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Relative Gene Expression of RND-Type Efflux Pumps in Tigecycline Resistant *Acinetobacter Baumannii* Isolated from Training Hospitals in Tehran, Iran

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Abstract

Background: Appearance of multi-drug resistance (MDR) *Acinetobacter baumannii* imposes limitation on antibiotic therapy in patients. Detection of MDR *A. baumannii* can play a crucial role to prevent MDR strains spreading in hospitals. The aim of this study was determination the efflux pumps gene expression in tigecyclin resistance strains in collected isolates from selected training hospitals in Tehran, Iran.

Materials and Methods: In this cross sectional study, *A. baumannii* was collected from July to February 2014. Tigecycline susceptibility testing has been prepared according to CLSI guide- lines after identification. Active efflux pumps have been detected by Carbonyl cyanide m-chlorophenyl hydrazone (CCCP) as an efflux pumps inhibitor. Gene expressions of these efflux pumps have been determined by Real- Time PCR.

Results: In this study 80 *A. baumannii* have been confirmed by conventional phenotypic methods. Tigecyclin resistant was confirmed according to antibiotic susceptibility testing results. The results of CCCP indicated that 22.5% of tigecycline resistant *A. baumannii* could include active efflux pumps. The results of Real- Time PCR indicated that *abeM* gene expression has been observed in the most of CCCP positive *A. baumannii* and *adeB* has been observed in the minimum number of strains.

Conclusion

According to the results of this study, Efflux pumps can play an important role in appearance of cross resistance and make MDR strains. Thus, the detection of antibiotic resistance related to active efflux pumps may be crucial to find a composition with efflux pump inhibitor effect by clinical usage.

Key Words: Acinetobacter baumannii, Efflux pump, Tigecycline.

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1- INTRODUCTION

Acinetobacter baumannii is one of the important gram-negative bacteria can cause healthcare associated infections (1-3). *A. baumannii* can survive in different hospital environments and also acquire antibiotic resistance genes (4, 5). Thus, it can make therapeutic complication and nosocomial infection among hospitalized patients (1- 5). Multi-drug resistance strains of *A. baumannii* are increasing worldwide and make therapeutic problems in healthcare centers (3- 5). Tigecycline is the first representative of the glycylcycline class of antibiotic to be marketed for treatment of complicated infection such as infectious disease caused by MDR *A. baumannii* isolates (6, 7). In some studies tigecycline has shown excellent in vitro activity against MDR *A. baumannii*. Tigecycline resistant strains of *A. baumannii* are rare worldwide (6, 7).

Hence, resistance to this antibiotic can be very considerable in healthcare system because of this antibiotic and also in some case with colistin can be remain a last choice for antibiotic therapy for treatment infections caused by MDR *A. baumannii* strains (6, 7). Efflux pumps are one of the antibiotic resistant mechanisms that can cause cross resistance in MDR *A. baumannii* (4, 8). RND-type efflux pump can be involved in resistance to tigecycline (9, 10). In this regards, investigation of efflux pump effect on tigecycline resistant maybe the first step to prevent this antibiotic resistant mechanism. This study aimed to determine the relative gene expression of RND-type of efflux pumps effect on tygecycline resistant in MDR *A. baumannii*.

2- MATERIALS AND METHODS

2-1. Bacterial strains

In this cross sectional study 80 *A. baumannii* isolates were collected from different clinical samples from July to February 2014. All isolated *A. baumannii* in microbiology laboratory of selected training hospitals were included in this study and non *A. baumannii* strains during samples collection were in exclusion criteria. The specimens were collected from pediatrics and adults. Identification of collected strains has been confirmed by conventional biochemical and microbiological tests such as; oxidase, TSI and growth on 42 ^{o}C (3, 4).

2-2. Antibiotic susceptibility testing

Tigecycline susceptibility testing has been conducted by disc diffusion method and minimum inhibitory concentration (MIC) according to CLSI guidelines (11). Antibiotic disc and tigecycline powder have been prepared from MAST Company and sigma (Sigma-Aldrich, cat No. PZ0021). *Pseudomonas aeruginosa* ATCC 27853 used as a control for antibiotic susceptibility testing and MIC.

2-3. PCR detection of efflux pumps

Conventional PCR was conducted for detecting *adeB* and *abeM* genes after DNA extraction. The primer sequences and PCR conditions have described previously (12, 13). Distilled water was used as negative controls and internal positive control after sequencing was used in this study. PCR products were run on 1% agarose gel (Sigma-Alderich, France) stained with DNA safe stain (SinaClon Co., Iran) at 85 V for one hour. Finally data were obtained by using Gel document. Direct sequencing of PCR amplified products was carried out using ABI 3730X capillary sequencer (Genfanavaran, Macrogen, Seoul, Korea). *A. baumannii* ATCC 19606 was used as reference stains.

2-4. Phenotypic detection of efflux pumps

Carbonyl cyanide 3-chlorophenylhydrazone (CCCP) (C2759 Sigma-Alderich, France) as an efflux

pumps inhibitor was used for phenotypic screening of active efflux pumps. Tigecycline MIC with CCCP (25 µg/ml) (14) in comparison with tigecycline MIC without CCCP were prepared for screening of active efflux pumps. Tigecycline for MIC, with and without CCCP, has been arranged from 0.5 to 256 µg/ml. At least four fold decrease of MIC with CCCP compared tigecycline MIC without CCCP shown presence of active efflux pumps (4, 8). *A. baumannii* ATCC 19606 was used as references stains.

