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Article

Study Gene Expression of *blaOXA-48* and*blaVIM-1*Genes Carbapenems Resistance in *Klebsiella Pneumoniae* isolated from urinary tract infections in Baghdad hospital's

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Abstract
Klebsiella pneumoniae has emerged as one of the eight most essential causes of urinary tract infection (UTI), with increasing resistance to antibiotics, especially carbapenems. About one hundred-eight urine samples were collected from inpatients and outpatients who attended. They were collected at three Baghdad hospitals: two from Karkh (AL-Yarmouk Hospital and Al-karamu Hospital) and one from Rusafa (AL-Baghdad Educational Hospital). A minimal inhibitory concentration (MIC) test was done using the Microtiter plate method and demonstrated different resistance levels against meropenem antibiotics. Gene expression of *blaOXA-48* and *blaVIM-1* genes was performed when treated with meropenem antibiotic using the Real-time PCR technique. The study showed that the highest value of gene expression in the *blaOXA-48* gene was recorded for the resistant group was (1.8705), and the lowest value of gene expression *in the OXA-48* gene before treated with the meropenem group was (1.0312). The gene expression value in the non-treated group was (0.97) control.
In contrast, A slight increase in gene expression value for the *blaVIM-1* gene was recorded for the meropenem-treated group (1.8705), and the sensitive group had a gene expression value of (1.00). The lowest value was in the gene expression of the meropenem-treated group (1.0312), as well as an increase in the value of the expression. The genotype of the *blaVIM-1*gene for the meropenem-treated group (1.828) is based on the 2-ΔCt method for both genes. When using the 2-ΔΔCt method, gene expression differed slightly in both genes. When relying on the 2-ΔΔCt method, both genes' gene expression was slightly different. It was concluded that the coexistence of *blaVIM-1* and *blaOXA-48* genes in four strains of *K. pneumoniae* indicated widespread *VIM-1* and *OXA-48* in Baghdad, Iraq.

Keywords: *Klebsiella Pneumonia, blaOXA-48,*and *blaVIM-1*, UTI infection

Introduction

Klebsiella pneumoniae is an encapsulated Gram-negative bacillus belonging to the *Enterobacteriaceae* family. It was first described in 1882 by Carl Friedlander from lung tissues of dead pneumonia patients. It presents a mucoid phenotype in media such as MacConkey or Eosin methylene blue agar. Moreover, it can ferment lactose and mannitol and reduce nitrate to nitrite. *K. pneumoniae* is a bacterium able to infect humans, causing different types of infections, including res-

piratory and urinary tract infections, soft tissue infections, surgical wounds, and sepsis [1]. This bacteria causes several diseases, including pneumonia, urinary tract infections (UTIs), bloodstream infections, and sepsis. Infections are particularly a problem among neonates, the elderly and immunocompromised individuals. *Klebsiella* is also responsible for a significant number of community-acquired infections. [2]. Urinary tract infection is among developing countries' most common infectious diseases (UTI). Antibiotic resistance has emerged due to the widespread use of antibiotics to treat uropathogens[3]. Because of its various impacts on the urinary tract and host immune system, urinary tract infection (UTI) is a serious health concern in diabetes people. Patients with an atypical genitourinary tract are more likely to have complicated UTIs. A thorough evaluation and rapid treatment are required to avoid morbidity and life-threatening conditions linked with UTI. [4]. Antibiotic resistance among Extended-Spectrum Beta-Lactamases (ESBLs)-producing uropathogenic bacteria to the most popular cephalosporins used in our hospitals has been reported in several local investigations[5]. The genetic identification of ESBL-producing organisms is vital for epidemiological application because the numerous Extended-Spectrum Beta-Lactamases (ESBLs) expressing genes in bacteria may disclose typical characteristics with antimicrobial resistance expression[6]. Treatment of multidrug-resistant bacteria has proven to be a therapeutic challenge. Extended-spectrum Beta-Lactamases (ESBL)-producing isolates are regarded as severe public health and financial challenges because physicians are limited in choosing appropriate antibiotics to treat ESBL infections effectively. Hence, the study's objectives are to In our study. We aimed to investigate the presence of *blaOXA-48* and *blaVIM-1* genes[7]. The development of carbapenem-hydrolyzing-lactamase is primarily responsible for *K. pneumonia* resistance to carbapenems. Recent research has been done on the prevalence of KPC, *OXA-48*, and Metallo-lactamase (*VIM-1*) generating strains of K. pneumoniae in various nations. Because these genes are found on mobile genetic components like transposons and plasmids, transmission to other gram-negative bacteria should be taken into account[8]. Nevertheless, with the high population density in Baghdad city and its high percentage of pollution due to the destruction caused by wars and military operations, the knowledge of carbapenemase is still limited. In Iraq, little attention has been paid to β-lactamases producing isolates. However, in Baghdad city, there is no information regarding the molecular studies on the occurrence of carbapenemase-producing *K. pneumoniae* recovered from clinical cases. This study aimed to determine the presence of serine and Metallo- β lactamase (MBL) genes, including *blaOXA-48* and *blaVIM-1* genes among carbapenem-resistant *K.pneumoniae* isolated from hospitalized patients in Three hospitals in Bagdad, Iraq.

