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Estimation the levels of two genes expression and their effects on tetracycline resistance of *Acinetobacter baumannii* isolated from different sources

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Abstract. *Al-Miyah SAF. 2023. Estimation the levels of two genes expression and their effects on tetracycline resistance of* Acinetobacter baumannii *isolated from different sources. Biodiversitas 24: 176-181. Acinetobacter baumannii* can cause severe infections of the blood, brain, and lungs. It may also results in infections of the urinary tract and wounds. It can be found on the skin, in food, drink, or soil, and can be transferred by direct contact. The pathogen *Acinetobacter baumannii* spreads quickly. This study aimed to find a relationship between the infection with *Acinetobacter baumannii* and the corresponding IL-17 level in patients' sera. To determine the effect of tetracycline exposure on the gene expression levels of the *TetA* and *AdeB* genes. This study included 150 samples from different patients suffering from burns, wounds, blood, and sputum. Samples have been tested by Vitek for the presence of *Acinetobacter baumannii*. Each isolate was then cultured on two separate Mueller-Hinton Agar (MHA) dishes, one with and one without tetracycline. RNA was extracted from all the cultured dishes and converted to cDNA. Quantitative real-time polymerase chain reaction (qRT-PCR) has then been done to evaluate the gene expression level of the *TetA* and *AdeB* genes and normalized to the results of the *16sRNA* gene. The results of gene expression showed that isolates grown with tetracycline in the medium had significantly higher gene expression than isolates grown without the antibiotic for both the *TetA* (2.49 vs. 1.023, respectively) and *AdeB* (5.051 vs. 1.023, respectively) genes. The results of IL-17 showed a higher level in infected patients (21.37 pg/mL) compared to non-infected subjects (14.76 pg/mL).

Keywords: *Acinetobacter baumannii*, *AdeB*, Genes expression, IL-17, *TetA*, qRT-PCR

INTRODUCTION

Infections such as bacteremia, meningitis, and pneumonia related to the use of ventilators, infections of the skin and soft tissues, peritonitis, and urinary tract infections are all brought on by the significant human pathogen *Acinetobacter baumannii* (Dahdouh et al. 2017). The multidrug resistance (MDR) of *A. baumannii's* capacity to endure and thrive in a variety of hospital settings and dry conditions has elevated it to the status of a major global source of nosocomial infections (Shirmohammadlou et al. 2018). One of the critical elements is *Acinetobacter baumannii's* ability to colonize and form biofilm on biotic and abiotic surfaces. Influencing chronic and persistent infections as well as antimicrobial resistance (Thummeepak et al. 2016). Acinetobacter species' capacity to endure in a hospital setting on a variety of dry and wet surfaces makes them a common, saprophytic, and significant nosocomial pathogen (Engür et al. 2014). Pneumonia, which frequently occurs in patients with tracheostomies or endotracheal tubes, UTIs, skin and wound infections, peritonitis in peritoneal dialysis patients, endocarditis, meningitis, and bacteremia are all human infections caused by *Acinetobacter* species (Momtaz et al. 2015).

One of the most recently discovered bacteria worldwide is *Acinetobacter* spp., and in particular *Acinetobacter baumannii* (Nguyen and Joshi 2021). *Acinetobacter baumannii* colonizes the skin, urinary tract, gastrointestinal, respiratory, and urinary systems. It commonly causes blood circulation infections, such as wound and urinary tract infections, meningitis, mediastinitis, osteomyelitis, and cholangitis (Feng et al. 2022). *Acinetobacter baumannii* is susceptible to both endogenous and acquired antibiotic and antiseptic resistance due to efflux pumps. Antibiotic intra-accumulation is decreased by bacterial efflux mechanisms, which raise the MIC, or minimal inhibitory concentration (Liu et al. 2018). The resistance nodulation division superfamily (RND) and the multidrug and compound extrusion family (MDE) (RND) (MATE) are parts of the efflux system of *A. baumannii* (Coyne et al. 2011). Tetracycline resistance genes in Gram-negative bacteria, such as *TetA*, have been especially addressed by the development of tigecycline in the latter half of the 20th century (Sumyk et al. 2021). *TetA* demonstrates an increase in antimicrobial potency against clinically significant infections (Jahantigh et al. 2020). Due to tigecycline's higher binding affinity for the ribosome than other tetracyclines, it has more antibacterial action. Notably, the antibiotic tigecycline has been able to evade *TetA-mediated* extrusion due to the bulky 9-tert-butyl-glycylamido moiety at position C9. This is probable because *TetA is sterically* hampered by this substituent (Sumyk et al. 2021).

