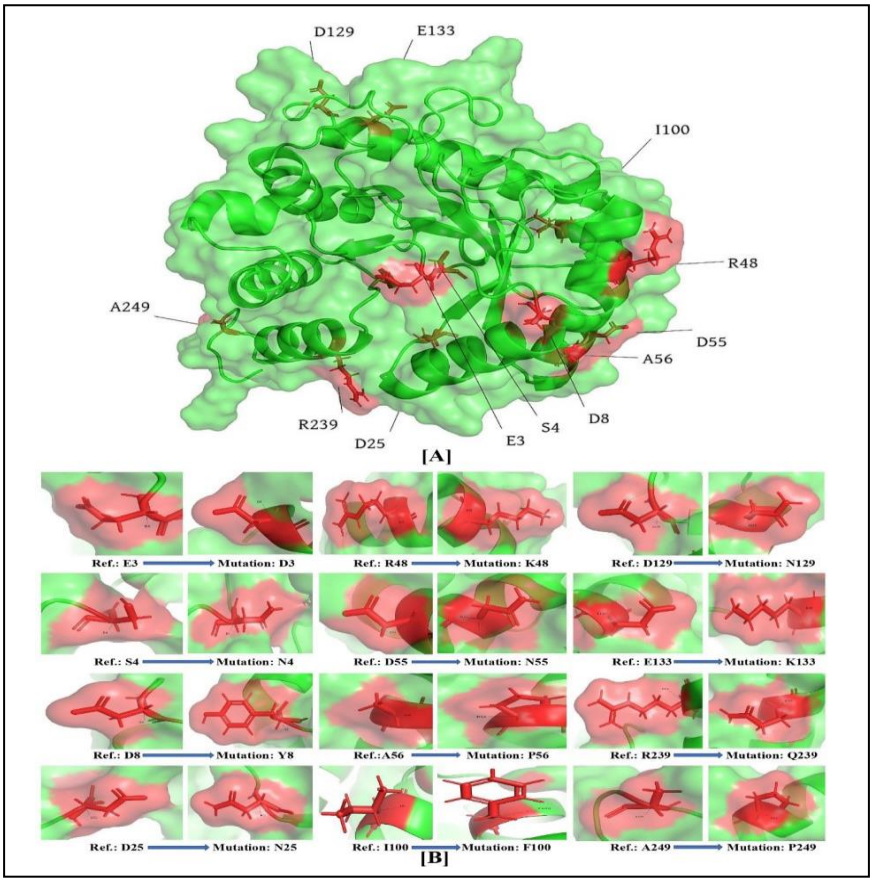

Figure 3. 3D structure models of the *sul1* gene are produced with PyMOL (http://www.pymol.org/). **A:** for reference sequence, **B:** Missense substitutions

Table 5. Nucleic acid substitutions and effect on amino acid of the *cat1* gene

NO.	Mutation isolates in *cat1*	Nucleotide		Amino acid		Type of Mutations
		Position	Change codon	Position	Change	
1.	EC48	12	ACG-ACC	4	T-T	Silent
2.	EC44	18	TTT-TTA	6	F-L	Missense
3.	EC44, EC47	21	TTA-TTT	7	L-F	Missense
4.	EC44, EC47	30	GTA-GTT	10	V-V	Silent
5.	EC39	52	TAT-AAT	18	Y-N	Missense
6.	EC47, EC48	85	ATG-GTG	29	M-V	Missense
7.	EC47, EC48	118	AAA-GAA	40	K-E	Missense
8.	EC48	126	GGT-GGA	42	G-G	Silent
9.	EC47, EC48	136	ATA-GTA	46	I-V	Missense
10.	EC48	183	ACT-ACA	61	T-T	Silent
11.	EC48	270	GGT-GGA	90	G-G	Silent
12.	EC48	291	CCT-CCA	97	P-P	Silent
13.	EC47, EC48	340	AGT-GGT	114	S-G	Missense
14.	EC47, EC48	439	ATG-GTG	147	M-V	Missense

