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Detection of Efflux Pump Using Ethidium Bromide-Agar Cartwheel Method in *Acinetobacter baumannii* Clinical Isolates

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ABSTRACT

Introduction: In the past decade, multidrug-resistant *Acinetobacter baumannii* has become one of the most critical challenges in treating infected patients. The AdeABC efflux pump is the most important among the various resistance mechanisms. This pump can force various antibiotics and ethidium bromide out of the bacterial cell to the surrounding environment. **Methods:** In this study, nine *A. baumannii* clinical isolates were isolated and identified using different biochemicals (catalase, oxidase, TSI, hemolysis, growth at 44°C, and indole) and molecular (bla_{Oxa51} gene) tests. Following the antibiogram test, the antibiotic resistance changes in the isolates in the presence and absence of efflux pump inhibitor (CCCP) were determined for tetracycline, and ciprofloxacin AdeABC efflux pump genes, including *adeA*, *adeB*, and *adeC*, were amplified by PCR. Finally, the presence of the AdeABC efflux pump was investigated using the agar ethidium-bromide cartwheel method (AEBCM). **Results:** According to the antibiogram test, all isolates were MDR. In the presence of efflux pump inhibitor, a reduced resistance for tetracycline was observed, but not for ciprofloxacin. The AdeABC efflux pump genes were detected in all isolates. An increase in the AdeABC pump activity in four isolates was confirmed using AEBCM. **Conclusion:** AEBCM, a fast and convenient tool for assessing the ethidium bromide secretion in various bacteria, provides a quick diagnosis and treatment of multidrug-resistant bacteria.

INTRODUCTION

Increasing multidrug resistance among bacterial isolates has become a significant challenge in health care settings for effective treatment. *Acinetobacter baumannii* is a bacterium with a wide antibiotic resistance mechanism range. Among them, efflux pumps are considered one of the most important mechanisms for high antibiotic resistance [1]. Efflux pumps in bacteria comprise five categories, including the following families: ATP binding cassette (ABC), small multidrug resistance (SMR), multidrug and toxic compound extrusion (MATE), resistance nodulation–cell division (RND), and major facilitator superfamily (MFS) [2]. Mutation in amino acid sequence or amino acid replacement can increase the expression of efflux pumps and cause resistance to a wide range of drugs by reducing the intracellular concentration of antibiotics [3].

One of the most crucial efflux pumps in bacteria is the resistance-nodulation-cell division superfamily (RND) efflux pump, having three different components. So far, three types of RND efflux pump, including AdeABC, AdeIJK, and AdeFGH, have been identified in *A. baumannii* [4, 5]. The AdeABC efflux pump is present in ~80% of *A. baumannii* isolates with high expression causing multidrug resistance (MDR). The AdeABC three-component efflux pump comprises the outer membrane protein encoded by the *adeC* gene, the membrane fusion protein encoded by the *adeA* gene, and the inner membrane protein encoded by the *adeB* gene [6]. The expression of this pump is influenced by a two-component regulating system called AdeR-AdeS, which is coded by the *adeRS* gene upstream of the AdeABC operon [5].

The AdeABC efflux pump is involved in resistance to different classes of antibiotics, including aminoglycosides, beta-lactams, tetracycline, fluoroquinolones, tigecycline, macrolides, chloramphenicol, and trimethoprim [7]. To investigate the role of efflux pumps in conferring antibiotic resistance, we can use different efflux pump inhibitors such as carbonyl cyanide m-chlorophenyl hydrazone (CCCP), valinomycin, dinitrophenol, verapamil, and omeprazole [8], among which CCCP is better known. This substance causes an uncoupling of the proton gradient established

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during the regular activity of electron carriers in the electron transport chain. This chemical acts as an ionophore and properly reduces the ATP synthase function [9].

