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Molecular Detection of *acrAB* and *oqxAB* Genes in *Klebsiella pneumoniae* and Evaluation the Effect of Berberine on their Gene Expression

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Abstract: One of the factors contributing to increase bacterial resistance to antimicrobial substances is the presence of multidrug efflux pumps. *AcrAB* and *OqxAB* are the most extensively researched efflux pumps genes in *K. pneumoniae* in relation to antibiotic resistance. The objectives of this study are Isolation and identification of *K. pneumoniae* from patient with urinary tract infections; detection the efflux pump genes (*acrAB, oqxAB*) in *K. pneumoniae* by Polymerase Chain Reaction using specific primers and then study the gene expression of one of the two pumps in the presence of the subinhibitory concentration of natural efflux pump inhibitor, Berberine. A total of 260 clinical specimens were collected from five of Baghdad hospitals. The identification of the isolates of *K. pneumoniae* depends on the biochemical tests. The Minimum Inhibitory Concentrations (MICs) of Berberine (plant – derived efflux pump inhibitors) against isolated bacteria was estimated with the microdilution broth method. Molecular Detection of *acrA, acrB, oqxA* and *oqxB* genes were carried out by polymerase chain reaction while the gene expression was done by real-time PCR. The results demonstrated that *Klebsiella pneumoniae* bacteria were found in 76 (29.2%) of all urine samples. The Minimum Inhibitory Concentrations (MICs) of Berberine HCL at the range 3.9-500 µg/ml. The detection of the efflux pump genes (*acrAB, oqxAB*) in *K. pneumoniae* revealed that all isolates have the 2 systems (100%). Relative quantification (RQ) was used to calculatethe fold change in gene expression using the delta delta Ct value and the gene expression of the isolates was calculated before and after treatment with the subinhibitory concentration of each isolate. In *oqxA* gene, by using the sub-MIC values of Berberine in *Klebsiella pneumoniae* resistant isolates before treatment with Berberine, the fold of gene expression was (1), and the fold of the resistant isolates after Berberine treatment was low (0.1-0.04). For *oqxB* gene Before Berberine treatment, the fold of gene expression was slightly higher (1). After Berberine treatment,the fold of gene expression was low (0.2-0.0001). In conclusion, the obvious activity of Berberine as efflux pump inhibitor in *K. pneumoniae* may be contributed in the management and control the multidrug resistance especially in life threatening infections.

Keywords: *K. pneumoniae*, Berberine, *acrAB, oqxAB*, gene expression.

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Introduction

Klebsiella pneumoniae is a Gram-negative, rod-shaped, capsulated bacterium that belongs to the family *Enterobacteriaceae* (1). It has become a significant opportunistic pathogen that causes nosocomial infections, particularly in the blood, lung, and urine systems (2). Since fewer therapeutic choices are available to treat such resistant germs, multidrug resistance is widely distributed among *K. pneumoniae* isolates and is generating major issues in clinical sites (3). Different mechanisms that result in antibiotic resistance to a variety of antibiotics may be acquired by *K. pneumoniae* isolates (4). One of the

factors contributing to increased bacterial resistance to many antimicrobial substances, including antibiotics, dyes, antiseptics, and detergents, is the presence of multidrug efflux pumps (5). *AcrAB* and *OqxAB* genes are the most extensively researched efflux pumps in *K. pneumoniae* in relation to antibiotic resistance (6). In the *acrRAB* operon, *acrA* encodes a lipoprotein (40 kDa) that spans the inner and outer cell membranes in bacteria, *acrB* produces an integral membrane protein (113.5 kDa) with 12 membrane-spanning-helices that are located in the cytoplasmic membrane. One of the major pumps responsible for the intrinsic resistance of *K. pneumoniae* isolates to fluoroquinolones, particularly ciprofloxacin, is the *AcrAB* efflux pump. Additionally, tetracycline, chloramphenicol, trimethoprim, macrolides, and -lactams are resistant to this pump (7). OqxAB has been commonly found chromosomally in *K. pneumoniae* and usually plasmid located in other Enterobacteriaceae species. This resistance determinant is responsible to develop reduced susceptibility and even resistance to olaquindox and other fluoroquinolone agents such as ciprofloxacin, norfloxacin, and flumequine (8). Many studies indicated that the presence of berberine at low concentrations exhibited inhibitory effect on gram negative bacteria such as *Klebsiella* and *Pseudomonas* and on the efflux pump systems of these bacteria (9,10). The aims of this study was the detection of efflux pump genes (*acrAB, oqxAB*) by PCR among *Klebsiella pneumoniae* isolated from UTIs to evaluate the role of berberine as antibacterial agent against this species and determine its role as efflux pump inhibitor for the 2 systems (*AcrAB* and *OqxAB*) by measurement the gene expression of these systems in the present of this inhibitor at subinhibitory concentrations.

