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Original Article

Prevalence of Efflux Pump and Porin-Related Antimicrobial Resistance in Clinical *Klebsiella pneumoniae* in Baghdad, Iraq

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Abstract

*Klebsiella pneumoni*ae is an opportunistic bacterium that causes many infections, including septicemia, pneumonia, urinary tract infection, and liver abscesses. There are many mechanisms for antibiotic resistance and *K. pneumonia* is considered a multidrug-resistant pathogen. This study aimed to find the correlation between the susceptibility of *K. pneumonia* to certain antibiotics with the porin-related resistance and pumps mechanisms. In total, two genes that are responsible for porin formation were considered in the current study: *OmpK-35*gene and *OmpK-36* gene, in addition to other four genes (*CfiaS*, *CfiaL*, *MFS*, and *MdtK* genes) related to an efflux pump mechanism of antibiotic resistance. The bacterial resistance was investigated towards five cephalosporins (Cefazolin, Cefoxitin, Ceftazidime, Ceftriaxone, and Cefepime) and two carbapenems (imipenem and ertapenem). Clinical samples, including blood, swabs, and urine, consisting of 20 specimens for each group, were collected from patients who attended three hospitals in Baghdad. The VITEK-2 system and genetic tests (polymerase chain reaction and sequencing) of bacterial isolates were applied to confirm the diagnosis of *K. pneumoniae* and detect the antibiotic sensitivity profile. The results showed that 51 (85%) and 15 (25%) of the total 60 isolates had positive results for *OmpK-35* and *Omp-K36* genes, respectively. The *MFS* and *MdtK* genes were observed (70-88.3%) in cephalosporin-resistant isolates of *K. pneumoniae*. There were no significant variations of bacterial resistance genes of antibiotics within the specimen groups. It was concluded that the bacterial resistance of the selected antibiotics was elevated markedly with the loss of the *OmpK-36* gene with a high expression of *MFS* and *MdtK* genes and a slight minimal occurrence in the new generation of carbapenems. The best antimicrobial agent was ertapenem with a percentage of 0% of resistance in all bacterial isolates.

Keywords: Carbapenems resistance, Cephalosporins, *Cfia* genes, *MFS* genes, OMPs, *Klebsiella pneumoniae*

1. Introduction

Klebsiella pneumoniae is a Gram-negative bacteria, which is a member of the family *Enterobacteriaceae*; characterized by rod-shaped and mucoid capsules. It ferments lactose and can grow easily on selective agar media without any motility. It causes many serious diseases, like urinary tract infection, septicemia, liver abscesses, and pneumonia (1). The emergence of multi-antimicrobial resistance should be controlled to help manage the health global issues caused by *K. pneumonia* which is leading to death annually (2). Porins, such as outer membrane protein (*OMP*) genes of *K. pneumonia* (*OmpK-35* gene and *OmpK-36* gene) are the vital route of therapeutics influx, such as cephalosporins and carbapenems into the bacterial cell (3). The OMPs are among the principle outer membrane proteins of gram-negative bacteria and are highly conserved among the *Enterobacteriaceae*. Efflux pumps and translocator proteins of *K. pneumoniae* are also important for the prevention of the

bacteria from being sensitized to the concentrations of drugs or preventing the accumulation of drug peptides via bacterial cell membrane (4).

Klebsiella pneumonia resistance towards antimicrobials increases due to their random use, especially carbapenems (e.g., meropenem, ertapenem, and imipenem), by the development of several strategies to inactivate these drugs, such as efflux pump mechanism (5). Membrane pumps can selectively transport drugs out of the bacteria under the controlling inner regulators. Pumps overexpression has led to extensive inactivation of antimicrobial drugs which had been active for decades, either for one type of these drugs or for much more than two categories, especially the clinical multidrug resistance (MDR) isolates (3).

In gram-negative bacteria, efflux systems are divided into two groups: the first one binds to adenosine triphosphate, and the other one is called the secondary transporter group (6). The secondary transporters consist of large numbers of proteins related to the MDR in clinical isolates which can be further subdivided into superfamilies based on the primary and secondary structures of these proteins. Three important superfamilies are called major facilitator superfamily (*MFS)*, *multidrug resistance protein (MdtK)*, and carbapenemase (*Cfia)* which are included in *K. pneumonia* resistance to carbapenems, as the overproduction of these genes results in carbapenems sand cephalosporins inactivation (7). In ordinary cases, clinically-isolated bacteria harbor one of the above-mentioned genes. However, after antibiotic treatment, they become resistant due to the insertion sequences in the upstream region of these genes as transporters or in the regulators of the genes; in a widely distributed form in *K. pneumonia* to carbapenems and cephalosporins (8).

Both *OmpK-35* and *OmpK-36* genes play an important role in the pathogenicity and drug sensitivity in *K. pneumonia* (5). Devoid *OmpK36* gene, or both *OmpK35* and *OmpK36* genes expressions result in a huge tendency toward tissue colonization and a high virulence in *K. pneumonia* clinical strains (9). Therefore, the loss of expression of *OmpK35* and *OmpK36* genes leads to the elimination of carbapenems and cephalosporins at the same time as the occurrence of other membranous resistance (7). These mutant populations (in either one or both *OMP* genes) of clinical *K. pneumonia* isolates are not only hard to study but also variable in terms of *in vivo* sensitivity profile.

It was noticed that the mutation of porins genes, *OmpK35* and *OmpK36*, has an obvious correlation with the increased minimal inhibitory concentrations (MICs) of the tested antibiotics (carbapenems, cephalosporins, fluoroquinolones, gentamycin, tobramycin, aminoglycosides, and tetracyclines). This correlation leads to significant MDR cases and mortality in case of treatment failure (10). Hence, this study aimed to evaluate the antibiotic resistance of clinically-isolated *K. pneumonia* in correlation with drug resistance and the prevalence of resistance genes and find the significant relation of antibiotic resistance with porin-resistance and efflux pump genes.

2. Materials and Methods

2.1. Sample Collection and Diagnosis

During the period from August to November 2020, 60 clinical samples were collected. The samples were divided into three major groups, namely blood, urine, and swabs. It should be mentioned that each group contained 20 specimens. Samples were collected from patients in three hospitals in Baghdad city, Iraq. The *K. pneumonias* that were isolated using differential and culture media were further diagnosed by VITEK-2 compact system. Both bacterial diagnosis and drug susceptibility tests were performed simultaneously. It should be mentioned that all VITEK-2 cards of diagnosis and antibiotic susceptibility tests were supplied by the Biomereux company (France).

