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Study of the Gene Expression of aacC1 Gene in *Acinetobacter baumannii* that Responsible for Aminoglycoside Resistance

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Abstract

Acinetobacter baumannii carries modifying enzymes, are resistant to many broad-spectrum antibiotic, including: - Amikacin, Tobramycin and Gentamycin (Aminoglycoside), the genes that encode the Aminoglycosides enzymes was aadA1 and aacC1 genes confer resistance to anti-group aminoglycoside.

Two Hundred clinical samples were collected from different sources of infection included (50 sample of blood, 55 sample of sputum, 50 samples of Urine , 10 samples of wound, 10 samples of burn ,10 samples of throat swab and 15 samples of body fluid from plural infusion).

Clinical samples were cultured in different media, Blood agar, MacConkey agar and CHROM agar and incubated at 37°C for 24–48 h. for diagnosis used biochemical tests including (oxidase and catalase) and microscopic examination, for final detection using Vitik2 compact system and API 20E . After identification we obtained 40 isolates of *Acinetobacter baumannii*, including 14 isolates from blood (35%) , 15 isolates from sputum (38%) , 4 isolates from wound

infection (10%) , 4 isolates from Urine (10%) , only one isolate from Throat swabs, Burn and plural effusion fluid (2.5%)

Minimum Inhibitory Concentrations detection to antibiotic. All isolates displayed resistant to Ticarcyclin 55%, Pipracillin / Tazobactam 55%, Trimethoprim 52.5%, Ticarciliin / Clavulanic acid 52%, Ceftazidim 52%, Cefipim52% , Imipinem 52%, Ciprofloxacin 52% , Gentamycin 40% , pipracillin 37% , Meropinem 37% , Colistin 15 % , Tobramycin 10% , Minocycline 5%.

Genotypic detection of *aacC1gene* using polymerase chain reaction (PCR). The results was appeared all isolates (20) have *aacC1* gene in percentage (100%) with molecular weight 465 base pair. Gene expression detection for *aacC1gene by using* Real Time Polymerase Chain Reaction (RT-PCR). The results of gene expression for *aacC1gene* was increasing when the isolate was treated by gentamycin antibiotic in concentration 1.2 µl/ ml.

Acinetobacter baumannii has aacC1 resistance gene in (100%). The results also showed that there was an increase in the gene expression of *aacC1* gene after treatment with Gentamycin.

Key words: Gene expression, *aacC1gene*, Aminoglycoside.

دراسة التعبيرالجيني لجين aacC1 في بكتريا *Acinetobacter baumannii* والمسؤول عن مقاومة مضادات الامينوكلايكوسايد

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الخلاصه

Acinetobacter baumannii تحمل انزيمات المحوره والمقاومة للعديد من المضادات الحيوية واسعة الطيف ، بما في ذلك Amikacin و Tobramycin و Gentamycin (Aminoglycoside) هذه الانزيمات Aminoglycosides تشفر من قبل جينات aadA1و aacC1 وتكون مسوولة عن مقاومة لمجموعة مضادات Aminoglycosides.

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جمعت 200 عينة سريرية من مختلف المصادر شملت 50 عينة دم (Blood) , 55 عينة من القشع (Sputum), 50 عينة من الادرار (Urine) من مرضى التهاب المسالك البولية ,10 عينات من مسحات الجروح (Wound swab)، 10 عينات من مسحات الحروق (Burn swab)،15 عينات من مسحات الحنجرة (Throat swab)، 10 عينة من سوائل الجسم (Pleural effusion).

