

ROLE OF RND-EFFLUX PUMP IN LEVOFLOXACIN RESISTANCE AMONG *ACINETOBACTER BAUMANNII*

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ABSTRACT

In this study out of 130 different clinical specimens, 70 *Acinetobacter baumannii* were collected from hospitalized patients. Then; all the isolates were characterized using biochemical tests and confirmed by using the Vitek II compact system. All isolates of *Acinetobacter baumannii* were subjected to the disc diffusion method to determine it's a susceptibility to 8 types of antibiotic disc, and the result showed these bacteria were resistant to Cefepime, Imipenem, trimethoprim, Piperacillin, tetracycline, Amikacin, ampicillin-sulbactam and levofloxacin. According to bacterial susceptibility test, all isolated were showed highly resistance to Trimethoprim (88.57%) and the lower resistance to tetracycline (47.14%). Minimum inhibitory concentration was determined only for levofloxacin resistant isolates of *Acinetobacter baumannii* using the broth dilution method. MIC shows these bacteria were resistant to levofloxacin in concentration ranging between 32µg/100µl to 128µg/100µl. The relation between *adeIK* and levofloxacin resistance was calculated by determining gene expression using real-time PCR. The result showed the mean value of *adeI* and *adeK* before exposing bacteria to levofloxacin was 2.3 and 0.42 respectively while the mean value of *adeI* and *adeK* after exposing bacteria to levofloxacin was 3.15 and 3.29 respectively. This study indicates that *adeI* has a non-significant role (p-value=0.11) in providing resistance to levofloxacin, while *adeK* plays a significant role (p-value= 0.008) in providing resistance to levofloxacin.

Keywords: disc diffusion method, gene expression, real-time PCR, MIC.

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دور مضخة تدفق RND في مقاومة الليفوفلوكساسين بين بكتريا *Acinetobacter baumannii*.

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باحث

قسم التقنيات الاحيائية، كلية العلوم، جامعة بغداد، بغداد، العراق.

المستخلص

في هذه الدراسة من أصل 130 عينة سريرية مختلفة ، تم جمع 70 من بكتيريا البوماني من المرضى المقيمين في المستشفى. تم توصيف جميع العزلات باستخدام الاختبارات البيوكيميائية والتأكد من ذلك باستخدام نظام Vitek II. تم إخضاع جميع عزلات *Acinetobacter baumannii* لطريقة الانتشار القرصي لتحديد مدى حساسيتها لثمانية أنواع من أقراص المضادات الحيوية ، وأظهرت النتيجة أن هذه البكتيريا كانت مقاومة لـ Cefepime و Imipenem و Tremethoprim و Piperacillin و tetracycline و Amikacin و ampicillin-sulbactam و levofloxacin. وفقا لاختبار الحساسية الجرثومية ، أظهرت جميع المعزولات مقاومة عالية لتريميثوبريم (88.57%) ومقاومة أقل للتتراسيكلين (47.14%). تم تحديد الحد الأدنى للتركيز المثبط فقط للعزلات المقاومة لليفوفلوكساسين من *Acinetobacter baumannii* باستخدام طريقة التخفيف المرق. أظهر MIC أن هذه البكتيريا كانت مقاومة لليفوفلوكساسين بتركيز يتراوح بين 32 ميكروغرام / 100 ميكرو لتر إلى 128 ميكروغرام / 100 ميكرو لتر. تم حساب العلاقة بين مقاومة *adeIK* و levofloxacin من خلال تحديد التعبير الجيني باستخدام PCR في الوقت الحقيقي. أظهرت النتائج أن متوسط قيمة كل من *adeI* و *adeK* قبل تعريض البكتيريا لليفوفلوكساسين كان 2.3 و 0.42 على التوالي بينما كان متوسط قيمة *adeI* و *adeK* بعد تعريض البكتيريا لليفوفلوكساسين 3.15 و 3.29 على التوالي. تشير هذه الدراسة إلى أن *adeI* له دور غير مهم (قيمة p = 0.11) في توفير مقاومة الليفوفلوكساسين ، بينما يلعب *adeK* دورًا مهمًا (قيمة p = 0.008) في توفير مقاومة الليفوفلوكساسين.

