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Role of OMP Expression in Carbapenem Resistance in Clinical Isolates of *Klebsiella Pneumoniae*

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Abstract

Klebsiella pneumoniae has been emerged as one of the most important causes of urinary tract infection UTI. The deficiency of porins *ompK35* and *ompK36* are important for the development of carbapenem-resistant *K. pneumoniae*. Clinical *K. pneumoniae* isolates has been characterized and investigated for the effect of meropenem on the *ompK*35 and *ompK*36 and their reduced expression to develop carbapenem resistance for six carbapenem resistant *K. pneumoniae* strains. One hundred eighty urine specimens were collected from impatiens and outpatients admitted to three hospitals in Baghdad, cultured and examined microscopically and identified by traditional biochemical tests, VITEK-2 system and molecular identification using the specific gene *tyrBK. pneumoniae* by polymerase chain reaction. Forty four isolates were identified as *K. pneumoniae* (24.4%) of the total collected bacteria causing UTI. Theantibiotic susceptibility and Minimal inhibitory concentration (MIC) test of 44 *K. pneumoniae* isolates towards meropenem antibiotic was examined using disc diffusion method and E-test method respectively, results showed that 6 (13.6%) were resistant to meropenem with MIC value (4-8 μg/ml), 35 (79.5%) were sensitive with MIC value (0.125-0.5μg μg/ml), while 3(6.8%) isolates were of intermediate resistant with MIC of (2 μg/ml). Poringene expression *ompK35* and *ompK36* genes was conducted using real-time quantitative PCR assay. Gene expression fold were recorded for three study groups, antibiotic sensitive group as control group, antibiotic resistant group before treatment with meropenem and resistant group after treatment with meropenm with a concentration of (1μg/ml) for all resistant isolates. The highest value of gene expression fold in *ompK35* gene was recorded for the sensitive group (1.00), andthe untreated meropenem group gene expression fold of *ompK35* (0.95), while the lowest gene expression fold of *ompK35* gene was for meropenem treated group (0.055).The highest value of gene expression fold in *ompK36* gene was recorded for the untreated meropenem group (1.33), and the gene expression fold of sensitive group of *ompK36* (1.00), while the lowest gene expression fold of *ompK36* gene was for meropenem treated group (0.007)depending on $2^{-\Delta ct}$ method. When depending on $2^{-\Delta\Delta ct}$ method, Gene expression fold had slight difference.

Introduction

Klebsiella pneumoniae is Gram-negative, encapsulated, rod-shaped non-motile, lactose-fermenting, gas-producing, facultative anaerobic bacterium. *K. pneumoniae* have the ability to grow with or without oxygen, esteem its facultative anaerobe (Jing *et al.,* 2012).*Klebsiella* is an opportunistic pathogen and this is associated with its owing an antiphagocytosis capsule (Umhe *et al*., 2006).*Klebsiella* causes a range of Nosocomial infections such as urinary tract infection,

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respiratory tract infection, surgical wounds,Osteoporosis, as well as it infects mucous membranes, necrosis, lung injury, and meningitis (Kumar and Talwar, 2010; Wiskur *et al*., 2008).*K. pneumoniae* strains are usually opportunistic pathogens involved in urinary tract infections and catheter-associated urinary-tract infections in hospitalized patients and compromised individuals. Some infections are from medical instrument like urinary catheters which considered the main location of *K. pneumoniae. K. pneumoniae* has been suggested to be involved in the formation of biofilms on these surfaces (Lavender *et al.,* 2004).*K. pneumoniae* is one of MDR organisms that identified as an instant threat to human health by World Health Organization (WHO) (Kidd *et al.,* 2017). *K.pneumoniae* has several mechanisms of antibiotic resistance, including 1 antibiotic inactivation/alteration 2 modification of antibiotic binding sites 3 efflux pump 4 biofilm formation and 5 changes in cell permeability resulting in reduced intracellular antibiotic accumulation .(Wilson, 2014; Wright, 2005). One of the major cause or the major contributing factor to antibiotic resistant phenotype in bacteria, especially Gram-negative species is the alteration in permeability of the outer membrane. In a number of *Enterobacteriaceae* members, including *K. pneumonia, Escherichia coli* and *Salmonella spp*., there are three major out membrane porins OMPs that were named in *E. coli* as *OmpC, OmpA* and *OmpF*. These porins permit the passage of nutrients and many other molecules in and out of the bacterial cell, including the passage of a variety of antibiotics. *K. pneumoniae* loss or reduced expression of the two major outer membrane porins*OmpK35* and *OmpK36* confers resistance to carbapenem in strains producing extended spectrum β lactamases or plasmid-mediated AmpC-type β-lactamases. (Hashemi *et al*., 2014). *OmpK35* has been reported to allow penetration of many β-lactam antibiotics including cefoxitin and cefotaxime and to a lesser extent meropenem and imipenem (Domenech-Sanchez *et al*., 1999). A study has reported that clinical exposure to cefuroxime can decrease the expression of *OmpK35* (Kallman *et al.*, 2008). Loss or reduced expression of *OmpK36* has been related with resistance to β-lactams, including the carbapenems antibiotic. It has been shown that *K. pneumoniae* strains that exhibits resistance to carbapenems, *OmpK36* is often lacked, suggesting that carbapenem antibiotics are capable of penetrating the cell through this porin (Kaczmarek *et al.,* 2006).