تم زراعه العينات السريرية على أوساط مختلفة ، اكار الدم ، اكار MacConkey واكار CHROM وحضنت عند 37 درجة مئوية لمدة 24-48 ساعة .للتشخيص استخدمت الاختبارات البيوكيميائية بما في ذلك (أوكسيديز والكتاليز) والفحص المجهري وللكشف النهائي تم استخدام نظام Vitik2 وAPI 20E . اظهرت نتائج التشخيص ان (40) عزلة تعود لبكتريا *Acinetobacter baumannii* وشملت (14) عزلة من عينات الدم و بنسبة (35%) , (15) عزلة من عينات القشع وبنسبة (38%) , اما من عينات الجروح فكانت (4) عزلات وبنسبة (10%) , عينات الادرار كانت 4 عزلات و بنسبة (10%) , اما عينات (الحروق , سوائل الجسم و مسحات الحنجرة) كانت عزلة واحده لكل منهم وبنسبة (2.5%) .

تم تحديد التركيز المثبط الادنى للمضادات الحيوية واظهرت النتائج أن جميع العزلات اظهرت مقاومة لـ Ticarcyclln)بنسبة ٪55 ، ٪55 Pipracillin / Tazobactam ، ٪52.5 Trimethoprim / Ticarciliin ، ٪52 Clavulanic acid ٪52 Ceftazidim، ٪52 Cefipim ، ٪52Imipinem ، ٪52Ciprofloxacin ، ٪40 Gentamycin ، ٪37pipracillin ، ٪37Meropinem ، ٪15Colistin ، ٪10Tobramycin ، ٪5Minocycline.

تم الكشف عن النمط الجيني لبعض الجينات المقاومة للمضادات الحيوية لجين *aacC1* باستخدام تفاعل البلمرة المتسلسل (PCR) ووجد ان نسبة تواجد الجين في 20 عزلة قيد الدراسة وبنسبة ٪100 و بوزن جزيئي 465 زوج قاعدي .

تم دراسة التعبير الجيني باستخدام تفاعل البلمره المتسلسل Real Time للجين *aacC1* اظهرت نتائج التعبير الجيني لهذا الجين زياده في التعبير الجيني عند معالجتها بالمضاد الحيوي Gentamycin بتركيز 1.2 مايكروغرام / مل .

الاستنتاجات: تمتلك *Acinetobacter baumannii* جين مقاومة aacC1 في (100%) وأظهرت النتائج أيضا أن هناك زيادة في التعبير الجيني للجين aacC1 بعد العلاج بالجنتاميسين.

الكلمات المفتاحية :- التعبير الجيني ، جين *aacC1* ، الامينوكلايكوسايد.

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Introduction

Aminoglycoside-modulating enzymes are one of the most important resistance mechanisms in A. baumannii. These enzymes can be divided into three parts: acetyl-transferases, adenyl-transferases and phospho-transferases. These enzymes are present within the transposon of pathogenic bacteria [1]. Several studies have shown that A. baumannii bacteria produce modified Aminoglycoside modifying enzymes [2].

A study conducted in China found that (4) clinical isolates belonging to *A. baumannii* carrying (Aminoglycoside modifying enzymes) [3]. In another study conducted in Greece, it was shown that all *A. baumannii* isolates carry transgenic enzymes of Aminoglycoside antibiotic [4]. *A. baumannii* which carries modifying enzymes, are resistant to many broad-spectrum antibiotic (ESA), including (Amikacin, Tobramycin) and Gentamycin (Aminoglycoside)) that attack bacteria in two stages, the first stage was absorbed by the bacteria during which one of the biological activities of the bacterium, and the second was that these antibiotics are associated with the ribosome and lead to inhibition of the protein synthesis process [5]. This group of antibiotics is very important for treating infections that occur due to infection in *A baumannii*. In recent years, it has been discovered that this bacteria possesses a number of resistance mechanisms against Aminoglycoside [2]. Resistance mechanisms of aminoglycosides group occur by have modifying enzymes which was most important resistance mechanisms in Gram-negative bacteria. As these enzymes bind with the amino group or the hydroxyl-groups of the aminoglycosides, which leads to block binding aminoglycosides with ribosomes [6].

A.baumannii possess some of the genes that encode the enzymes Aminoglycosides the *aadA1* and *aacC1* genes confer resistance to anti-group aminoglycoside genes, and these genes encode modified Aminoglycoside enzymes and in turn are resistant to the same group antagonists. [7].