كلمات مفتاحية: طريقة الانتشار القرصي، التعبير الجيني، real-time PCR، أقل تركيز تثبيطي

INTRODUCTION

Acinetobacter is gram negative when stained with gram stain and is non-motile, non-lactose fermentative and are strictly aerobic bacteria which mean it cannot live in the absence of oxygen (3, 18). *Acinetobacter* genus contains a different number of species, but the most clinically important species among this genus is *Acinetobacter baumannii* (2). *A.baumannii* is commonly found in the soil, water and particularly in the hospitals environment (10). *A.baumannii* has quickly emerged as a major opportunistic pathogen and a major cause of morbidity and mortality in healthcare settings in recent years (11, 13). This bacterium is able to cause infection in a deferent site of the human body which is able to cause UTI, bloodstream infection, Pneumonia and wound infection (4, 6). *A.baumannii* it has been classified as one of the major causes of nosocomial infection that belong to the ESKAPE organisms (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* species) a group of pathogens of clinical significance with the ability for antimicrobial resistance (9). These bacteria have a number of mechanisms that enable them to providing resistance to antibiotics among of these mechanisms is the presence of efflux pump (1). Efflux pump is found in both gram negative and gram positive and are responsible for avoiding antibiotic from affecting bacteria by secreting it outside of their cell (14). Each efflux pump consists of three components which is the transporter of the inner membrane and the channel of the outer membrane and finally lipoprotein of periplasmic space. The efflux pump can be classified into 5 superfamilies based on the similarity in the amino acid and energy source, this classification includes; resistance-nodulation cell division (RND), major facilitator superfamily (MFS), multidrug and toxic compound extrusion (MATE), the ATP-binding cassette (ABC) and small multidrug resistance (SMR), all these family identified in *A.baumannii* (8). Among of these efflux pump, the RND superfamily is the most important pump in *A.baumannii*, and these superfamily includes *adeABC*, *adeFGH* and *adeIJK* pump

that are the most prevalent in these bacteria (20). The first pump of RND superfamily was *adeABC* first discovered in 2001 by Magnet (15), and they are located in the chromosome of *A.baumannii*, and it is transcription regulated by *adeRS*. The second pump of RND super family was *adeIJK* was first described in *A.baumannii* by Damierpiolle in 2008 (8). The transcription of *adeIJK* is regulated by *adeR* and this pump found in all strain and it is responsible for providing resistance to large number of antibiotics. While *adeFGH* was described in 2010 by coyne (7), it is transcription is regulated by *LysR-type* transcriptional regulator *AdeL*. This study was aimed to determine the role of two genes (*adeIK*) of the RND-*adeIJK* efflux pump in providing resistance to levofloxacin.

MATERIALS AND METHODS

Isolation and identification of *A.baumannii*

The study was approved by the ethical committee of Collage of Science University of Baghdad (CSEC/0121/0010). In this study, 70 *A.baumannii* isolates were collected from 130 different specimens (urine, respiratory secretions, catheters, wound and burned surface) that were collected from different hospitals in Baghdad city during the 3 months from August 2020 to November 2020. All isolates identified with biochemical test and confirmed with the Vitek II device.

Antibiotic susceptibility

Disk diffusion method was conducted to study the susceptibility of all isolates of *A.baumannii* using eight disks including Ampicillin-sulbactam, Trimethoprim, Tetracycline, Piperacillin, Cefepime, Amikacin, Levofloxacin, and Imipenem. The results were interpreted based on the CLSI guideline 2020 (6)

Minimum inhibitory concentration (MIC)

The MICs of Levofloxacin for *A. baumannii* were determined using a broth Microdilution method in which antibiotic dissolved in Muller Hinton broth, after that it was transferred to the microdilution plate with a starting concentration of approximately 2, 4,8,16, 32, 64 and 128µg/100 µl. The result of MIC was interpreted according to the guideline of CLSI (2020) after 18hrs of growth (6).

Extraction of DNA: Genomic DNA was extracted from all bacterial isolates using DNA extraction Kit (WizPrep Company, Korea).

Molecular detection of *adeI* and *adeK* genes of RND

Conventional PCR was carried out for detection the presence of *adeIK* genes of the RND efflux pump. The sequence of primers used in this study is shown in the table (1-1).

