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Study The Gene Expression Of *bla*oxa23 And *bla*oxa24 Genes In Imipenem Resistant *Acinetobacter baumannii* Isolated From Burn Wounds

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Abstract

The aim of this study was to identify changes in the gene expression of *bla*OXA genes in *Acinetobacter baumannii*, isolated from burn wounds, in response to subinhibitory concentrations of Imipenem. The gene expression of *bla*OXA genes was conducted by using real-time quantitative PCR assay. Ten isolates were chosen which had high resistance to Imipenem with minimum inhibitory concentrations (MICs) from 16 to >256 µg/ml and also contained the two *bla*OXA genes 23 and 24. It was found that the highest value of gene expression fold was recorded for the gene *bla*OXA23 (6.96) in the local isolate K5 in contrast with the Imipenem untreated samples, while the highest value of fold for *bla*OXA24 gene was 3.68. It was obvious there was a direct proportion between MICs values and folds of gene expression, therefore the increase of antibiotic concentration in the growth medium led to increasing of gene expression. The results of 16S rRNA gene expression, which was used as a reference gene, demonstrated that this gene was well suited as housekeeping gene because of the minimal variations of expression of this gene whether in Imipenem treated and untreated samples. It was concluded that the resistance of *A. baumannii* to Imipenem was related to the genes *bla*OXA23 and *bla*OXA24 but the main role may be due to *bla*OXA23. The presence of both genes increases the resistance of this species to Imipenem.

Key words: *Acinetobacter baumannii*, gene expression, *bla*OXA.

INTRODUCTION

A. baumannii is an opportunistic pathogen, which has been successfully spreading worldwide nosocomial infections and causing outbreaks in hospitals [1]. It has also emerged as an important cause of burn infections in burn units which identified *A. baumannii* as the most common cause of burn site infection and the most of isolates demonstrating multidrug resistance [2]. The most alarming problems encountered are the ability of this species to have different mechanisms of resistance and the emergence of strains that are resistant to all commercially available antibiotics coupled with the lack of new antimicrobial agents [3]. One of the main mechanisms for antibiotic resistance in *A. baumannii* is beta-lactamase production and the most widespread beta-lactamases are carbapenem hydrolysing oxacillinases (OXA) belonging to molecular class D (CHDLs) [4]. The OXA carbapenemases of *Acinetobacter* spp. are divided into four phylogenetic subgroups: OXA-23-like; OXA-40-like; OXA-51-like and OXA- 58-like [5]. It has become obvious that increased expression of *bla*OXA genes plays a major role in multidrug resistance of *A. baumannii*, therefore the main purpose of this study was to measure the gene expression of the *bla*OXA genes (*bla*OXA23 and *bla*OXA24) and compare the gene expression in the present of the Imipenem antibiotic and in the absence of it in order to improve the role of these genes in the resistance of *A. baumannii* to carbapenemes .

MATERIALS AND METHODS

Acinetobacter baumannii isolates

Ten isolates of *A. baumannii* were selected for this study which recovered from burn wounds infections from patients in Baghdad hospitals, Iraq. The clinical isolates were identified according to the conventional biochemical tests and molecular methods. Antimicrobial susceptibility tests were performed using disc diffusion method and Minimal Inhibitory Concentrations (MICs) by using microdilution method based on the results reported by the Clinical and Laboratory Standards Institute (CLSI) guidelines. The ten isolates were multidrug resistant and carried the tow genes *bla*OXA23 and *bla*OXA24 according to PCR results [6].

*Bla*OXA genes expression using RT PCR technique

This experiment was designed by using ten isolates of Imipenem resistant *A. baumannii* which have different values of MIC ranged from 16 to > 256 µg/ml and also have the two *bla*OXA genes 23 and 24. The measurement of gene expression of the two genes in the resistant isolates was done before the treatment with the antibiotic, Imipenem and after the treatment. The concentrations of antibiotic used in the treatment were in the dose under the MIC value to allow the bacterial growth with induction of resistance.

Total RNA extraction

This took place after growing the *A. baumannii* isolate in Mueller Hinton broth (without antibiotic) and incubated overnight at 37°C to detect *bla*OXA gene expression by using SV Total RNA Isolation System kit according to the manufacture instructions (Promega, USA).

Preparation of primers

Specific primers were obtained (Table 1) according to the previous studies [7, 5] for detection of the gene expression.

Table 1. Sequences of primers that used to gene expression.