Materials and methods

Bacteria, media and chemicals

A total of 260 clinical samples from urinary tract infection ,76 of positive cultures was identified as *K. pneumoniae*; Selective and differential media were used for isolation of *K. pneumoniae* from Baghdad hospitals, Iraq. VITEK 2 system was used for the confirmation of bacterial species identification. Berberine hydrochloride was purchased from Nootropics Depot/ USA.

Minimum inhibition concentration (MIC) of Berberine

Minimum inhibition concentration (MIC) value of *K. pneumoniae* was determined by broth dilution method with microtiter plate method described in the National Committee for Clinical Laboratory Standards (11). 1:2 serial dilutions of Berberine HCL in Mueller Hinton Broth (MHB) were placed in a 96-well round-bottom plate at concentrations ranging from 500, 250, 125, 62.5 ,31.25, 15.6,7.81, 3.9 µg/ml. The bacterial inoculum was prepared from a subculture of *K. pneumoniae* in MHB.The bacteria suspension was diluted to $1x10^8$ colony forming units (CFU)/mL, to obtain a turbidity equivalent to 0.5 on the McFarland scale, confirmed by spectrophotometry upon reaching an absorbance between 0.08–0.1 at a wavelength of 625 nm; then a 1:200 dilution in MHB was performed to obtain a final concentration of $5x10^5$ CFU/mL. The diluted bacterial suspension was added to the 96-well plate containing the serially diluted peptides. The final volume of 200 µL per well consisted of

100 µl of the compound and 100 µL of diluted bacteria suspension. Negative and positive growth controls were performed by adding only MHB or *K. pneumoniae* with MHB to the wells, respectively. After incubation for 24 h at 37 °C, resazurin (0.015 %) was added to all wells (20 µl per well), and further incubated for 2–4 h for the observation of colour change. On completion of the incubation, columns with no colour change (blue resazurin colour remained unchanged) were scored as above the MIC value. At the end of the incubation time, MIC was determined as the lowest compound concentration at which no bacterial growth was observed.

PCR primers and conditions

For the purpose of PCR assay, the specific primers of *K. pneumoniae* efflux pump genes (*oqxA, oqxB, acrA,* and *acrB*) as shown in Table (1).

Table (1). Oligonucleotide primers sequences of efflux pump genes used in this study

Gene	PrimerName	Sequence (5-3)	Productsize (bp)	References
oqxA	*oqxA*(F)	GGTGCTGTTCACGATAGATG	144	Razavi *et al*., (12)
	oqxA (R)	GAGACGAGGTTGGTATGGAC		
oqxB	*oqxB* (F)	CGGCCAGTTCTACAAACAGT	136	
	oqxB (R)	GGTAGGGAGGTCTTTCTTCG		
acrA	*acrA* (F)	TGATGCTCTCAGGCAGCTTA	226	
	acrA(R)	GCCTGGATATCGCTACCTTC		
acrB	*acrB*(F)	CGTCTCCATCAGCGACATTAAC	219	
	acrB (R)	GAACCGTATTCCCAACGCGA		
16S rRNA	16S rRNA (F)	TGGAGCATGTGGTTTAATTCGA	159	Gou *et al*., (13)
	16S rRNA (R)	TGCGGGACTTAACCCAACA		