2.2. Molecular Detection and Sequence Analysis

Molecular diagnosis was performed by the detection of the 16s rRNA gene. This technique is based on the amplification of a part of housekeeping genes by polymerase chain reaction (PCR), followed by the sequencing of amplified fragments to detect the genus of K. pneumoniae. The advantage of this technique is

that it is a highly discriminatory method (11). Genotypic assays were performed for DNA extraction by Wizard DNA extraction kit (Promega/ USA) followed by the use of PCR as a precise tool for the investigation of *16s rRNA* gene, porin-related genes, and efflux pump genes by using the Green Master Mix kit (manufactured by Promega, USA).

Two primers (forward and reverse primers for each gene) were designed in this study by using Genius Software Program (Macrogen/Korea). They were used to amplify each of the housekeeping (*16s rRNA*) and target genes (*Omp* genes and efflux pump genes*)* for 40 cycles of PCR (Table 1). Gel electrophoresis, agarose (Promega) of 2% was performed to analyze the PCR products and evaluate the positivity of the results (occurrence of both genes) among bacterial isolates. The optimal conditions of PCR and electrophoresis with the sequences of primers are summarized in table 1.

Table 1. Optimal conditions and sequences of forward (F) primer and reverse primer (R) of studied genes

Initial Denaturation: 94° for 5 minutes					
Denaturation: 94° for 1 minute					
Gene symbol	**Forward primer sequence (5′…3′)**	**Reverse primer sequence (5′…3′)**	**Size bp**	**Annealing temperature**	**Electro-phoresis duration min.**
Housekeeping gene					
16s rRNA	AGAGTTTGATCCTGGCTCAG	TACGGTTACCTTGTTACGACTT	1500	60	90
Efflux pump genes					
CfiaS	TGACGGCAAAAATCACCA	TGACGCAGCAAAAGATCA	276	51.6	45
CfiaL	ATGCAGGATAAAAGGTGAACC	AAGGATTTTTAGTCTGGCTGG	680	57.4	75
MFS	TGGCGATTTTGCTGGTTTA	ATTTCGCGCTAAACACCA	615	53.4	60
Mdtk	GATGATAAATCCGCACCAGAA	GCTGTTCTTTGAAGTCACTCT	538	57.4	60
Porin genes (Outer membrane proteins)					
OMP-K35	TGAGTTTTACCAGCGAAGTG	AAACGGCAACAAACTGGA	780	58.5	75
OMP-K36	TCAACATATTTCAGCAGGTCC	ACCGTAACTCTGATTTCTTCG	1317	57.4	60

Extension: 72° for 1 minute
Final Extension: 72° for 5 minutes

In total, 5 µL of ethidium bromide dye (manufactured by Promega, USA) was used for every 100 ml of melted agarose for visualizing DNA bands under a UV-illuminator in comparison with the size of DNA Ladder (1500 bp) with loading dye (6X) (Promega). The PCR products were sent for Sanger sequencing using ABI3730XL, automated DNA sequences (manufactured by Macrogen Corporation, Korea). The results were analyzed using Geneious software. Pairwise comparison was performed between each studied gene with the same gene of a standard strain of *K. pneumoniae* with specific National center for biotechnology information (NCBI) codes, which was applied to analyze the identity of each pair of compared genes.

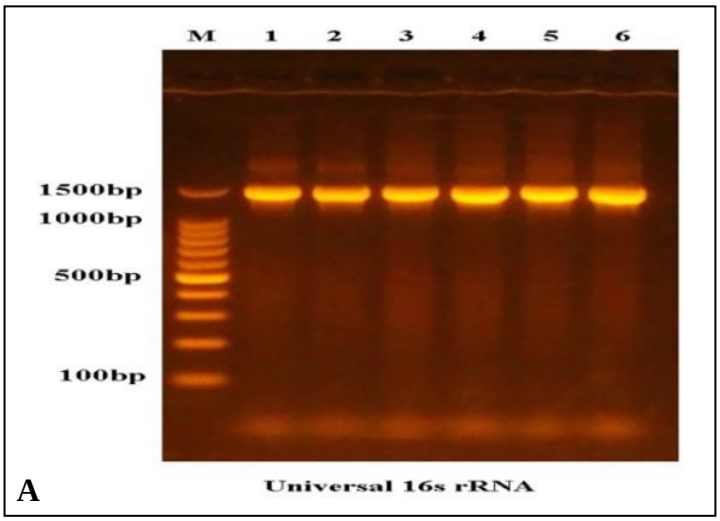
2.3. Statistical Analysis

Statistical analysis was performed using SPSS statistical package for Social Sciences (version 20.0 for Windows, SPSS, Chicago, IL, USA). It should be mentioned that the qualitative data are represented as count and percentage. The chi-square test was used to test the relation of qualitative data. Spearman's rho correlation test was used to test the relation between qualitative data. The p-values less than 0.05 were considered statistically significant.

3. Results and Discussion

After being diagnosed by the VITEK-2 system, gene detection of *16srRNA* was applied for molecular diagnosis of *K. pneumoniae* to ensure species

identification level which was supported by the identification of *K. pneumoniae* via phenotypic methods. The results showed 60/60 (100%) positivity among isolates with the amplified size of 1500 bp as illustrated in clear bands, compared to the DNA ladder (100bp) shown in figure 1A. As reported in sequence analysis studies, 16S could be used to identify *K. pneumoniae* subsp. pneumoniae to the species level by the amplified highly conserved *rRNA* gene (16s) which was an ideal candidate for bacterial identification and evolutionary studies (12). Figure 1B represents an identical *16s rRNA* gene of *K. pneumoniae* with a standard strain (CP009775) of NCBI, with an identical percentage of 99.8%.



