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Impact the some Antibiotics on Gene Expression Efflux Pump of *Klebsiella pneumoniae* Isolated from Clinical Sources

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Abstract

One hundred thirty bacterial samples were collected from different clinical sources, including (diuresis, sputum, wounds, blood, and burns) from different hospitals in Baghdad, including Imamain Al-Kadhimain (PBUT) Medical City, Al-Karkh General Hospital, Al-Kadhimiya Children's Hospital, The burns Hospital, Ghazi Al Hariri Hospital and Baghdad Hospital. The isolates were diagnosed with phenotypic, microscopic, and biochemical tests, and molecular diagnosis was performed by detecting the *16SrRNA* gene. After the final diagnosis, 50/130 (38.4%) of *K. pneumoniae* bacteria were obtained. The results of the study showed that the highest percentage of isolation of *K. pneumoniae* bacteria was from sputum samples of patients, which amounted to 14/60 (46.67%), followed by 25/60 (41.67%) of urine, 2/5 (40%) of burns, 6/20 (30%) of wounds and 3/15 (20%) of blood. A pattern of multi-drug resistance (MDR) was found among all *K. pneumoniae* isolates at 34 (68%). The results of the statistical analysis showed highly significant differences ($p \leq 0.001$) between most of the treatments studied for gene folding in resistant isolates, and of the treatment with the antibiotic ceftazidime was recorded the highest folding of *acrAB,* gene was (4.88±2.73), followed by the combination of the antibiotics ceftazidime + gentamicin together, in *mdtK* gene (4.52±0.92). The other treatments recorded increased and decreased in folding, but it was not significant ($p>0.05$).

Keywords: *Klebsiella pneumonia*e, Efflux pump genes, Antibiotics, folding.

تأثير بعض المضادات الحيوية على التعبير الجيني لبعض جينات مضخة التدفق في الكلبسيلا الرئوية المعزولة من المصادر السريرية

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الخلاصة

تم جمع مائة وثلاثين عينة بكتيرية من مصادر سريرية مختلفة بما في ذلك (إدرار ، البلغم، الجروح، الدم، الحروق) من مستشفيات مختلفة في بغداد، بما في ذلك مدينة الإمامين الكاظمين (عليهما السلام) الطبية، ومستشفى الكرخ العام، ومستشفى الكاظمية للأطفال، ومستشفى الحروق، ومستشفى غازي الحريري ومستشفى بغداد، وتم تشخيص العزلات بالاختبارات المظهرية والمجهرية والكيميائية الحيوية والتشخيص الجزيئي عن طريق

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الكشف عن جين *16SrRNA*. وبعد التشخيص النهائي، تم الحصول على 130/50 (38.4٪) من بكتيريا *Klebsiella pneumoniae*. أظهرت نتائج الدراسة أن أعلى نسبة عزل بكتيريا الكلبسيلا الرئوية كانت من عينات القشع 30/14 (46.67٪) تلتها 60/25 (41.67٪) من البول، 5/2 (40٪) من الحروق 20/6 (30٪) من الجروح و 15/3 (20٪) من الدم. تم العثور على نمط من مقاومة الأدوية المتعددة بين جميع عزلات الكلبسيلا الرئوية بنسبة 34 (68٪). أظهرت نتائج التحليل الإحصائي وجود فروق ذات دلالة إحصائية عالية ($p\leq0.001$) بين معظم المعاملات المدروسة للتعبير الجيني في العزلات المقاومة، وسجلت المعاملة بالمضاد الحيوي سيفتازيديم أعلى طي للجين *acrAB* حيث بلغ (4.88±2.73)، يليه مزيج المضادين الحيويين سيفتازيديم + جنتاميسين معاً، في الجين *mdtK* حيث بلغ (4.52±0.92)، وسجلت المعاملات الأخرى زيادة ونقصان في الطي ولكن لم يكن ذا دلالة إحصائية ($p>0.05$).

1. Introduction

The genus *Klebsiella* belongs to the intestinal family Enterobacteriaceae [1]. In 1834, Edwin Klebs discovered the genus *Klebsiella* and gave his name [2, 3].

The genus *Klebsiella pneumoniae* is characterized as a negative for Gram stains ranging in length between (0.6-6) µm and its width ranges between (0.3-1) µm, arranged individually or in pairs, or short chains [4, 5].

Colonies appear on the blood agar, gray-white, non-hemolytic, and mucous colonies. However, some studies have reported that some of them are able to produce hemolysin on blood agar and appear as dark pink mucous colonies on (EMB) [6]. These bacteria are commonly found in water, plants, soil, and mammalian mucous surfaces [7].

The prevalence of *K. pneumoniae* may be a pathogen in hospitals in Europe and the USA due to acquiring antibiotic resistance markers that give it a selective advantage in a hospital environment. It is responsible for 6-17% of urinary tract infections (UTIs), 4-15% of septicemia cases, 7-14% of pneumonia cases, 2-4% of wound infections, 4-17% of nosocomial infections in intensive care units and 3-20% of all neonatal septicemia [8, 9].