The complexity of bacterial efflux pumps is brought on by clinically relevant antibiotic resistance, bacterial colonization, and bacterial persistence in the host (Ranjbar et al. 2020). A chromosome-encoded pump is a member of the RND-type superfamily of tripartite efflux machinery (Alav et al. 2021). The *adeA* (membrane fusion), *AdeB* (multidrug transporter), and *adeC* (outer membrane protein) genes make up the AdeABC efflux pump. Two-component regulatory systems, adeR and adeS, are present to regulate these genes in close proximity (Ranjbar et al. 2020). The highly immunostimulatory lipopolysaccharide (LPS) molecule on the surface of a Gram-negative bacterium such as *A. baumannii is* expected to cause potent reactions in designed human cells, including mouse splenocytes and the human monocytic cell line THP-1. Other cell subsets, including T cells, cytotoxic CD8+ T cells, innate tissue-specific cells, innate lymphoid cells (ILCs), and myeloid cells, have the ability to release IL-17A. Host defense and survival against infection depend on IL-17A-mediated inflammation. Additionally, IL-17A has been linked to immunopathology and has been shown to worsen fetal inflammatory responses. Multiple inflammatory diseases, such as sepsis, pneumonia, systemic lupus erythematosus, rheumatoid arthritis, allograft rejection, and cancer, are associated with high levels of IL-17A. We hypothesize that the T helper cells that produce IL-17, based on the compelling evidence supporting the IL-17 pathway's regulation of neutrophil recruitment, are involved in mediating the clearance of *A. baumannii* (Feng et al. 2022).

MATERIALS AND METHODS

Samples collection

This study included 150 samples isolated from different patients suffering from burns, wounds, and also patients having chronic kidney failure. Patients who were suspected to have infection with another species have been excluded, those patients were attending to Al-Yarmook Teaching Hospital/ Baghdad, Iraq. All the isolates of bacteria were cultivated by the streak method on blood agar and incubated for 24 hours in a temperature of 37°C. The test tube has added standard sterile saline (3.0 mL). To transfer sufficient pure-crop colonies and suspend the isolated colonies in the normal saline, an application stick or sterile swab was used. The turbidity (0.5-0.63) of McFarland was adjusted and Densi ChekTM was used. Then the prepared bacterial suspension transferred to the GN carb which was sealed and placed in the VITEK system (BioMe'rieux) to detect the positivity of the samples for *Acinetobacter baumannii*. Serum samples was also collected from each patients and kept at -20°C for analysis of IL-17 serum level estimation.

Antibiotic resistance test

Disk diffusion method was used to detect the resistance capability against tetracycline (30 µg) antibiotic based on recommendations from the Clinical and Laboratory Standards Institute for 2015. This was accomplished using the Medium Mueller-Hinton agar from Merck (Germany).

Evaluation of the genes expression

The strain was raised in two MH dishes for expression research. Agar at 37°C. The first agar dish was supplemented with 14 µg/mL tetracycline which is concentrations represent therapeutic concentrations. The other dish of agar has left without antibiotic, as a control for gene expression technique.