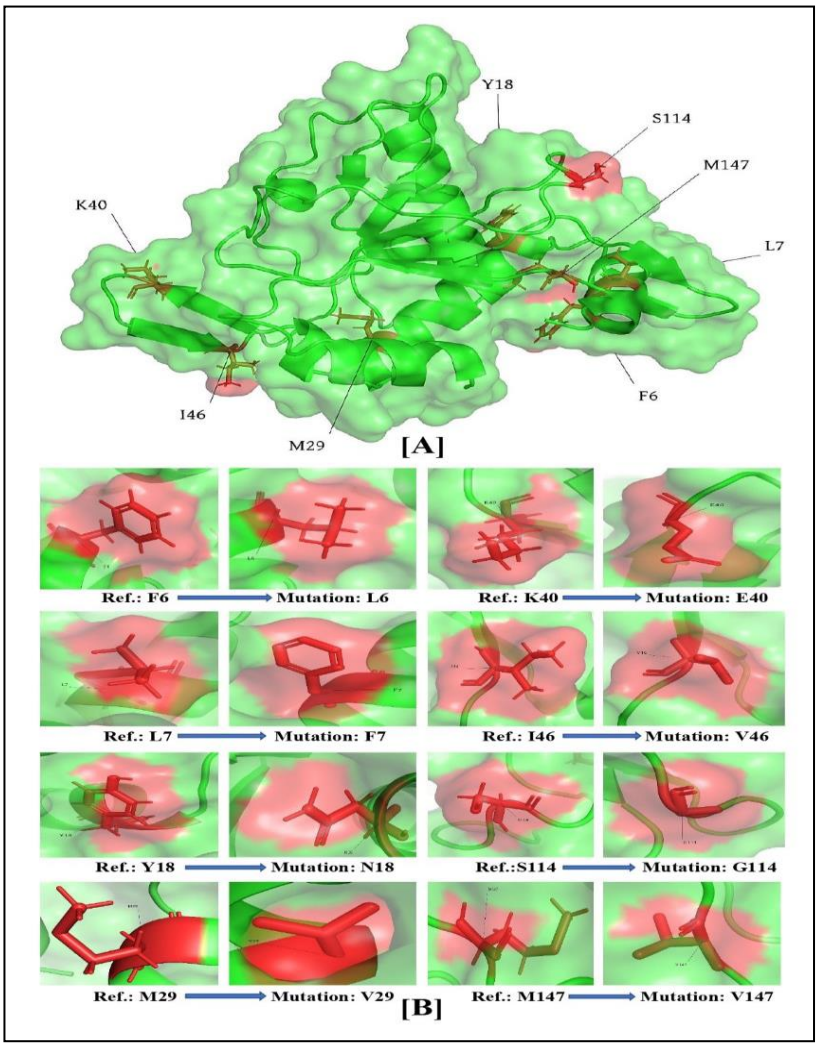

Figure 4. 3D structure models of the *cat1* gene are produced with PyMOL (http://www.pymol.org/). **A:** for reference sequence, **B:** Missense substitutions

4. Discussion

The *16s rRNA* gene is considered one of the essential criteria in the classification since it is characterized by highly conserved regions and is unable to change over time. Moreover, it contains areas of high covariance among types of bacteria that provide a specific sequence to each type. That is to say, this gene may play a key role in diagnosis and typing when the diagnostic methods are ended (6). Furthermore, this technique of molecular diagnosis using the *16s rRNA* gene was confirmed by Brooks, Butel (7) as the final bacterial diagnosis. Aminoglycoside group of antibacterial is recommended as the first-line empiric therapy in Uropathogenic Escherichia coli (UPEC) through their activity against common urinary pathogens, such as *E. coli*, *K. pneumonia,* and some other Gram-negative bacteria (8-10).