Using 25µL of PCR reaction, 2.5 µl DNA template (100 ng/µl) is amplified by using 12.5 µl of ONETaq(NEB)® green master mix 2X (Promega, USA) and 1 µl of each primer (10 pmol/µL) for each specific gene, up to the final volume 25 µl with nucleases free water. The extracted DNA, primers and PCR premix is thawed at 4°C, vortexes and centrifuged briefly to bring the contents to the bottom of the tubes.

Table (2). Conditions of uniplex PCR reaction for efflux pump genes and *16S rRNA* detection

Target gene	PCR conditions	NO of cycles	References
16S rRNA	Initial denaturation at 95°C for 4 min 95°C for 1 min 60 °C for 1 min 70 °C for 1min a final extension at 70 °C for 5 min	1 cycle 32 cycle 1 cycle	Gou *et al*., (13)
***oqxAB* genes**	Initial denaturation at 94° C for 4 min 94°C for 1 min 57 °C for 1 min 72°C for 1 min a final extension at 72 °C for 5 min	1 cycle 32 cycle 1 cycle	Razavi *et al*., (12)
***acrAB* genes**	Initial denaturation at 95°C for 4 min 95°C for 30 sec 59 °C for 1 min 70 °C for 30 sec a final extension at 70 °C for 5 min	1 cycle 32 cycle 1 cycle	Razavi *et al*., (12)

Gene expression using RT- PCR technique

The experiment was designed using 5 isolates of resistant *K. pneumoniae* that had the two *oqxA* and *oqxB* genes. The gene expression of the two genes in the resistant isolates were measured before treatment with the Berberine and after the treatment. The concentrations of Berberine HCL ranged from (3.9-62.5) µg/ml used in the treatment were below the MIC value to allow the bacterial growth with induction of resistance. To examine the effect of sub-inhibitory concentrations of the powder extract on the efflux pumps gene expression.

RNA isolation by TRIzol™

500 µl from bacterial culture was added into a 1.5 ml tube containing 700µl Trizol for isolate. The chloroform was added as 0.15 mL of TRIzol™ Reagent used for lysis. The Incubation for 2–3 minutes. The sample was centrifuged for 15 minutes at 12,000 × g. The mixture was separated into a lower red phenol-chloroform, interphase.. The RNA was precipitated byadding 0.45 mL of isopropanol to the aqueous phase. The mixture was incubated for 10 minutes. Then Centrifuged for 10 minutes at 12,000 × g. Total RNA precipitate forms a white gel-like pellet at the bottom of the tube. The pellet gets resuspended by 0.75 mL of 75% ethanol. Then the vortex was used to dissolve the pellet and centrifuge for 5 minutes at7500 × g. Then the pellet was resuspended by 20 µl of RNase- Free water. The RNA concentration was measured by Qubit 4.0.

RT-qPCR protocol

Synthesis of cDNA from RNA through a specific primer for *oqxA*, *oqxB* and 16S rRNA transcripts and protoscript cDNA synthesis kit was used. Five microliters from each extracted total RNA sample added into the new PCR tube. Protoscript reaction mix that contain dNTPs, buffer and other essential components added as 10 µl for each sample. MuLV Enzyme added into reaction as 2 µl per sample. Two microliter oligoT, and the volume completed up to 20 µl by adding 1 µl. The mixture was incubated for 1 hour at 42 ^{O}C by using thermocycler and this followed by 80 ºC for inactivation of enzyme. The second section of this protocol it's done by choosingthe cDNA sample from the patient and control at the same run, for each sample, there are three PCR tubes, one tube for each gene, *oqxA*, *oqxB* and 16sRNA which is considered as (HKG) gene in this study. The detection of quantity is based on the fluorescent power of SyberGreen (Table 3). PCR tubes were spine to removethe bubbles and collect the liquid (1 minute at 2000g), and then the program for Real-Time PCR was set up with indicated thermocycling protocol as shown in Table (4). The result was collected and analyzed by Livak formula.