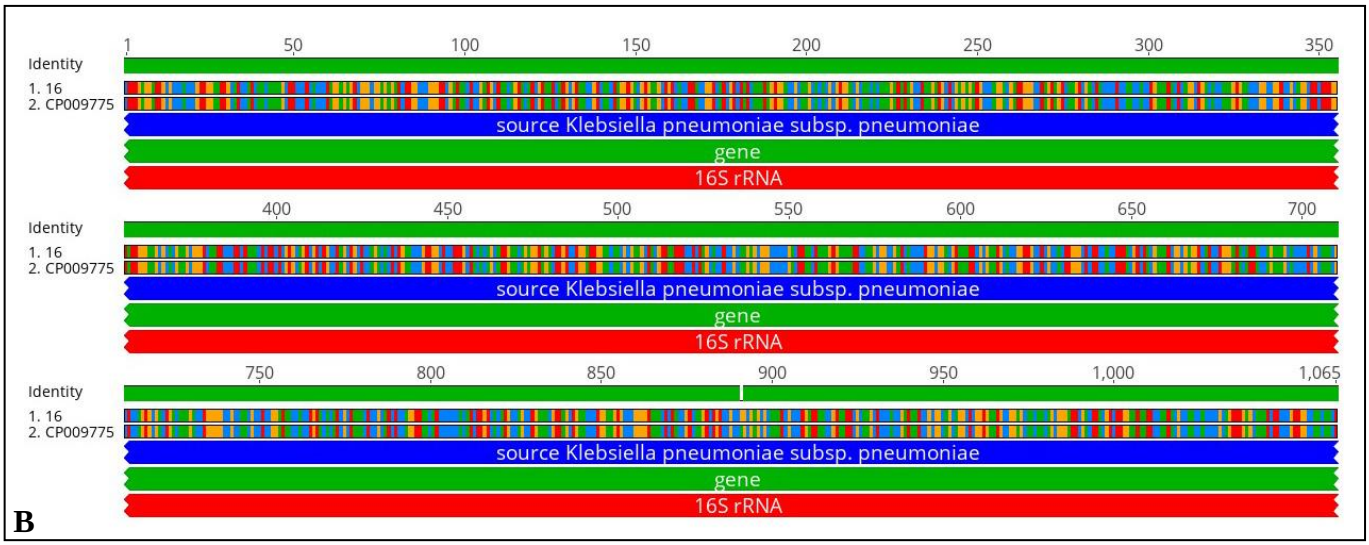
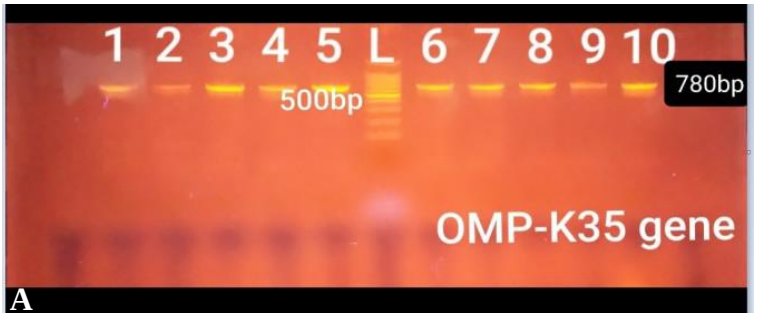


Figure 1. A) Gel electrophoresis of polymerase chain reaction product of *16s rRNA* gene (1500bp amplicon) of *Klebsiella pneumonia* by using 2% agarose, lane 1 (M) 100-1500bp of DNA ladder, while lane 1-6 represents positive results for *16s rRNA* gene. **B)** Pairwise identity of *16s rRNA* gene with a standard strain of *K. pneumoniae* (CP009775)

The results of PCR of porin-genes in K. *pneumoniae* showed that 51 (85%) and 15 (25%) isolates of *K. pneumoniae* that possessed *OmpK-35* and *Omp-K36* gene were amplified, respectively. Figures 2A and 3A clarify the positive results of both genes under UV-illuminator apparatus after electrophoresis of their amplicons, while figures 2B and 3B clarify the comparison of *OmpK-35* and *OmpK-36* genes with the same genes of standard strains of NCBI as their identity percentages were 99.9 and 99.7, respectively, with no more two gaps of nucleotides in both gene sequences.



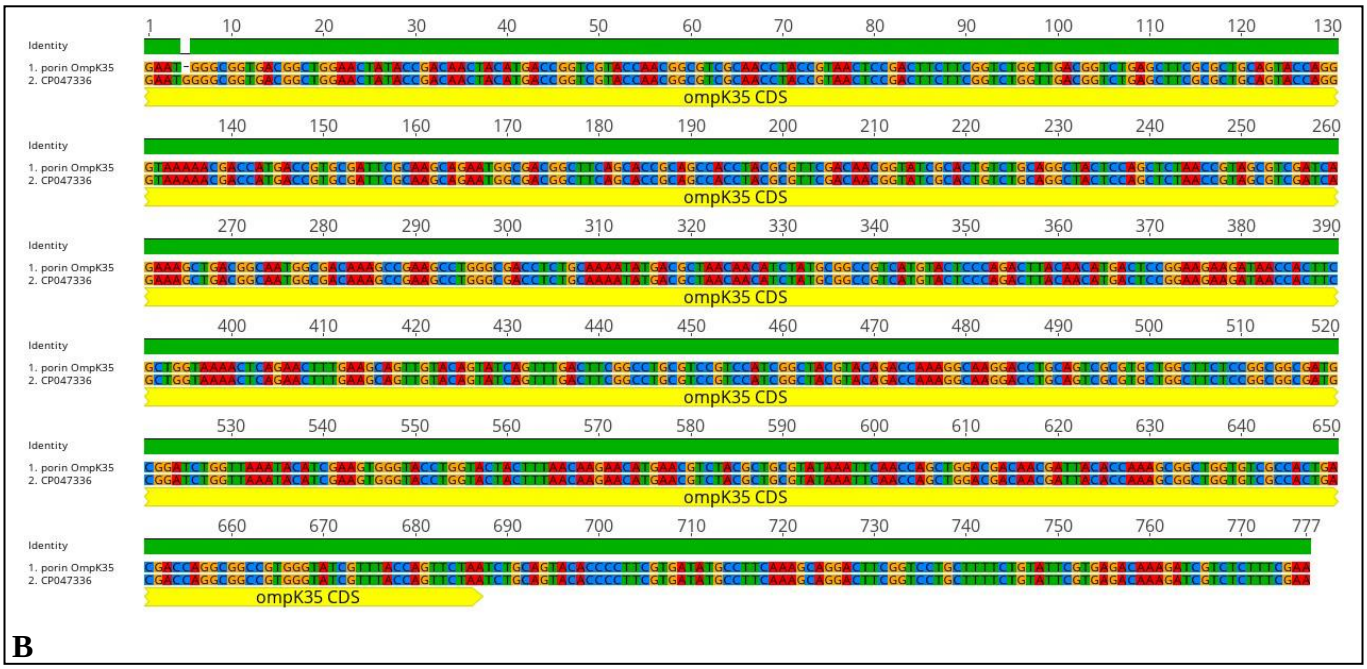


Figure 2. A) Gel electrophoresis of polymerase chain reaction product of *OmpK-35* gene (780bp amplicon) of *Klebsiella pneumoniae* by using 2% agarose gel. Lane L,100-1500 bp of DNA ladder, all 10 lanes give a positive result for the *OmpK-35* gene. **B)** Comparison between *K. pneumoniae OmpK-35* gene with the *OmpK-35* gene of NCBI strain (CP047336)

