Bionatura
Latin American Journal of Biotechnology and Life Sciences

Bionatura Issue 1 Vol 8 No 1 2023

Article

Study the Gene Expression of *bla-IMP* and *bla-VIM* Genes In Meropenem-resistant *Klebsiella pneumoniae* Isolated from Urinary Tract Infections

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Available from: http://dx.doi.org/10.21931/RB/CSS/2023.08.01.82

ABSTRACT: Background: The emergence of carbapenem-resistant Enterobacteriaceae, especially *Klebsiella pneumoniae*, which causes infection associated with multidrug-resistant strains, is a primary clinical and public health concern. Objectives: This study aimed to evaluate the expression level of *bla-IMP* and *bla-VIM* genes in the presence and absence of meropenem antibiotics. Methods: Eighty *K. pneumoniae* isolates were obtained from 250 urine samples collected from patients in Baghdad Hospitals, Iraq, from November 2020 to March 2021. The minimum inhibitory concentrations (MICs) were measured for the isolates by microdilution method. Real-time PCR detected the presence of *bla-IMP* and *bla-VIM* resistance genes. The *blah-IMP* and *bla-VIM* gene expression levels were measured by real-time PCR in the presence and absence of meropenem antibiotics. Results: The results of this study showed that the higher expression level of *bla-IMP* and *bla-VIM* increased in resistant strains in the presence of meropenem, where the range of fold for gene expression of *bla-VIM* was 1.3 to 8.3, while for *bla-IMP* gene was 1.5 to 8.4. Conclusion: In conclusion, the overuse of meropenem and imipenem antibiotics in the treatment of *K. pneumoniae* may lead to an increase in the resistance of this species to carbapenems and cephalosporins, and There was a correlation between the meropenem resistance in *K. pneumoniae* isolates and the gene expression of the tow genes *bla-IMP* and *bla-VIM*

Keywords: Carbapenem Resistance, *Klebsiella pneumoniae*, *bla-IMP*, *bla-VIM*, Gene expression, UTI.

Introduction

Urinary tract infections (UTIs) are one of the most widespread bacterial infections requiring antimicrobial treatment [1]. Klebsiella pneumoniae, a Gram-negative rod, belongs to the Enterobacteriaceae family. It is an opportunistic pathogen, causing septicemia, pneumonia, urinary tract infections, meningitis, diarrhea, and soft tissue infections. The rising multi-drug resistance (MDR) of K. pneumoniae isolates has led to limited antibiotic therapy and infection control treatment options[2]. The World Health Organization (WHO) has recognized antimicrobial resistance (AMR) as a significant global health problem [3]. Carbapenems are a primary class of β-lactam antibiotics for treating severe infections in Gram-negative bacteria. Carbapenemase-producing Enterobacteriaceae (CPE) in hospitalized patients has been a significant concern

[4]. Carbapenemases are a member of molecular classes A, B, and D β-lactamases that could hydrolyze β-lactam antibiotics [5].

Carbapenemases are commonly found on mobile genetic components and can spread worldwide [6]. Most carbapenemase-producers (CPs) are MDR pathogens carrying multiple resistance determinants to other antimicrobial agents [7]. The most common carbapenemases are K. pneumoniae carbapenemases (KPC), New Delhi metallo-β-lactamase (NDM), imipenem-resistant carbapenemases (IMP), Verona integron-encoded metallo-β-lactamase (VIM), and oxacillinase (OXA-48-like) types. They are encoded by blaKPC, blaNDM, blaIMP, blaVIM, and blaOXA-48 genes, respectively [8]. The IMP and VIM have two characteristics that separate them from the other functional group 2 enzymes. First, these enzymes are found on transferable plasmids; they can be transferred from one bacterium to another, from one person to another, and from one country to another; blah-imp and bla-VIM, clinically essential and active against many β-lactam antibiotics, including the cephalosporins and carbapenems. Carbapenemase enzymes destroy the antibiotics before they have a chance to have an effect. Due to the potential for transmission of these genes, recognition of KPC-producing organisms is essential for controlling their prevalence in nosocomial and long-term-care settings [9]. This study's objective was to evaluate the expression level of bla-IMP and bla-VIM genes in the presence and absence of meropenem antibiotics among K. pneumoniae isolated from Baghdad Hospital.