Table (3): RT-qPCR protocol and volumes

Component	20 µl Reaction
Luna Universal qPCR Master mix	10
Forward primer (10 µM)	1
Reverse primer (10 µM)	1
Template DNA	5
Nuclease-free Water	3

Table (4): Thermocycling protocol and conditions

Cycle Step	Temperature °C	Time	Cycles
Initial Denaturation	95	60 seconds	1
DenaturationExtension	95 60	15 seconds 30 seconds (+ plate read)	40-45
Melt Curve	60-95	40 minutes	1

Delta delta Ct (ΔΔCt) method

The cycle number (Ct) at which signals crossed a threshold set within the logarithmic phase was recorded. The differences in cycle threshold (ΔCt) and fold changes evaluated between the treated groups and calibrators of each gene. These values were normalized to House Keeping gene (*16S rRNA*) expression as showed below: Relative quantification:

- Delta delta Ct (ΔΔCt) method
- ΔCT =CT gene - CT House Keeping gene (HKG)
- ΔΔCT =ΔCT Treated - ΔCT Control
- Folding $=2^{-\Delta\Delta CT}$

Firstly, the ΔCt between the target gene and the HKG 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 (14). The samples were analyzed in duplicates and standardized against *acrAB, oqxAB* 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 *Klebsiella pneumoniae*.

Results and discussion

The results of Minimum Inhibitory concentrations for Berberine against *K. pneumoniae* isolates (Five multidrug resistant isolates were selected) revealed that the MICs of these isolates were at the range 7.81 to 62.5 µg/ml as showed in Table (5).

Table (5). Minimum Inhibitory concentrations for Berberine against 5 multidrug resistant *K. pneumoniae* isolates

The code ofisolate	MIC (µg/ml)
K3	7.81
K23	62.5
K24	62.5
K26	62.5
K27	62.5

In a current study of (15) observed that the presence of Berberine at low concentrations (25 and 50 µg/ml) led to greater minimal inhibitory concentrations of efflux-related antibiotics such as Rifampicin and Azithromycin. Additionally, the potential danger ofits usage against gram negative bacteria at low doses was discovered. Numerous medicinal plants containBerberine, a natural isoquinoline alkaloid that has been shown to have

antibacterial and antifungal properties either by itself or in conjunction with other medications (9). When used with several antibiotics, berberine increases their ability to suppress microorganisms. According to a prior study, berberine disrupts MRSA cell surface in a dose-dependent manner and alters the amounts of saturated and unsaturated fatty acids, which in turn compromises membrane integrity. Alkaloids, quinones, tannins, and flavonoids are just a few of the beneficial secondary metabolites that are abundant in plants. Secondary metabolites found in plants are the subject of numerous investigations as a possible source for new antibiotics (16).

Molecular detection of *K. pneumoniae* efflux pump genes by PCR

DNA extraction and purification

DNA was extracted from clinical isolates using genomic DNA purification kit. Extraction of genomic DNA from 76 isolates was confirmed by gel electrophoresis as bands. Concentrations and purity of DNA were measured by Qubit 4, all of the isolates had concentration between (10- 100 ng/µl) with high purity.

Molecular identification of *K. pneumoniae* by detection of *16S rRNA* gene

In order to detect the presence of *K. pneumoniae 16S rRNA* gene (159bp), a gene used for identifying *K. pneumoniae*, polymerase chain reaction for each extracted DNA sample has been done. The PCR products were confirmed by the analysis of bands with gel electrophoresis and by comparison of bands molecular size with DNA Ladder of 100 bp. The results of PCR reaction for *16S rRNA* gene shown in Figure (1). 76 clinical samples were identified as *K. pneumoniae* which confirmed the results obtained from the VITEK2 system.