Table (1.1) all primer sequences used in this study

Gene name		Primer sequence 5'→3'	Product size	References
<i>AdeI</i>	F	GTAGCAAAGGCTCCGATGAG	233 bp	Designed according to NCBI
	R	GAGCTCATAAAGCGCCTGAC		
<i>FOR qPCR</i>	R2	GCTGAAGTACGGCCTGAAAG	130 bp	
<i>AdeK</i>	F	CACAGAACAACCAGCTTCCA	222 bp	
	R	TTGAGTCGAATCACGAGCAC	130 bp	Designed according to NCBI
<i>FOR qPCR</i>	R2	GCAGTTACACCCAAACCTACTTG		
<i>gyrA</i>	F	AAATCTGCCCGTGTCTGTTGGT	344bp	(12)
	R	GCCATACCTACGGCGATACC		

The conventional PCR program for detecting the presence of *adeIK* was begun with an initial denaturation step at 95°C for 5 min, only one cycle, followed by denaturation for 30 seconds at 95°C, 30 cycles. After that, the reaction mixture enters into 35 cycles of annealing step (annealing temperature was 58 °C for *adeI* while for *adeK* was 60 °C) for 30 seconds. Then the reaction mixtures proceed to enter 35 cycles of extension step for 1 minute. Finally, the amplification stops after 5 minutes of final extension step at 72°C. A 2% agarose gel containing ethidium bromide was used to detect the PCR products and then the gel was observed using UV light.

Extraction of RNA

Extraction of RNA from *A.baumannii* is necessary to determine the gene expression for the *adeIK* gene of RND-efflux pump. In this study, RNA was extracted by using RNA extraction kit (TransGen Biotech Company, China).

Complementary DNA synthesis from Mrna

The conversion of mRNA into complementary DNA was done with specific KIT (EasyScript® Company, china). All components that required to convert mRNA into cDNA was mixed together and the conversion was done using Conventional PCR, in three steps. the step; is started with 25 °C for 10 minutes in which random primers anneal to mRNA than

the reaction mixture proceeds into step two in which reaction temperature began to rise into 42 °C for 30 minutes to allowing the enzyme reverse transcriptase mRNA into cDNA. Finally, the reaction mixture proceeds to enter step three in which the temperature raised into 85 °C for 5 minutes in order to terminate the reaction.

Quantitative Real-Time PCR (qRT-PCR):

Gene expression of *adeIK* genes of the RND-efflux pump and its correlation with levofloxacin resistance in *A.baumannii* was determined by using real-time quantitative PCR. In this technique SYBR green dye was used as fluorescence dye, this dye can bind only with double-strand DNA and emit light after binding with DNA. The fluorescence light can be measured by real-time PCR. this technique was performed using Corbett Real-time PCR System (German) in this step 10µl of 2x QPCR master mix (contain SYBER GREEN) are mixed with 2µl of the primer, 2µl cDNA and the volume was completed into 20µl through the addition of 6µl of nuclease-free water. The qPCR program began with initial denaturation step at 95 °C for 30 seconds (for enzyme activation), followed by denaturation for 5 seconds at 95°C, 40 cycles. After that, the reaction mixture enters into 40 cycles of annealing and extension step (annealing temperature was 58 °C for *adeI*

while for *adeK* was 60 °C as well as for *gyrA* was 58-60 °C) for 30 seconds. Finally, the amplification stops after 1 minute of dissociation step at 95 °C.

RESULTS AND DISCUSSION

In this study, from 130 specimens only 70 bacterial isolates were diagnosed as *A.baumannii* by biochemical test and confirmed with CHROMagar and Vitek 2 compact. The distribution of *Acinetobacter baumannii* was as following: 25.38% from the burned skin surface, 10% was from medical equipment while it was 60% from wounds as well as it was 4% from UTI as shown in Figure (1.1)

Results of antibiotic susceptibility:

Out of 70 *A. baumannii* isolates, most of them were resistant to Trimethoprim in percentage (88.57%) and this rate of resistance was decreased in Piperacillin were become (82.85%), The rate of resistance continued to decrease in other antibiotics as the resistance of bacteria to Cefepime was 71.42% while its resistance to Ampicillin-Sulbactam and Amikacin was in the same percentage 61.42%, Imipenem 54.28% and Tetracycline 47.14%. Finally, 36 isolates out of 70 isolate it was resistance to levofloxacin in the percentage of 51.42%. Trimethoprim and Piperacillin were observed to be the most antibiotics effective against *A.baumannii* as shown in the figure (1.2).