Primers		Primer sequence	Product size (base pair)
House-keeping gene (16S Rrna)	F	5-CAGCTCGTGTCGTGAGATGT-3	150bp
	R	5- CGTAAGGGCCATGATGACTT-3	
BlaOXA 23	F	5- GAT CGG ATT GGA GAA CCA GA - 3	501bp
	R	5- ATT TCT GAC CGC ATT TCC AT - 3	
BlaOXA 24	F	5- GGT TAG TTG GCC CCC TTA AA - 3	246bp
	R	5- AGT TGA GCG AAA AGG GGA TT - 3	
BlaOXA 58	F	5- AAG TAT TGG GGC TTG TGC TG - 3	599bp
	R	5- CCC CTC TGC GCT CTA CAT AC - 3	

One step Quantitative Real-time PCR Assay (QRT-PCR) By using GoTaq® 1-Step RT-qPCR System (Promega, USA), Amplification of fragment of mRNA was preformed with the following master amplification reaction with the program of One-Step RT-PCR list in Table (2) and the program in Table (3). Several experiments were done for more appropriate synthesis of cDNA and annealing temperature**.**

Table 2. One-Step quantitative RT-PCR Reaction Mix.

Component	Volume per 20µl Reaction	Volume per 50µl Reaction
qPCR Master Mix, 2X	10µl	25µl
Forward Primer, 10X	2µl	5µl
Reverse Primer, 10X	2µl	5µl
RT Mix for 1-Step RT-qPCR, 50X	0.4µl	1.0µl
RNA Template (500fg–100ng) (or	4µl	10µl
Nuclease-Free Water	to 20µl	to 50µl

Table 3. Thermocycler program for One-Step quantitative RT-qPCR .

Step	Temperature	Time	Cycles
Reverse Transcription	≥37 °C	15 minutes	1
RT inactivation/Hot-start activation	95 °C	10 minutes	1
Step qPCR a. Denaturation b. Annealing c. Extention	 95 °C 55 °C 72 °C	 10 seconds 30 seconds 30 seconds	40
Dissociation	60–95 °C		1

Delta delta Ct (ΔΔCt) method

This method is the simplest one, as it is a direct comparison of 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 samples and also for the calibrator sample).

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 $2^{-\Delta\Delta Ct}$ and this value can be used to compare expression levels in samples [8].

The samples were analyzed in triplicates and standardized against 16S rRNA gene expression. The relative changes in mRNA expression levels were determined using comparative threshold cycle (CT) method ($2^{-\Delta\Delta Ct}$) between the antibiotic-exposed and antibiotic non-exposed *A. baumannii.*

RESULT AND DISCUSSION

***Bla*OXA genes expression by quantitative real-time PCR**

Real time PCR quantification applied in the present experiment utilizes the SYBR green; a fluorescent dye which recognizes any double stranded DNA including cDNA. The amplification was recorded as Ct value (cycle threshold). The house keeping gene used in the present study was 16S rRNA. The purpose of using this gene in molecular studies is that its expression remains constant in the cells or tissues under investigation and different conditions [9]. The experiment of quantitative PCR reaction was done by using 10 carbapenem-resistant isolates of *A. baumannii* which have *bla*OXA24 and *bla*OXA23 together. These isolates are chosen with different MIC values to Imipenem (16 - > 256 µg/ml). In the present study, quantitative RT- PCR assay analyzed the mRNA expression of *bla*OXA genes by comparing the treated and untreated samples of bacterial growth with Imipenem antibiotic by using the concentration below the dose of MIC for each sample. The Ct values of genes amplification were recorded from the software of quantitative RT PCR. The calculation of gene expression fold change was done by using relative quantification (RQ) from delta delta Ct value as shown in figures 1 and 2.

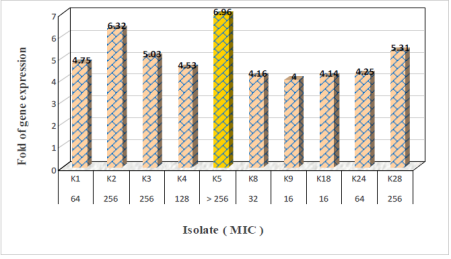

Figure 1. Fold of gene expression of blaOXA23 gene depending on ΔΔCt method.

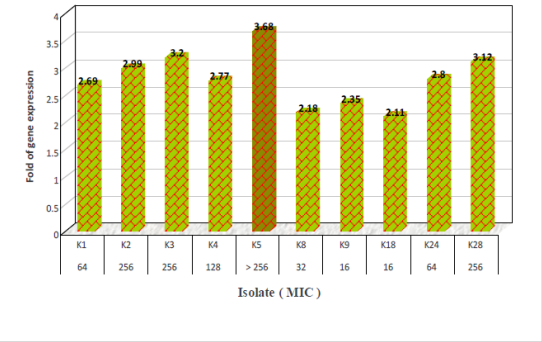

Figure 2. Fold of gene expression of *bla*OXA24 gene depending on ΔΔCt method.