Materials and Methods

Collection of samples

About one hundred-eight urine samples were collected from inpatients and outpatients who attended. They were collected at three Baghdad hospitals: Karkh (AL-Yarmouk Hospital and Al-karamu Hospital) and Rusafa (AL-Baghdad educational hospital).

Quantitative MIC

1. Using the micropipette, dispense 100 µl of the medium into all microtiter plate wells.
2. Pipette 100 µl of appropriate antibiotic solutions into the wells in column A (far left of the plate).
3. Using the micropipette set at 100 µl, mix the antibiotics into the wells in column A by sucking up and down 6-8 times. Do not splash.
4. Withdraw 100 µl from column A and add this to column B. This makes column B a tenfold dilution of column A, transfer 100 µl to column C, and repeat the procedure to column H only. The same set of tips can be used for the entire dilution series.
5. Discard 100 µl from column H.
6. Dispense bacteria into all wells With a similar micropipette set to 100 µl.
7. Incubate the plates at 37C.
8. When satisfactory growth is obtained (24 hours).
9. After incubation, 20 µl of resazurin dye was added to all the wells and incubated for 30 minutes to observe any color changes. The Minimum Inhibitory Concentrations were determined visually in broth micro dilutions as the lowest concentrations of the extracts at which no color changed from blue to pink in the resazurin broth assay[9,10].

Total RNA Extraction

Before and after treatment, the total RNA was carefully extracted using commercially available RNA extraction of three *K. pneumoniae* isolates. The study resulted in a high concentration of total RNA, which was dependent on the extraction conditions and required aseptic procedures. Trizol Reagent was used to extract total RNA from bacterial cells, and the findings were promising. Using nuclease-free water, RNase-free equipment such as tips and microfuge tubes, and UV radiation to decontaminate the working area could all be working aids in achieving the optimal outcome. There was a link between a high RNA concentration and an absence of DNA.

Preparation of primers:

Specific primers were obtained in Table (1) according to [11] for detection of the gene expression. Lyophilized forward and reverse primers were suspended with a suitable volume of nuclease-free water as the Macrogen company protocol recommended. Lyophilized primers were dissolved in nuclease-free water to give a final concentration of (100 pmol/µl) (as the stock solution) to prepare 10 pmol concentration as a work solution. It was prepared by pipetting 10 µl from a stock

solution in 90 µl of nuclease-free water to reach a final concentration (10 pmol/µl) and stored in the deep freezer until use.

Genes	Sequences 5-3	PCR product size(bp)	Reference
16srRNA	**F**-5 -GGACGGGTGAGTAATGTC-3	150	[11]
	R-5-TCTCAGACCAGCTAGGG ATCG-3		
*bla*OXA-48	**F**-5- TATCG-GAATGCCTGCGGTAG-3	200	[12]
	R-5-GCTTGGTTCGCCCGTTT AAG-3		
*bla*VIM-1	**F-**5-AAGTGCCGCTGTGTTTTT CG -3	389	[12]
	R-5-GGTGTTTGGTCGCATAT CGC -3		

Table 1: Primer Sequences used qRT-PCR in this study.

