Gene Expression of biofilm formation genes (plcN, bap) of Acinetobacter baumannii from wounds and burns patients in Baghdad, Iraq

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Abstract
Acinetobacter baumannii has a high propensity to form biofilm and frequently causes medical device-related infections with multiple-drug-resistance in hospitals. The aim of this work is to study antimicrobial resistance and the role of plcN, bap genes in biofilm formation by A. baumannii to understand how this pathogen persists in the hospital environment and determine the minimum inhibition concentration (MIC) of IMIPENEM and CHALCONE. As well as comparing the effect before and after of using the antibiotic and chalcone. Two hundred and fifty (250) samples were collected from various sites in Baghdad/Iraq hospitals and divided into two groups based on their source: clinical (150) and environmental (100) samples, collected from(October 2021) to (March 2022). Antibiotic resistance profile of isolates of A. baumannii was evaluated by VITEK-2 system and in vitro biofilm-forming ability was evaluated by micro titer plate methods, respectively. Isolates were tested for the presence of plcN, Bap genes Forty (40) A. baumannii isolates were identified by microscopic examination and biochemical tests. The identification of (40) isolates was verified using the VITEK-2 system and a molecular approach based on the rplB gene, which is an essential gene found in this genus. Antibiotic susceptibility testing was performed on all (40) identified isolates, and the findings revealed that 36 isolates had Multi-Drug Resistance (MDR). This study found that 16 isolates from these 16 isolates carrying the rblB gene and 6 isolates from 6 carrying plcN that confirmed by molecular detection methods, and 38 isolates from these 40 isolates had the ability to form biofilm in the micro titer plate method, and this study show there is down expression of plcN and bap with the antibiotic while overexpression of this genes with chalcone. In conclusion, there was strong association between presence of plcN, bap genes and biofilm formation by A. baumannii isolates. In addition, multidrug resistant isolates produced stronger biofilm than other isolates. These results indicate importance of biofilm in resistance of isolates and effect of the presence of plcN ,bap genes in biofilm formation by A. baumannii strains. and exposed to imipenem and chalcone decreased their biofilm gene expression level(plcN.bap).
Keywords: genes; patients in Baghdad; Acinetobacter baumannii

1. introduction

Acinetobacter baumannii is a Gram-negative coccobacilli opportunistic extracellular human pathogen that is non-motile 1. It has emerged as a significant nosocomial pathogen, causing a wide range of infections in patients. A. baumannii has developed resistance to nearly all antibacterial agents currently available. Multidrug-resistant strains of A. baumannii are infamous for spreading among hospitalized patients and causing outbreaks, which have been documented globally 2. Several studies have shown that A. baumannii has a higher intrinsic human virulence capacity than other Acinetobacter spp. Many conventional antibiotics have been used to treat infections caused by A. baumannii in the past, but it has recently developed resistance to large groups of antibiotics such as tetracycline, fluoroquinolone, carbapenem, chloramphenicol, penicillin, cephalosporin, and aminoglycosides. In addition to its increased prevalence and the growth of resistance at a significant rate. Contagion strains of A. baumannii are notable for both inherent antibiotic resistance and the ability to acquire genes encoding resistance determinants. The approach to treating this bacterial infection is limited due to its high susceptibility to clinically effective antibiotics 3.Biofilm formation is another way for bacteria to survive in the presence of antibiotics, particularly for A. baumannii, which causes biofilm-related medical devicecontamination 4 . forming has been an active area of study in recent years, Biofilm with separation of bacterial organisms from biofilms and discovery of genetic determinants to regulate this complex procedure. Biofilms are complex, highly ordered heterogeneous colonies of bacteria contained within a polymeric conglomerate of polysaccharides and proteins with combined metabolic activities that produce sessile phenotypes distinct from their planktonic counterparts 5. Bacterial biofilms have been detected on the surfaces of plastics, glass, and a variety of surgical instruments, as well as a variety of other hospital surfaces 6.