The fold of gene expression of *bla*OXA23 gene for the ten isolates ranged from 4 to 6.91 with mean value 4.9, this result was higher than the fold of *bla*OXA24 gene (2.11-3.68) with mean value 2.79. When we compared the Imipenem MIC values of the isolates used in the gene expression with the results of fold, it was found that there was a parallel elevation in the two parameters and a significant correlation was demonstrated for the two genes *bla*OXA23 and *bla*-OXA24 and it was obvious that the highest values of fold was recorded in the isolate K5 with the highest MIC value (>256) for the two genes. To summarize, despite the variations in the expression levels of genes of the ten isolates, exposure to Imipenem at high concentrations induced the expression of *bla*OXA genes in all isolates of *A. baumannii* studied. The large expression of extended spectrum beta-lactamase among gram negative bacilli due to increasing use of carbapenemes, especially in intensive care unit (ICU), the increased use of carbapenemes enables the expression of these antibiotics due to selective pressure [10]. The results of gene expression in the current study indicated to the important role of *bla*OXA23 gene in the resistance of cabapenemes. The previous findings indicated to the main role of *bla*OXA23 gene in acquiring carbapenem-resistance in *A. baumannii* clinical isolates [11]. The transposon-like structure of *bla*OXA23 gene enhances the expression of this gene; hence the ISAba1 or ISAba4 elements that are upstream of *bla*OXA23 genes provide promoter sequences that enhance their expression [12].

Internal qPCR controls used 16s rRNA gene to ensure differences seen are due solely to alterations in the target gene expression and not due to mRNA quality or quantity.

Some of reports indicated the role of *bla*OXA72 gene expression in Imipenem resistance, hence this gene represents one of the important variants in our local isolates, Kuo *et al*. (2013) [13] demonstrated to contribution of *bla*OXA72 gene in Imipenem resistance when mRNA overexpression of this gene was responded to the presence of this antibiotic.

Real time PCR quantification of 16S rRNA expression

The Ct values of 16S rRNA**,** the housekeeping gene used in the present study, were shown in Table 4.

Reference genes are used to eliminate sample to sample variation. It is therefore essential to identify stably expressed reference genes, as variation in reference gene expression can create false positives or mask real positives [14]. In eukaryotic cells, a number of stably expressed reference genes have been identified that can be used for routine normalization during quantitative expression analysis, including β-*actin* and GAPDH [15]. However, no such standard set of reference genes has been determined for prokaryotic cells, as expression of typical reference genes in prokaryotes has been shown to be highly variable under different experimental conditions [16]. However, rRNA expression has been shown to be highly dependent on the physiological status of the bacterial cell [17]. But 16S rRNA may not be a reliable reference gene. There is therefore, a need to identify reference genes that are expressed stably in *Acinetobacter baumannii*. To do this utilizing the 2^{-Ct} value and the ratio of 2^{-Ct} treated and untreated samples [8] as shown in Table 1. The small variations in gene fold expression treated and untreated samples renders 16S rRNA gene a useful control gene. The results reference gene expression experiment revealed the fold of this gene was convergent values (0.98-1.014) under different concentrations of antibiotic. The current study supported the using of 16S rRNA (150 bp) as reference gene in the gene expression studies of *A.baumannii*.

The findings of *bla*OXA genes expression indicated highest fold gene expression of the *bla*OXA23 gene followed by *bla*OXA24 and the fold of gene expression was increased with the increasing of Imipenem antibiotic in the growth medium.

The results proved that 16S rRNA gene (150 bp) gave ideal results when used a housekeeping gene in the gene expression experiment with the minimal variation in different conditions.

Table 4. Ct values and fold of gene expression of housekeeping gene 16S rRNA at different MIC values for *A. baumannii* clinical isolates.

MIC	No.	Ct value of calibrator (untreated with antibiotic)	Mean	$2^{-\Delta Ct}$	Ct value of sample (treated with antibiotic)	Mean	$2^{-\Delta Ct}$	Sample / calibrator	Fold of gene expression
16	2	14.72 14.80	14.76	3.6E-5	14.71 14.77	14.74	3.6E-5	3.6E-5/3.6E-5	1.00
32	1	14.77	---	3.56E-5	14.78	---	3.55E-5	3.56E-5/3.55E-5	0.99
64	2	14.74 14.71	14.725	3.69E-5	14.71 14.73	14.72	3.7E-5	3.7 E-5/3.69 E-5	1.002
128	1	14.80		3.5E-5	14.78		3.55E-5	3.55E-5/3.5 E-5	1.014
256	3	14.76 14.72 14.75	14.743	3.64E-5	14.71 14.72 14.80	14.743	3.64E-5	3.64E-5/3.64E-5	1.00
> 256	1	14.77		3.57E-5	14.79		3.52E-5	3.52E-5/3.57E-5	0.98

REFRENCES

[1] Kempf M, Rolain JM. 2012. Emergence of resistance to carbapenems in *Acinetobacter baumannii* in Europe: clinical impact and therapeutic options. Int. J. Antimicrob. Agents. 39: 105–114.