Materials and methods

Isolation and identification of*K. pneumoniae*

In this study the bacterial isolates were collected from three hospitals in Baghdad, Iraq, between September 2018 and December 2018. Out of 180 Urine sample, a total 44 isolates were identified as *K.pneumoniae.* The isolates were collected from impatients and outpatients suffering from UTI .MacConkey (Himedia) and blood agar (Himedia) were used for the isolation of *K.pneumoniae*.All of the isolates were identified by using the traditional biochemical tests, VITEK2 system (bioMerieux, France), according to the manufacturers recommendations.

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Molecular identification of *K.pneumoniae* (DNA extraction and identification of *K. pneumoniae tyrB* specific gene)

Identification was confirmed with molecular identification using *K. pneumoniae tyrB* specific gene. First DNA was extracted from all the *K.pneumoniae* isolates using genomic extraction Kit (Promega/ USA). Concentrations and purity of the extracted isolates was monitored by Nanodrope (Thermo Scientific/USA). Polymerase chain reaction was used to detect the *K.pneumoniae tyrB*gene. The primers (Alpha/Canada) used to amplify this gene was: F (5-GGCTGTACTACAACGATGAC-3)and R (5-TTGAGCAGGTAATCCACTTTG-3).The amplification reaction mixture was carried out in a 25 µl reaction volume. Reaction mix was of 2.5 µl DNA template, 0.5 of (10 pmol/µl) of each forward and reverse primers, 12.5 µlG2 Green master mix (Promega / USA). Up to a 25 µl volume with nuclease free water. Cycling conditions were as follows: initial denaturation at 95 C˚ for 5 minutes, followed by 35 cycle of denaturation at 95 C˚for 1 minuet, annealing at 55 C˚ for 1 minuet, extension at 72 C˚ for 1 minuet, and a final extension step at 72 for 10 minutes. PCR products resulted from this run were run in 2% agarose gel within 1X TBE with 2µl of (20000X) red safe, electric field was applied at 5 volt for each cm of the gel for 120 min. DNA bands together with (100bp) ladder (Intron/Korea) were visualized using UV- Transiluminater and photographed.

Antibiotic susceptibility test

Antimicrobial susceptibility test was performed using Kirby-Bauer method for meropenem antibiotic. All isolates of *K. pneumonae* were grown on MacConkey agar overnight. One or two colonies of each isolate were resuspended in normal saline. The turbidity of the suspension is adjusted to 0.5 MacFarland, then the suspension was used to inoculate the preprepared Mueller-Hinton agar (Himedia). The antibiotic disc used in this study was meropenem (MEM) of (10µg). This antimicrobial disc was placed on the medium. The plates were incubated at 37°C overnight .The inhibition zones diameter were measured and interpreted according to CLSI breakpoint interpretive criteria (CLSI, 2014) into susceptible, intermediate and resistant.

Determination of Minimum inhibitory concentration (MIC)

All the 44 *K. pneumoniae* isolates that were previously detected as resistant or sensitive to meropenem antibiotic were subjected to E-test (diagnostic liofilchem/Italy).*K. pneumonae* were grown on MacConkey agar overnight. Similar colonies of each isolate were resuspended in normal saline. The turbidity of the suspension is adjusted to 0.5 MacFarland, then the suspension was used to inoculate the preprepared Mueller- Hinton agar (Himedia). The antibiotic meropenem (MEM) strips were of (0.125-8µg/ml). Strips were placed on the medium. The plates were incubated at 37 °C overnight .The inhibition zones were interpreted according to CLSI breakpoint interpretive criteria (CLSI, 2014).