Quantitative Real-time PCR Assay (qRT-PCR) reaction

Quantitative RT-PCR (qPCR) was performed using Luna Script RT Master Mix Kit (5X) (Bio Labs, England). The RT-PCR mixture contained 10 µl of the master mix, 0.5 µl of each forward Primers, and reverse primers. 5 µl of cDNA from each sample was added, then the volume was completed to 20 µl by adding 4 µl of nuclease-free water. The GAPDH gene was used as the endogenous control. A qPCR was performed at 95°C for 1 minute for polymerase activation, followed by 45 cycles of 95°C for 15 seconds for denaturation of the double-stranded cDNA and 60°C for 20-sec annealing steps with channel scanning, and melting curve analysis was performed based on the separation characteristics of double-stranded cDNA during cycles with increasing denaturing T.M.

The ct value of target miRNAs was standardized to the GAPDH reference gene, and the expression of miRNAs was determined by the relative quantitative method using the comparative Ct formula: Folding $=2^{\wedge-\Delta\Delta CT}$ where.

Amplification of a fragment of cDNA was performed in a Real-time PCR thermal cycler (Transgene / Chine) with the following master amplification reaction listed in Table (2) and the program of RT-PCR shown in Tables (3), (4), and (5).

Several experiments were done for a more appropriate annealing temperature.

Component	Volume per 20µl Reaction
qPCR Master SYBR	**10 µl**
Forward Primer, 10X	**0.5 µl**
Reverse Primer, 10X	**0.5 µl**
cDNA	**5 µl**
Nuclease-Free Water	**4 µl**

Table 2: Real-time quantitative RT-PCR Reaction Mix.

Step	Temperature	Time	Cycles
Initial denaturation (hold)	95 °C	10 minutes	**1**
Denaturation	95 °C	15 seconds	**45**
Annealing	60°C	30 seconds	
extension	**90 °C**	**15 seconds**	**1**

Table 3: Thermocycler Program for One-Step quantitative RT-qPCR for the *16srRNA* (house-keeping) gene.

Step	Temperature	Time	Cycles
Initial denaturation (hold)	95 °C	10 minutes	**1**
Denaturation	95 °C	15 seconds	**45**
Annealing	60 °C	30 seconds	
extension	**90 °C**	**15 seconds**	**1**

Table 4: Thermocycler Program for One-Step Quantitative RT-qPCR for the *blaVIM-1* gene.

Step	Temperature	Time	Cycles
Initial denaturation (hold)	95 °C	10 minutes	**1**
Denaturation	95 °C	15seconds	**45**
Annealing	60 °C	30 seconds	
Extension	**90 °C**	**15 seconds**	**1**

Table 5: Thermocycler Program for One-Step Quantitative RT-qPCR for the *blaOXA-48* gene.

Delta - delta Ct (ΔΔCt) method

This method is the simplest one as it directly compares Ct values between the target gene and the reference gene. Relative quantification involves the choice of a calibrator sample. The calibrator sample can be the untreated sample or any sample

wanted to be compared to the unknown.

Firstly, the ΔCt between the target gene and the reference gene is calculated for each sample (for the unknown and calibrator samples).

ΔCt = Ct target – Ct reference gene

Then, the difference between the ΔCt of the unknown and the ΔCt of the calibrator is calculated, giving the ΔΔCt value:

ΔΔCt = (Ct target – Ct reference) sample – (Ct target – Ct reference) calibrator

The normalized target amount in the sample is then equal to (2-ΔΔCt), which can be used to compare expression levels in samples. The samples were analyzed in triplicates and standardized against *16srRNA* gene expression. The relative changes in mRNA expression levels were determined using the comparative threshold cycle (C.T.) method (2-ΔΔCt) between the antibiotic-exposed and non-exposed *K. pneumoniae.*

Result

Results of quantitative Real-Time PCR

This experiment used the SYBR green fluorescent dye for real-time PCR quantification. This fluorescent dye recognizes and absorbs all double-stranded DNA, including cDNA.