2. Materials and Methods

2-1 Bacterial isolation and identification Through the period extending from September 2021 till March

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2022, (250) clinical (Burns, wound infection, and blood) regardless age and gender, the choice of samples differs according to the clinical manifestations; (i.e. inflammation, fever, abscesses, pain, rapid breathing, irritation at site of infection). And environmental specimens (beds, tables, sinks, floors and instruments) collected as swabs and transported in sterilized transport medium containers. The samples were streaked on blood agar, MacConkey and CHROM agar and incubated at 37°C for 24 hrs. Identification of A.baumannii by manual biochemical tests that were used catalase test and oxidase test. For final confirmation biochemical tests embedded in VITEK2 compact system.

2-2 Antibiotic susceptibility test

These tests were done by VITEK 2 Compact Instrument using antibiotic sensitivity test number (AST- N222) cards according to the manufacturer's instructions, which included antimicrobial agents as follows: Amikacin (AK), Aztreonam (AZT), Cefepime (CPM), Ceftazidime(CAZ), Trimethoprim/ Sulfamethoxazole (TMP/SMX), Ciprofloxacin (CIP), Gentamicin (GM), Imipenem (IMI), Meropenem (MEM), Piperacillin (PRL), Ticarcillin/clavu lanate (TIM), Tobramycin (TM), Colistin (cs), Pefloxacin(PEF), Rifampicin (RA), Minocyclie (MNO) and Piperacillin/tazobactam (PTZ) **7**.

2-3 Molecular method

DNA Extraction Genomic DNA was isolated from bacterial growth according to the protocol of Wizard Genomic DNA Purification Kit, this kit was designed for the isolation of DNA from various biological samples. DNA was extracted by this kit using bacterial protocol (for gram negative bacteria).

Conventional polymerase chain reaction for rblB and PlcN genes

Concerning the conventional PCR reaction, designed primers were used for detection of each gene. Provided in lyophilized form, dissolved in sterile deionized distilled water to give a final concentration of 100 Pico mole/îl as recommended by the provider. The specific primers for the plcN gene (plcN - F 5'GTTATCGCAACCAGCCCTAC 3' and(plcN-R 5'AGGTCGAACACCTGGAACAC 3') and the rplB gene (rplB -F, 5'-GTA GAG CGT ATT GAA TAC GAT CCA AAC C-3' and rplB - R, 5'-CAC CAC CAC CGT GCG GGT GAT C-3') were used (Table 1). Using 20ìL of PCR reaction volume protocol, 3 µl DNA template (100 ng/µl) was amplified using 10 µl of Go Taq® green master mix 2X (Promega, USA) and 1 µl of each primer (10 pmol/µL) for each specific gene, up to the final volume 20 µl with nucleases free water. The extracted DNA, primers and PCR premix was thawed at 4°C, vortexed and centrifuged briefly to bring the contents to the bottom of the tubes. Negative control contained all material except DNA, that D.W. was added instead of template DNA. PCR programs were set on Thermal cycler gradient PCR (Thermo fisher/ USA), the plcN gene was amplified under the following conditions: 95°C for five min, then 30 cycles of 95 °C for 30 sec, 58°C for 30 sec, 72 °C for one min, followed by final extension 72°c for 7 min. Also, PCR condition for rplB gene was as follow: 95°C for five min, then 30 cycles of 95°C for 30 sec, 50°C for 30 sec, 72°C for one min (30 cycles), followed by final extension 72°C for 7 min. The PCR products were separate in 1.5% agarose gel.

Table 1: Primer sequences used PCR in this study.

NO	Primer name	Sequence	Product size
1	PlcN F	'GTTATCGCAACCAGCCCTAC 3'5	466
2	PlcN R	'AGGTCGAACACCTGGAACAC 3'5	466
3	rblB F	-'5'GTA GAG CGT ATT GAA TAC GAT CCA AAC C-3	475
4	rblB R	-'CAC CAC CAC CGT GCG GGT GAT C-3'5	475