RNA was extracted by utilizing the Quick-RNA Fungal/Bacterial Miniprep Kit (ZYMO, USA). Concentration and purity of RNA were done for samples by using Quantus™ Fluorometer (Promega/ USA) and PromegaQuantiFluor® Dye Systems (Promega/ USA). The 1X TE buffer was prepared by diluting 20X Tris EDTA (TE) Buffer (pH 7.5) to 1X with nuclease-free water. The QuantiFluor® Dye working solution was diluted till 1:400 with 1X TE buffer. The blank sample was prepared by adding 200 µL of QuantiFluor® Dye working solution prepared in to a 0.5 mL PCR tube (without RNA sample or standard). For the QuantiFluor® ONE dsDNA System standard, 1 µL of the standard was added to 200 µL of QuantiFluor® ONE dsDNA Dye. Mixture was mixed three times by pipetting slowly. Tubes were centrifuged at 2,000 × g for 5-10 seconds to collect liquid at the bottom of the tube and remove any bubbles present. Tubes were Incubated at room temperature for 5 minutes, protected from light. Then the prepared tubes of blank and standard placed in the Quantus apparatus to be read and calculate the standard to blank ratio.

The samples then prepared by adding 1 µL of the RNA to 200 µL of the prepared working assay, then placed in the calibrated Quantus apparatus to be read. Eluted RNA converted to cDNA by adding 8µL of the RNA to a new sterile PCR tubes then the volume completed to 10 µL by adding 2 µL of the commercial kit prime script provided by Takara (Japan, Cat#RR014A), then the tubes contain reaction mixture was incubated under 37°C, for 15 minutes (Reverse transcription) then 85°C, for 5 sec (Inactivation of reverse transcriptase with heat treatment) 4°C. The prepared cDNA then kept until the day of the PCR in the deep freezer.

Using certain primers, specific regions of the *Acinetobacter baumannii* genome have been amplified. Table 1 lists the primer sequences for each pair (1). Prior to being dissolved in free ddH2O, the primers were lyophilized to create a stock solution with a 100 pmol/L ultimate concentration. They were then kept in a stock. A final volume of 100 was created by combining 10 L of the stock solution with 90 L of free ddH2O. 10 L of the stock solution was then heated to -20 to form a suspended work primer solution with a concentration of 10 pmol/L.

The Real-time PCR reaction mixture was prepared using 10 µL of KAPPA SYBR® FAST master mix (Kappa research, USA), 5 µL of DNA, and 0.5 µL of forward primer, 4 µL of nuclease-free water and 0.5 µL of reverse primer in a brand-new tube. The tubes were then sealed and placed in the thermal cycler device, which was designed to do 35 cycles, beginning at 94°C for 35 s, 60°C for 1 minute, and finishing at 72°C for 7 minutes. Real-time PCR is utilized to quantify the amplification process which has been detected using the FAM channel since the KAPA master mix contains sybr green dye to detect the amplification.

Serum level of IL-17 have been measured by following the instruction provided by the manufacturer of ELISA kit (Cat.No.:E-EL-H0105) by Elabscience® (USA).

Statistical analysis

Microsoft Excel and SPSS version 24.2 (SPSS Inc. Chicago, Illinois, United States) were both utilized in the statistical analysis for this investigation. Two categorical parameters have been analyzed using the chi-square approach. P-value is predicted to occur with a chance of 0.05. Pearson's correlation was calculated using SPSS to evaluate the relations between each studied parameter. T-test test was also done to evaluate the differences among more than two groups.

RESULTS AND DISCUSSION

Results

The distribution of the samples according demographic characteristics is shown in table 2. According to age group, the higher number of samples were ranged between 21-30 (24 samples) of which 4 were positive, followed by age group older than 61 (22) and 11 were positive, age group 51-60 (18) and 6 of those samples were positive, 41-50 years (17 samples) and 5 were positive, age group 31-40 years (14) in which 8 samples were positive, and the lower number of samples were shown in age group younger than 20 (5 samples) from which only one sample was positive.