The virulence factors of *K. pneumoniae* isolates are mediated mainly by four main groups of virulence factors: capsular polysaccharide (CPS), lipopolysaccharide (LPS), fimbriae (type 1 and type 3), and siderophores. Several additional factors, such as urease production, cytotoxins, enterotoxins, hemolysin, protein-tyrosine kinase, heat-labile endotoxin, and phosphotyrosine-phosphatase protein [10]. MDR is defined as the resistance of pathogens to one or more antimicrobial agents [11]. Several health organizations, such as the World Health Organization (WHO), the Centers for Disease Control and Prevention (CDC), and the European Centre for Disease Prevention and Control (ECDC), have warned that treating bacterial infections with multiple antibiotics has become difficult and expensive with the use of costly drugs and require long treatment systems [12]. One of the most effective mechanisms that confer multi-drug resistance bacteria is multi-drug efflux pumps, which prevent the accumulation of antibiotics inside the cell, thereby reducing their concentration to levels below toxicity [13]. Efflux pumps enhance host resistance to antimicrobial peptides in other bacteria [14], an essential component of the host's innate immune system. This mechanism likely plays a similar role in the virulence of *K. pneumoniae,* which has efflux pumps extended over the membrane to secrete toxic compounds ranging from heavy metal ions to organic chemicals, including antibiotics [15, 16]. The overall structure of these efflux pumps is highly preserved, with an inner membrane energy transfer subunit coupled via a transducer protein to an outer membrane channel subunit that enables the expulsion of toxic compounds into the environment [17]. This study aimed to detect the resistance mechanisms in *K. pneumoniae* isolates, determine the folding of bacteria that is MDR, and detect efflux pump genes in the bacteria.

2. Methods

Collection of Sample

One hundred thirty bacterial samples were collected from different clinical sources, including (diuresis, sputum, wounds, blood, and burns) from different hospitals in Baghdad, including Imamain Al-Kadhimain (PBUT) Medical City, Al-Karkh General Hospital, Al-Kadhimiya Children's Hospital, The burns Hospital, Ghazi Al Hariri Hospital and Baghdad Hospital. The isolates were diagnosed with phenotypic, microscopic, and biochemical tests, and molecular diagnosis was performed by detecting the *16SrRNA* gene.

Isolation and Identification

Cultural identification

Bacterial isolates developed in different culture media (MacConkey agar, eosin methylene blue agar, blood agar) were initially diagnosed based on their culture characteristics, including shape, structure, color, size, edges, and height of isolated bacterial colonies [18].

Microscopic identification (Gram Stain)

Part of a single colony was stained with Gram stain [18].

Biochemical tests:

Oxidase, catalase, indole, methyl red, Voges-Prosakaur and citrate (IMViC), glucose fermentation, and haemolysin tests, many of these biochemical tests have been performed to diagnose *K. pneumoniae* bacteria [19].

Molecular study

DNA extraction

The ABIOpure Extraction Protocol (table 1) was used to extract DNA from bacterial isolates. The process involved preparing a broth medium, inoculating *K. pneumoniae* isolates, and incubating them for 24 hours at 37°C. The bacterial suspension was then taken and placed in sterile Eppendorf tubes. Protein digestion and cytolysis were performed using 20 µg/mL proteinase K solution and 200 µL buffer BL. Absolute ethanol was added to the sample, and the mixture was transferred to a mini-column. The tubes were centrifuged for one minute at 6000, and the precipitate was replaced with a new one. Buffer BW, Buffer TW, and Buffer AE were added, and the collection tubes were then centrifuged at maximum speed to remove the remaining wash buffer.

Table 1: PCR mixture component

No	Materials	Size µl
1	Forward primer	1
2	Reverse primer	1
3	Master mix	12.5
4	DNA template	2
5	Nuclease –free water	7.5
	Total volume	24

Quantitation of DNA

A Quantus Fluorometer was used to detect the concentration of extracted DNA to detect the quality of these samples for molecular applications. This estimation was performed according to information provided by Promega QuantiFluor dsDNA System Kit from each DNA sample, 1 µl was combined with 200 µl of QuantiFluor dsDNA 1X dye. They are mixed well and

incubated in the dark at room temperature for 5 minutes, and then the concentration values are read.

Quantitative PCR

The primers used the genes *acrAB, TolC, oqxA, oqxB,* and *mdtk,* shown in Table 2, and each gene has its program, as shown in Table 3, to perform calculations and experiments; Real-time PCR and gene expression used KAPA SYBR® FAST qPCR Master Mix (2X) Kit as shown in Table 4, and the gene expression values of the target genes were calculated after the completion of quantum polymerase reaction cycles. The threshold cycle values were obtained for each sample of S.aureus bacteria.

Threshold limit values were also calculated for the *16srRNA* gene. After obtaining the threshold limit values, the variations in the threshold limit values in the target genes were calculated by subtracting the threshold limit value of the *16srRNA* gene from the target gene values for each gene to obtain the ΔCt value. The variations values of the *16SrRNA* control samples were then subtracted from the variations values of the treated sample values to obtain a value of ΔΔCt [20].

Table 2: Sequencing of primers used in gene expression.

Primer	Sequence 5`-3`	Annealing Temp. (°C)	Product Size (bp)	Reference
acrAB-F	ATCAGCGGCCGGATTGGTAAA	52	312	[21]
acrAB-R	CGGGTTCGGGAAAATAGCGCG			
toIC-F	ATCAGCAACCCCGATCTGCGT	52	527	[21]
toIC-R	CCGGTGACTTGACGCAGTCCT			
oqxA-F	CTCGGCGCGATGATGCT	60	392	[22]
oqxA-R	CCACTCTTCACGGGAGACGA			
oqxB-F	TTCTCCCCCGGCGGGAAGTAC	60	512	[22]
oqxB-R	CTCGGCCATTTTGGCGCGTA			
mdtk-F	GCGCTTAACTTCAGCTCA	43	453	[21]
mdtk-R	GATGATAAATCCACACCAGAA			
16S-F	GCAAGTCGAGCGGTAGCACAG	58	260	[23]
16S-R	CAGTGTGGCTGGTCATCCTCTC			

Table 3: Optimum conditions for Real-Time PCR reaction for genes *16SrRNA, acrAB, TolC, oqxA, oqxB, mdtk*

No.	Phases	Temperature °C	Time	Number of Cycles
1	Activation of the enzyme RT	37	15 min	1
2	Initial Denaturation	95	5 min	
3	Denaturation	95	20 sec	40
4	Annealing			
5	*16SrRNA*	58		
6	*acrAB*	52	20 sec	
7	*TolC*	52		
8	*oqxB*،*oqxA*	60		
	mdtk	43		
9	Extension	72	20 sec	

Table 4: Real-Time PCR reaction mixture.