The aims of study:-. Study the gene expression of *aacC1* gene in *Acinetobacter baumannii* with 1.2 µl/ ml concentration of gentamicin

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Materials and Method

Collection of sample

Two hundred collection sample of *A. baumannii* was obtained from different causes from several Hospitals in Baghdad including :- (Teaching Baghdad Hospital, Pediatric Hospital, AL Shahid Kazy AL-harery and teaching clinical laboratories, AL Yarmok hospital and AL Kadhimia hospital from the period 15/11/2018 to 19/2/2019. The Sample included 50 sample of blood, 55 sample of sputum, 50 samples of Urine, 10 samples of wound, 10 samples of burn, 10 samples of throat swab and 15 samples of body fluid from plural infusion.

Identification of isolates

Clinical samples were cultured in different media, e.g., Blood agar, MacConkey agar (oxide (England)) and CHROM agar (Chromo agar (France) and incubated at 37°C for 24–48 h. for diagnosis used biochemical tests including (oxidase (N-N-N-N –tetra methyle –p-phenyle diamine dihydrochloried) (Difco (England)) and catalase (H_2O_2 3%) (BioMerieux (France)) and microscopic examination, for final detection of isolates using Vitik2 compact system (BioMerieux (France)) and API 20E Test include (Suspension Medium, API , Mineral Oil, Incubation Boxes and indicator TDA, Jemes , VP1and VP2) BioMerieux (France) [8].

MIC detection

MIC detection to antibiotic including (Pipracillin\Tazopactam , Pipracillin (break point **>=128** µl/ml), Ticarcicllin (break point >=128 µl/ml) , Ticarcicllin\ Clavulanic acid, Ceftazidem (break point **>=84** µl/ml), Cefepim (break point **>=64** µl/ml), Imipenem (break point **>-16** µl/ml), Meropenem (break point **>=16** µl/ml) , Gentamycin (break point **>=16** µl/ml),Tobramycin (break point 8 µl/ml) , Ciprofloxacin (break point **>=4** µl/ml) , Minocycline, Colistin (break point **<=0.5**µl/ml) and Trimethoprim **>=32**µl/ml) (Minimum Inhibitory Concentrations if the value was equal or more than breakpoint, that means the bacterium was resistant to that antibiotic) by using Vitik2 compact system (France)

Molecular detection of the *aacC1* gene

The primers that used to detect *aacC1* genes (F: ATGGGCATCATTCGCA CATGTAG, R: TTAGGTGGCGGTACTTGGGTC) 465bp [9] The solutions were prepared of the Stock solution according to the instructions of the manufacturer Alpha DNA (Canada) using deionized nuclease free water to obtain a concentration of 100 picomol / microliter. The solution of each primers was presented separately at 10 picomol / Microliter by taking 10 microliters of each stock solution and added to 90 microliters of distilled water and mixed well with Vortex mixture, kept with stock solutions at -20°C and mixed the primers solution after removed it from ice. PCR mixture of the genes using in this study preparation with a total volume of (25) µl including the forward (1) µl and the Revers (1) µl, (2) µl of DNA template, (8.5) µl of Nuclease-free water Deionized (Go Taq master mix) with 12.5 µl., they were mixed with a Vortex and placed in the PCR. Thermal-cycling system depending on the conditions and temperatures of the *aacC1* gene optimum [10].

Steps of PCR reaction were:- step 1 (Only one cycle for 5 minutes at a temperature of 95°C for the primary DNA denaturation), step 2 (30 cycle included: A: 30 sec at 95°C for DNA template denaturation, B: 30 sec at 53°C for the primers to bind to DNA template annealing, C: 40 sec. at 72°C for the associated primers to be elongated. Step3 (Only one cycle for 7 minute at 72 ° C for the final elongation of the double DNA strip. After that, 5 µL of the product of the PCR was transferred to the electrophoresis on an agarose gel at a concentration 2%.

