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Molecular detection of *adeB*, *tetA* and *tetB* efflux pump genes in clinical isolates of *Acinetobacter baumannii* and evaluation of their role in resistance to ciprofloxacin and tetracycline

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ABSTRACT

Multidrug-resistant *Acinetobacter baumannii* are increasing and cause nosocomial infections with high morbidity and mortality rate, especially in long- hospitalized and immunocompromised patients. Among different antibiotic resistance mechanisms, efflux pumps play a vital role. This descriptive cross-sectional research was conducted in the first six months of 2018. In this study, 50 isolates of *A. baumannii* were obtained from patients admitted to different ICU sections. The antibiotic resistance pattern was investigated by the disk diffusion method according to CLSI guideline (2015). Out of 50 *A. baumannii* isolates, 9 of them identified as extensively drug resistant (XDR). Minimum inhibitory concentration was measured for ciprofloxacin and tetracycline in the presence and absence of CCCP for XDR isolates. The prevalence of *tetA*, *tetB*, and *adeB* efflux pump genes was investigated using polymerase chain reaction for all isolates. Moreover, the expression of the *adeB* gene was investigated by Real-time PCR and Ethidium bromide agar cartwheel method for tetracycline-resistant isolates. In this study, the highest antimicrobial resistance was to cefotaxime/clavulanic acid (92%) and no isolate was resistant to colistin. The prevalence of MDR and XDR isolates was 68 and 18% respectively. In XDR isolates, the MIC of tetracycline reduced in the presence of CCCP whereas the MIC of ciprofloxacin was unchanged. The prevalence of *tetA*, *tetB* and *adeB* was 10, 62 and 100%, respectively. Real-time PCR and EtBr-agar cartwheel results showed that *adeB* was overexpressed in tetracycline-resistant isolates. According to our results, using efflux pump inhibitors can be useful in the treatment of nosocomial infection due to MDR and XDR *A. baumannii*. Moreover, EtBr-agar cartwheel is a reliable method for the evaluation of the efflux pumps expression in clinical laboratories.

Keywords: *Acinetobacter baumannii*, antimicrobial resistance, efflux pump.

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INTRODUCTION

A. baumannii is an opportunistic pathogen that causes nosocomial infections including ventilator-associated pneumonia, bloodstream infections, wound and surgical site infections, etc. with high mortality and morbidity rates especially in immunocompromised and long-hospitalized patients (Howard et al., 2012). This bacterium uses different mechanisms such as efflux pump up regulation, porin down regulation, production of β-lactamases enzymes, modification of aminoglycosides, and alteration of antibiotic target sites to become resistant to antibiotics (Lee et al., 2017). Among these different mechanisms, efflux pumps upregulation plays a crucial role by extruding antibiotics and toxic compounds from the bacterial cell to the external environment in an energy-dependent manner (Rumbo et al., 2013).

The ATP-binding cassette (ABC) superfamily, the major facilitator superfamily (MFS), the multidrug and toxic-compound extrusion (MATE) family, the small multidrug resistance (SMR) family, and the resistance nodulation division (RND) family are five major families of efflux-

pumps in bacteria (Piddock 2006 ; Yoon et al., 2013).

In the case of *A. baumannii*, the most important efflux pumps are MFS and RND families. The *tetA* efflux pump confers resistance to tetracycline and *tetB* efflux pump confers resistance to both tetracycline and minocycline belong to MFS superfamily (Piddock 2006). Moreover, the AdeABC efflux system that belongs to the RND family can be responsible for the failure of antibiotic therapy in antibiotics resistant *A. baumannii* isolates (Coyne et al., 2011; Beheshti et al., 2014).

Overexpression of the efflux pumps can increase the minimum inhibitory concentrations (MICs) of antibiotics in comparison with susceptible strains. There are limited options for the treatment of MDR *A. baumannii* infections so identification and development of new therapeutic strategies are crucial. Efflux Pump Inhibitors (EPIs) can be considered as potential treatment options along with antibiotic therapy (Adabi et al., 2015). Carbonyl Cyanide 3-chlorophenylhydrazone (CCCP) is categorized as a member of the EPI family that makes resistant strains susceptible to some antibiotics (Park and Ko, 2015). However, CCCP is used as an experimental agent with no therapeutic value clinically. This efflux pump inhibitor interferes with proton motive force alongside the cytoplasmic membrane and reduces the ATP production as well as increase membrane permeability in the bacterial cell (Osei Sekyere and Amoako, 2017).