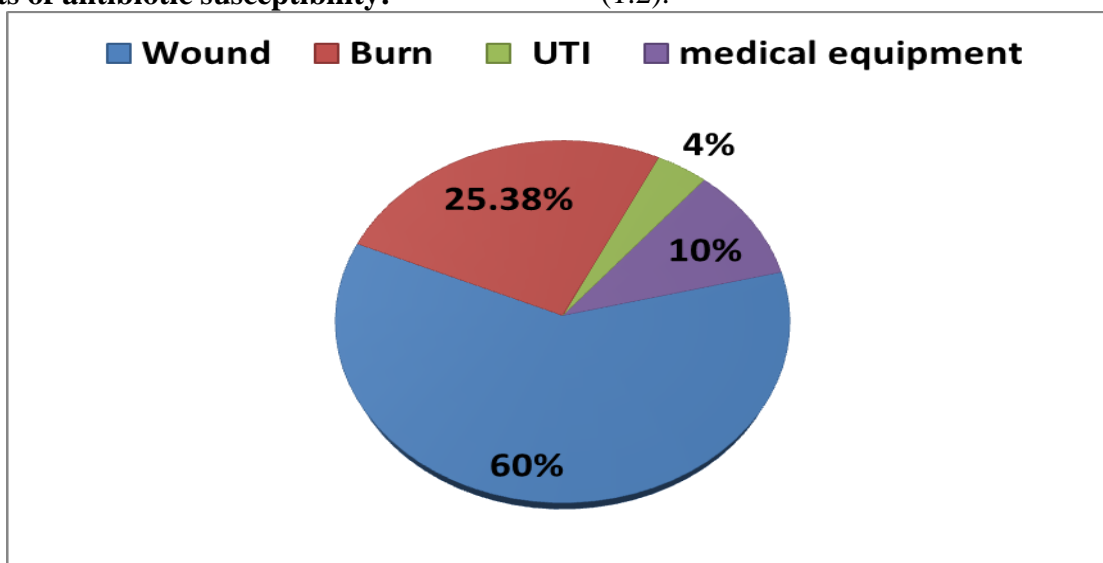


Figure (1.1) Distribution of *A.baumannii* Isolates based on the site of infection

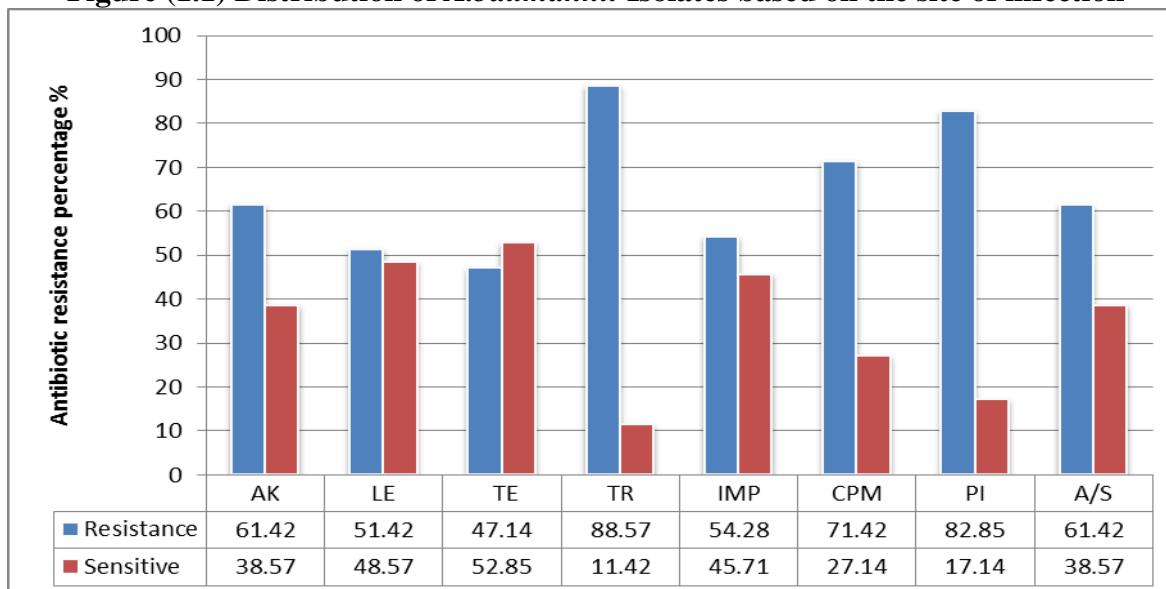


Figure (1.2) The pattern of antibiotic resistance of *Acinetobacter baumannii*.