The primary purpose of this step was to quantify gene expression of the *blaOXA-48*and *blaVIM-1*genes and compare the quantity of gene expression in the presence of the meropenem antibiotic in its absence to identify the role of these genes in the carbapenems-resistance of *K. pneumonia*.

Due to accurate, sensitive, and fast results, reverse transcription-quantitative PCR (RT-qPCR) is distinguished from other methods for gene expression. This technology has established itself as a brilliant criterion in gene expression analysis.

In the present quantitative R.T.- PCR assay analysis experiment, the mRNA expression was conducted using three isolated meropenem-resistant from *K. pneumoniae* with *blaOXA-48* and *blaVIM-1* genes. These isolates were chosen with different sub-MIC values for meropenem antibiotic sensitivity (8 µg/ml) and three resistance isolates for meropenem antibiotic as control. Study the gene expression results of *blaOXA-48 and blaVIM*-1 for the carbapenem-resistance gene present in *K. pneumoniae*. The Ct values of amplification of the genes were recorded from the software of quantitative RT PCR.

The effects of meropenem antibiotics on the gene expressions for *blaOXA-48, blaVIM-1,* and *16srRNA* housekeeping genes were evaluated in a study with the following qRT-PCR results.

Group	Ct *16srRNA*	CT *OXA-48*	ΔCT	2^-ΔΔ CT	Fold Change
1-B.T.	13.6	27.1	13.1	0.11	0.97

2-B.T.	15.1	28.1	13.0	0.1	0.93
3-B.T.	13.3	29.1	13.2	0.12	0.9553
K36-A.T. MEM	16.8	30.47	13.67	0.2	1.8705 ±0.12
K46-A.T. MEM	16.4	34.4	13.5	5	1.0312 ±0.006
K48-A.T. MEM	20.3	33.31	12.8	-0.2	1.148 ± 0.17

Table 6. Comparison between different groups in Ct value of *OXA-48* gene and *16srRNA* housekeeping gene expressions
***B.T.:Before treatment, A.T.: After treatment, MEM: Meropenem antibiotic.**

The results mentioned in Table (6) show the value of gene expression for the (*OXA-48* and *16srRNA*) genes after treatment with the sub-MIC of Meropenem, which was obtained in previous steps and according to the paragraph (sub-MIC). The following was shown:

The range of Ct value for *OXA-48* in resistant isolates before treatment with the meropenem group (Control) was (27.1-29.1) with a mean fold change value shown (0.97),(0.93), and (0.955), respectively, for isolates (K36-K46-K48). The range of Ct value for *OXA-48* in sensitive isolates after treatment with the meropenem group was (30.47-34.4) with mean ±SE Ct values of High expression (1.8705 ±0.12), (1.0312±0.006), (1.148±0.17), respectively in the Table (6).

The mean Ct values in resistant isolates after meropenem treatment were more significant than those in resistant isolates before meropenem treatment, which was somewhat higher than those in sensitive isolates, indicating that the genes are present in mRNA samples. These findings show that the sensitive group has the most significant copy number of target genes on mRNAs, indicating higher expression. The resistant group has the lowest copy number of target genes carried on mRNA following treatment with Meropenem, indicating Higher expression[16]. The reason may be that resistant bacteria that carry the resistance gene *blaOXA-48* were not affected by this meropenem antibiotic, as previously studied for resistance genes [17].

This review highlights the new mechanisms that enhance antibiotic resistance and the evolutionary background of multidrug resistance; some promising points for controlling or reducing the occurrence and spread of antimicrobial resistance are also proposed [18].