Advanced tools such as Real-Time PCR for investigating the increased expression of efflux pumps are available but costly and time-consuming. Hence, the use of alternative methods seems necessary in underprivileged laboratories. The agar ethidium-bromide cartwheel method (AEBCM) can be a suitable tool for the fast and accurate detection of efflux pumps in MDR bacteria. Bacterial growth in a culture medium containing ethidium bromide causes the accumulation of this substance within the bacterium [10]. This accumulation can be easily studied using ultraviolet (UV) light. In the case of higher activity of the AdeABC efflux pump, the accumulation of ethidium bromide inside the bacterium is reduced compared to the strains with low AdeABC expression. As a result, a decreased fluorescence occurs due to the removal of ethidium bromide from the bacterium [11].

Unfortunately, the nosocomial infections caused by this bacterium have increased over the past decade, especially in patients admitted to intensive care units, burns, and surgery wards [2]. This study aimed to evaluate the activity of the AdeABC efflux pump using the AEBCM to accelerate the identification of multidrug-resistant *A. baumannii* in clinical isolates.

MATERIAL AND METHODS

Collection and identification of bacterial isolates. In this cross-sectional descriptive study, nine *A. baumannii* clinical isolates were obtained from a teaching hospital laboratory in Tehran, Iran, in 2018 and studied using biochemical tests including catalase, oxidase, TSI, hemolysis, growth at 44°C, and indole. Then, the identity of the isolates was confirmed using the PCR amplification bla_{Oxa51} gene.

Determination of antibiotic resistance pattern. Antibiotic resistance pattern for identification of MDR isolates by disk diffusion method for tetracycline (30 µg), ciprofloxacin (5 µg), imipenem (10 µg), amikacin (30 µg), gentamicin (10 µg), cefotaxime/clavulanic acid (30.10 µg), ceftazidime/clavulanic acid (30/10 µg) (MAST, UK company) were determined according to the criteria presented by the Institute of Laboratory and Clinical Standards (CLSI 2018). The changes in the inhibition zone diameter in the antibiogram method for two antibiotics, using tetracycline and ciprofloxacin antibiotic disks, were performed in the presence and absence of CCCP (Sigma Aldrich Company) efflux pump inhibitor for all nine *A. baumannii* isolates. For this purpose, a concentration of 2 µg/ml of CCCP was added to plates containing Müller-Hinton agar medium.

DNA extraction and identification of efflux pump genes. According to the manufacturer's instructions, DNA extraction was performed using a commercial kit (Kiagen, Iran). The amplification program for AdeABC efflux pump genes (*adeA*, *adeB*, and *adeC*) at the specific annealing temperature of each pair of primers (Table 1) was done according to the following program: 10 min at 94 ° C, 1 min at 94 ° C, 1 min at a specific primer annealing temperature of each gene, 1 min at 72 ° C, and 10 min at 72 ° C in a volume of 25 µl. The obtained product was then examined on 1% agarose gel with UV light and a gel imaging device. The Kiagen's bp100 marker was used to identify DNA fragments.

Table 1. Sequences, length, and annealing temperature of primers were used in this study.

Primer sequence	Gene	Length (bp)	Annealing temperature (°C)	Reference
F:ATCTTCCTGCACGTGTACAT R:GGCGTTCATACTCACTAACC	*adeA*	513	59	**[12]**
F:TTAACGATAGCGTTGTAACC R:TGAGCAGACAATGGAATAGT	*adeB*	541	59	**[12]**
F:TACGGACTGCTACGCTTAAT R:AACAGGATGACCTGCTAACA	*adeC*	527	58	**[12]**
F:TAATGCTTTGATCGGCCTTG R: TGGATTGCACTTCATCTTGG	*oxa51*	451	58	**[13]**

Agar ethidium bromide cartwheel method. The AEBCM was used to identify the phenotypic AdeABC efflux pump activities. For this purpose, we put a concentration of 1 µg per milliliter of ethidium bromide in the Müller Hinton agar plate and planted bacteria next to the negative control strain as a cartwheel (samples from the side of the plate to near the center of the plate in a straight line) followed by incubation at 37 ° C for 24 hours. Then, the plates were investigated under UV light using the Uvitec Cambridge gel dock device. The clinical isolation of *A. baumannii* ATCC 19606 was used as a negative control [11, 14].