Master mix component	Volume
qPCR Master Mix	5
RT mix	0.25
$MgCl_2$	0.25
Forward primer	0.5
Reverse primer	0.5
Nuclease Free Water	2.5
RNA	1
Total volume	10

Determination of Sub-MIC concentration of Gentamicin and Ceftazidime antibiotics
Bacterial inoculum preparation

One ml of sterile normal saline solution was transferred to a transparent polystyrene test tube with a size of 12×75 mm. Then, bacterial suspension was prepared after adjusting bacterial suspension concentration to McFarland concentration (0.5×10^8) cells/ml using a DensiCHEK Plus Meter.

Quantitative MIC

One- hundred µl of the medium was distributed to all the wells of the microtiter plate using the micropipette; two plates of the microtiter plate were used, one for the Gentamycin and the second for the Ceftazidime. Then 100 µl of Ceftazidime was placed at a concentration of 500 µg/ ml and Gentamycin (100 µg/ ml) in well column No. 1 (far left of the plate), and mix the materials in the wells in the first column using a micropipette with a size of 100 µl and mix the materials by pull-up and down method 6-8 times. After that, 100 µl were pulled from the first column and added to the second column. Then, 50 µl bacterial suspension was added to each well of the plate, with column No. 12 remaining containing the medium and bacterial suspension (positive control) and the last row containing the medium and antibiotic without the bacterial suspension (negative control). Finally, the plate was incubated at a temperature of 37 °C for (18-24) hours, after which 20 µl of resazurin stain, which was prepared previously, was added to each well in the plate and incubated for an additional two hours at a temperature of 37 °C. The color change was observed and recorded, and then the MIC concentrations at which the color change occurred were taken as the minimum inhibitory concentration (MIC).

RNA Purification

RNA was extracted from bacterial isolates according to the instructions of the TRIzol™ Reagen group. RNA precipitation is done by adding 0.5 ml of isopropanol to the aqueous phase, incubating for 10 minutes, and centrifuging using a centrifuge at a speed of 12000 rpm for 10 minutes. 0.5 ml of ethanol was added to wash the RNA, then centrifuged using a centrifuge at 10,000 rpm for 5 minutes. RNA was rehydrated in 50 µl nuclease-free water and then incubated in a water bath or thermal mass at 55-60°C for 10-15 minutes.

Quantus Fluorometer was used to determine RNA concentration to detect sample quality for the final assay of 1 µl of RNA. 200 µl of diluted QuantiFluor reagent to QuantiFluor buffer was mixed and incubated for 5 minutes at room temperature in a dark place, and RNA concentration values were determined.

Calculation of RTT-PCR results

The relative levels of expression obtained by amplifying the genetic yield by the cycle threshold (Ct) value, according to Livak and Schmittgen [20], were interpreted as follows:-

ΔCt (patients) = Ct (patients) - Ct (*16S rRNA*)

ΔCt (controls) = Ct (controls) - Ct (*16S rRNA*)

ΔΔCt = ΔCt (patients) - ΔCt (controls)

Folding = $2^{-(\Delta\Delta Ct)}$

Statistical analysis

The data was tabulated in a datasheet of IBM SPSS version 25.0, which was utilized to do the statistical analysis. The mean and standard errors of continuous variables were reported, and significant differences were tested using the analysis of variance (ANOVA) test, followed by the least significant difference (LSD) test. Statistical significance was defined as a probability value ($p \leq 0.05$).

3. Result and Discussion

Isolation and detection of strains from clinical samples

One hundred thirty samples were collected from patients hospitalized in several hospitals in Baghdad, including (Imam Al-Kadhimin Hospital, Al-Karkh General Hospital, Kadhimiya Children's Hospital, Burns Hospital, Ghazi Al-Hariri Hospital and Baghdad Hospital). They included 60 samples of urine, 30 samples of sputum, 20 samples of wounds, 15 samples of blood, and 5 samples of burns, as shown in Table 5. After the isolation, the bacterial isolates were diagnosed with phenotypic, microscopic, and biochemical tests. The molecular diagnosis was done by detecting the *16SrRNA* gene, and after final diagnosis, 50 (38.4%) of *K. pneumoniae* bacteria were obtained from the total samples. The results of the study showed that the highest percentage of *K. pneumoniae* bacteria isolation was from sputum samples, which amounted to 14/30 (46.67%) of the total isolates (130), 2/60(41.67%) of urine, 2/5(40.00%%) of burns, 6/20(30.00%) of wounds, 3/15(20.00%) of blood, as shown in Table (5).

Table 5: Source of isolation, number, and percentages of *K. pneumoniae* isolates.