2-4 Biofilm formation assay

The biofilm formation ability of A. baumannii isolates was determined by 96 well micro titer plate assay based on the crystal violet staining method. Briefly, each 96-well flat-bottomed sterile polystyrene micro titer plate well containing 199µLof Mueller–Hinton broth supplemented with 1% glucose were inoculated with 1µL from suspended bacterium of 0.5-0.7 McFarland, micro titer plate are incubated 24 h at 37°C. The liquid media was discarded and the adherent cells were washed twice with phosphate buffered saline (PBS) and wells are dried at60°C for 1 h or less. After that it was stained with 150µL of crystal violet for 15 min. Then the crystal violet - stained wells of micro titer plate were washed twice with PBS to discharge crystal violet stain. After air drying process of wells of micro titer plate, dye of biofilms that lined the walls of the micro titer plate is re-solubilized by 150µL of96% ethanol. After 5-10min micro titer plate is measured spectrophotometrically at 570 nm by a micro titer plate reader 8. The optical density cut-of value (ODc) was established as three standard deviations (SD) above the mean of the optical density (OD) of the negative control as shown in the following formula: ODc = average OD of negative control + (3×SD of negative control). The results were divided into four categories according to their optical densities as1- Strong biofilm producer (4 × ODc< OD) 2- Medium biofilm producer (2 × ODc< OD ≤ 4 × ODc) 3- Weak biofilm producer (ODc< OD ≤ 2 × ODc) Non-biofilm producer (OD ≤ ODc) 2-5 Minimum inhibitory concentration (MIC) Using the micropipette, dispense 100µl of medium into all wells of a micro titer plate-1

2- Pipette 100 µl of appropriate antibiotic solutions into the wells in column A (far left of 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 repeat the procedure down to column H only. The same set of tips can be used for the entire dilution series

5- Discard 100 µl from column H

With the similar micropipette set to 100 µl, dispense bacteria into all wells 6-

Incubate the plates at 37C -7

)When satisfactory growth is obtained (24 hours- 8

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

2-6 Quantitative Real Time-PCR

RNA Extraction

RNA was extracted from the isolates before and after treating with the antibiotic. Total RNA was extracted using TRIzol™Reagen.

Preparation of primers

Specific primers were obtained (Table2) according to the previous studies for detection of the gene expression.

Primer sequences used qRT-PCR in this study. Table 2:

NO	Primer	sequence	Product size
1	PlcN F	5' CATAAGGTAGCGCCCGTGAT'3	466
2	PlcN R	5'AAACAAACACGTCTGCAGGC 3'	466
3	Bap F	5'TAGACGCAATGGATAACG 3'	405
4	Bap R	5' TTAGAACCGATAACGATACC 3'	405
5	RbIB F	5'GTA GAG CGT ATT GAA TAC GAT CCA AAC C-3	475
6	RbIB R	CAC CAC CAC CGT GCG GGT GAT C-3'5	475

Quantitative Real-time PCR Assay (QRT-PCR)

By using Qubit® 1-Step RT-qPCR System (Qubit®-USA), Amplification of fragment of mRNA was performed with the following master amplification reaction with the Qubit of two -Step RT-PCR list in Table (3) and the (Qubit®- USA) in Table (4). Several experiments were done for more appropriate synthesis of cDNA and annealing temperature.

Table (3): quantitative RT-PCR Reaction Mix.

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

Table (4): Quantitative RT-PCR Reaction Mix.

Cycle Step	Stage	Temperature	Time
1	Initial Denaturation	95 °C	60 seconds
40-45	DenaturationExtension	95°C60 °C	15 seconds30seconds (+plate read)
1	Melt Curve	60-95 °C	40 minutes

Delta delta Ct method 11

Delta delta Ct (ΔΔCt) method is the simplest one, as it is a direct of Ct values comparison 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, optimum temperature 37 °C or any sample wants to compare the unknown samples. 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) as: shown in the following equation.

ΔCt = Ct target gene - 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) control

The normalized target amount in the sample is then equal to 2−ΔΔCt and this value can be used to compare expression levels in samples[14] .The relative changes in mRNA expression levels were determined by using a comparative threshold cycle (CT) method (2−ΔΔCt).The result was collected and analyzed by Livak formula.

Statistical analysis

The Statistical Analysis System-SAS (2012) program was used to detectthe effect of difference factors in study parameters.Least significant difference –LSD test (Analysis of Variation-ANOVA) was used to significant compare between

means. Chi-square test was used tosignificant compare between percentage (0.05 and 0.01 probability) in this study.

3. 3-Results and Discussion

Identification of A. baumannii

Using the VITEK 2 system's Gram-negative strain identification card74 clinical isolates were identified as A. baumannii. This method has been used in a number of previous studies and has produced satisfactory resultsin terms of biochemical test diagnosis and validation. This automated device could determine the antibiograms of A. baumannii isolates 12.