Agarose Gel Electrophoresis

After PCR amplification, a migration of Agarose gel electrophoresis was performed to confirm the presence of DNA. The Agarose gel was concentration 2% with Ethidium bromide (0.5) µg/ ml. (5) µl of the PCR product was transferred to the pits designated for it, as well as loaded 5 µl of DNA Ladder (100) bp. at a voltage (100) volts for (80) minutes. The gel was examined by using a UV-Transilluminator (300 nm) [11].

RNA extraction

RNA extraction (Thermo Fisher Scientific, USA) according [12].

Real Time Polymerase Chain Reaction (RT-PCR) and gene expression

As a result of (Real time PCR) experiments, the kit One Step RT- qPCR real time PCR includes:- (SYBR FAST DNA ,buffer, dNTPs, SYBR Green I dye, $MgCl_2$, Polymerase reaction *16srRNA* (as control) was used (One Step RT-qPCR) manufactured by (Promega, USA) (qPCR Master Mix 5 µl , RT mix 0.25 µl, $MgCl_2$ 0.25 µl , Forward primer 0.5 µl , Reverse primer 0.5 µl , Nuclease Free Water 1 µl, final Total volume 10 µl) Aliquoroer for single rxn 9µl of Master mix per tube and add 1µl of Template.

Then placed in a Real time PCR. Steps were step 1 (Only one cycle for 15 minutes) at a temperature of 37°C for c DNA and for the Initial denaturation of DNA (5 minutes) at 95°C, Step 2 (40 cycle included: A: 20 sec at 95°C for DNA template denaturation, B: 20 sec at 53°C for the primers to bind to DNA template annealing, C: 30 sec. at 72°C for the associated primers to be elongated. Step3 (three cycle for one sec. at 72°C to 95°C for the Melted green. Calculate the amount of change in the level of gene expression as shown by the following equations: -

$$\text{Folding} = 2^{-\Delta\Delta CT}$$

$$\Delta\Delta CT = \Delta CT \text{ Treated} - \Delta CT \text{ Control}$$

$$\Delta CT = CT \text{ gene} - CT \text{ House Keeping gene } (\textit{16SrRNA}).$$

Result and discussion

After identification we obtained 40 isolates of *Acinetobacter baumannii*, including 14 isolates from blood (35%) , 15 isolates from sputum (38%) , 4 isolates from wound infection (10%) , 4

isolates from Urine (10%) , only one isolate from Throat swabs, Burn and plural effusion fluid (2.5%)

Minimum inhibition concentration (MIC) was determined using Ticarcillin, Ticarciliin / Clavulanic acid, Pipracillin, Pipracillin / Tazobactam, Ceftazidime, Cefipim, Imipenem Trimethoprim, Meropenem ,Gentamicin , Tobramycin , Ciprofloxacin , Minocycline and Colstin . The result showed the ratio of antibiotic resistance of bacteria (Ticarcycllin 55%, Pipracillin / Tazobactam 55%, Trimethoprim 52.5%, Ticarciliin / Clavulanic acid 52% , Ceftazidim 52% , Cefipim52% , Imipinem 52%, Ciprofloxacin 52% , Gentamycin 40% , pipracillin 37% , Meropinem 37% , Colistin 15 % , Topramycin 10% , Minocycline 5% as shown in Figure (1).