Source of isolation	No. of *K. pneumoniae* isolates/ total bacteria isolates	Percentage
Urine	25/60	41.67%
Sputum	14/30	46.67%
Wound	6/20	30.00%
Blood	3/15	20.00%
Burns	2/5	40.00%
Total number	50/130	38.46%

The results of the study showed that the highest percentage of *K. pneumoniae* bacteria isolation was from sputum samples for patients, as it amounted to 14/30 (46.67%) of the total isolates. The results were consistent with Mohammed *et al.* [25], who found that a total of 120 samples from different clinical sources were obtained from Anbar Teaching Hospital, the highest percentage of *K. pneumoniae* isolates were obtained from sputum samples were 60 isolates (50%), followed by urine samples were 40 isolates (33.33%), 10 isolates (8.33%) of blood samples, and 10 isolates (8.33%) of wound samples.

The results differed from Razavi *et al.* [24] study; it was found that the highest infection of *K. pneumoniae* bacteria was from the urine samples, where 64 isolates (58.18%) were obtained

from 110 *K. pneumoniae* samples in urine samples, 31 isolates (28.18%) of sputum samples, and 15 isolates (13.63%) of blood samples. The reason for the appearance of *K. pneumoniae* bacteria at a higher rate in sputum is because it is a typical sign of pneumonia caused by this species of bacteria [26, 27].

Diagnostic tests for isolates

Phenotypic identification

The results showed that the bacteria *K. pneumoniae* growing on MacConkey agar medium appeared to be bright pink colonies with a mucous structure, which was a characteristic of these bacteria because it was lactose fermented [28], which is non-motile, non-spore-forming, oxidase negative, containing a prominent polysaccharide capsule of wide thickness, which gives a mucous appearance to the colonies on the culture media [4, 5]. They also grow rapidly on nutrient agar and appear large, pink, round, and mucous colonies on MacConkey agar, indicating lactose fermentation and acid production. It also grows rapidly on nutrient agar media, blood agar media, and eosin methylene blue agar (EMB) [29, 30].

Microscopic identification

The results of the microscopic examination of the bacteria under light microscopy were micro-negative bacilli of Gram stains with single or double chains or in short chains, which is consistent with the results of Mahon and Lehman [31].

Biochemical identification

The results of the biochemical tests showed that all isolates were negative for the oxidase, the indole, the motility, and the methyl red tests. The isolates were positive for the urease, citrate, catalase, and Voges-Proskauer tests, and all results were consistent with those of Mohammed *et al.* [32].

Molecular diagnostics

After *K. pneumoniae* isolates were diagnosed phenotypically, microscopically and by biochemical tests, all *K. pneumoniae* isolates were subjected to molecular diagnosis using PCR technology to confirm the diagnosis using the *16S rRNA* gene because it is characterized as a stable gene and low variability for long periods in the bacterial species, the results of electrophoresis showed that all *K. pneumoniae* isolates (50 isolates) by 100% possess the *16S rRNA* gene, as shown in Figure 1, and by comparing the replication bands and the DNA ladder, the resulting bands were found to have a molecular weight of 260 base pairs.