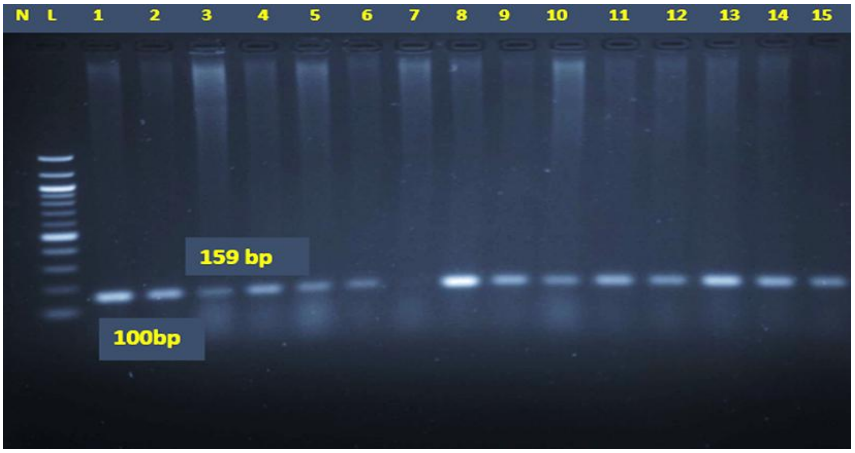

Figure:(1): Agarose gel electrophoresis of PCR products , *16S rRNA* gene in 15 selected isolates of *Klebsiella pneumoniae.* Lane L :100 bp DNA ladder, ,Lane (1-15) PCR produt for *16S rRNA* . (50 A, 70 V for 120 min).

Molecular detection of Efflux pumps genes

Molecular Detection of *acrAB* genes

In order to detect the presence of *K. pneumoniae acrAB* genes, *acrA* (226bp), *acrB* (219bp) efflux pumps genes. The PCR products were confirmed by the analysis of bands with gel electrophoresis and by comparison of bands molecular size with DNA Ladder of 25 bp. The results of PCR reaction for acrAB genes shown in

Figure (2) and (3) 76 clinical samples were identified as *K. pneumoniae* which confirmed the results obtained from the VITEK2 system.

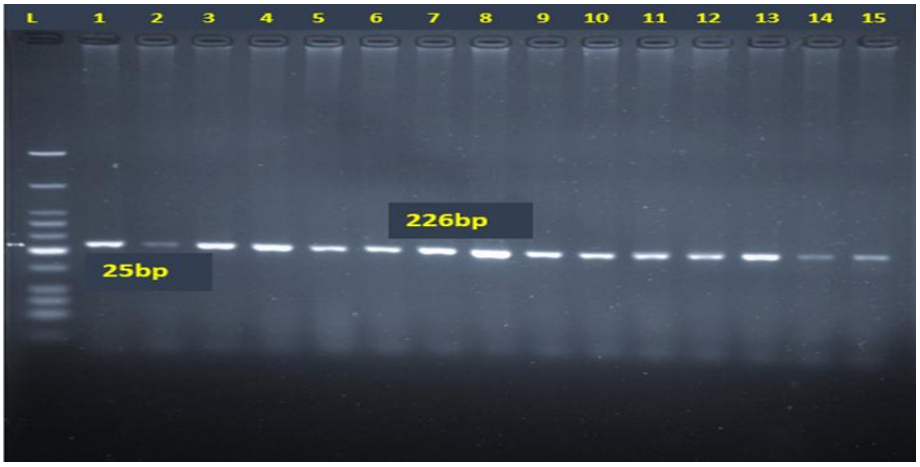

Figure (2) Agarose gel electrophoresis of PCR products , *acrA* gene in 15 selected isolates of *Klebsiella pneumoniae* Lane L :25 bp DNA ladder, ,Lane (1-15) PCR produt for acrA . (50 A ,70 V for 120 min).