2-5. RNA Extraction

The strains were cultured in Brain Hurt Infusion (BHI) medium (5mL) (Merck, Germany) and were grown to mid-exponential phase (OD600 = 1.5-2.0). Then, the bacteria ($5*10^8$) were added to 0.5 mL of RNeasy bacteria protect solution (Qiagen, 74104, Germany) to extract RNA according to the supplier's instructions. Furthermore, DNA was eliminated using 20U of RQ1 RNAse-free DNAse (Promega, Madison, WI, Korea), and was suspended in 50 µL of DEPC-treated water (0.1% v/v). *A. baumannii* ATCC 19606 was used as reference strains.

2-6. cDNA Synthesis

The RNA sample (1 µg) was incubated with 250 ng random hexamer primers (Sigma-Alderich, France) and was added to the pre-mix cDNA synthesis kit (BioNEER, Cat. No. K- 2041. Korea). The reaction was performed for 60 seconds at 15 ^{o} C, 60 min at 55 $^{\circ}$C and then at 95 ^{o} C for 5 min.

2-7. Relative gene expression of efflux pumps by Real-Time PCR

Semi quantitative Real- Time PCR has been prepared to express *adeB* and *abeM* genes; 16 srRNA (14) and *A. baumannii* ATCC 19606 have been used as a housekeeping gene and reference strain, respectively. The qPCR primers recommended by other authors were used, as shown in **Table.1**.

A Rotor Gene RT-PCR machine (Corbett Research, Sydney, Australia; Model RG3000, software version 6) was used for the duplicated PCR reactions with the Quanti Test SYBR Green RT-PCR Kit (Qiagen, Cat. No. 204243, Germany). After activation of the modified Taq polymerase at 95 ^{o} C for 12 minutes, 40 cycles of 15 sec at 95 ^{o} C, 30 sec at each gene annealing temperature set up and 30 sec at 72 ^{o} C were performed. Then, the ΔΔCT values were used for data analysis (15). *A. baumannii* ATCC 19606 was used as references strains.

Table.1: Sequencing of primers in this study.

Target	5′–3′	Refernces
adeB	Forward AACGGACGACCATCTTTGAGTATT	12
	Reverse CAGTTGTTCCATTTCACGCATT	
abeM	Forward TGCCAATTGGTTTAGCTGTG	13
	Reverse TACTTGGTGTGCGGCAATAA	
16srRNA	Forward AACGGACGACCATCTTTGAGTATT	14
	Reverse CAGTTGTTCCATTTCACGCATT	

3- RESULTS

In this study 80 *A. baumannii* isolates have been identified and confirmed by conventional phenotypic methods. According to antibiotic susceptibility testing and MIC results, 98% (78/80) of strains were resistant to tigecycline. The results of CCCP indicated that 22.5%(18/80) of tigecycline resistant *A. baumannii* isolates can include active efflux pumps with a minimum 4 fold decrease of MIC + CCCP in contrast with MIC of tigecycline without CCCP. Efflux pump genes have been detected in 98% of tigecycline resistant strains by PCR as a molecular method. The results of Real-Time PCR indicated an *abeM* gene expression increase from 16 to more than 256 times compared to ATCC strain and *adeB* which increased the gene expression from 2 to 8 times in comparison with ATCC strains. Fifty percent and 70% percent of strains with CCCP positive test include an increase in *adeB* and *abeM* gene expression respectively, according to Real- Time PCR results. Actually, 4 strains showed over gene expression in both two efflux pumps.

4- DISCUSSION

The appearance of MDR and / or XDR *A. baumannii* is an increasing problem all over the world (4, 5). Several antibiotic resistance mechanisms can lead to the existence of MDR and XDR strains such as active efflux pumps (4, 5, 8). Efflux pumps can make resistance to several antibiotics simultaneously even in different families of antibiotics and they can be one of the important causes of the appearance of cross resistance in MDR strains (4, 8- 10, 16, 17). Tigecycline is used for treatment of MDR *A. baumannii* (9, 10), but tigecycline resistant strains have been reported in some studies (16-20). These studies show the involvement of gene expression increase in *adeB* and *abeM* efflux pumps for resistance to tigecycline in *A. baumannii* (18- 20). In this study, 18% of tigecycline resistant *A. baumannii* isolates included an increasing gene expression at least in one tested efflux pumps and 16% of them showed an increase of gene expression in more than two efflux pumps. In Peleg et al. and Deng et al. study on tigecycline, non-susceptible *A. baumannii* showed that increase of expression in *adeB* and *abeM* in tigecycline non-susceptible isolates (18, 19). Yuhan et al. showed linear relationship between tigecycline MIC and *adeB* expression (20). Also, the results of this study indicated that increase in *adeB* in 50% of tigecycline resistant strains that have CCCP positive test. According to the results of this study and other studies (4, 8-10, 16- 21), efflux pumps can play an important role in resistance to tigecycline and make more complication in antibiotic therapy in *A. baumannii* as a one of important threats worldwide (22).

5- CONCLUSION

In this regards, the detection of *A. baumannii* includes active efflux pumps may be the first step to prevent the spread of the MDR strains in healthcare centers. On the other hand, the result of this study can be helpful for finding a natural substance that can inhibit efflux pumps in clinical usage. Because of inactivation of this active efflux pumps can be helpful for increasing the chance of patient's treatment especially in pediatrics.

6- CONFLICT OF INTEREST: None.

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