According to blood group, the higher samples number are shown in O+ (30 samples and 15 were positive) followed by AB+ (25 samples and 7 were positive), B- (15 samples and 3 were positive), A+ (13 samples and 2 were positive), A- (7 samples and 4 were positive), B+ (5 samples and 3 were positive), AB- (4 samples and 1 was positive) and the lower number shown by O- (1 sample which showed negative results).

According to gender, the male samples were slightly higher (56 samples) than females (44 samples). But results of infection detection showed higher number of female samples were positive than male 25 vs.

The results of the real-time PCR cycle were shown as logarithmic curves that showed the amplification of the chosen genomic sequence. The outcomes depicted in Figure 1. 35 samples were found to be positive for the *16sRNA* gene. The sample was judged positive if the real-time PCR curve indicated an increase.

The results of disc-diffusion method revealed that from the 35 positive samples by the results of *16sRNA* gene amplification in the RT-PCR 26 (74%) of them were resistant to tetracycline antibiotic and the rest of samples were 9 samples were sensitive to the antibiotic (26%) as shown in the Figure 2.

The results of gene expression showed a significant higher gene expression in isolates that grown with a tetracycline in the medium than the isolates that grown without the antibiotic for both *TetA* (2.49 vs. 1.023, respectively) and *AdeB* (5.051 vs. 1.023, respectively) genes. The results are presented in Figure 3.

The results showed higher serum level of IL-17 in subjects that showed a positive infection to *Acinetobacter baumannii* (21.37± 0.39 pg/mL) compared to none-infected subjects (14.76 ± 0.13 pg/mL). The difference between the two groups was significant (P-Value= <0.001) (Figure 4).

In order to investigate the relation among each of the studied parameters a pearson's correlation has been calculated (table 3). The results showed a significant positive correlation between *TetA* gene expression and IL-17 level (r= 0.659, P-Value= 0.018). IL-17 also showed a significant correlation with the gene expression level of *AdeB* gene (r=0.823, P-Value= 0.043). The correlation between gene expression levels of the two genes *TetA* and *AdeB* failed to show a significant relation (r=-0.10, P-Value= 0.568).

Discussion

Interleukin-17 which plays a role in both inflammation and protective antimicrobial immunity, is particularly interesting in terms of periodontitis' pathogenesis (Khader et al. 2009). In the latter regard, it has been shown that IL-17 mediates the body's response to extracellular pathogens (Hernández-Santos and Gaffen 2012) and another cytokine produced by Th17 and other IL-17-expressing cells is IL-22 that can stimulate the synthesis of antimicrobial peptides believed to be protective in periodontitis, should be used in conjunction with this (Liang et al. 2006). The results of this study agreed with those facts, since the patients who have a positive diagnosis of *Acinetobacter baumannii* showed a higher level of IL-17.

Table 1. Sequences of primers used for qRT-PCR

Gene	Nucleotides sequences	Reference
TetA	CGGTCTTCTTCATCATGCAACTC GTCCCAGTGAAAGCGATCC	Møller et al. (2016)
AdeB	AACGGACGACCATCTTTGAG CAGTTGTTCCATTTCACGCA	Rafiei et al. (2022)
16sRNA	CAGCTCGTGTCGTGAGATGT CGTAAGGGCCATGATGACTT	Rafiei et al. (2022)

Table 2. Distribution of samples according to demographic characteristics

Parameter		Number	Positive infection (%)
Age	<20	5	1 (2)
	21-30	24	4 (11)
	31-40	14	8 (22)
	41-50	24	5 (14)
	51-60	44	6 (17)
	>61	39	11 (31)
Blood	A+	23	2 (5)
	A-	17	4 (11)
	B+	10	3 (17)
	B-	15	3 (8)
	AB+	50	7 (2)
	AB-	4	1 (3)
	O+	30	15 (34)
	O-	1	0 (0)
Gender	Male	76	10 (28)
	Female	74	25 (71)