Cefepime (CPM), Ampicillin-Sulbactam (A/S), Imipenem (IPM), Levofloxacin (LE), Piperacillin (PI), Tetracycline (TE), Trimethoprim (TR) and Amikacin (AK).

There are many reasons that make this bacteria resistance to all tested antibiotics, but the most important reasons for these bacteria is they have efflux pump especially RND- efflux pump which is divided into more than one pumps such as *adeABC*, *adeFGH* and *adeIJK* all of these pump responsible for specific role as well as all of them responsible for providing resistance to a specific groups of antibiotics (16).

PCR result for *adeI* and *adeK* genes

The conventional PCR was successfully performed and 222 bp and 233 bp bands were amplified as *adeK* and *adeI* genes, respectively. The results showed that the frequencies of *adeK* and *adeI* were present in all isolates of *A.baumannii* with one hundred percent. Figure (1.3) and (1.4) show the presence of *adeK* and *adeI* in all isolate of *Acinetobacter baumannii*. The obtained result about the presence of these genes in all isolate of *A.baumannii* was consistent with the work of (19).

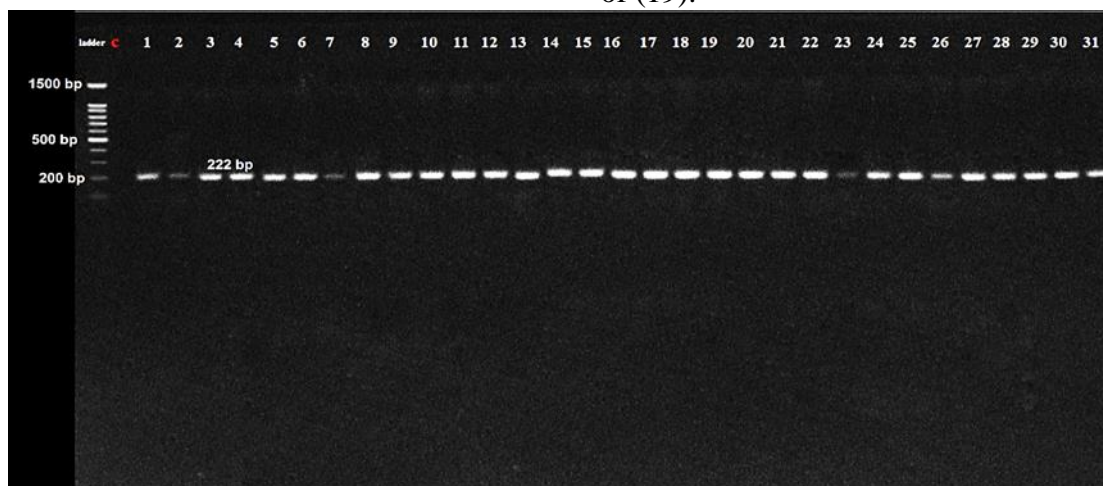


Figure (1.3a) The presence of *adeK* (222 bp) in all isolate of *A.baumannii*. The picture taken after the amplified product was electrophoresed in 2% of agarose and the gel was electrophoresed at high speed for 45 minutes using bioneer gel electrophoresis (Korea) and screened using a UV transilluminator.