Aminoglycoside adenylyl transferase (*aadA*) genes mediate resistance to streptomycin and spectinomycin (11). In addition, the rate of gentamicin resistance was decreased in the last few years (12). The resistance of *E. coli* to Trimethoprim-sulfamethoxazole due to chromosomal mutations (often single point mutations) in the *dhfr* or *dhps* genes is commonly the cause of resistance to these drugs (13). Moreover, the *cat1* gene which is a variant of *cat* genes encodes chloramphenicol acetyltransferases, a significant mediator of chloramphenicol resistance. Furthermore, *E. coli* has been reported to be associated with a decreased passive accumulation of antibiotics regulated by the multiple antibiotic resistance locus or inactivation of the drug by an enzyme acetyltransferase which converts the acetyl group of acetyl CoA in chloramphenicol to a primary (C-3) hydroxyl group and restricts its binding to the bacterial ribosome (14).

The increased incidence of transversional and transitional mutations has important implications in epidemiological studies. Any changes which may occur in this gene can lead to enhanced effectiveness of the gene and increased virulence of the bacteria, causing epidemic diseases (15). It is worth noting that the occurrence of genetic mutations in genes is essential in understanding the molecular basis of mutations. These mutations occur in a particular gene to cause changes in the structure and the function of this gene. Moreover, they may exert effects on gene expression, leading to the replacement of amino acids in the protein. Sometimes, the replacement of amino acids in a specific region of the protein does not significantly affect the formation and function of the protein since the genetic code consists of three nitrogenous bases, while the change may occur in one base without any change in the amino acid which encodes it (16-18).

Substitution mutations are point mutations whose occurrence leads to a change in one nitrogen base in the gene sequence, and this change is copied during replication to cause continuous changes in the sequence of the nitrogen bases of the gene. These mutations include a transversion, in which a purine base is replaced by a pyrimidine or vice versa. As for the transition mutation, another purine base replaces a purine base, or a pyrimidine base is replaced by another pyrimidine base (19). Several missense mutations lead to an alteration in substrate specificity of the enzyme so that resistance is increased. These mutations may alter by influencing the accessibility or orientation of the reactive groups within the active site (20).

The precise point (Missense) mutation (Tables 2-5 and Figures 1-4) in the four genes *aadA1*, *dfrA1*, *sul1*, and *cat1* in the expected sequence is the target site of a site-specific recombination mechanism. A previous study by Clark, Olsvik (21) suggested that the PCR product was not large enough to include other cassettes, and it was assumed that the *aadA1* gene was the only resistance gene inserted within the integron. Even so, it is unclear whether this integron is a component of a larger transposon; however, it seemingly originated from an enteric organism based on the sequence data. Most prokaryotes must produce their own folate. The silent expression of 4-amino-4-deoxybranch acid cleasthase on the folic acid metabolism pathway may influence folic acid synthesis and decrease adaptability (22). Chromosome mutations in the mar locus, for example, in *E. coli*, can result in resistance to

chloramphenicol and structurally unrelated antibiotics as part of the MAR phenotype mediated by a reduced drug uptake mechanism (23).

5. Conclusion

Antibiotic resistance causes significant morbidity, mortality, and economic costs each year by rendering bacteria resistant to antibiotics. The sequencing method enhances our ability to detect and investigate antibiotic resistance. The precise point (Missense) mutation in the four genes *aadA1*, *dfrA1*, *sul1*, and *cat1* in the expected sequence is interpreted to be the target site of a site-specific recombination mechanism that led to antibiotics resistance in *E. coli* isolates.

Authors' Contribution

Study concept and design: R. K. M.
Acquisition of data: R. K. M.
Analysis and interpretation of data: R. K. M.
Drafting of the manuscript: A. A. I.
Critical revision of the manuscript for important intellectual content: R. K. M.
Statistical analysis: A. A. I.
Administrative, technical, and material support: R. K. M.

Ethics

The study design was approved by the ethics committee of University of Baghdad, Baghdad, Iraq.

Conflict of Interest

The authors declare that they have no conflict of interest.

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