In previous study, few data are available on the phenotypic and genotypic characterization of resistance to these bacteriostatic drugs among *A. baumannii* strains in Iran, and tetracycline resistance is not routinely studied for multidrug resistant *A. baumannii*. Based on an E-test (MIC50 = 32 g/mL and MIC90 = 512 g/mL), the majority of the *A. baumannii* strains in this investigation (n = 89/100; 89%) were provisionally classed as tetracycline resistant. MIC values of 16 g/mL, the suggested clinical breakpoint of CLSI to indicate resistance to tetracycline, minocycline, and doxycycline, were later used to confirm the isolates' resistance (Maleki et al. 2014). The same study disagreed with this study in respect to the prevalence of tetracycline which showed lower frequency of tetracycline resistance (74%) in this study.

Table 3. Correlation between each of TetA gene expression, AdeB gene expression and IL-17

Parameter		**IL-17**	***TetA***	***AdeB***
IL-17	Pearson Correlation	1	0.659	0.823
	P-Value	0.0	0.018	0.043
TetA	Pearson Correlation		1	-0.100
	P-Value			0.568
AdeB	Pearson Correlation			1
	P-Value			

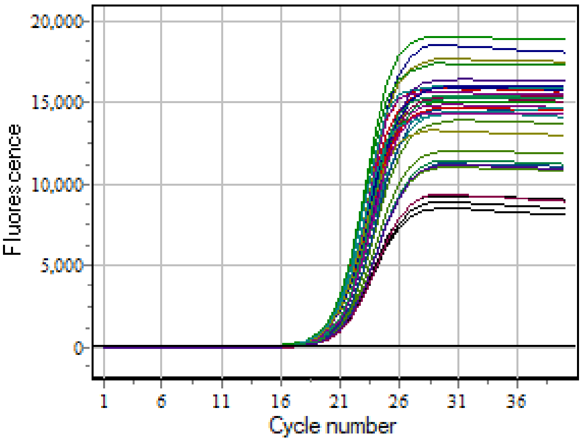

Figure 1. Real time-PCR amplification plot results

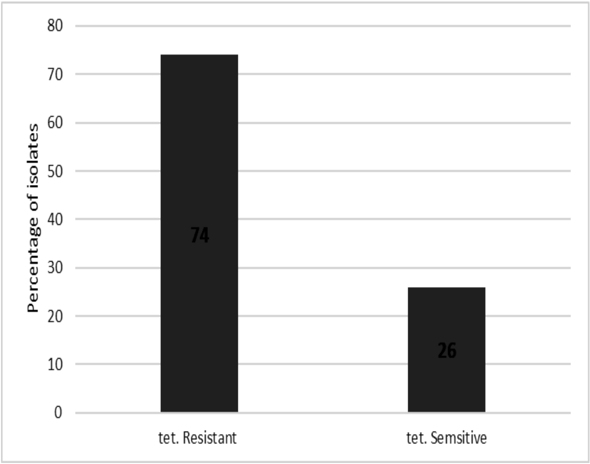

Figure 2. percentage of resistance and sensitive isolates to the tetracycline

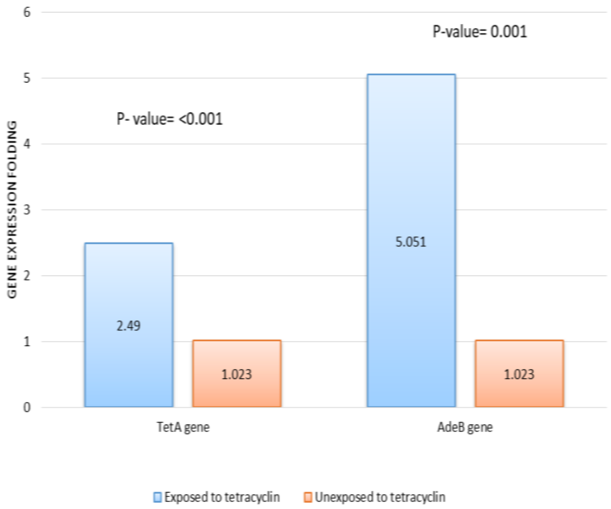

Figure 3. Comparison between gene expression level of TetA and AdeB gens in isolates exposed to antibiotic (tetracycline) and unexposed isolates Molecular Epidemiology