[2] Keen EF, Robinson BJ, Hospenthal DR, Aldous WK, Wolf SE, Chung KK, Murray CK. 2010. Prevalence of multidrug resistant organisms recovered at a military burn center. Burns. 36: 819–825.

[3] Urban C, Segal-Maurer S, Rahal JJ. 2003. Considerations in control and treatment of nosocomial infections due to multidrug resistant *Acinetobacter baumannii*. Clin. Infect. Dis. 36:1268-1274.

[4] Rossolini GM, Mantengoli E, Docquier JD, Musmanur A, Cortaza G. 2007. Epidemiology of infections caused by multiresistant gram-negatives: ESBLs, MBLs, panresistant strains. New Microbiol. 30: 332-339.

[5] Woodford N, Ellington MJ, Coelho JM, Turton JF, Ward ME, Brown S, Amyes SG, Livermore DM. 2006. Multiplex PCR for genes encoding prevalent OXA carbapenemases in *Acinetobacter* spp. Int. J. Antimicrob. Agents. 27: 351-353.

[6] Ghaima KK, Saadedin SMK, Jassim KA. 2016. Prevalence of blaOXA Like Carbapenemase Genes in Multidrug Resistant *Acinetobacter baumannii* Isolated From Burns and Wounds in Baghdad Hospitals. Res. J. Pharmaceut. Biologic. Chem. Sci. 7(3): 1247.

[7] Higgins PG, Wisplinghoff H, Stefanik D, Seifert H. 2004. Selection of topisomerase mutations and overexpresson of adeB mRNA transcripts during an outbreak of *Acinetobacter baumannii*. J. Antimicrob. Chemother. 54: 821-823.

[8] Schmittgen TD, Livak KJ. 2008. Analyzing real-time PCR data by the comparative (CT) method. Nat. Protoc. 3: 1101–1108.

[9] Rebouças EDL, Costa JJDN, Passos MJ, Passos JRDS, Hurk RVD, Silva JRV. 2013. Real time PCR and importance of housekeeping genes for normalization and quantification of mRNA expression in different tissues. Brazilian Archives of Biology and Technology. 56(1): 143-154.

[10] Rawat D, Nair D. 2010. Extended-spectrum β-lactamases in Gram Negative Bacteria. J. Glob. Infect. Dis. 2(3): 263–274.

[11] Higgins PG, Dammhayn C, Hackel M, Seifert H. 2010. Global spread of carbapenem-resistant *Acinetobacter baumannii.* J. Antimicrob. Chemother. 65:233–238.

[12] Corvec S, Poirel L, Naas T, Drugeon H, Nordmann P. 2007. Genetics and expression of the carbapenem-hydrolyzing oxacillinase gene *bla*OXA-23 in *Acinetobacter baumannii*. Antimicrob. Agents Chemother. 51: 1530-1533.

[13] Kuo HY, Yang SP, Lee YT. 2013. Dissemination of imipenem-resistant *Acinetobacter baumannii* with new plasmidborne *bla*OXA-72 in Taiwan. BMC Infectious Diseases.13: 319.

[14] Dheda K, Huggett J, Chang J, Kim L, Bustin S. 2005. The implications of using an inappropriate reference gene for real-time reverse transcription PCR data normalization. Anal. Biochem. 344: 141–143.

[15] Huggett J, Dheda K, Bustin S, Zumla A. 2005. Real-time RT-PCR normalisation; strategies and considerations. Genes Immun. 6: 279–284.

[16] Vandecasteele S, Peetermans W, Merckx R, Van Eldere J. 2001. Quantification of expression of *Staphylococcus epidermidis* housekeeping genes with Taqman quantitative PCR during in vitro growth and under different conditions. Journal of Bacteriology. 183: 7094-7101.

[17] Hansen M, Nielsen A, Molin S, Hammer K, Kilstrup M. 2001. Changes in rRNA levels during stress invalidates results from mRNA blotting: Flourescence in situ rRNA hybridization permits renormalization for estimation of cellular mRNA levels. J. Bacteriol. 183(16): 47-51.