Over the past decades, resistance to fluoroquinolones and tetracycline has exponentially increased in clinical isolates of *A. baumannii* in the many regions of the world (Biglari et al., 2017; Smiline Girija, 2019). Therefore, the aim of the present study was molecular detection of *adeB*, *tetA*, and *tetB* efflux pump genes in clinical isolates of *A. baumannii* obtained from patients admitted to different parts of ICU sections in Imam Khomeini hospital in Tehran, Iran and evaluation of their role in resistance to ciprofloxacin and tetracycline.

METHODOLOGY

Isolation and identification of *A. baumannii* strains

The present study was descriptive cross-sectional research and conducted on 50 isolates of *A. baumannii* obtained from alveolar secretion, blood, urine, skin lesion, and CSF samples from patients admitted to different ICU sections in Imam Khomeini hospital in Tehran, Iran in the first six months of 2018. All collected samples transferred to the laboratory using the proper transfer medium. Following 24 hours of incubation in blood agar medium at 37°C, the isolates were identified using the biochemical tests and were confirmed using the PCR method for the identification of the $bla_{OXA\text{-}51}$-like gene (Golanbar et al., 2011). In this part, *A. baumannii* ATCC19606 was used as a positive control.

Determination of antibiotic resistance patterns

The disk diffusion method was performed according to the methodology described in the Clinical and Laboratory Standards Institute (CLSI-2015) guidelines to identify antibiotic resistance patterns. The antibiotic disks (MAST UK company) used for this purpose include cefotaxime-clavulanic acid (30/10 µg), ceftazidime-clavulanic acid (30/10 µg), amikacin (30 µg), gentamicin (10 µg), tetracycline (30 µg), ciprofloxacin (5 µg), colistin (10 µg), rifampin (5 µg), and imipenem (10 µg). *Pseudomonas aeruginosa* ATCC 27853 was used as quality control for this assay.

By definition, isolates that are resistant to more than three classes of antibiotics are defined as multidrug resistant (MDR) and bacterial isolates that remain susceptible to only one or two antimicrobial categories are defined as extensively drug-resistant (XDR) (Falagas et al., 2006).

Evaluation of minimum inhibitory concentration for ciprofloxacin and tetracycline in the presence and absence of CCCP

The MIC of the 9 isolates of *A. baumannii* which were resistant to all antibiotics except colistin was carried out using the micro dilution method as described by CLSI standard guideline for ciprofloxacin and tetracycline (MAST UK company) (ESCMID, 2003). In the next step, the minimum inhibitory concentration was carried out in the same way described above except that the efflux pump inhibitor called CCCP (Sigma Aldrich Company) was added with the final concentration of 2 µg/ml. The plates were incubated for 18 hours at 37°C, and the MIC was recorded.

Identification of *adeB*, *tetA*, and *tetB* efflux pump genes

The DNA extraction was performed using (KIAGEN™) kit as described by the manufacturer. Nucleotide primers used for all of the PCR reactions in this study listed in Table 1. Briefly, 10 pm of reverse and forward primers added to 1 µl of extracted DNA, 12.5 µl of master mix 2x, and 9.5 µl of distilled water.

Investigating the function and expression of *adeB* efflux pump gene by EtBr-agar cartwheel method and Real-time PCR

In this part, 9 isolates of *A. baumannii* from sub-MIC of tetracycline containing media were selected. Briefly, Mueller Hinton agar plates with 0, 0.5, 1, and 2 µg/ml concentrations of ethidium bromide were prepared. Subsequently, each isolate was cultured on the plates like a wheel. Afterward, the plates were incubated for 24 hours at 37 °C and consequently assessed using the transilluminator. In this part, *A. baumannii* sensitive to all antibiotics and had only one *adeB* gene (without *tetA*, *tetB*, *tetM* genes) used as the negative control.