RESULTS

Determination of antibiotic resistance pattern. All nine clinical of *A. baumannii* isolates were resistant to tetracycline, ciprofloxacin, imipenem, tigecycline, amikacin, gentamicin, cefotaxime/clavulanic acid, ceftazidime/clavulanic antibiotics. By definition, isolates resistant to three or more representatives of the following

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classes of antibiotic (carbapenems, aminoglycosides, third-generation cephalosporins, and fluoroquinolones) are considered MDR [15]. Hence all isolates investigated in the present study were MDR. The effect of efflux pumps on reducing resistance to ciprofloxacin and tetracycline was investigated using the disk diffusion method in the presence and absence of CCCP as the efflux pump inhibitor. The obtained results showed that in the case of tetracycline antibiotics, a change in the resistance pattern was observed from the resistant to the semi-sensitive or susceptible form. However, no change in the diameter of the inhibition zone with ciprofloxacin was observed in the isolates (Fig. 1). The results of changing the pattern of resistance of clinical isolates to tetracycline are shown in Table 2. Moreover, the electrophoresis results of the efflux pump genes are shown in Fig. 2.

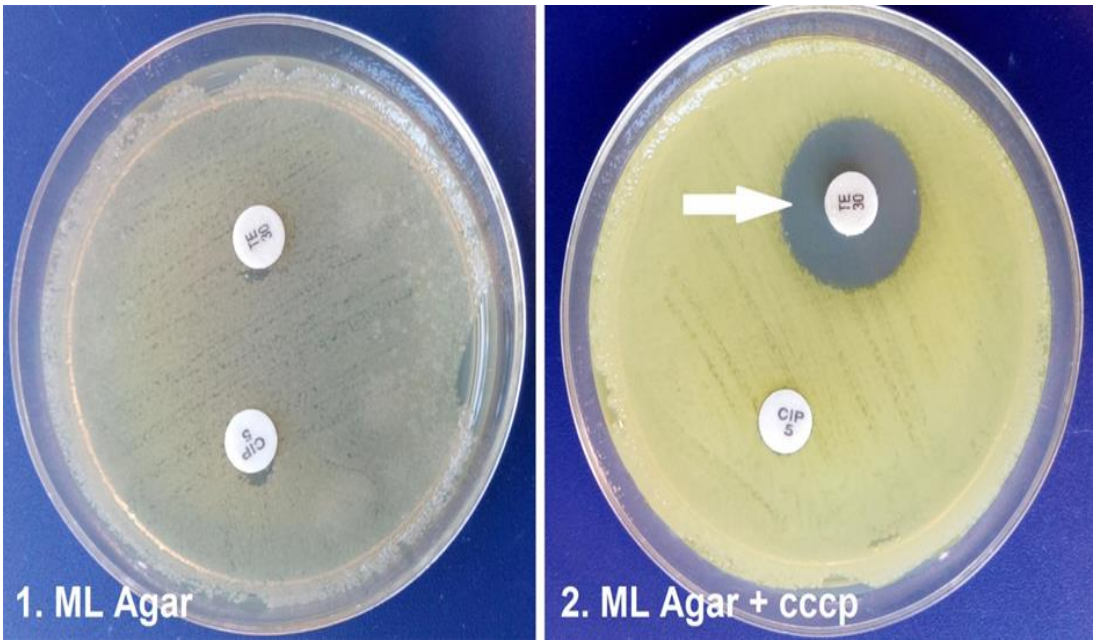

Fig. 1. Antibiotic susceptibility test using tetracycline and ciprofloxacin disks for *A. baumannii* in the absence (1) and presence (2) of the CCCP, an inhibitor on Müller Hinton agar plate.