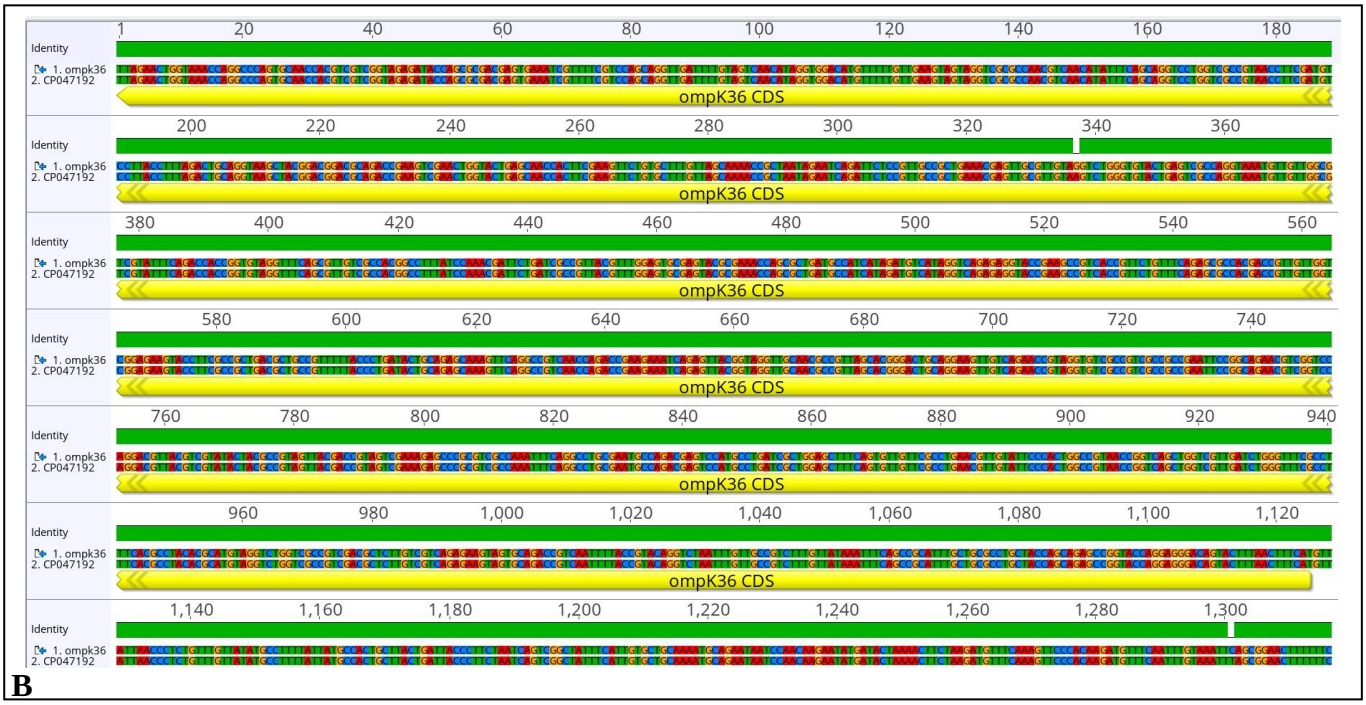
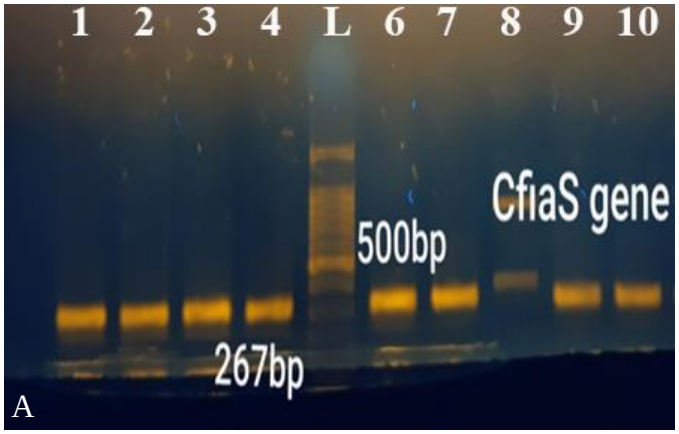


Figure 3. A) Gel electrophoresis of polymerase chain reaction product of *OmpK-36 gene* (1317bp amplicon) of *Klebsiella pneumonia* by using 2% agarose gel. Lane L,100-1500 bp of DNA ladder, only lane six gives a positive result for the *Omp-K36* gene. **B)** Pairwise identity with *K. pneumoniae* NCBI strain (cp047192)

The PCR bands of efflux pump genes are shown in figure 4A for the *CfiaS* gene, figure 5A for the *CfiaL* gene, figure 6A for the *MFS* gene, and figure 7A for the *MdtK* gene. Figures 4B, 5B, 6B, and 7B show the pairwise identity of each four genes above. The identity percentages were 99.2%, 99.4%, 99.3%, and 99.0% with very few gaps of nucleotides, compared with NCBI strains for *CfiaS*, *CfiaL*, *MFS*, and *MdtK* genes.