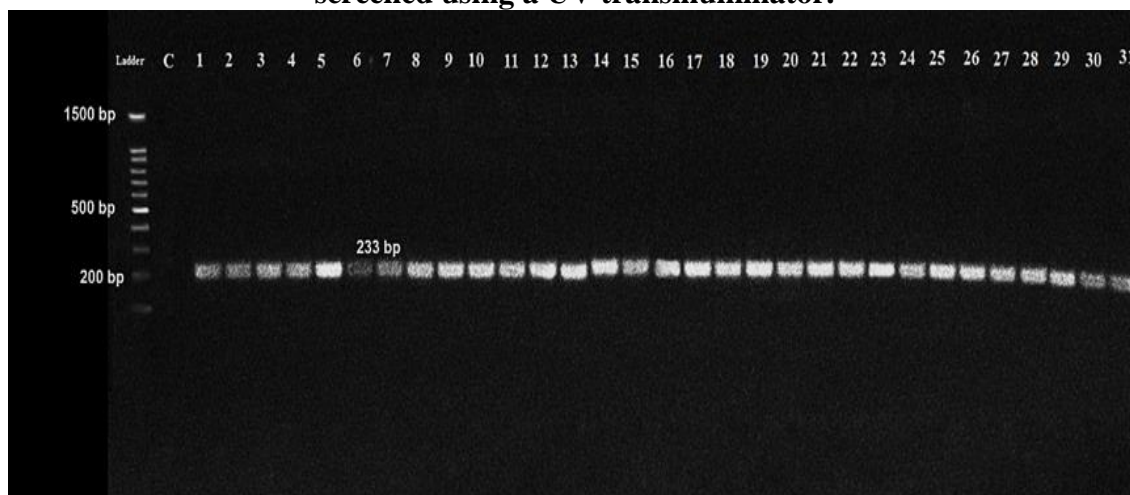


Figure (1.3b) The presence of *adeI* (233 bp) in all isolate of *A.baumannii*. The picture taken after the amplified product was electrophoresed in 2% of agarose and the gel was electrophoresed at high speed for 45 minutes using bioneer gel electrophoresis (Korea) and screened using a UV transilluminator

Result of minimum inhibitory concentration

The minimum inhibitory concentration was determined for only some bacterial isolate because these isolates were resistance to all tested antibiotics (extensively drug resistance (XDR) *A.baumannii*. The result showed that

the MICs of Levofloxacin against clinical isolates of XDR *A. baumannii* were ranged between 32/100 μ l to 128/100 μ l. While the result showed that the MICs of Levofloxacin in sensitive strains of *A.baumannii* was 2/100 μ l. The purpose of determining the

minimum inhibitory concentration of levofloxacin is to exposing some resistance strain and sensitive strain of *A.baumannii* isolates under the pressure of this antibiotic in order for determining the gene expression of *adeK* and *adeI* and its correlation with levofloxacin resistance

Gene expression of *adeI* and *adeK* genes

The gene expression of *adeK* and *adeI* was measured by using real-time quantitative PCR utilizing SYBR green dye. The amplification of genes was recorded as CT (Cycle Threshold), in which increased CT values indicate lower gene expression and lower CT

values indicate higher gene expression. The relative mean of gene expression of *adeI* and *adeK* genes before exposure to levofloxacin was 2.3 and 0.42 respectively while the relative mean of gene expression of *adeI* and *adeK* genes after exposure to levofloxacin was 3.1 and 3.2 respectively. The results showed no statistically significant difference in the *p-value* ($P=0.1$) of the *adeI* gene but, there was a significant difference in the *p-value* ($P=0.008$) of the *adeK* gene between the resistance *A. baumannii* before exposure and after exposure to levofloxacin.



Figure (1.5) Microdilution plate indicates that the growth of XDR *A.baumannii* was inhibited at 32/100µl, 64/100µl and 128/100µl. While sensitive *A.baumannii* was inhibited at 2/100µl