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Analysis of Gene Expression by Real-Time RT-PCR

Real-time reverse transcription (RT)-PCR for analyzing theexpression of the *ompK35*, *ompK36* was carried out by extracting RNA from *K. pneumoniae*isolates using RNA extraction Kit (DSBIO/China). Concentrations and purity of the extracted isolates was monitored byNanodrope (Thermo Scientific/USA). RNA samples were reverse transcribed into cDNA by usingWizScript RT FDmix (Hexamer) kit (WizBio /Korea), which is a complete system for thesynthesis of cDNA from RNA template. All the reagents necessary for successful cDNA synthesis in an individually aliquot and lyophilized in single-tube. In each tube 5µl of extracted RNA and 15µl of nuclease free water was added to make a final volume of 20µl. The reaction mix were run in thermal cycler to allow the reaction take place. Reaction conditions of the cDNA synthesis is as follows: annealing at 25°C for 10 min, activation of enzyme at 42°C for 30 min and elongation at 85°C at 4 min. Amplifications were performed in triplicate on a SmartCycler using WizPure ™ qPCR Master (SYBR)WizBio/ Korea). The reaction volume was 20µl,of 10 µlSYBER Green, 2.5 µlof cDNA, 0.5 µl of (10 pmol/µl) of upstream and downstream primers (Alpha/Canada) of each target genes and up to 20µl with nuclease free water. Each gene was run in its own reaction conditions. Data were analyzed using theΔCtand ΔΔCt method, in which the *rpoB* gene was chosen as a referencegene. All primers used and its reaction conditions are listed in Table (1) and Table (2). All the three genes were with the same dissociation curve of 60 - 95 °C for 2-5 sec.

Table (1): Sequences of primers used in gene expression.

Primer	Sequence(5'-3')	Product size
rpoB	F: AAGGCGAATCCAGCTTGTTCAGC R: TGACGTTGCATGTTCGCACCCATCA	100bp
ompK35	F: GCAATATTCTGGCAGTGGTGATC R: ACCATTTTTCCATAGAAGTCCAGT	62bp
ompK36	F: TTAAAGTACTGTCCCTCCTGG R: TCAGAGAAGTAGTGCAGACCGTCA	85bp

Table (2): Reaction conditions of each gene used in gene expression.

Gene	Step	Temperature	Time	Cycles
rpB	Initial denaturation	95°C	5 min	1
	Denaturation	95°C	20 sec	40
	Annealing	64°C	25 sec	1
OmpK35	Initial denaturation	95°C	5 min	1
	Denaturation	95°C	20 sec	40
	Annealing	64°C	30 sec	1
ompK36	Initial denaturation	95°C	5 min	1
	Denaturation	95°C	20 sec	40
	Annealing	62°C	30 sec	1

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Results and discussion

Collection and diagnosis of isolates

A 180 isolates were collected from midstream urine samples, isolates that formed large mucoid colonies on MacConkey and were non-hemolysis on blood agar suspected to be*K. pneumoniae*, these isolates were negative for Oxidase, Indol, Methyl red and Motility tests, and were positive for Catalase, Citrate utilization, Urease and Voges-poskaur tests, these traditional tests revealed that 48 isolates were *K. pneumoniae*. Confirmation of the identification of sample were done using VITEK 2 system that including 47 tests, the results revealed that 44 (24.4%) of the isolated bacteria were *K. pneumoniae*. The molecular identification that were done using *K. pneumoniae tyrB* also showed 44 (24.4%) isolates were *K. pneumoniae*.Many studies revealed the same percentage of *K. pneumoniae*causing UTI such as in a local study for Al-Zubaidi, (2012) on the Role of plasmids in bacteriocin production from *Klebsiella* spp. revealed that 22% of bacteria causing UTI was *K. pneumoniae*, also a study on urinary tract infections and antimicrobial sensitivity among diabetic patients Hamdan *et al.,* (2015) showed nearly similar result, 23% isolates of *K. pneumoniae* as causative agent of UTI.