Materials And Methods

K. pneumoniae isolates

This study was conducted in Baghdad Hospital, Iraq. The isolates of K. pneumoniae from urine samples of patients admitted to different wards between November 2020 and March 2021. Eighty K. pneumoniae isolates were obtained from 250 urine samples.

Five isolates of K. pneumoniae were selected for this study, which recovered from UTI patients in Baghdad hospitals, Iraq. The clinical isolates were resistant to carbapenems according to the Minimum Inhibitory Concentrations (MICs) using the microdilution method based on the Clinical and Laboratory Standards Institute (CLSI) guidelines. The five isolates were multidrug-resistant and harbored the two genes bla-IMP and bla-VIM according to PCR results [10]

Gene Expression of bla-IMP and bla-VIM genes expression using RT PCR technique

This experiment was designed by using five isolates of Meropenem-resistant K. pneumoniae, which have different values of Sub-MIC ranging from 0.12 to 32.2 µg/ml and have the two bla-IMP and bla-VIM genes. The measurement of gene expression of the two genes in the resistant isolates was done before the treatment with the antibiotic meropenem and after the treatment.

Total RNA extraction

After growing the K. pneumoniae isolate in Mueller Hinton broth (with and without antibiotic) and incubated overnight at 37°C to evaluate bla-IMP and bla-VIM gene expression by using Total RNA Isolation System kit according to the manufacture instructions (Promega, USA).

Synthesis of cDNA from RNA

Using All-In-One 5X RT MasterMix (ABM, Kanada), the Amplification of a fragment of mRNA was performed with the following master amplification

reaction. Protocol RT reactions should be assembled in an RNase-free environment. Using of kit abm (kanada).

Component	Volume (µl)
All-In-One 5X RT Master Mix	4
Total RNA extraction	10
Nuclease-free H2 O	6
Total Volume	20

Table 1. Synthesis of cDNA from RNA.

Step	Temperature (°C)	Time(min)	No. of cycles
Step1	37	15	1
Step2	60	10	1
Step3	95	3	1

Table 2. Program PCR converted RNA to cDNA.

RT-PCR Technique Molecular identification of bla-IMP and bla-VIM genes

In K. pneumoniae, reverse transcription-polymerase chain reaction (RT-PCR) was applied to evaluate isolates with positive results. Specific primer pairs, targeting bla-IMP and bla-VIM genes Table 3.

Target Gene	Primer name	Oligonucleotide primer Sequence(5-3)	Tm melting	Amplicon size (bp)
16S rRNA	16S-F	GCCCAGTAATTCCGATTA	60	100
	16S-R	CCTCATCGATTGACGTTA		
blah-IMP	IMP-F	GCAGCAGTTTGTTGATTG	61	137
	IMP-R	CGACGGCATAGTCATTTG		
blah-VIM	VIM-F	CCCTATGGAGTCTTGATGTTA	62	138
	VIM-R	GACCGGAATTTCGTTGAC		

Table 3. Specific primer pairs targeting *bla-IMP,bla-VIM* and *16S rRNA* genes.

Real-Time PCR Assay

The Real-time PCR was used to determine the role of bla-IMP and bla-VIM genes in resistance to carbapenemases in the presence and absence of meropenem in 5 antibiotic-resistant clinical strains containing two genes. Quantitative PCR (qPCR) was also performed according to the manufacturer's instructions. SYBR Green and 2X RT-PCR Master Mix Green were used in Volumes and concentrations of the qPCR reaction mix table (4). RT-PCR program table 5 was examined.

Component	Volume(µl)
qPCR Master Mix	12.5
Forward primer	1
Reverse primer	1
Template cDNA	4
Nuclease-free Water	6.5
Total volume	25

Table 4. Volumes and concentrations of the qPCR reaction mix.

The program for Real-Time PCR was set up with the indicated thermocycling protocol, as shown in Table 5.

Cycle Step	Temperature	Time	Cycles No
Initial Denaturation	95 °C	60 seconds	1
Denaturation	95 °C	15 seconds	40
Annealing	60	30 seconds	
Extension	60-95 °C	40 seconds	

Table 5. RT-PCR Cycling Program.

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.

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, and this value can be used to compare expression levels in sample [11].

The samples were analyzed in triplicates and standardized against 16S rRNA gene expression. The relative changes in mRNA expression levels were determined using the comparative threshold cycle (CT) method (2-ΔΔCt) between the antibiotic-exposed and antibiotic-non-exposed K. pneumoniae.