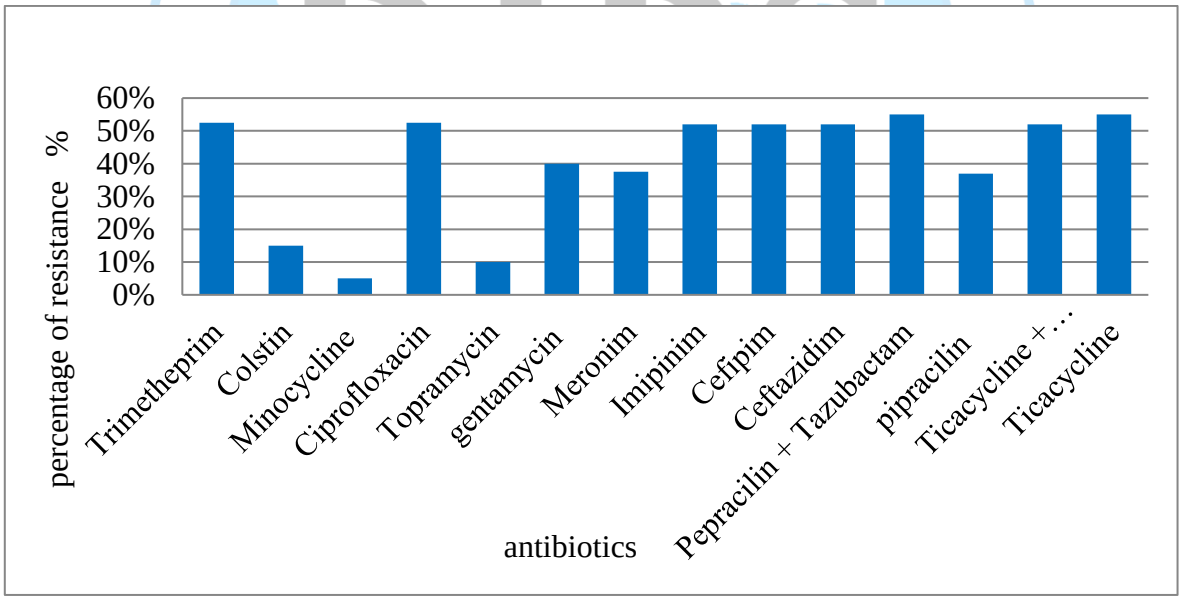

Figure 1: Resistance percentage for different antibiotics of A. baumannii bacteria by using MIC value.

The minimum inhibitory concentration (MIC) was resistance to Ceftazedim and Imipenim (52%) for each of them, in another study the percentage of resistance of *A. baumannii* to Ceftazedim and Imipenem 100% and (85%) respectively [9]. While the results of [13] show that the percentage of resistance of *A. baumannii* to Ceftazedim and Imipenim was (98.4%) and

(90.6%) respectively. While the bacteria resistance of Cefipem was 52%. In another study, the percentage of resistance to the same antibiotic (100%) and (97.7%) as indicated by the [14, 15] respectively.

The current study showed that *A. baumannii* resistance to Ampicillin, , Ceftazedime , Cifepim, Imipinem and this resistance was due to the possession of (β - lactam) and this bacteria production β-lactamase [16] . β- Lactam ring, which leads to inactivation of the antibiotic. *A. baumannii* may be resistant to β- lactam antibiotic (due to mutations that alter target sites such as penicillin binding proteins) [17].

The current study showed that the percentage of resistance of bacteria to Tobramycin and Gentamycin was (40%) and (10%), respectively. In another study, [18] showed that the percentage of resistance of *A. baumannii* bacteria to Tobramycin and Gentamycin were 61% and 41% respectively, While the study of [6] showed that the rate of bacterial resistance to the antibiotics Gentamycin and Tobramycin was (86%), (63%) respectively. The reason for the resistance of the bacteria to the antibiotics Gentamycin, and Tobramycin is due to the binding of these enzymes to the amine or hydroxyl Aminoglycoside group and lead to the degradation of these antibiotics. Modified enzymes, including (Acetylases, Adenylases, Phosphorylases) which encode certain genes, inhibit the activity of Aminoglycoside [9].