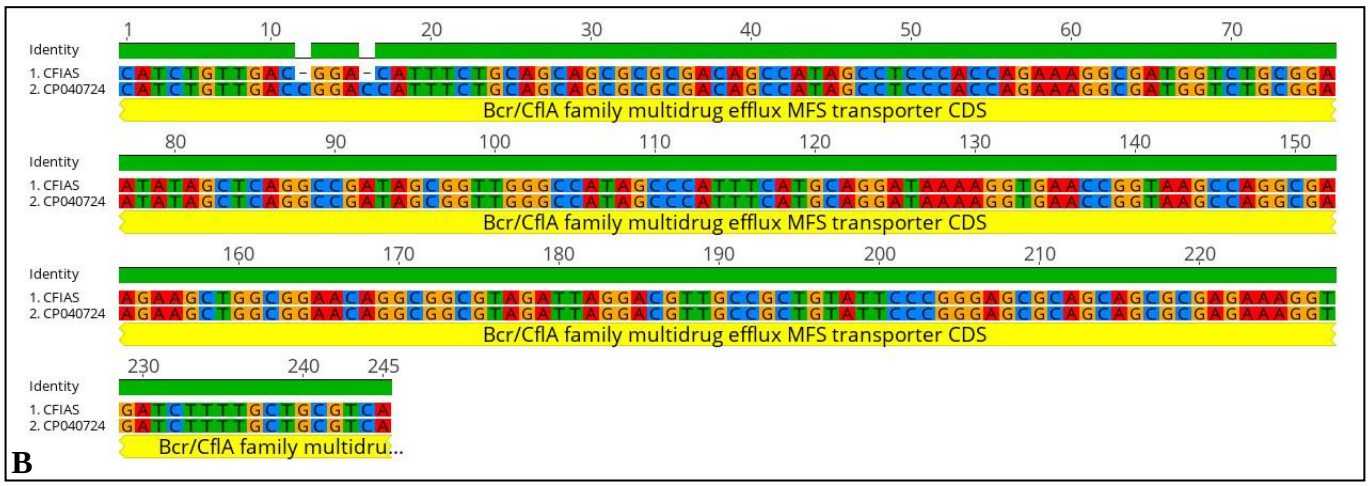
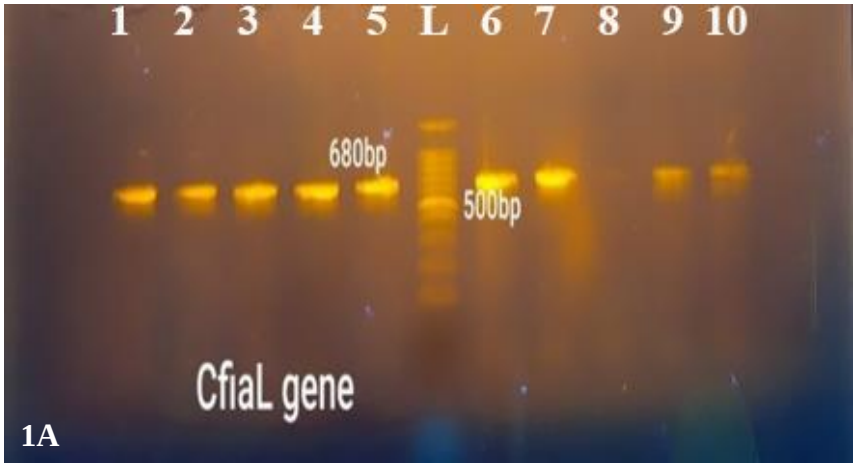


Figure 4. A) Gel electrophoresis of polymerase chain reaction product of *CfiaS* gene (267bp amplicon) of *Klebsiella pneumonia* by using 2% agarose gel. lane L, 100-1500 bp of DNA ladder, while 2, 3, 4, 5, 6, 7, 9, and 10 lanes represent positive results for the *CfiaS* gene. **B)** Pairwise comparison of *CfiaS* with NCBI gene of (CP040724) strain



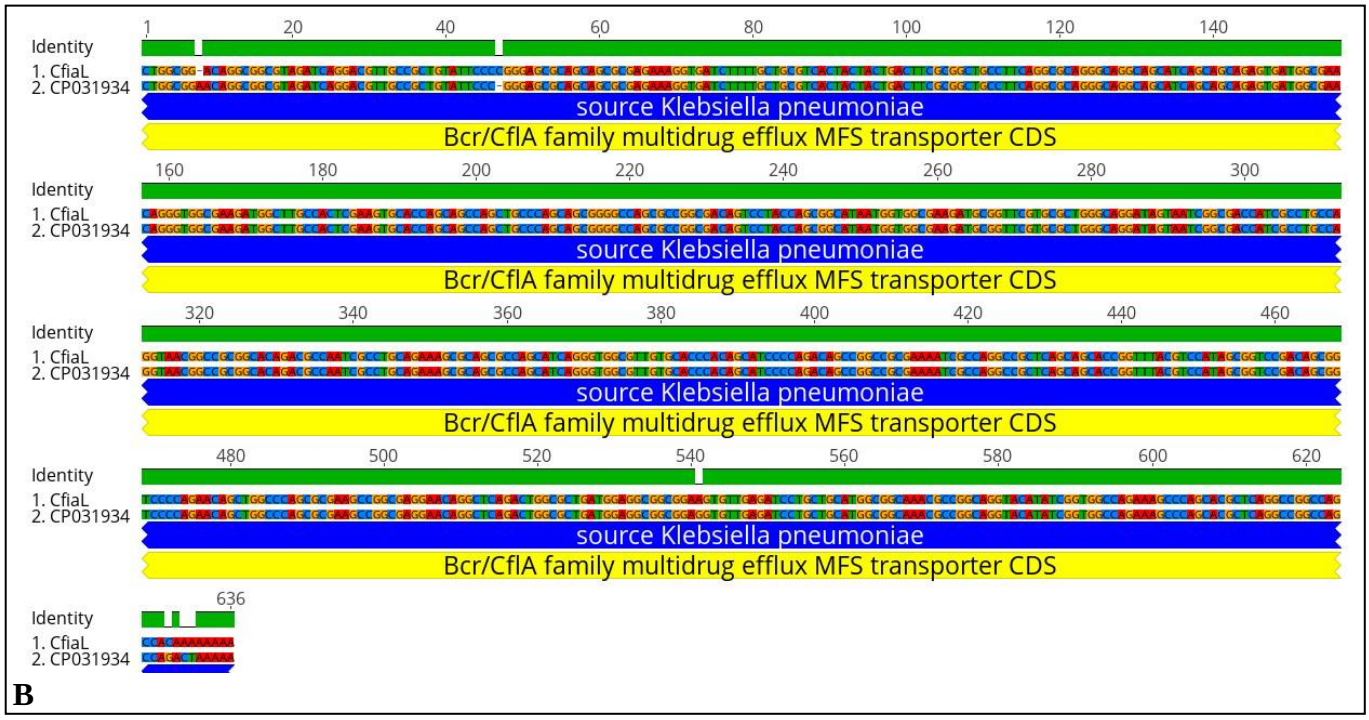
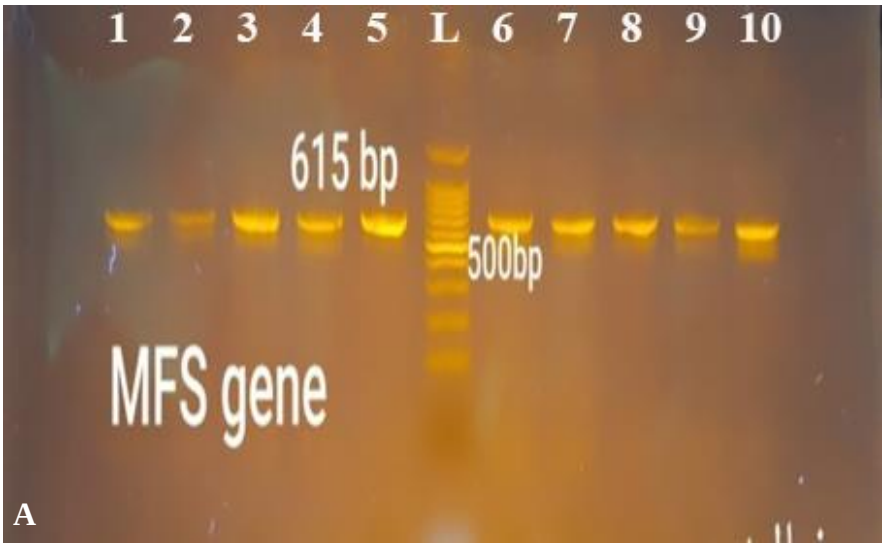