Total RNA of 9 *A. baumannii* isolates was collected from exponentially growing cells (optical density at 600 nm [OD600], 0.8 to 1.2). cDNA synthesis was performed with reverse transcriptase (Roche Diagnostic GmbH, Mannheim, Germany) and Real-time PCR performed using the SYBR PCR Master kit (Roche) with gene-specific primers according to the manufacturer's instructions. In this part, 16 s rRNA was used as internal control.

RESULTS

Isolation and identification of *A. baumannii*

In this study, 50 isolates of *A. baumannii* were obtained from clinical samples including alveolar secretion (54%), blood (26%), urine (10%), skin lesion (6%), and CSF (4%). The antimicrobial resistance patterns were as follow: cefotaxime/clavulanic acid (92%), ciprofloxacin

Table 1. The nucleotide sequence of primers used in this study.

No.	Primer Sequence	Gene	Size (bp)	Annealing temperature (°C)	Reference
1	F: GTAATTCTGAGCACTGTCGC R: CTGCCTGGACAACATTGCTT	*tetA*	957	57	Henriksen (1973)
2	F: GTAAAGCGATCCCACCACCA R:ACCACCTCAGCTTCTCAACG	*tetB*	548	58	Baumann et al. (1968)
3	F: GTTAAATAGTGTTCTTGGAG R:CTAAGATATGGCTCTAACAA	*tetM*	700	52	Henriksen (1973)
4	F: TTAACGATAGCGTTGTAACC R:TGAGCAGACAATGGAATAGT	*adeB*	541	54	Baumann (1968)
5	F:GGATTATGGCGACWGAAGGA R:AATACTGCCGCCAATACCAG	*adeB* (Quantitative)	180	59	This study
6	F: CAGCTCGTGTCGTGAGAT R:CGTAAGGGCCATGATGACTT	16 s rRNA (Quantitative)	130	59	This study
7	F: TAATGCTTTGATCGGCCTTG R: TGGATTGCACTTCATCTTGG	*OXA51*	451	56	Baumann (1968)

(90%), ceftazidime/ clavulanic acid (86%), tetracycline (80%), imipenem (76%), rifampin (70%), gentamicin (64%), and amikacin (44%). None of the strains was resistant to colistin. The results of the antibiogram test showed that 68 and 18% of the studied isolates were MDR and XDR respectively.

Evaluation of minimum inhibitory concentration for ciprofloxacin and tetracycline in the presence and absence of CCCP

Minimum inhibitory concentrations (MICs) of ciprofloxacin in the presence of CCCP remained unchanged whereas the MIC of tetracycline reduced in 8 isolates. The results of minimum inhibitory concentrations (MICs) of 9 isolates were shown in Table 2.

Identification of *adeB*, *tetA*, and *tetB* efflux pump genes

The PCR results indicated that the frequency of *tetA, tetB,* and *adeB* was 10, 62 and 100%, respectively. No strain was positive for the *tetM* gene (Table 2 and Figure 1).

Investigating the function and expression of *adeB* efflux pump

Fluorescence reduction was observed in 4 samples (A. b $_{1, 5, 7, 8}$) while in other samples (A. b $_{2, 3, 4, 6, 9}$) did not change significantly.

Results of Real time-PCR showed that A. b $_{1, 5, 7, 8}$ isolates express *adeB* gene at a high level in the presence of tetracycline. These isolates showed an upper than 400-fold increase in *adeB* gene expression. Moreover, A. b $_{2, 4, 9}$ isolates showed a 10 to 30 times increase and A. b $_{3, 6}$ strains didn't show any changes in *adeB* gene expression (Table 2).

DISCUSSION

A. baumannii has become an important agent that causes nosocomial infections in healthcare settings especially in intensive care units around the world. Therefore, finding new strategies seems necessary to combat this problem (Huang et al., 2018). This bacterium can cause infections at various anatomical sites of the body (Gonzalez-Villoria and Valverde-Garduno 2016). In this study, we isolated *A. baumannii* from alveolar secretion, blood, urine, skin lesion, and CSF samples from patients admitted to the different ICU sections. According to obtained results, the most frequency of *A. baumannii* isolates was in alveolar secretion and the least frequency was in CSF.