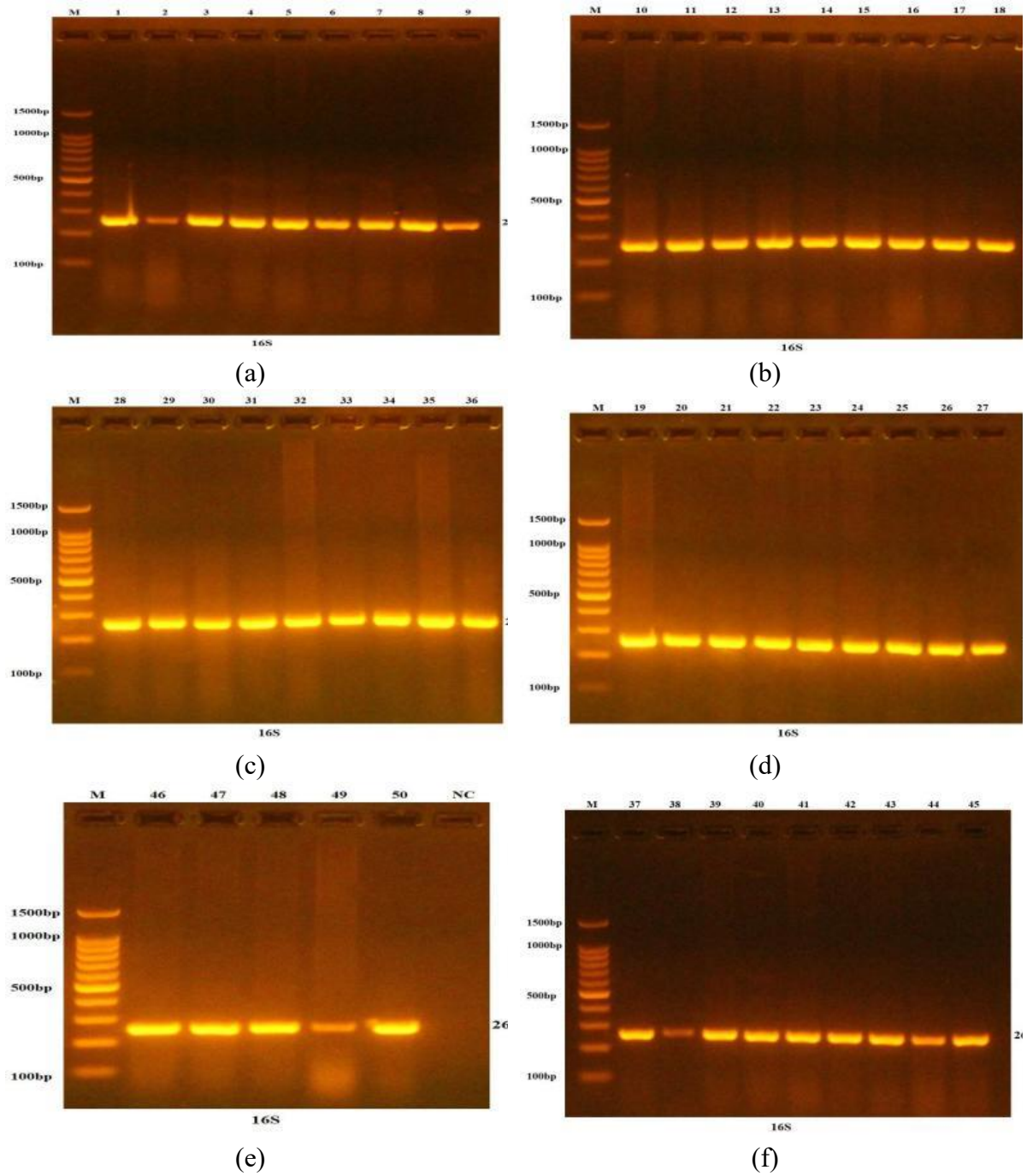

Figure 1: Electrophoresis of the PCR product of the *16S rRNA* gene (260 base pairs) of *K. pneumoniae* isolates on agarose gel at a concentration of (0.2%) and a potential difference of 100 volts for 60 minutes. Line M (volume index) 100 – 1500 base pairs. Lines (K1-9) in Fig. (A4), (K10-18) in Fig. (B4), (K19-27) in Fig. (C4), (K28-36) in Fig. (D4), (K37-45) in Fig. (E4), (K46-50) in Fig. (F4).

Detection of multidrug-resistant isolates of K. pneumoniae

The results of the study showed that the pattern of multi-drug resistance (MDR) was 34 (68%) of the *K. pneumoniae* isolates studied. The result of the study agreed with the study carried out by Obaid and Hasson [33], in which they found that the percentage of antibiotic resistance among *K. pneumoniae* isolates was 50 (66.6%). Zaidan *et al.* [34] also showed in their study that 54% of *K. pneumoniae* isolates were resistant to many drugs where they were resistant to at least one antibiotic from three or more different groups of antibiotics [35]. The

appearance of MDR isolates limits the effectiveness of existing antibiotics. The risk becomes more complex when MDR-producing ESBL isolates spread worldwide, leading to a public health challenge and treatment failure [36].

Minimum Inhibitory Concentrations (MICs) of efflux pump inhibitor

Minimal inhibitory concentration (MIC) is defined as the lowest concentration of an antimicrobial agent that can inhibit the visible growth of a particular microorganism after 24-hour incubation. Clinically, antibiotic doses for specific infections are determined according to the MIC. Therefore, a reliable evaluation of MIC will provide the physician with valuable information about the choice of therapeutic strategy [37]. The minimum inhibitory concentrations MIC of Gentamycin and Ceftazidime antibiotics were determined using the microdilution method in the Mueller-Hinton broth, and the results were interpreted 24 hours after incubation at 37°C according to the Clinical Laboratory Standards Institute [38]. The result of MIC Sub was 25 µg/ml for antibiotic-resistant isolates (11, 22, 25, 33, 41) for Gentamycin, as in Figure 2. The results of Sub MIC for antibiotic-resistant isolates to Ceftazidime was 3.9 µg/ml for four bacterial isolates (22, 25, 33, 41), while for the isolate (11) was 1.9 µg/ml, as shown in Figure 3.

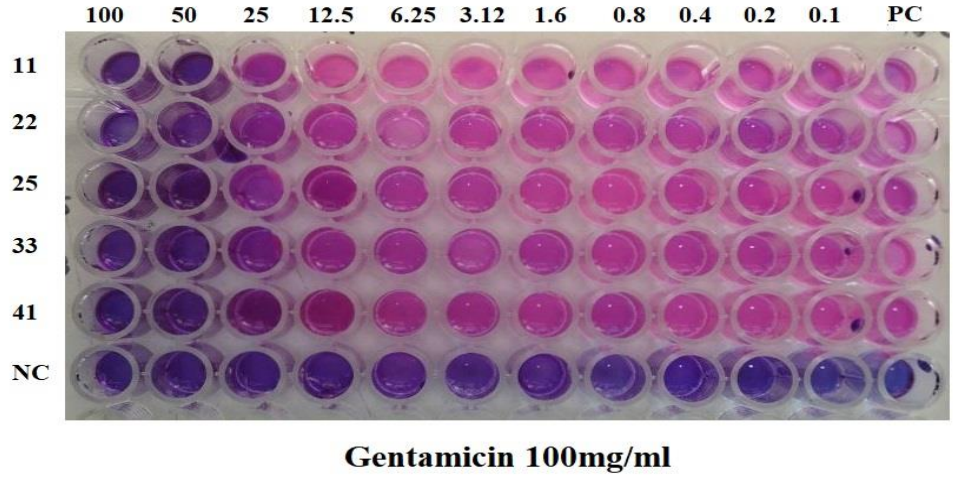

Figure 2: Minimum inhibitory concentrations (MICs) of Gentamycin antibiotic at concentrations (0.1-100 µg/ml) against *K. pneumoniae* isolates by Microtiter Plate test with Resazurin dye.