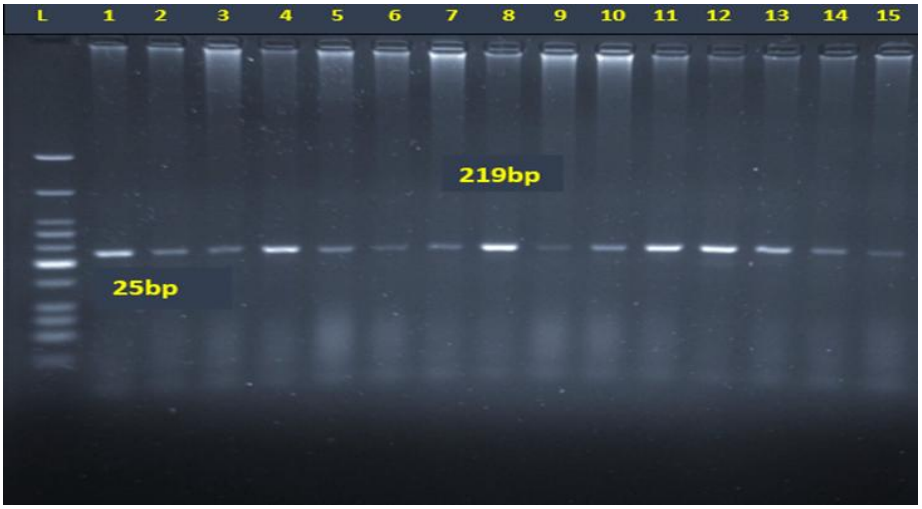

Figure (3) Agarose gel electrophoresis of PCR products , *acrB* gene in 15 selected isolates of *Klebsiella pneumoniae* Lane L :25 bp DNA ladder, ,Lane (1-15) PCR produt for acrB . (50 A ,70 V for 120 min).

Molecular detection of oqxAB genes

In order to detect the presence of *K. pneumoniae* oqxAB genes, *oqxA* (144bp), *oqxB* (136bp) efflux pumps genes. The PCR products were confirmed by the analysis of bands with gel electrophoresis and by comparison of bands molecular size with DNA Ladder of25 and 100 bp. The results of PCR reaction for *oqxAB* genes shown in Figure (4) and (5) 76 clinical samples were identified as *K. pneumoniae* which confirmed the results obtained from the VITEK2 system.

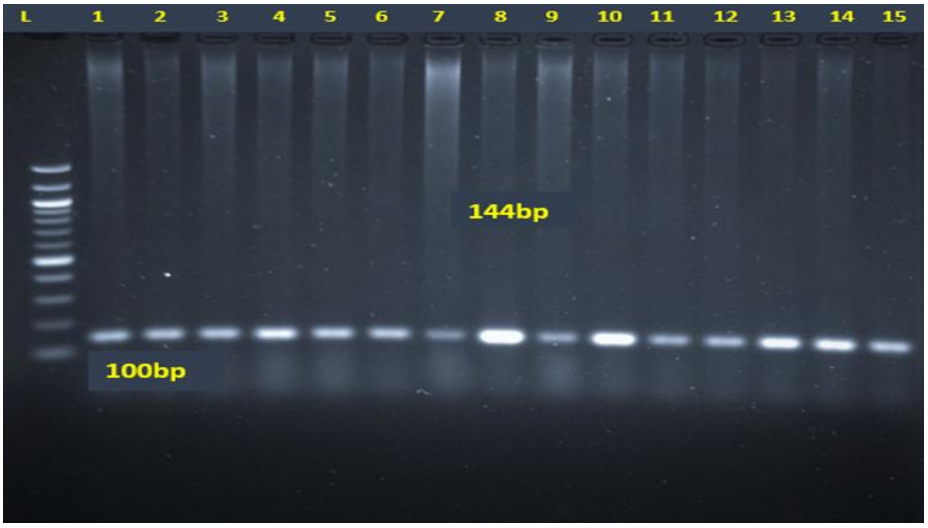

Figure (4) Agarose gel electrophoresis of PCR products , oqxA gene in 15 selected isolates of *Klebsiella pneumoniae.* Lane L :100 bp DNA ladder, ,Lane (1-15) PCR produt for oqxA . (50 A ,70 V for 120 min).