The antibiotic sensitivity test

Antimicrobial susceptibility test was performed for all 44 *K. pneumoniae* isolates to meropenem antibiotic, using disc diffusion method on Mueller-Hinton agar. Of the 44 isolates, 35(79.5%) isolates were sensitive, 3 (6.8%) isolates showed intermediate resistance and 6 (13.6%) isolates were resistant to meropenem antibiotic. Our findings were higher than a local study conducted by Ibrahim and Hameed, (2015) whom stated that less than 8% of *K. pneumoniae* isolates were resistant to meropenem antibiotic. In one study by Saad *et al.,* (2014), demonstrated 0% *K. pneumoniae* isolates were resistant to meropenem. Another study in Egypt by Barwa and Shaaban, (2017) showed that the resistant rate of *K. pneumoniae* were 8% of urine samples. In comparison with the results of previous local studies, the current study demonstrated the increasing of antibiotic resistance to meropenem, which is the drug of choice for the treatment of *K. pneumoniae* infections. The high rates of antibiotic resistance shown in the present study may be due to many factors including recent use of broad-spectrum antibiotics, hospitalization, exposure of patients to a self-prescribed antibiotic because of the availability of medicines without prescription and the transfer of resistance genes by transport means. Meanwhile, our results were in agreement with results of Jarallah and Abbas, (2014), which revealed that 17.6% *K. pneumoniae* isolates were resistant to meropenem antibiotic. Also in another study in Egypt by Wassef *et al.,* (2015), it was found that 18.7% of *K. pneumoniae* clinical isolates were resistant to meropenem. Also the results of one research in Taiwan showed that the resistant rate of *K. pneumoniae* to meropenem was 16% (Lee *et al.,* 2018).

Results of Minimum inhibitory concentration

MIC for meropenem antibiotic were determined using the (E-test) method, Results of MIC had confirmed the previous results of antibiotic disc diffusion test, where the current study showed that the resistant isolates had high MIC values, while sensitive isolates had lower MIC values to

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meropenem antibiotic.The results of the antimicrobial were interpreted according to Clinical and Laboratory Standards Institute (CLSI). Number of isolates that gave MIC values (4-8 µg/ml) were 6 (13.6%) of clinical isolates and 35(79.5%) of clinical isolates were with MIC value of (0.125-0.50 µg/ml). These values ensure resistance of six studied *K. pneumoniae* isolates for meropenem antibiotic. These findings were concordant with the results of Al-Sultani and Al-Taai (2019) a local study in diyala,Iraq on the Detection of NDM-1 in Cabapenem-Resistant *K. pneumoniae* which revealed that the MIC of *K. pneumoniae* for meropenem reached (8 µg/ml) in resistant isolates. Also in another study in Iran on the Molecular epidemiology of *K. pneumoniae* in an Iranian hospital by Solgi *et al*. (2018), had shown an MIC rang of (4-8 µg/ml) in resistant isolates for meropenem.Different MIC values for Meropenem have been reported by several researchers. In a local study in Baghdad by Mohammed (2015), who reported the MIC values for meropenem (≥16 µg/ml). In Malaysia the results of Low *et al.,* (2017) reported a meropenem MIC value (>32 µg/ml).

Expression of *ompK53* and*ompK36* genes

Total RNA was successfully extracted from all the 44 isolates. Concentrations and purity of RNA were measured by Nanodrop spectrophotometer, all of the isolates had concentration between (30- 80 ng/µl) and the RNA purity was (1.6-1.9). RNA samples were reverse transcribed into cDNA, the efficiency of cDNA concentration was assessed through the efficiency of qPCR conducted later. The amplification by quantitative RT-PCR was recorded as a Ct value (cycle threshold). The lower Ct value indicates the presence of higher copies of the target and vice versa. In terms of gene expression, high Ct values indicate low gene expression and low Ct value indicates a high gene expression (Livak and Schmittgen, 2008) (Nolan *et al.,* 2006).In the present experiment, quantitative RT- PCR assay analyzed the mRNA expression of *ompK35* and *ompK36* genes by comparing the untreated and treated group of samples of resistant bacteria grown with meropenem antibiotic by using (1 µg/mL) of meropenem for each sample and considering the sensitive group of samples as control.Results of mean fold of gene expression in relation with mean MIC value are shown in Figures (1), (2).