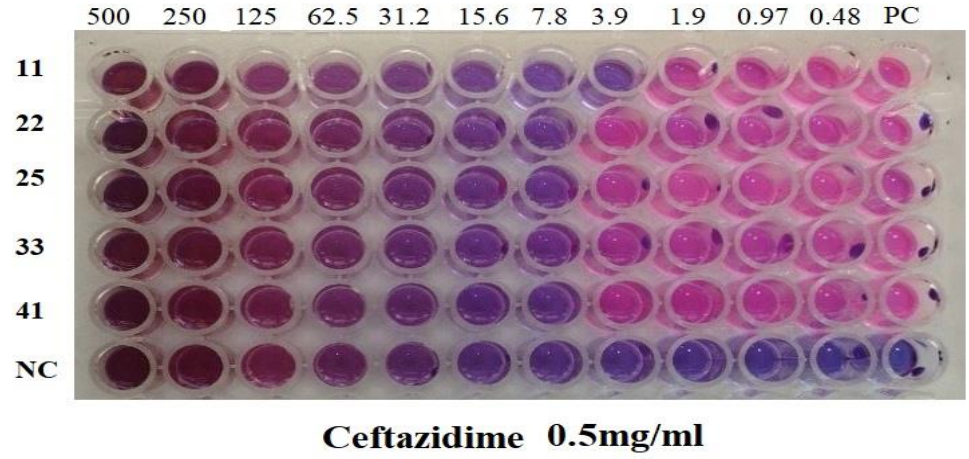

Figure 3: Minimum inhibitory concentrations (MICs) of the antibiotic Ceftazidime at concentrations (0.48-500 µg/ml) against *K. pneumoniae* isolates by Microtiter Plate test with Resazurin dye.

Narimisa *et al.* [39] indicated in their study of the Gentamicin and Ceftazidime antibiotics MIC against the bacteria *K. pneumoniae* ATCC 13883 using the broth microdilution method, they found MIC for two antibiotics 0.5 and 0.25 mg/ml, respectively. The studies confirmed that their results showed a decrease in bacterial cells after exposure to the sub-MIC levels of Gentamicin and Ceftazidime, indicating a greater effect of sub-MIC levels of Gentamicin and Ceftazidem on the growth of *K. pneumonia*. Since the concentration of antibiotics in different tissues is often lower than the concentration of MIC, exposure to lethal doses of antibiotics appears necessary for the effective treatment of *K. pneumoniae* infection [40].

Gene Expression

Five isolates of *K. pneumonia* that were multi-drug resistant were selected to study the effect of both the antibodies Ceftazidem and Gentamicin and synergized together on the expression of the five efflux pump genes *acrAB, TolC, oqxA, oqxB,* and *mdtk*. Sub-MIC concentrations were selected for the tested isolates to study their effect on gene expression because these concentrations do not affect bacterial growth, and the *16SrRNA* gene was used as the housekeeping gene in the present study because its expression remains constant in the studied cells under different conditions [41]. The results of gene expression after statistical analysis (Table 6 and Figure 4) showed significant differences $p \leq 0.002$ between most of the studied treatments for gene folding in resistant isolates. The isolates treated with Ceftazidime recorded the highest gene expression in *acrAB* gene (4.88±2.73) compared with control and other antibiotics treatments, followed by the isolated treated with the combination of antibiotics Ceftazidime+Gentamicin together in *mdtk* gene (4.52±0.92) compared with control and other antibiotics treatments, while the other treated genes with the antibiotics recorded increased or decreased in the gene expression but it was non-significant differences ($p > 0.05$). Antibiotics of aminoglycosides and cephalosporins classes are commonly used to treat inflammatory conditions caused by *K. pneumoniae,* and the choice of antibacterial depends on the patient's health, medical history, and severity of disease [42, 43]. *K. pneumoniae* isolates show resistance to a number of major classes of antibiotics, including those of cephalosporins and aminoglycosides, leading to antibiotic failure [44]. Active efflux pumps of all kinds are widely found in the genome of *K. pneumoniae*. These pumps selectively or non-selectively pump drugs or substrates present in bacteria out of the body, resulting in a decrease in the concentration of antibacterial drugs in the body and drug resistance [45].

Through the results, it is clear that the genes for *oqxA, acrAB*, *tolC,* and *mdtK* efflux pumps recorded the highest folding in the isolates of resistant *K. pneumoniae* bacteria and treatment with Ceftazidime compared with control treatment, which belongs to the family of cephalosporins, followed by the combination of antibiotics Ceftazidime+Gentamicin together. This may be because the efflux pumps are transport proteins involved in the excretion of toxic substrates from inside cells to the extracellular environment. These efflux proteins can modify the permeability of the bacterial membrane by excreting the drug, thus creating resistance to antibiotics such as Ceftazidime [46]. The study of Bialek-Davenet *et al.* [47] confirms that cephalosporins are substrates of both the *OqxAB* and the *AcrAB* efflux pumps in the bacteria *K. pneumoniae* due to their increased gene expression when detected in the bacteria *K. pneumoniae* resistant to a number of antibiotics, which is indicated by the current study.