Distribution of A. baumannii according to sample typeAccording

to Tables 5, A. baumannii was included in 74(29.6%) of the 250 clinical and environmental samples tested. The samples were taken from variousenvironmental and medicinal sources, including wounds and burns, sputum, and inpatients' body fluids (peritoneal fluid) (Table5).

Source	No. of samples	Positive samples	Nagtive sample
Clinical samples			
Wounds	75(50%)	32 (12.8%)	43
Burns	75 (50%)	29 (11.6%)	46
Environmental samples			
beds	25 (10%)	5 (2.0%)	20
tables	25 (10%)	4 (1.6%)	21
sinks	25 (10%)	3 (1.2%)	22
loors	25 (10%)	1 (0.4%)	24
Total	250 (100%)	74 (29.6%)	

Susceptibility to antibiotic

test the automatic VITEK 2 Compact device was used to screen all 83 A. baumannii isolates for antibiotic susceptibility. All samples were cultured on MacCkonkey agar plates and each isolate was given a McFarland 0.5 standard suspension in 0.45 percent sodium chloride. To obtain the results, a liquid suspension of all isolates was loaded into the VITEK machine and left overnight. The Gram Negative Susceptibility card used in the VITEK 2 Compact device was used to test 14 different antibiotics(Table6).

Table 6: Antimicrobial susceptibility test of 83 *A. baumannii* isolates to 14 antimicrobial agentsBiofilm			
Antibiotic	Resistant	Intermediate	Sensitive
Piperacillin/tazobactam(PTZ)	38 (51.30%)	2 (2.70%)	34 (45.94%)
Colistin(cs)	5 (6.75%)	9 (12.16%)	61 (82.42%)
Trimethoprim/Sulfamethoxazole (TMP/SMX)	17 (22.97%)	2 (2.70%)	55 (74.32%)
Tobramycin(TM)	24 (32.43%)	13 (17.56%)	37 (50.0%)
Piperacillin (PRL)	38 (51.35%)	0 (0.0%)	36 (48.64%)
Meropenem(MEM)	30 (40.54%)	13 (17.56%)	31 (41.89%)
Minocycline (MNO)	5 (6.75%)	4 (5.40%)	65 (87.83%)
Imipenem (IMI)	20 (27.02%)	21 (28.37%)	33 (44.59%)
Cefepime(CPM)	49 (66.21%)	0 (0.0%)	25 (33.78%)
Ceftazidime(CAZ)	70 (94.59%)	0 (0.0%)	4 (5.40%)
Ciprofloxacin(CIP)	36 (48.64%)	3 (4.05%)	35 (47.29%)
Gentamicin(GM)	30 (40.54%)	0 (0.0%)	44 (59.45%)
Ticarcillin	25 (33.78%)	13 (17.56%)	36 (48.64%)
Ticarcillin /clavulanate(TIM)	23 (31.08%)	10 (13.51%)	41 (55.40%)

Formation

In this study, determining the ability of 40 *A. baumannii* isolates to adhere and produce a slime layer (Biofilm formation) was experienced by using micro titer plates (MTP) methods. The results abstained by micro titer plates reader (GloMax® Discover Micro plate Reader), the results showed that 38 isolates (95%), have the ability to adhere and produce slim layer with significant differences in thickness degrees (strong, moderate and weak) where the variation in biofilm thickness may be due to differences in isolates ability to produce biofilm whereas 2 isolates with no change in OD over the control were detected as non-biofilm former and these isolates did not have the ability to adhere and to produce slim layer the results in Table 6 shown that 26 isolate gave strong biofilm and 11 isolate gave moderate biofilm and 3 isolate gave weak biofilm. **13** used phenotypic approaches to the evaluate biofilm-forming capability of *A. baumannii* isolated from clinical samples in Baghdad hospitals and found significant differences between 83 *A. baumannii* isolates.