As for the Ct, the value of *the 16srRNA* housekeeping gene before treatment without meropenem antibiotic was (13.6),(15.1), and (13.3). After treatment with Meropenem, antibiotics found Ct values were (16.8),(16.4), and (20.3) respectively. A non-significant difference was found between these groups regarding the mean Ct value of *16srRNA* ($p=0.0001$: $p<0.01$) with an LSD value of (15.29). Where it was noticed that there was no significant expression in the level of gene expression of gene *OXA-48* and *16srRNA* housekeeping gene before and after

treatment with meropenem antibiotic, the results of the mean fold of gene expression concerning the mean sub-MIC value are shown in Figure(1).

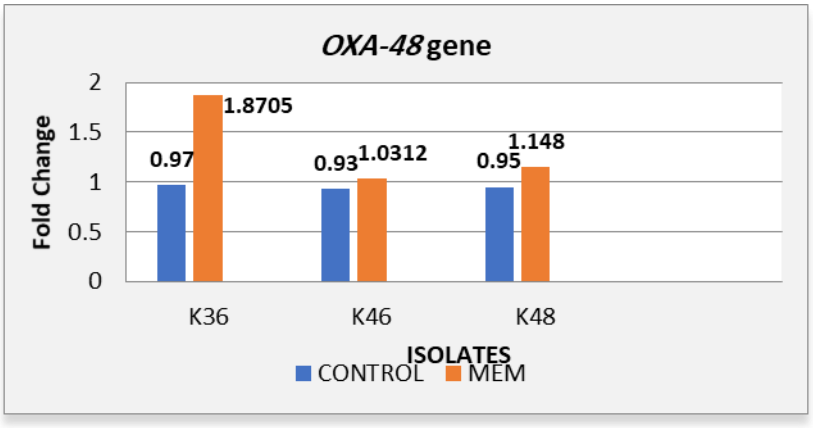

Figure 1: The mean fold of gene expression of *blaOXA-48* gene after and before treated with the Meropenem compared with control.

Results of qRT-PCR evaluation of gene expression blaVIM-1 and 16srRNA housekeeping gene with Meropenem antibiotic

This study compared the untreated and treated groups of resistant bacteria grown with meropenem antibiotic and quercetin extracts for each sample (1 µg/ml), with the sensitive group of samples serving as a control, to assess the mRNA expression of the *blaVIM-1*genes and *16srRNA* housekeeping gene. RT-PCR quantitative software was used to record the Ct values for gene amplification. Relative quantification based on the delta Ct value was used to calculate the mean fold change in gene expression (details shown in materials and methods). The Table shows the results of a mean fold of gene expression of *blaVIM-1* genes with mean sub-MIC values in Table (7).

Group	Ct *16srRNA*	C.T. *blame-1*	ΔCT	2^-ΔΔCT	Fold Change Mean± SE
1-B.T.	13.6	26.8	12.9	0.11	0.991
2-B.T.	15.1	28.1	13.0	0.1	1.022
3-B.T.	13.3	27.3	13.12	0.12	1.00
K36-A.T. MEM	16.8	23.6	6.8	1.8	1.2871 ±0.03
K46-A.T. MEM	16.4	25.1	8.7	3.7	1.0769 ±0.005
K48-A.T. MEM	20.3	23.8	3.5	-1.5	1.828 ±0.16

Table 7. Comparison between different groups in Ct value of *VIM-1* gene and *16srRNA* housekeeping genes expressions
***B.T.:Before treatment, A.T.: After treatment, MEM: Meropenem antibiotic**

The results are described in Table (7) and demonstrate the value of gene expression for the *blaVIM-1* and *16srRNA* genes following treatment with the sub-MIC of Meropenem, obtained in earlier steps. According to the paragraph (sub-MIC), it was proved that.

The range of Ct value for *blaVIM-1*gene in resistant isolates before treatment with the meropenem group (Control) was (26.8-28.1) with a mean fold change value shown (0.991),(1.022), and (1.00), respectively, for isolates (K36-K46-K48). The range of Ct value for *blaVIM-1*gene in sensitive isolates after treatment with the meropenem antibiotic group was (23.6-25.1) with mean ±SE Ct values of High expression (1.2871 ±0.03),(1.0769 ±0.005),(1.828 ±0.16), respectively .see Table (5). There was a significant difference in the mean Ct values between the different study groups (p=0.0001). The Ct value of the *16srRNA* housekeeping gene before treatment without Meropenem was (13.6),(15.1), and(13.3). After treatment with Meropenem antibiotic, the Ct value was (16.8),(16.4), and(20.3) respectively. A non-significant difference was found between these groups regarding the mean Ct value of *16srRNA* (p=0.0001: p<0.01) with an LSD value of (9.7244).