Results

Real-time PCR quantification in the present experiment utilizes the SYBR green, a fluorescent dye that recognizes any double-stranded DNA, including cDNA. The Amplification was recorded as a Ct value (cycle threshold). The housekeeping gene used in the present study was 16S rRNA. The purpose of using this gene in molecular studies is to maintain its expression in the cells or tissues under investigation and different conditions [9]. The experiment of the quantitative PCR reaction was done by using 5 carbapenem-resistant isolates of

K. pneumoniae, which have bla-IMP and bla-VIM together. These isolates have different Sub-MIC values to meropenem (0.12 – 31.2 µg/ ml). In the present study, a quantitative RT-PCR assay analyzed the mRNA expression of bla-IMP and bla-VIM genes by comparing the treated and untreated samples of bacterial growth with meropenem antibiotics by using the concentration below the dose of MIC for each sample. The Ct values of gene amplification were recorded from the software of quantitative RT-PCR. Gene expression fold change was calculated using relative quantification (RQ) from delta Ct value as shown in Figures 1 and 2 and Table 6.

The results of bla-IMP gene expression revealed that the range of fold in the five isolates was from 1.5 to 8.4 in contrast with the control (the fold was 1), and the highest value of the fold was recorded for the isolate number 2 (8.4), while the lowest for the isolate number 5 (1.5). The results of bla-VIM gene expression revealed that the range of fold in the five isolates was from 1.3 to 8.3 in contrast with the control (the fold was 1), and the highest value of the fold was recorded for the isolate number 2 (8.3), while the lowest for the isolate number 9 (1.3). Also, isolates number 3 and 25 demonstrated higher values for the gene bla-VIM in comparison with the gene bla-MP.

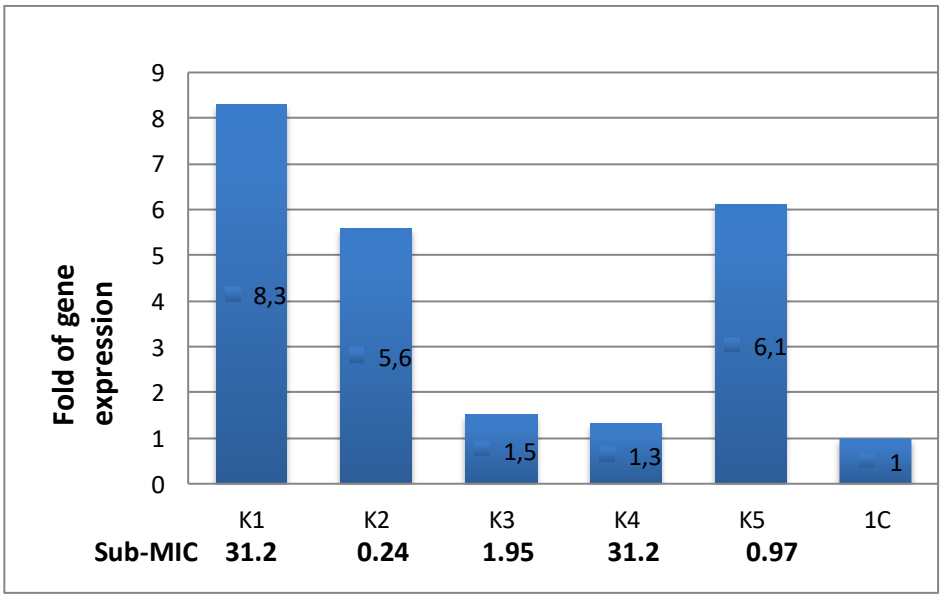

Figure 1. Fold of gene expression of *bla-IMP* gene in *meropenem resistant K. pneumonia*e isolates depending on ΔΔCt method.