The percentage of antibiotic resistance to Ciprofloxacin was (52%), while the percentage of bacteria resistance to Ciprofloxacin and Norfloxain was (71.87%), (62.5%), respectively, according to [19,20] , stated that the mechanism of action of the (Fluoroquinolones) is that it binds to an enzyme (DNA gyrase and topoisomerase) that plays an "important" role in the process of DNA replication in bacterial cells, as these antibiotics inhibit the process of DNA replication, which Inhibits the process of bacterial cell division. The mechanism of bacterial resistance to Ciprfloxacin and Norfloxacin is due to mutations that occur in DNA gyrase encoding *gyrA*, *gyrB*, *parC* and *parE* as subspecies [20, 22].

As for the Colistin, the current study showed that the percentage of bacteria resistance was (15%). In another study conducted by [12], they showed that the resistance of *A. baumannii* to Colistin was 15%. While the [12] showed that the rate of bacterial resistance to Colistin was (10%) [22] Clarified that Colstin was the first treatment in the treatment of Infection with *A.baumannii*, while other studies have shown that *A. baumannii* are resistant to Colistin, [23]. It is one of the components of the outer membrane of the cell and is the target of the Colistin, which led to the bacteria's resistance to this antibiotics [24]. The antibiotic resistance of *A.baumannii* to Ticarcyclin was (55%). while the percentage of bacterial resistance to the same antibiotic was (100%) [25]. The percentage of resistance to *A. baumannii* to Trimethoprim was (52.5%)." In another study conducted by [26], they showed that the proportion of bacteria *A baumannii* isolated from intensive care units to (66.3%). As for *A. baumannii* bacteria isolated from the surgical corridors, the percentage of antibiotic resistance to Trimethoprim was (59.9%), the resistance of *A. baumannii* to meropenim, was 37.5% in the current study. In another study conducted by [27], the percentage of bacterial resistance to the same antibiotic was 63.6%, while the percentage of bacterial resistance to Meropenim in a 2014 study at the Medical Research Institute in Malaysia was (57.3%) [28].

The percentage of antibiotic resistance to Piperacillin / tazobactam, in our current study was 55%. While the rate of antibiotic resistance to Piperacillin / tazobactam was (94%) in a study conducted by [29]. In another study the rate of resistance to Piperacillin / tazobactam was (85%) [30].

Detection of the *aacC1* gene:

Twenty isolates of *A. baumannii* were resistant to Aminoglycosides group (Gentamycin, Tobramycin, Amikacin), the results of the current study showed that 20 (100%) bacterial isolates were carrying the (*aacC1*) gene. It encodes the modified Aminoglycoside enzymes as shown in Figure (2) and Table (1), while the [6] showed in their study that *A. baumannii*

possesses the *aacC1* gene by (60.46%). The bacteria are resistant to Aminoglycoside because they possess *aacC1* genes that encode enzymes (Aminoglycoside modifying enzymes) including acetylases, adenylases and phosphorylase that degrade Aminoglycoside antibiotic, [31,32] showed that the genes encode to Aminoglycoside modified enzymes are located within Plasmids and Transposons, or among the first class of Integrons especially in *A. baumannii*, isolates that have high antibiotics resistance.