Presence of *plcN*,*bap* and *rblB*

PCR analysis confirmed the existence of the *rplB* gene in all 16 (100%). *A. baumannii* isolates, confirming the conventional diagnosis of culture, biochemical tests, and Vitek-2 tests. In the electrophoresis procedure, the formed amplicons of this gene appeared clearly at 475 bp on an agarose **14** as shown in Fig. 1. PCR assay was used to

determine the virulence gene in all 6 MDR *A. baumannii* isolates. PCR assay was used to detect a single gene using particular primers. The findings of this analysis showed that 6 isolates carried the phospholipase C (*plcN*) as shown in Figure 2, Results were reported in a study of mousl, Iraq by **15**. The presence of *plcN* gene in their isolates were ten isolates, 52.6%, at a molecular size of 466 base pairs.

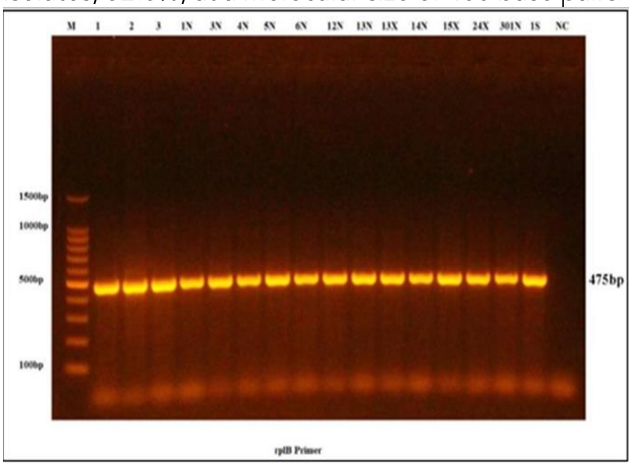

Figure 1: The results of the amplification of rplB gene of Acinetobacter baumannii samples were fractionated on 1.5% agarose gel electrophoresis stained with Eth.Br. M: 100bp ladder marker. Lanes 1-1S resemble 475bp PCR product.

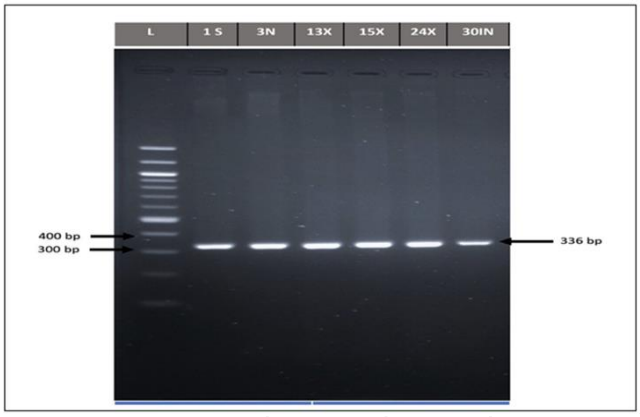

Figure 2: the results of the amplification of plcN gene of Acinetobacter baumannii samples were fractionated on 1.5% agarose gel electrophoresis stained with Eth.Br. M: 100bp ladder marker. Lanes 1-30N resemble 336 bp PCR product.

Results of RT-qPCR for *bap* and *plcN* and *genes*

Real time PCR quantification applied in the present experiment utilized the SYBR green, a fluorescent dye which recognizes and intercalate every double stranded DNA including cDNA. 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 **15** .

The main purpose of this step was to quantify gene expression of the *rblB*, *plcN* and *bap* genes and compare the quantity of gene expression in the presence of the antibiotic and in its absence in order to identify the role of these genes in the resistance of *A. baumannii*. Due to accurate, sensitive and fast results, reverse transcription quantitative PCR (RT-qPCR) is distinguished from other methods for gene expression. This technology had established itself as the brilliant criterion in gene expression analysis. It is very important to realize that in relative quantification studies, all experiments are usually concerned with comparing the expression level of a particular gene amongst different samples **16**.

The *rblB* gene, as the housekeeping gene, was used in the present experiment because its expression remains constant in the investigated cells under different conditions .

These isolates were chosen with sub MIC values to antibiotic (31.25 µg/ml), two isolates treated with 125 µg/ml chalcone and two sensitive isolates as control. In the present experiment, quantitative RT- PCR assay analyzed the mRNA expression of *plcN* and *bap* genes by comparing the untreated and treated group of resistant bacteria samples grown with antibiotic and chalcone.