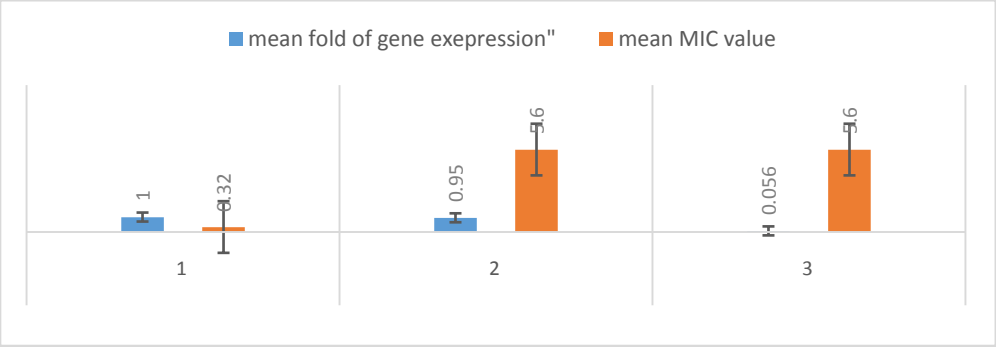

Figure (1) Mean fold of gene expression of *ompK35* gene in relation with the mean of MIC values depending on ΔΔCt method, (1) sensitive group, (2) resistant group before treatment with meropenem, (3) resistant group after treatment with meropenem.

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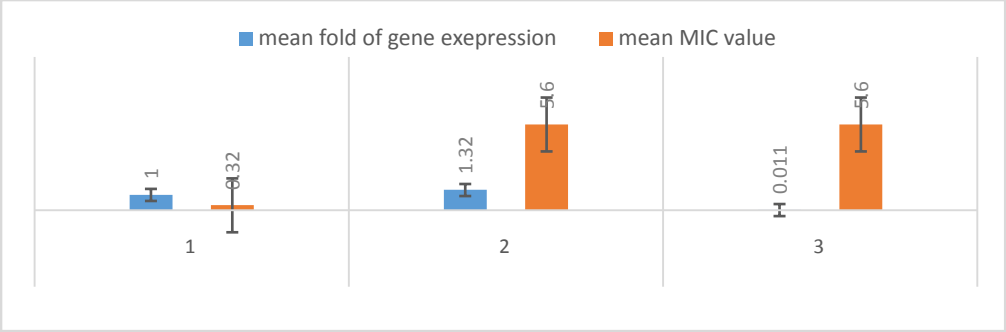

Figure (2) Mean fold of gene expression of *ompK36* gene in relation with the mean of MIC values depending on ΔΔCt method, (1) sensitive group, (2) resistant group before treatment with meropenem, (3) resistant group after treatment with meropenem .

When the mean value of MIC was low (0.32 µg/ml) for the sensitive isolates the mean fold of gene expression of*ompK36* gene was high (1.00), and when the MIC mean value was high (5.6 µg/ml) in resistant isolates before treatment with meropenem the mean fold of gene expression was slightly higher (1.32) and the mean fold of the resistant isolates after treating them with meropenem the fold of gene expression was low (0.011). And for *ompK35* gene when the mean value of MIC was low (0.32µg/ml) for the sensitive isolates the mean fold of gene expression was high (1.00), and when the MIC mean value was high (5.6 µg/ml) in resistant isolates before treatment with meropenem the mean fold of gene expression wasslightlylower (0.95) and the mean fold of the resistant isolates after treating them with meropenem the fold of gene expression was low (0.056). The fold of gene expression of*ompK36* gene in resistant isolates after treatment with meropenem lower than the fold of gene expressionof resistant isolates before treatment with meropenem and this in turn was higher than those in sensitive isolates, and the fold of gene expression of *ompK35* gene in resistant isolates after treatment with meropenem was lower than the fold of gene expression of resistant isolates before treatment with meropenem which was slightly lower than those in sensitive isolates. This is important in reflecting the gene copy number present in mRNAs molecules in the samples. It is evident from these results that least copy number of *ompK35* and *ompK36* genes carried on mRNA in resistant isolates after treatment with meropenem reflecting its lowest expression.The results showed difference in fold of gene expression between *K. pneumoniae* sensitive, resistant isolates before treatment and resistant isolates after treatment, and it is important evidence that *ompK35* and *ompK36* gene expression decreases in resistant isolates after treatment with meropenem antibiotic, and this will subsequently contribute in the increase of *K. pneumoniae* resistance to carbapenems.These results agreed with El Din *et al.*, (2016) their results indicated that there were reduced expression of *ompk35*and *ompK36*genes in a significant number of resistant isolates. This study findings were consistent as well as those of Netikul and Kiratisin, (2015), whose results indicated that carbapenem resistant isolates showed various degree of decreased expression of *ompk35* and *ompK36* genes. Within that respect, Nicolas-Chanoine *et al*., (2018). Whom studied the interplay between membrane permeability and enzymatic barrier leads to antibiotic resistance in *K. pneumoniae* and measured the expression of porin genes*ompK35* and *ompK36* under ertapenem pressure also showed decreased expression in *ompK35* and *ompK36* genes.