Disk diffusion results demonstrated that the highest resistance was to cefotaxime/clavulanic acid, ciprofloxacin, and ceftazidime/ clavulanic acid whereas no isolates were resistant to colistin. Carbapenem is generally used as a promising antibiotic against

Table 2. Comparison of *adeB* efflux pump expression changes and the presence of tetracycline resistance genes beside MIC changes in the presence of CCCP inhibitors for different isolates.

No. of isolate	MIC of tetracycline (µg/ml)		Fold in reduction of MIC	Resistance gene					
	cccp-	+cccp		*adeB*	*tetM*	*tetB*	*tetA*	EtBr-agar cartwheel	Over Exp of *adeB* gene
A.b$_{1*}$	≥512	256	≥2	+	-	+	-	+	415
A.b$_2$	512	64	8	+	-	+	-	-	10
A.b$_3$	8	≤4	≥2	+	-	-	+	-	-
A.b$_4$	512	64	8	+	-	+	-	-	26
A.b$_5$	128	16	8	+	-	+	-	+	439
A.b$_6$	512	512	0	+	-	-	-	-	2
A.b$_7$	512	32	16	+	-	+	+	+	501
A.b$_8$	≥512	256	2	+	-	-	-	+	537
A.b$_9$	512	128	4	+	-	+	-	-	34

** A. baumannii.*

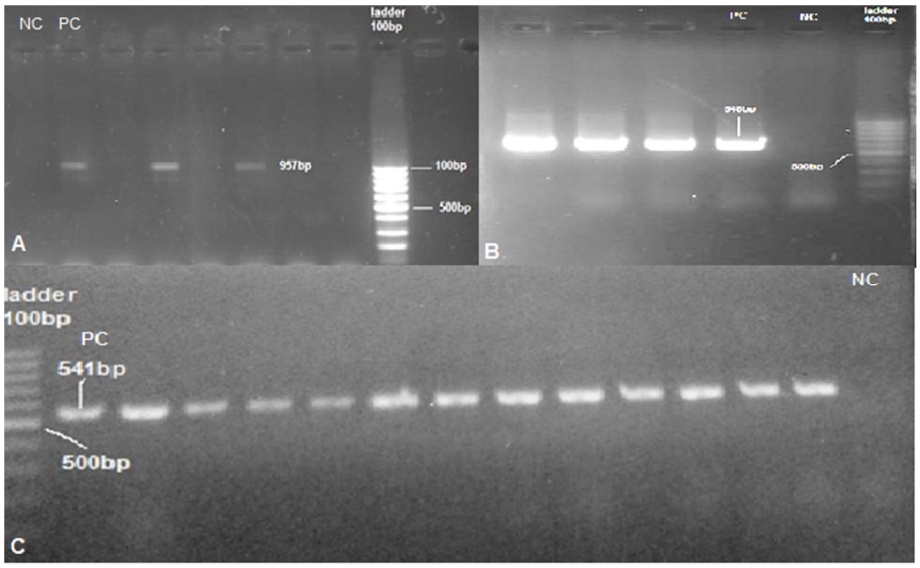

Figure 1. Agarose Gel electrophoresis of PCR products generated by amplification of the *tetA* gene (A) (957 bp), *tetB* gene (B) (548 bp) and *adeB* gene (C) (541 bp). 100-bp DNA ladder size marker, PC= Positive Control NC= Negative Control.

A. baumannii infections. However, resistance to these antibiotics is increasing worldwide (Higgins et al., 2010; Huang et al., 2018). Using unconventional antibiotics such as fluoroquinolones and tetracycline is another strategy against this bacterium. However, there are little data about their therapeutic efficacy (Tan et al., 2007).