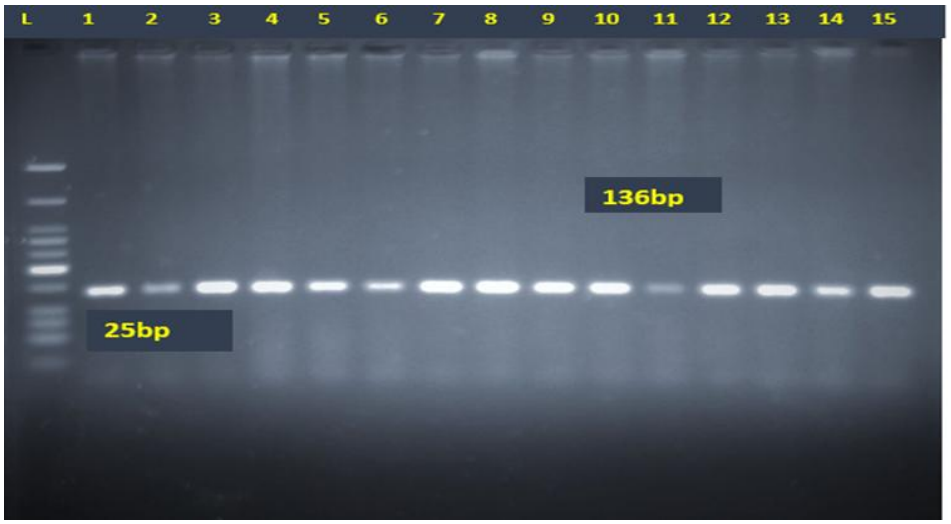

Figure (5) Agarose gel electrophoresis of PCR products , oqxB gene in 15 selected isolates of *Klebsiella pneumoniae.* . Lane L :25 bp DNA ladder, ,Lane (1-15) PCR produt for oqxB . (50 A ,70 V for 120 min).

The results of genes detection by PCR demonstrated that these efflux pump genes from the 2 systems *acrAB, oqxAB* were found in all *K. pneumoniae* tested isolates .

The previous findings reveal that the *AcrAB* and *OqxAB* efflux pumps have a high frequency among clinical isolates with a major role in the antibiotic resistance of multidrug resistance *K. pneumoniae* isolates (6).

Gene expression

Total RNA extraction

Total RNA was extracted from all the 76 isolates. Concentrations and purity were measured by Qubit 4.0 and all of the isolates had concentration between (10- 100 ng/µl) and the RNA purity was (1.6-1.9).

Gene expression by quantitative Real Time PCR

Complimentary DNA reverse

transcription was managed on the same day of RNA extraction. The efficiency activity of cDNA concentration was assessed through the activity of qPCR conducted later. All steps were associated with perfect yield reflecting efficient reverse transcription.

The amplification 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 (17,18). The main purpose of this step was to quantify gene expression of the *oqxA* and *oqxB* genes and compare the quantity of gene expression in the presence of Berberine HCL (at subinhibitory concentration) and in its absence in order to identify the effect of Berberine to the efflux pump genes in the of *K. pnumoniae*. In the present study, quantitative RT- PCR assay analyzed the mRNA expression of *oqxAB* genes by comparing the treated and untreated samples of bacterial growth with Berberine HCL by using the concentration (0.2µg/ml) 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 Ct value (show the details in materials and methods) as showed in Tables (6) and (7).