Real time PCR quantification of *plcN* expression

The range of Ct value for *plcN* in isolates before treatment with group was (14.76-15.28). The range of Ct value for *plcN* in isolates after treatment with antibiotic group was (14.61-15.28). While the range of Ct value for in isolates after treatment with chalcone group was (13.34-13.57) see Table 7 . there was significant difference in the Ct values between the different study groups as shown in the table 7 The Ct values in isolates after treatment with antibiotic were higher than the Ct value of isolates before treatment and after treatment with chalcone and this in turn was slightly higher than those in isolates which reflects that the genes are present in mRNAs samples. It is evident from these results that the antibiotic group is associated with the highest copy number of target gene on mRNAs reflecting its lowest expression, and least copy number of target gene carried on mRNA in isolates after treatment with chalcone reflecting its higher expression.

It was reported that all 30 *A. baumannii* isolates were investigated for the presence of virulence factors genes (*plc-N* and *lasB* genes) and results showed that, 16 (53.33%) were harboring *lasB* genes while 7 (23.3%) isolates were harboring *plcN* gene The presence of any of these gene enhance the killing ability of *A. baumannii* strain and increased invasiveness in A549 cell line **17**.

Table 7: Gene expression of plcN comparison between Chalcone, antibiotic and control

Sample	Ct *rblB*	Ct *plcN*	ΔCt	ΔΔCt	fold
1N (BT)	12.58	14.76	2.18	0	1
1X(BT)	12.02	15.28	3.26	0	1
2N (AT) chalcone	10.74	13.34	2.6	0.42	1.337928
2X (AT) chalcone	8.86	13.57	4.71	1.45	0.366021
3N(AT) A.b	9.3	14.61	5.31	3.13	0.114229
3X(AT) A.b	9.25	15.28	6.03	2.77	0.146604

Real time PCR quantification of *bap* Expression

The range of Ct value for *bap* in resistant isolates before treatment with group was (34.54-34.99). The range of Ct value for *bap* in isolates after treatment with antibiotic group was (35.0). While the range of Ct value for *bap* in isolates after treatment with chalcone group was (45.98-33.5) (see Table). There was a significant difference in the Ct values between the different study groups as shown in the table 8.
The Ct values in isolates after treatment with antibiotic were higher than the Ct value of isolates before treatment and after treatment chalcone and this in turn was slightly higher than those in isolates which reflects that the genes are present in mRNAs samples. It is evident from these results that the antibiotic group is associated with the highest copy number of target gene on mRNAs reflecting its higher expression, and least copy number of target gene carried on mRNA in isolates after treatment with chalcone reflecting its lowest expression. Biofilm associated protein expression in a population of *A. baumannii* cultured iron-limiting media (M9 medium with 20 M FeCl3); the outcome was compared to that of the control strain *A. baumannii* ATCC19606. *bap* expression in 20 M Fe was four-fold higher in several *A. baumannii* isolates that formed robust biofilms than in the same isolates cultured in 50 M Fe **18**.

Table 8: Gene expression comparison between chalcone, antibiotic and control of the bap gene

Sample	CT *rblB*	CT *bap*	ΔCT	ΔΔCT	fold
1N (BT)	12.58	34.54	21.96	0	1
1X(BT)	12.02	34.99	22.97	0	1
2N(AT)chalcone	10.74	45.98	35.24	13.28	0.00010054
2X(AT)chaclone	8.86	33.5	24.64	1.68	0.31208264
3N(AT) A.b	9.8	35	25.2	3.24	0.10584316
3X(AT) A.b	9.52	0	0	0	0

4. Conclusions

A.baumannii is an emerging member of multi-resistant pathogen increasingly know to causes nosocomial infections in Iraqi hospitals,in this study the plcN and bap genes play role in biofilm formation, also concluded the Sub MIC of antibiotic for samples (N,X) at a concentration (31.25) while the concentration of chalcone for the same samples is 125,so the gene expression was measured Real Time-pcr it was found that the plcN and bap genes the treatment with antibiotic were higher than the Ct value of isolates before treatment and after treatment with chalcone and this in turn was slightly higher than those in isolates which reflects that the genes are present in mRNAs samples. It is evident from these results that the antibiotic group is associated with the highest copy number of target gene on mRNAs reflecting its lowest expression, and least copy number of target gene carried on mRNA in isolates after treatment with chalcone reflecting its higher expression.

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