Figure 5. A) Gel electrophoresis of polymerase chain reaction product of *CfiaL* gene (680bp amplicon) of *Klebsiella pneumonia* by using 2% agarose gel. Lane L, 100-1500 bp of DNA ladder, all lanes gave a positive result for *CfiaL* gene. **B)** Pairwise identity with *CfiaL* gene of a standard strain (CP031934)



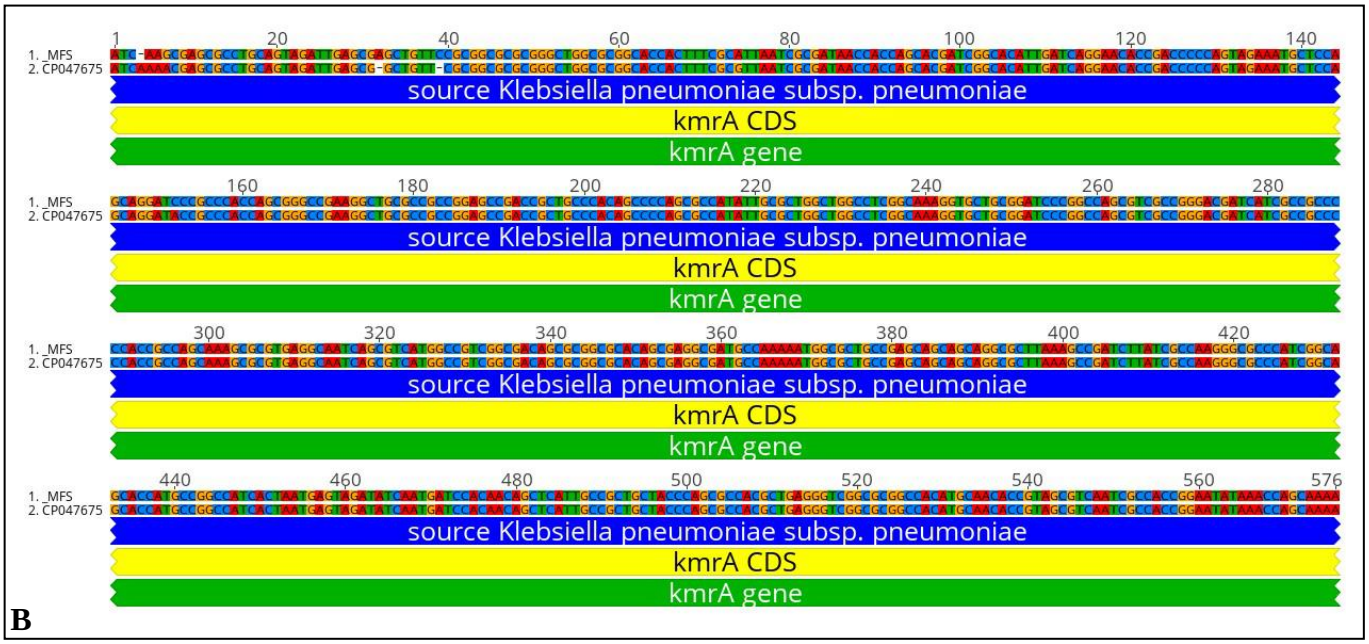
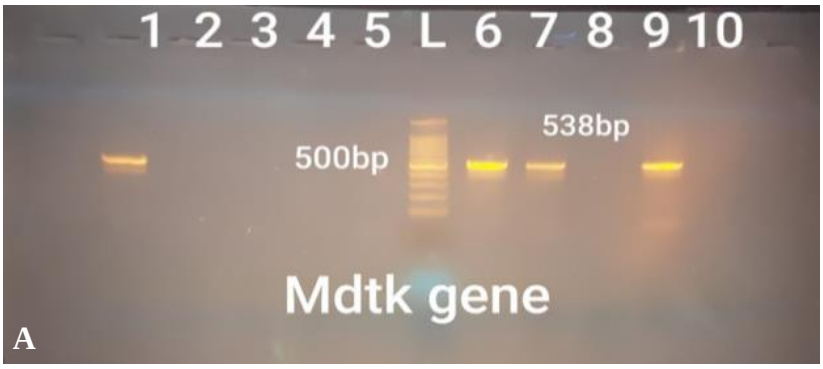


Figure 6. A) Gel electrophoresis of polymerase chain reaction product of the major facilitator superfamily *(MFS)* gene (615bp amplicon) of *Klebsiella pneumonia* by using 2% agarose gel. Lane L represents 100-1500 bp of the DNA ladder. All lanes gave a positive result for the *MFS* gene. **B)** Comparison of *MFS* gene with *K. pneumoniae* NCBI strain (CP047675)