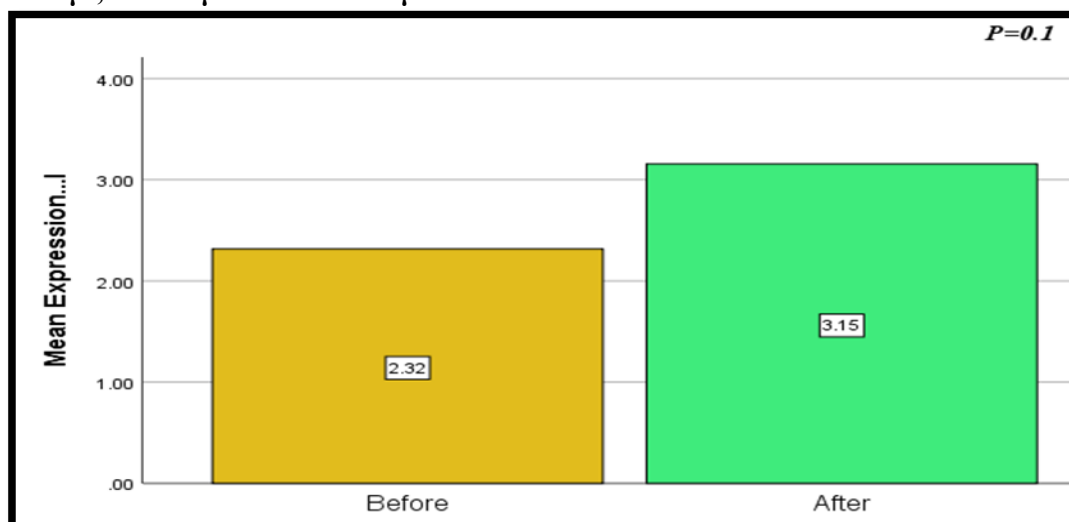


Figure (1.5) The expression level of *adeI* gene before exposure to levofloxacin was 2.3 and after exposure to levofloxacin was 3.2 with *P-value* 0.1 which show there are no significant difference

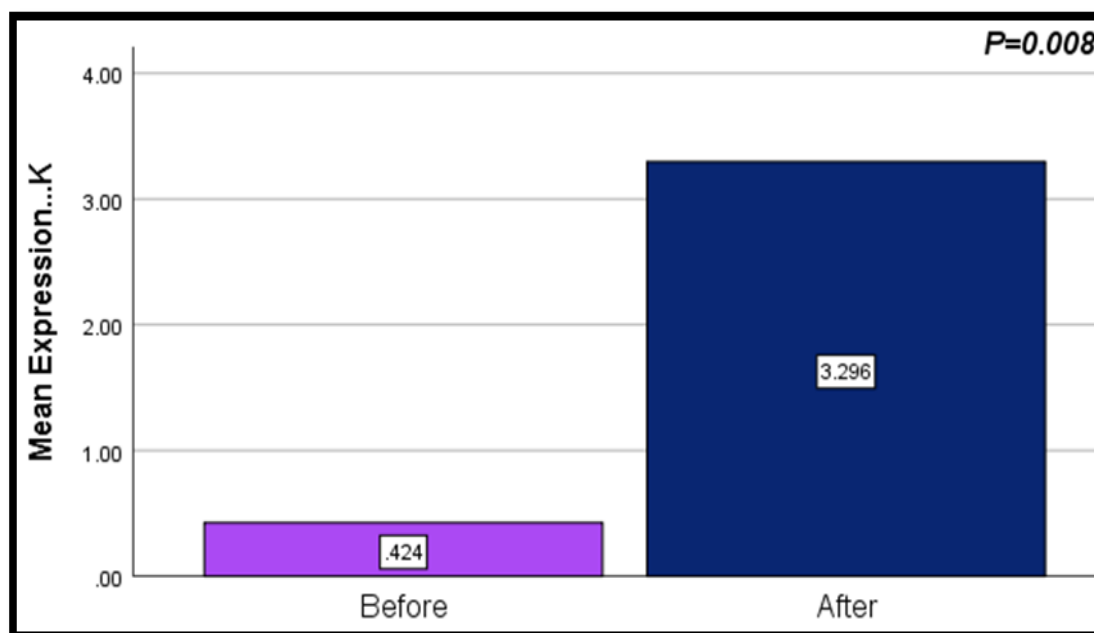


Figure (1.6) The expression level of *adeK* gene before exposure to levofloxacin was 0.4 and after exposure to levofloxacin was 3.29 with *P*-value 0.008 which show there was large significant difference

The most prevalent cause of nosocomial infections is gram negative bacteria. These infections have been one of the main medical issues. *Acinetobacter baumannii* is an opportunistic pathogen in hospitals that causes a large spectrum of nosocomial infections. Owing to the indiscriminate use of large spectrum antibiotics by people, we are observing a strong resistance to antibiotics caused by this bacterium. The strong antibiotic resistance of this bacterium is associated with the dissemination of several antibiotic resistance genes. Different experiments indicate that *Acinetobacter baumannii* resists most antibiotics from and quinolones and beta lactam and increases its resistance to aminoglycoside (5). Hence, we noted an increase resistance of *A.baumannii* to levofloxacin which promotes us to study the role of *adeI* and *adeK* genes of RND efflux pumps. In this study 70 isolate of *A.baumannii* was undergone for molecular detection of these genes and result show the presence of these genes in all isolates. After detection was done the gene expression of these genes were measured in order to determine the role of these gene with levofloxacin resistance and the result showed *adeI* gene not participate in providing resistance to levofloxacin while *adeK* play a significant role in providing resistance to levofloxacin with *p*-value 0.008.

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