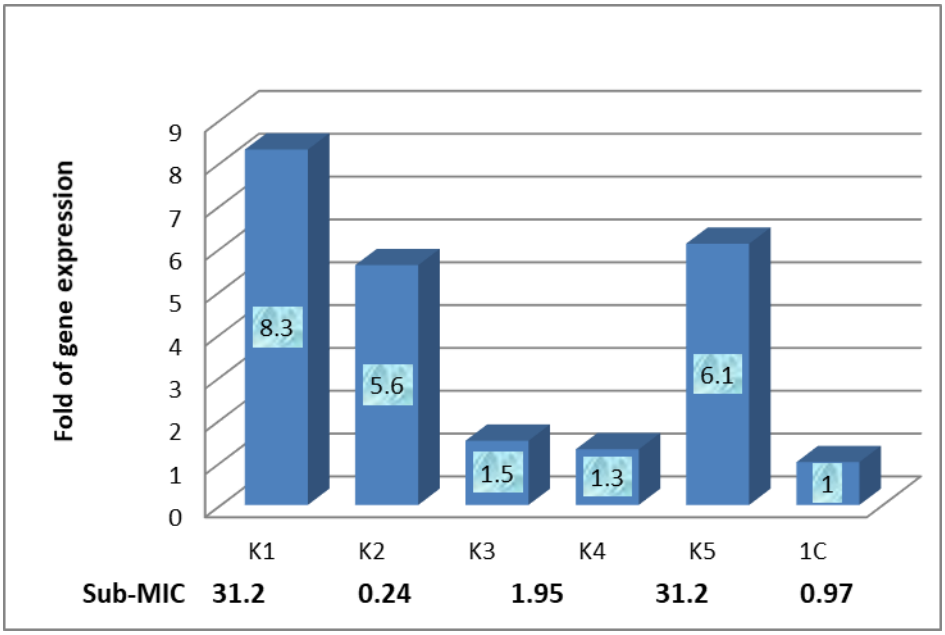

Figure 2. Fold of gene expression of *bla-VIM* gene in *meropenem resistant K. pneumonia*e isolates depending on ΔΔCt method.

Sub-MIC Conc.	Isolate Number	Fold *of bla-IMP*	Fold *of bla-VIM*
31.2	K2	8.4	8.3
0.24	K3	2.0	5.6
1.95	K5	1.5	1.5
31.2	K9	2.1	1.3
0.97	K25	3.2	6.1

Table 6. Fold of gene expression of *bla-IMP and bla-VIM* genes *K. pneumonia*e isolates at sub-MIC concentrations.

From the preceding, it was evident that there was a correlation between the meropenem resistances in K. pneumoniae isolates and the gene expression of the two genes bla-IMP and bla-VIM, and this indicated the role of carbapenemases production with the resistance of carbapenems antibiotics.

Discussion

The prevalence of carbapenemase-producing K. pneumoniae in hospitals is associated with increased mortality among patients with multi-drug resistant infections [12]. Carbapenems are often applied as first-line treatment for drug-resistant pathogens, while the increasing frequency of KPC-producing organisms has decreased the efficacy of these antibiotics [13]. This study is the first report on the expression of bla-IMP and bla-VIM genes from K. pneumoniae strains of Baghdad Hospitals, Iraq. Our findings revealed the high prevalence of IMP and VIM genes encoding carbapenem resistance among K. pneumoniae isolates. The gene expression level of bla-IMP and bla-VIM was correlated with the meropenem resistance among K. pneumoniae isolates. Bandari et al. (2019), in Iran, one hundred and eighty-one K. pneumoniae strains were collected from patients presenting to Firoozgar Hospital of Tehran, Iran; these strains were tested for the presence of bla-KPC and bla-GES resistance genes by RT-PCR.

The bla-KPC and bla-GES gene expression level was measured by real-time PCR in the presence and absence of β-lactam antibiotic. This study showed that the expression levels of bla-KPC and bla-GES were increased in resistant strains in the presence of imipenem antibiotic [14]. The study by Dhabaan et al. (2015) showed the overexpression of 12 pilus genes in resistant Acinetobacter baumannii isolates by three folds when treated with a sub-MIC of imipenem [15].

Conclusion

In conclusion, the overuse of meropenem and imipenem antibiotics in treating K. pneumoniae may increase this species's resistance to carbapenems and cephalosporins. Due to the high resistance of K. pneumoniae isolates to common antibiotics used in treating severe infections, identifying carbapenemase-producing isolates is essential for antibiotic therapy. Also, revisiting the antibiotic therapy protocols for preventing and controlling the spread of resistant bacteria is an effective strategy.

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Received: May 15, 2023/ Accepted: June 10, 2023 / Published: June 15, 2023
Citation: Hamad, S.T.; Ghaima, K.K. Study the Gene Expression of bla-IMP and bla-VIM Genes In Meropenem Resistant Klebsiella pneumoniae Isolated from Urinary Tract Infections. Revis Bionatura 2023;8 (1) 82. http://dx.doi.org/10.21931/RB/CSS/2023.08.01.82