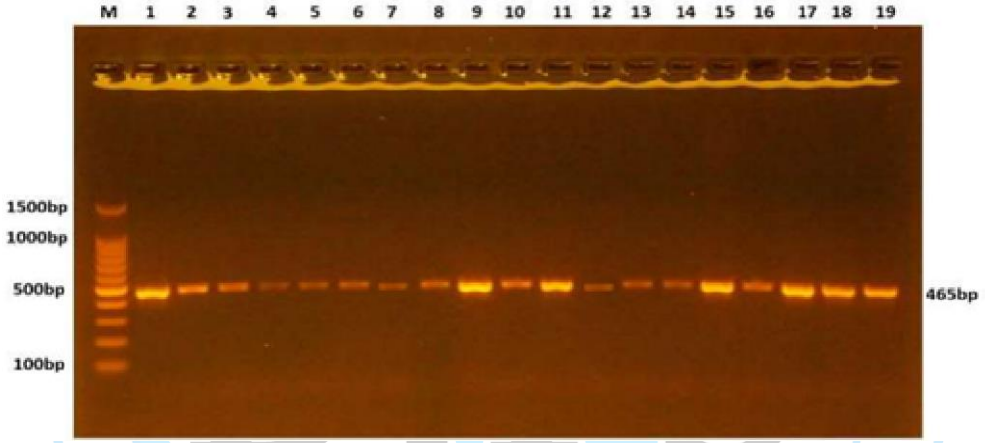

Figure 2: Agarose gel electrophoresis (2% agarose, 100 V/Cm for 80 minutes) and ethidium bromide staining to detect *aacC1* gene size product (465bp). Molecular size DNA Ladder (100 bp DNA Ladder); (1-6) isolated From *A.baumannii* (1-19) sample showed positive PCR.

Table 1: distribution bacteria's resistance and *aacC1* gene

No. of isolates	Antibiotics resistance	**No. (%)**	***Gene`s have aacC1***
1,4,9,10,11,16,17,18,20	Gentamycin , Amikacin	9 (45)	+
2,3,5,6,7,8,12,13, 14,15,19	,Gentamycin , Amikacin, Tobramycin	11 (55)	
Total resistance		20 (100)	100 %

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Measurement gene expression in the *aacC1* gene

The gene expression of the *aacC1* gene was measured by real time PCR as showed in Figure (3) and Table (2), an increase in gene expression was observed when the isolate was treated with the gentamycin antibiotic in concentration 1.2 µl/ ml.

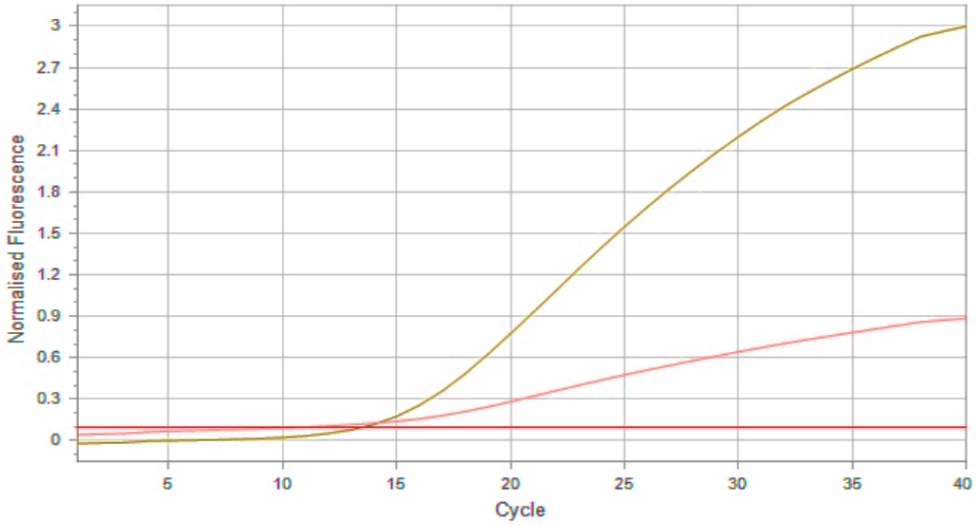

Figure 3: Gene expression result *aacC1* gene. , the yellow color represents the sample treated with the antibiotic gentamicin, the pink color represents the sample without treatment

Table 2: Ct, ΔCt, ΔΔCt values and fold ratio of *16srRNA gene and aacC1gene*

Samples	*16srRNA* Housekeeping gene CT	*aacC1* gene CT	ΔCT	ΔΔCT	Folding
positive control	7.15	13.60	6.45 -	0.00	1.00
Treatment with gentamicin antibiotic	12.67	11.50	1.18	7.63	198.12

Ct (cycling threshold), ΔCT (delta cycling threshold), ΔΔCT (delta delta threshold).

Conclusions

Acinetobacter baumannii has aacC1 resistance gene in (100%). The results also showed that there was an increase in the gene expression of *aacC1* gene after treatment with Gentamycin.

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