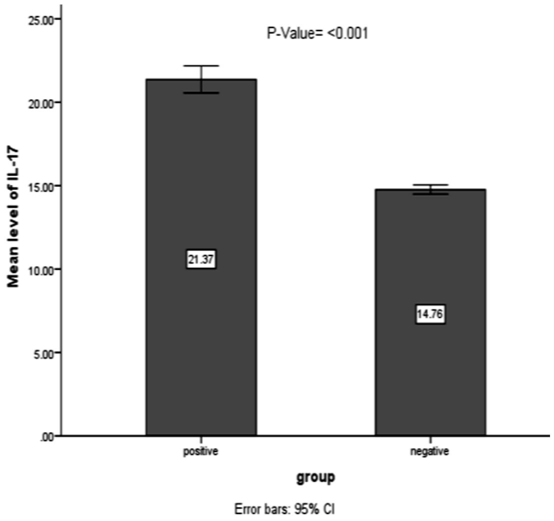

Figure 4. Comparison between IL-17 serum levels in infected subjects and none-infected subjects

Tetracyclines are bacteriostatic antibiotics that prevent the production of bacterial proteins by attaching itself reversibly to the 30S ribosomal subunit's a site (Chopra and Roberts 2001). Tetracyclines are widely utilized in treating human and animal illnesses because of their wide range of therapeutic effects and affordable price. Tetracyclines are added to livestock feed in many nations for the purpose of promoting growth at subtherapeutic doses for metaphylaxis (Granados-Chinchilla and Rodríguez 2017). It was discovered that tetracyclines are expelled from Gram-negative bacteria in a cooperative manner by single component efflux pumps (such as *TetA*) and tripartite Resistance Nodulation cell Division (RND) type efflux pumps, both of which are protons in motion (Foong et al. 2020). *TetA first* moves tetracyclines from *A. baumannii's* cytoplasm to periplasm, where efflux pumps of the RND type then move the antibiotics through the outer membrane. The results of this study corroborated those of an earlier investigation that showed *TetA* expression is induced by sub-inhibitory tetracycline doses (Takahashi et al. 1986).

The results of *AdeB* gene expression also showed an increased level of gene expression in patients than control subjects this results agreed with a previous study that shown the upregulation of the *AdeB* gene in *A. baumannii* clinical isolates and a more than fourfold decrease in tigecycline resistance with CCCP treated isolates (inhibiting efflux pumps). The efflux pumps of the RND type are potential targets for inhibition. (+) Usnic acid is an efflux pump inhibitor that can be added to MDRAb as an adjuvant. To enhance the effectiveness of antibiotics in the treatment of infectious illnesses, chemicals originating from plants can be used as adjuvants (Bankan et al. 2021). The results also agreed with another study that proven a correlation between the antibiotic effects of Tigecycline on *AdeB* gene expression and the frequency of *AdeB* gene in the *A. baumannii* isolates were 100% (Rafiei et al. 2022). To the best of our knowledge this is the first study that seek into the correlation between the expression level of two antibiotic resistance genes and the level of IL-17 in the sera of positive patients and the results showed a significant positive correlation between the IL-17 level and each of the genes expression.

In conclusion, from the results of this study we can conclude the effect of infection with *Acinetobacter baumannii* on the immune system of the patient and especially the IL-17 level. We can also conclude the role of *TetA* and *AdeB* gene in tetracycline antibiotic resistance since higher frequency of both *TetA* and *AdeB* in the resistance isolates.

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