It was noticed that there was no significant expression in the level of gene expression of the *blaVIM-1* gene and *16srRNA* housekeeping gene before and after treatment with meropenem antibiotic. The results of the mean fold of gene expression concerning the mean MIC value are shown in Figure (2). After treatment, the mean Ct values of the *K. pneumoniae*-resistant group were higher than those of the *K. pneumoniae*-sensitive group, which were slightly higher than those of the *K. pneumoniae*-resistant group before treatment. This is essential since this reflects the number of copies of genes present in mRNA samples[19].

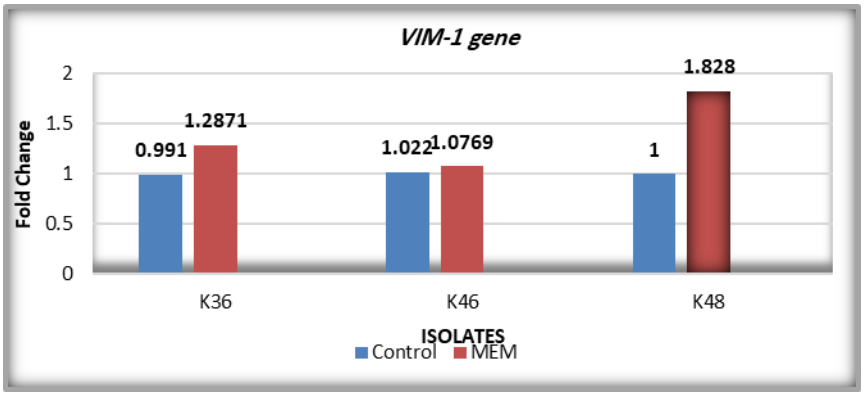

Figure 2. The mean fold of gene expression of *blaVIM-1*gene after and before being treated with Meropenem compared with control.

Discussion

The mRNA amplification the presence of more copies of the target gene, and vice versa. High Ct values indicate low gene expression, while low Ct values indicate high gene expression [13].

It is essential to realize that in relative quantification studies, all experiments are usually concerned with comparing the expression level of a particular gene amongst different samples [14]

16srRNA gene, the housekeeping gene, was used in the present experiment as a control because its expression remains constant in the investigated cells under different conditions[15].

The fold change of gene expression was calculated using relative quantification from the delta-delta Ct value (details shown in materials and method[16]).

Conclusions

This study attracted attention to *K. pneumoniae* as one of the essential causes of UTI in a patient of different ages, as the present study showed that most local clinical isolates of *K. pneumoniae* had high percentage resistance to meropenem and imipenem antibiotics, which is a potent antibiotic of carbapenems used for the treatment of UTIs caused by *K. pneumoniae*. Gene expression of *blaOXA-48* and *blaVIM-1* indicated a lower fold of gene expression than *blaOXA-48* and *blaVIM-1* gene in the meropenem antibiotic. At the same time, gene expression fold was significantly decreased in both genes *blaOXA-48* and *blaVIM-1* in the antibiotic-treated resistant group to increase antibiotic resistance by decreasing the entrance of antibiotic molecules through membrane porins. Both *blaOXA-48* and *blaVIM-1* Porin genes differed slightly in gene expression fold in the antibiotic-sensitive group.

Acknowledgment

We are grateful to the staff of the Medical Laboratory in the hospital for providing the clinical samples. These findings show that sensitive and resistant isolates have a high copy number of target genes on mRNAs before treatment with meropenem antibiotics. Resistance isolates have a Higher copy number of target genes on mRNAs after treatment with meropenem antibiotic, showing high expression.

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