In addition, the antibiotic Ceftazidime belongs to the group of β-lactams, so *K. pneumoniae* produces β-lactamases, which include the enzymes cephalosporinase and penicillinase. These enzymes break down the beta-lactam ring, inhibiting the action of antibiotics belonging to the penicillin and cephalosporins groups [48]. The current study is consistent with the study of Kumar [49] in the United States, where the isolates of *K. pneumoniae* showed resistance to a number of cephalosporins, including the ceftazidime, where this resistance was attributed to

the increased expression of the *OqxAB* efflux pump that includes the *oqxA* and *oqxB* genes. Rodríguez-Martínez [50] study was almost compatible with the current study for the *oqxA* gene, where the expression of this gene in the isolates of *K. pneumoniae* was 2.80 after treatment with β-lactamase antibiotics, while this same study did not correspond to the current study for the *oqxB* gene with expression of 2.82.
The current study is also consistent with Razavi *et al.* [24] in Iran, where isolates treated with cephalosporins recorded high gene expression for *oqxA* and *acrAB* gene expression and moderate gene expression for *oqxB* gene. Albarri *et al.* [51] also showed resistance of *K. pneumoniae* bacteria to a number of antibiotics, including cephalosporins, and this study showed high gene expression for both the *acrAB*, *oqxA,* and *oqxB* genes in isolates resistant to cephalosporins. It also did not agree with the study of Amereh *et al.* [52]; the *oqxB* gene appeared in 2.24% of the isolates of *K. pneumoniae* treated with the cephalosporins antibiotics Chloramphenicol and Ceftriaxone.

As for the *tolC* gene, it is the outer membrane efflux protein (OEP) of bacterial cells and is associated in *K. pneumonia* with the multi-drug efflux systems OqxAB-TolC and AcrAB-*TolC*. The *tolC* gene is coding separately from other efflux systems and is shared between different efflux systems and plays a crucial role in antibacterial resistance due to its function with super-family vectors (ATP-binding cassette) ABC, Resistance-nodulation-cell division (RND) and major facilitator (MF). The function of these vectors depends on the presence of OEP similar to them and does not work if their OEP does not exist. It has been observed that when mutations occur in the *tolC* gene, *K. pneumoniae* becomes highly sensitive to multiple antibiotics [53, 54]. The result of the study is consistent with several other studies that stated that the presence of the genes of *tolC*, *AcrAB,* and *mdtK* is strongly associated with multi-drug resistance patterns in *K.pneumoniae* bacteria, where these studies showed that the AcrAB-*TolC* multi-drug efflux pump system is responsible for resistance to quinolones, tetracycline, TGC, and beta-lactam antibiotics, including the Ceftazidime in many multidrug-resistant isolates [55].

The result did agree with Ferreira *et al.* [44] study conducted in Brazil on isolates of *K. pneumoniae* bacteria with multi-drug resistance to a number of antibiotics, including Ceftazidime, where the *tolc* gene recorded high gene expression of isolates. The *mdtK*, belongs to the major facilitator superfamily within the efflux pump proteins, which increases resistance to *K.pneumoniae*, as the high production of this gene leads to inactivation of carbapenems and cephalosporins [56].

The increased use of cephalosporins leads clinical isolates to increase the expression of efflux genes, including the *mdtK* gene, either by directly binding to antibiotic molecules or by promoting multiple inhibitors that inhibit the action of antibacterial agents [57]. This response can be achieved through the acquisition of genetic elements, such as plasmids or jumping genes, which enhance efflux genes or mutate the regulatory network to express efflux pumps as well as be located inside the inhibiter and change the genes of efflux pumps as in the *mdtK* gene or its regulators [58]. As for the treatment of the antibiotic Gentamicin, which belongs to the family of aminoglycosides, the *mdtK* gene recorded the highest folding (1.85±0.37) in the isolates of *K. pneumoniae* bacteria, followed by the *tolC* gene with a folding of 1.10±0.41, then the *oqxA*, *oqxB,* and *acrAB* genes with folding (0.78±0.35, 0.58±0.34, 0.62±0.28) respectively. Resistance of *K.pneumoniae* isolates to aminoglycosides (Amikacine and Gentamycin) may be the result of modifications in cell permeability due to changes in AcrAB-TolC efflux pump systems and due to hypothetical loss of purine KpnO. Disturbances in AcrAB-TolC may also increase bacteria's sensitivity to Gentamicin. Methyl enzymes *16SrRNA* encoded on plasmids confer resistance to all aminoglycosides. Mutations that confer resistance by modulating the target can also be a possible cause attributed to increased resistance of *K. pneumoniae* to most

aminoglycosides antibiotics [59, 60]. The results of Table (6) also showed that the folding of the *mdtK* gene (4.52±0.92) was the highest in treating the mixture of Ceftazidime+Gentamicin together compared to the rest of the treatments for resistant isolates. The lowest genes folding were 0.51±0.24, 0.51±0.28, 0.93±0.77 and 0.96±0.53 in genes *tolC, oqxB*, *oqxA,* and *acrAB,* respectively, in the treatment of the combination of Ceftazidime+Gentamicin antibiotics together. The use of a mixture of two antibiotics may be a promising treatment against antibiotic-resistant *K. pneumoniae* bacteria, as in the study of Shi *et al.* [61], where the antibiotic Avibactam, an antibiotic manufactured from the non-β-lactam group (Diazabicyclooctane) with the antibiotic Ceftazidime, was found that this antibiotic enhances the antibacterial activity of Ceftazidime against intestinal bacteria and some non-fermented gram-negative bacilli by inhibiting carbapenems without affecting the activity of the antibiotic Ceftazidime towards living organisms that sensitive to Ceftazidime or most anaerobic bacilli gram-negative. This is observed in the current study where most efflux genes except the *mdtK* gene were reduced on isolates of antibiotic-resistant *K.pneumoniae* that were treated with Ceftazidime+Gentamicin.

Table 6: Percentage of gene folding in antibiotic-treated resistant isolates.