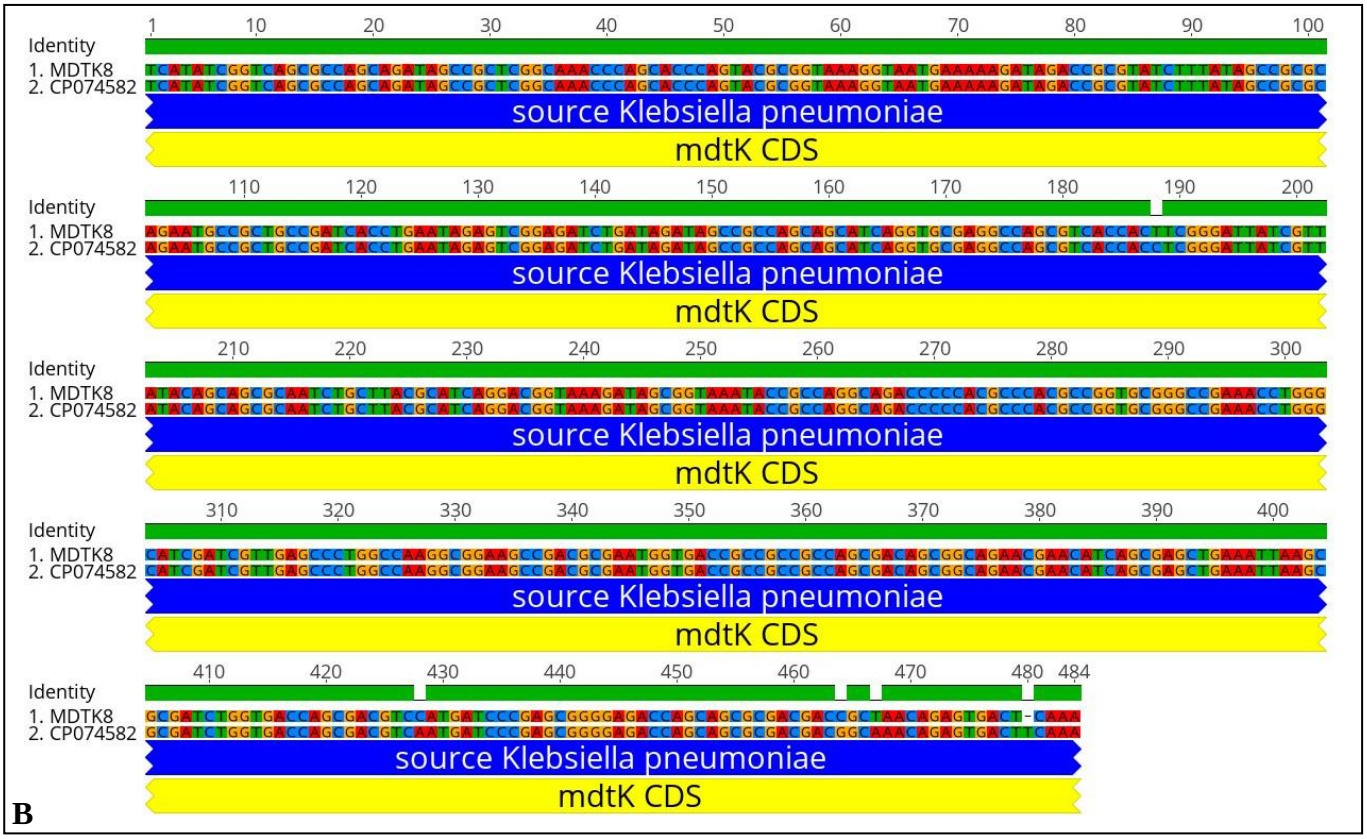


Figure 7. A) Gel electrophoresis of the polymerase chain reaction product of multidrug-resistant protein (*Mdtk* gene, 538 bp amplicon) of *Klebsiella pneumonia* by using 2% agarose. Lane L,100-1500 bp of DNA ladder, 1, 6, 7, and 9 lanes give a positive result for the *Mdtk* gene. **B)** Pairwise comparison of *MdtK* gene of standard strain (CP074582)

As summarized in table 2, the results showed no significant variation regarding the resistance genes with the type of specimen (P<0.05). The *OmpK-35* gene was expressed in 16 isolates in both swabs and blood samples as well as all urine isolates. Moreover, *OmpK-36*gene was present in seven positive isolates of swabs, and there were only four positive results in both blood and urine samples. For efflux pump genes, *MdtK* was presented in about half of the total isolates (n=60). The *MFS* genes were ranked the highest percentage of 88.3% among the total isolates, while *CfiaS* and *CfiaL* were 78.3% and 70%, respectively.

In table 3, the antibiotics which were suspected to be resisted by porin and efflux mechanisms were recorded based on VITEK-2 results according to the given information for patient data and specimen type.

The results showed that there was no significant variation regarding antibiotic resistance with the specimen type (P<0.05). Therefore, the resistance was the same towards any used antimicrobial agent within any group of samples.

To find the relationship between phenotype and genotype, resistance percentages of the selected antibiotics were recorded with each resistance gene. As clarified in table 4, the highest numbers were observed in the presence of *MFS* and *MdtK* genes among efflux pump genes. In the presence of *OMPs* genes, the sensitivity was not the lowest as it is suspected that the expression of porins enables the antibiotics to enter the bacterial cells and be accumulated for further activity. For ertapenem, all resistance percentages in the presence of the six genes were 0.