Table (6): **Folding of gene expression of efflux pumps gene (*oqxA*) in *Klebsiella pneumoniae* isolates before and after treatment with Berberin**

Isolate code	state	*16SrRNA*	oqxA	Delta ct	Delta delta ct	Folding
3K	treat	6.91	19.63	12.72	3.2	0.108819
23K	treat	7.36	21.42	14.06	1.89	0.269807
24K	treat	7.72	18.55	10.83	1.6	0.329877
26K	treat	6.82	17.59	10.77	3.77	0.073302
27K	treat	8.49	22.96	14.47	4.45	0.045753
3K	untreat	6.56	16.08	9.52	0	1
23K	untreat	7.64	19.81	12.17	0	1
24K	untreat	8.26	17.49	9.23	0	1
26K	untreat	7.17	14.17	7	0	1
27K	untreat	9.61	19.63	10.02	0	1

Table (7): **Folding of gene expression of efflux pumps genes (*oqxB*) in *Klebsiella pneumoniae* isolates before and after treatment with Berberin**

Isolate code	State	*16SrRNA*	oqxB	Delta ct	Delta delta ct	Folding
3K	treat	6.91	21.1	14.19	2.04	0.243164
23K	treat	7.36	26.52	19.16	6.69	0.009685
24K	treat	7.72	22.92	15.2	6.78	0.009099
26K	treat	6.82	23.78	16.96	4.39	0.047696
27K	treat	8.49	29.09	20.6	12.88	0.000133
3K	untreat	6.56	18.71	12.15	0	1
23K	untreat	7.64	20.11	12.47	0	1
24K	untreat	8.26	16.68	8.42	0	1
26K	untreat	7.17	19.74	12.57	0	1
27K	untreat	9.61	17.33	7.72	0	1

In oqxA gene when the MIC value was high (62.5 µg/ml) in resistant isolates before treatment with Berberine, the fold of gene expression

was slightly higher (1) and the fold of the resistant isolates after treating them with Berberine was low (0.1-0.04). For *oqxB* gene when the MIC value was high (62.5 µg/ml) in resistant isolates before treatment with Berberine, the fold of gene expression was slightly higher (1) and the fold of the same isolates after treating them with Berberine was low (0.2-0.0001). The gene expression analysis approach is confounded by the fact that the effect of many efflux inhibitors could be at the protein level or they may be simply acting by diffusing the ionic gradient required for driving the ant port activity (19). The real time PCR would help to determine if known efflux pumps are under expressed due to the activities of inhibitory compounds. For example (9), investigated the effect of two EPIs Berberine and Palmatine on the expression patterns of *mexA, mexB, mexC, mexD, mexE, mexF* and *mexX* in *Pseudomonas aeruginosa* isolated from burn infections. It revealed that the effect of these EPIs was more on the MexAB-OprM operon (20).

Another study (21) for a purpose to investigate the antimicrobial effects of berberine and thioridazine, as well as their effect on the gene expression of the AdeABC efflux pump system in Multidrug-Resistant (MDR) *Acinetobacter baumannii* (*A. baumannii*) isolates, The results showed that treatment of strains with thioridazine alone and in combination with berberine and ciprofloxacin significantly ($p<0.05$) decreased the expression of *adeB* efflux pump gene. Reverse transcription quantitative PCR (RT-qPCR) stands out from other approaches forgene expression due to its accurate, sensitive, and quick results. In the field of gene expressionanalysis, this method had already proven to be an excellent standard. It is crucial to understandthat in relative quantification investigations, every experiment typically focuses on comparingthe level of gene expression in various samples (22, 23). Due to the possibility that many efflux inhibitors work at the protein level or by merely diffusing the ionic gradient necessary to drive the ant port function, the gene expression analysis approach is complicated (19). Real-time PCR would be useful in figuring out whether known efflux pumps are under expressed as a result of inhibitory drug activity (20). The impact of two EPIs, palmatine and berberine, on the expression patterns of *mexA*, *mexB*, *mexC*, *mexD*, *mexE*, and *mexX* in bacteria isolated from burn illnesses. This research showed that the effect of these EPIs was more on the MexAB-OprM operon (20). Berberine shows a bactericidal effect that is likely to be useful for the treatment of clinical infections. Berberine may be important for the future development of antibiotics against *K. pneumoniae*, especially in multidrug-resistant and refractory infections.

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