Colistin is the last line for MDR *A. baumannii* treatment. Sensitivity to colistin has been reported in many studies (Dizbay et al., 2008; Hernan et al., 2009; Morovat et al., 2009). According to a systematic review and meta-analysis results (Kengkla et al., 2018), combination therapy of colistin with sulbactam is the best choice for the treatment of MDR and XDR *A.baumannii* infections. However, the usage of this antibiotic has limitations because of its side effects such as neurotoxicity and nephrotoxicity (Hernan et al., 2009).

In this study, 68 and 18% of *A. baumannii* isolates were MDR and XDR respectively. Consistent with our results, the frequency of MDR *A. baumannii* strains was reported from 84 to 100% (Azizi et al., 2016; Hatami 2018) and the frequency of XDR *A. baumannii* strains was reported 48% (Higgins et al., 2010) in different parts of Iran. In another study in India, the frequency of MDR and XDR strains was 71.23 and 39.72%, respectively (Smiline Girija, 2019).

The minimum inhibitory concentration of 9 *A. baumannii*

isolates that were resistant to all studied antibiotics except colistin was measured in the presence and absence of CCCP for ciprofloxacin and tetracycline. These results demonstrated that the MIC of ciprofloxacin did not change in the presence of CCCP while the MIC of tetracycline reduced in 8 isolates. In the study by Beheshti and colleagues, the MIC of tetracycline decreased in the most isolates in the presence of CCCP as an efflux pump inhibitor (Beheshti et al., 2014). According to these results, they suggested that active efflux pump had an important role in tetracycline resistance *A. baumannii* isolates. Despite our results, in the study by Ardebili and colleagues, the MIC of ciprofloxacin reduced by 81% in resistant *A. baumannii* isolates when they used CCCP as an efflux pump inhibitor (Ardebili et al., 2014). Using other ciprofloxacin resistance mechanisms can justify this difference between the studies.

Based on these present results, the frequency of *tetA* and *tetB* genes were 10 and 62% respectively. Non-isolate has the *tetM* gene. In the study by Maleki et al., the frequency of *tetB* and *tetA* were 87.6 and 2.2% that is consistent with our findings (Maleki et al., 2014). The study by Ribera et al. (2003) demonstrated the low frequency of the *tet M* gene in clinical *A. baumannii* isolates.

Out of the 9 isolates tested for the adeABC efflux pump, 4 isolates (A. $b_{1, 5, 7, 8}$) showed high expressions, and also the reduction in the fluorescence property in the EtBr-agar cartwheel method as well as 2 to 16 times decreasing in MIC in the presence of CCCP these data could indicate the direct effect of this pump in tetracycline resistance.

Research on MDR *A. baumannii* isolates in Taiwan demonstrated that the *adeB* gene was overexpressed in these isolates and tigecycline was the strongest inducer of gene expression for efflux pump (Lin et al., 2017). In a study by Yoon, *adeB* gene was detected in 13 of 14 MDR *A. baumannii* isolates and significant overexpression of *adeB* gene was observed in 10 strains (Rumbo et al., 2013). Similarly, Yang demonstrated that the expression level of the *adeB* gene was significantly increased in MDR *A. baumannii* strains (Yang et al., 2015). These results indicate the importance of this efflux pump is creating antibiotic-resistant strains.

The results of the EtBr-agar cartwheel method showed that this method can be used as a quick and practical method for examining the high expression of this pump. In a study by Martins the EtBr-agar cartwheel method was used as a quick and convenient method for detecting MDR *Escherichia coli*, *Acinetobacter*, *Enterobacter aerogenes*, and *Salmonella enterica* strains at different ethidium bromide concentrations (Martins et al., 2013).

Our study had limitations because the sample size was small and samples were collected from 1 hospital. Further studies are needed for identification of the exact role of the efflux pump in antibiotic-resistant *A. baumannii* isolates.

Conclusion

Among different mechanisms of antibiotic resistance, efflux pumps are very important. The aim of this study was the detection of 3 important efflux pumps of MDR *A. baumannii* and evaluation of their role in antibiotic resistance. We found that CCCP as an efflux pump inhibitor can reduce the MIC of tetracycline as well as this antibiotic is a strong inducer for efflux pump expression.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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