	Resistant isolates					
Genes Treatments	***oqxA***	***oqxB***	***acrAB***	***tolC***	***mdtK***	**p-value**
Control	1.00±0.00[a]	0.80±0.20[a]	1.00±0.00[a]	1.00±0.00[a]	1.00±0.00[a]	
Ceftazidime	2.78±1.14[a]	1.00±0.46[a]	4.88±2.73[b]	1.90±0.55[a]	1.18±0.43[a]	
Gentamicin	0.78±0.35[a]	0.58±0.34[a]	0.62±0.28[a]	1.10±0.41[a]	1.85±0.37[a]	0.002[*]
Mixed	0.93±0.77[a]	0.51±0.28[a]	0.96±0.53[a]	0.51±0.24[a]	4.52±0.92[b]	

The different small letters indicated the presence of significant differences at P≤0.05.
The same small letters indicated non-significant differences at P>0.05.

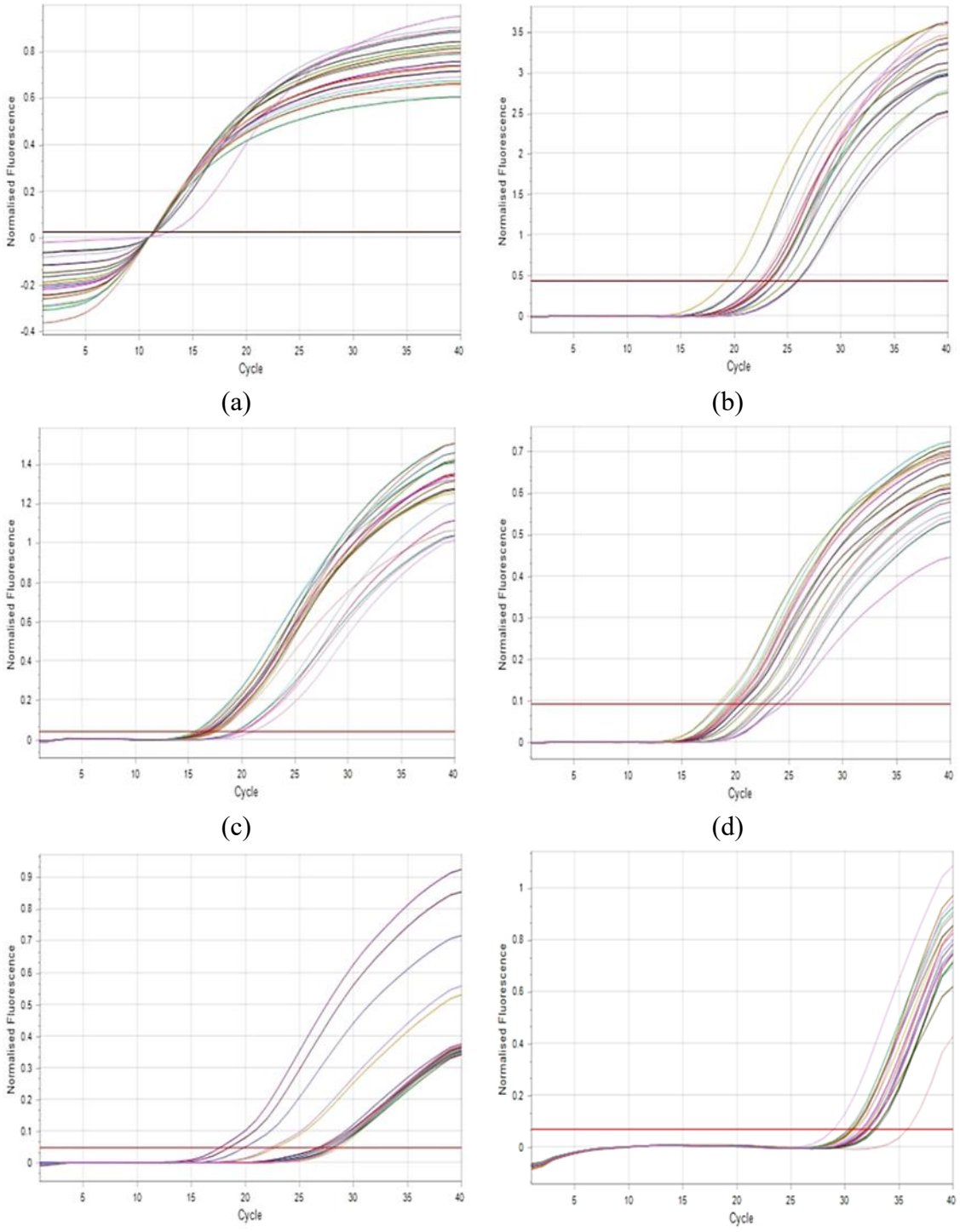

Figure 4: Quantitative polymerase chain reaction curve of *16SrRNA, acrAB, TolC, oqxA, oqxB, mdtk* genes for resistant isolates (11, 22, 25, 33, 41).

4. Conclusion

Most *K. pneumoniae* isolates were categorized as MDR. There are highly significant differences between most of the studied treatments for gene folding in resistant isolates and the highest folding in *acrAB* gene treated with Ceftazidime antibiotic and *mdtK* gene treated with combination of antibiotics Ceftazidime+ Gentamicin. In contrast, the other genes (*oqxA*, *acrAB,* and *tolC*) were non-significant $p>0.05$.

Ethical approval

The study protocol was approved by the College of Education for Pure Science (Ibn Al-Haitham) at the University of Baghdad and the Iraqi Ministry of Health and Environment, which numbered (39891) on 19/10/2023**.**

Conflict of interest

The authors declare that they have no conflicts of interest.

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