Table 2. Distribution of porin gene expression within the clinical groups

Sample / Gene	Blood samples	Swabs (Burn, wound, and ear swabs)	Urine samples	Percentage of positivity of Total isolates (%)	*P*-value
CfiaS	14	15	18	78.3%	0.279
CfiaL	18	14	10	70.0%	0.062
MFS	19	19	15	88.3%	0.075
MdtK	15	11	6	53.3%	0.060
OmpK-35	16	16	20	86.67%	0.099
OmpK-36	4	7	4	25%	0.449

Table 3. Antibiotic resistance of blood, urine, and swab (burn wound and ear) isolates for five selected cephalosporins and imipenem (as carbapenem), followed by chi-squared test results and a *P*-value of 0.01

Antibiotic / Sample 20 for each	Cefazolin CZ\30 µg	Cefoxitin Fox\30µg	Ceftazidim CAZ\30µg	Ceftriaxone CRO\30 µg	Cefepime CPM\30 µg)	Imipenem (IMP\10 µg	Ertapenem ETP\10 µg
Urine	15	9	15	12	12	7	0
Blood	18	10	18	18	18	8	0
Swabs	18	15	18	18	18	15	0
Total (60)	51	34	51	48	48	30	0
P-value	0.308	0.122	0.308	0.240	0.240	0.226	-

Table 4. Percentages of antibiotic resistance within the resistance genes groups

Antibiotic / Gene	Cefazolin (CZ\30µg)	Cefoxitin (Fox\30µg)	Ceftazidim (CAZ\30µg)	Ceftriaxone (CRO\30µg)	Cefepime (CPM\30µg)	Imipenem (IMP\10µg)	Ertapenem (ETP\10µg)
CfiaS	85.1	61.7	85.1	78.7	78.7	53.2	0
CfiaL	85.7	54.8	85.7	81.0	81.0	45.2	0
MFS	90.6	56.6	90.6	88.7	88.7	50.9	0
MdtK	87.5	68.8	87.5	81.2	81.2	59.4	0
OmpK35	82.7	53.8	82.7	76.9	76.9	46.2	0
OmpK36	86.7	60.0	86.7	80.0	80.0	46.7	0

The results of the present study are consistent with those of the studies performed by Ahmed, Hadi (13), which showed the clinical isolates of *K. pneumoniae* in the phenotypic aspect of antibiotic susceptibility profile in Iraq. Moreover, the findings were in line with those of the studies conducted by Yan, Pu (14), Boszczowski, Salomão (15) and Al-Bayati (16) regarding the genetic resistance to carbapenems and cephalosporins. Wasfi, Elkhatib (5) found that the absence of the genes that code for outer membrane proteins (porin-genes and pumps) are related to MDR isolates regardless of the source of specimens, and also showed variation in their expression in blood, sputum, wounds, and urine samples. The minimum inhibitory concentration (MIC) is markedly increased in recent years in Iraqi hospitals to imipenem, but not the newer antibiotics, like ertapenem (17).

Although resistance to drugs, such as carbapenems and cephalosporins, is mediated enzymatically, the porin-mediated resistance is also present as a result of mutation achieved by replacement of porins with more selective channels as well as the deletion or insertion to decrease the expression of porins, or protein structure alteration, or inactivation of outer membrane genes, namely *OmpK-35* and *OmpK-36* (18). The strategy of losing such porin proteins by *K. pneumoniae* aims to prevent drugs from entering or accumulating inside the bacterial cell (12).

Another accompanied resistance strategy is to alter the structure and charge of the lipopolysaccharide (LPS) layer by adding some chemical groups. It decreases the LPS affinity towards antibiotics and reduces its binding to many drugs and decreases the frequency in the expression of porins simultaneously (14). Furthermore, *K. pneumonia* could be impermeable to multi-drugs via changing the shape, number, or even the affinity of the pores as a non-specific route; hence, a limited number of antibiotics can enter the bacterial cells (16).

It has been documented that if *K. pneumoniae* strains did not express *OmpK-35* and *OmpK-36* genes, there would be either insertion elements or nucleotide insertion that make these strains much more virulent with complex numerous drug resistance (19). The single deletion mutation of the *OmpK-36* gene could influence the antimicrobial resistance by increasing minimal inhibitory concentrations (MICs) of both carbapenems and cephalosporins due to the absence of the *OmpK-36* route while the loss in the *OmpK-35* gene alone could affect the virulence of *K. pneumonia*. The downregulation of both *OmpK-35/OmpK-36* genes could markedly cause more bacterial virulence and elevated mortality rates as a result of increased drug resistance (20). This might be due to the fact that the downregulation of one or both porin genes decreases the accumulation of a significant concentration of cephalosporins and carbapenems with a direct relationship with other mechanisms of permeability resistance (21).

Moreover, the bacterial cells could remain sensitive to drugs even if they possessed resistance genes within their genome and the drug resistance might still be inactivated even if there was an exposure to antibiotics because of the role of regulatory regions in *K. pneumoniae* resistance genes (22). In this study, the distribution of efflux-resistant genes was significantly high in the studied isolates (88.3%) with no resistance towards ertapenem. It has been reported that *K. pneumoniae* strains that resist carbapenems and cephalosporins by efflux system are strongly associated with other mechanisms as a complex, non-specific, and numerous defense (21). Unfortunately, a strategy used by *K. pneumonia* to overcome the high concentration of drugs inside is the induction of efflux and the development of some chemical regulators to prevent antibiotic accumulation in novel therapeutic strategies (23).

Although the carbapenems and cephalosporins represent the first-line treatment of serious *K. pneumonia* infections, the elevated resistance leads to treatment failure and high rates of morbidity and mortality, except for the newest carbapenems, like ertapenem (17). Increased use of carbapenems and cephalosporins leads the clinical isolates to

overexpression of efflux genes either by direct binding to antibiotic molecules or by enhancing multiple entire repressors that inactivate antimicrobials (13). This response could be achieved by the acquisition of genetic elements, like plasmids or transposons, to confer efflux resistance genes or via mutation incidences in the regulatory network of efflux pumps expression in addition to being within the repressor to alter pumps genes, like *MFS* and *MdtK* genes or their regulators as a consequence especially in the promoter activity as a newly discovered single- base-pair mutation (24).

In conclusion, the *K. pneumoniae* resistance of carbapenems and cephalosporins is elevated markedly with the loss of *OmpK-36*gene with the prevalence of *MdtK* and *MFS* genes *in vivo* regardless of the source of the clinical isolate. Therefore, *OMPs* and pumps profile is a good parameter for the evaluation of the susceptibility to carbapenems and cephalosporins in terms of the occurrence of many types of resistance mechanisms. Furthermore, the sequence gaps of the studied genes were few when they were compared with the same genes of NCBI strains recording a high percentage of pairwise reaching more than 99.0% of identity. Translation process should be performed to ensure variants that cause the conversion of different amino acids via stable mutations.

Authors' Contribution

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

Ethics

This study was approved by the Research Ethics Committee of Mustansiriyah University, Baghdad, Iraq

Conflict of Interest

The authors declare that they have no conflict of interest.

Acknowledgment

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