Meanwhile the expression level of *rpoB* housekeeping gene showed non-significant difference in mean Ct values between study groups, the fold of gene expression of *rpoB* for the sensitive

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isolates, resistant isolates before treatment with meropenem and resistant isolate after treatment with meropenem was (1.00), (0.97) and (1.01), which had led to considering it a reliable housekeeping gene as mentioned in (Gomes *et al.,* 2018) that used*rpoB* as normalizer in gene expression in *K. pneumoniae.*. The calculation of gene expression fold change was made using relative quantification (Livak and Schmittgen, 2008). This depends on normalization of Ct values calculating the ΔCt which is the difference between the mean Ct values of replica of *ompK35*and *ompK36*cDNA amplification of each single case and that of the *rpoB*.To calculate the gene expression folds in relation to the housekeeping gene the result of $2^{-\Delta Ct}$ of each group was measured in relation to that of sensitive group. The $2^{-\Delta\Delta Ct}$results was also applied to calculate the relative gene expression of *ompK35* and *ompK36* genes. A calibrator was used and it was one of the samples of the controls with high expression of *ompK35* and *ompK36*. All results of gene expression fold depending on both ΔCt and ΔΔCt methods are shown in table (3) and (4).

groups	Means Ct of *ompK35* gene	Means Ct of *rpoB*	ΔCt (Means Ct of*ompK35* gene - Means Ct of *rpoB*)	$2^{-\Delta Ct}$	Fold of gene expression	Mean ΔCt Calibrator (ct *ompK35* gene -ct *rpoB*	ΔΔCt	$2^{-\Delta\Delta Ct}$	Fold of gene expression
***ompK35* B.T.**	**29.7**	**22.15**	**7.55**	**0.00533**	**0.95**	**11.32**	**-3.77**	**13.64**	**0.95**
***ompK35* A.T.**	**33.7**	**22.09**	**11.61**	**0.00031**	**0.055**	**11.32**	**0.29**	**0.81**	**0.056**
***ompK35* S.**	**29.6**	**22.11**	**7.49**	**0. 00556**	**1.00**	**11.32**	**-3.83**	**14.22**	**1.00**

Table (3): Fold of *ompK35* gene expression. Depending on$2^{-\Delta Ct}$ and $2^{-\Delta\Delta Ct}$Methods

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Table (4): Fold of *ompK36* gene expression. Dependingon$2^{-\Delta Ct}$ and $2^{-\Delta\Delta Ct}$Methods

groups	Means Ct of *ompK36*gene	Means Ct of *rpoB*	ΔCt (Means Ct of *ompK36* gene - Means Ct of *rpoB*)	2-ΔCt	Fold of gene expression	Mean ΔCt Calibrator (ct ompK36 gene -ct rpoB	ΔΔCt	$2^{-\Delta\Delta Ct}$	Fold of gene expression
ompK36 B.T.	27.87	22.15	5.72	0.0189	1.33	11.5	-5.78	54.9	1.32
ompK36 A.T.	34.61	22.09	12.52	0.0001	0.007	11.5	1.02	0.49	0.011
ompK36 S.	28.24	22.11	6.13	0.0142	1.00	11.5	-5.37	41.3	1.00

Conclusions

Present study showed that most local clinical isolates of *K. pneumoniae* had high percentage resistance to meropenem antibiotics that considered a potent antibiotic of carbapenem used for treatment of UTI caused by *K. pneumoniae*. The *tyrB* gene is reliable in molecular identification of *K. pneumoniae*. The *ompK35* gene expression indicated lower fold of gene expression than *ompK36* gene in meropenem untreated group, and the *ompK36* gene expression indicated lower fold of gene expression than *ompK35* gene in meropenem treated group. Gene expression fold was decreased in both porin genes *ompK35* and *ompK36* in the antibiotic treated resistant group.In antibiotic sensitive group both *ompK35* and *ompK36* porin genes had slight difference in gene expression fold.The use of *rpoB* gene gave an ideal results when used as housekeeping gene in the gene expression experiment with the minimal variation in different conditions.

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