Table 2. Evaluation of changes in bacterial resistance pattern for tetracycline in the presence and absence of CCCP.

Tetracycline + CCCP (mm)	Tetracycline (mm)	*A. baumannii* isolates
INT(16)	R(0)	**A.B$_1$**
INT(19)	R(0)	**A.B$_2$**
INT(20)	R(14)	**A.B$_3$**
INT(17)	R(12)	**A.B$_4$**
S(20)	R(10)	**A.B$_5$**
R(14)	R(0)	**A.B$_6$**
S(21)	R(0)	**A.B$_7$**
R(14)	R(0)	**A.B$_8$**
INT(16)	R(10)	**A.B$_9$**

R= Resistant, INT=Inter mediate, and S=Sensitive.

DNA extraction and identification of AdeABC efflux pump genes. The PCR assay detected AdeABC efflux pump genes, *adeA*, *adeB*, and *adeC* in all nine *A. baumannii* isolates (Fig. 1).

Agar ethidium bromide-agar cartwheel method. Of the nine isolates, 4 (No. 1, 5, 7, 8) showed an increased expression in the AdeABC efflux pump, resulting in the increased outflow of ethidium bromide from the bacteria, which decreased fluorescence compared to the control. However, there were no significant changes in the fluorescence in other samples compared to the control (Fig.1).

DISCUSSION

Today, the emergence of multidrug resistance in different bacteria has become a significant challenge in treating patients, especially in ICUs. As a result, rapid identification of MDR bacteria such as *A.baumannii* is crucial [3, 16]. This bacterium currently causes

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nosocomial infections, especially in intensive care units worldwide. Therefore, finding novel strategies to deal with this problem seems necessary.

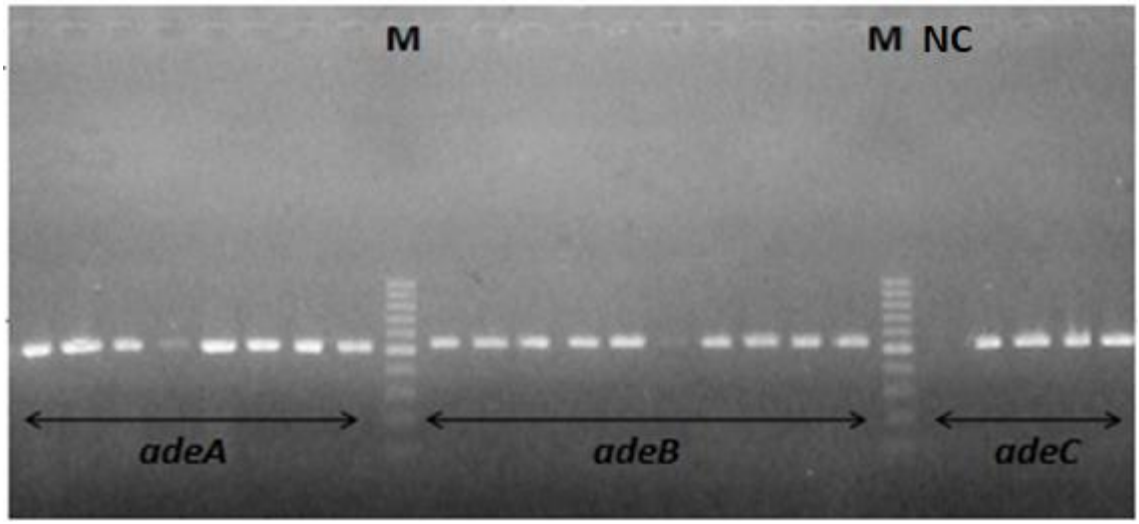

Fig. 2. Electrophoresis of amplified AdeABC efflux pump genes *adeA*, *adeB*, and *adeC* in *A.baumannii* isolates (M: Marker, NC: Negative Control).

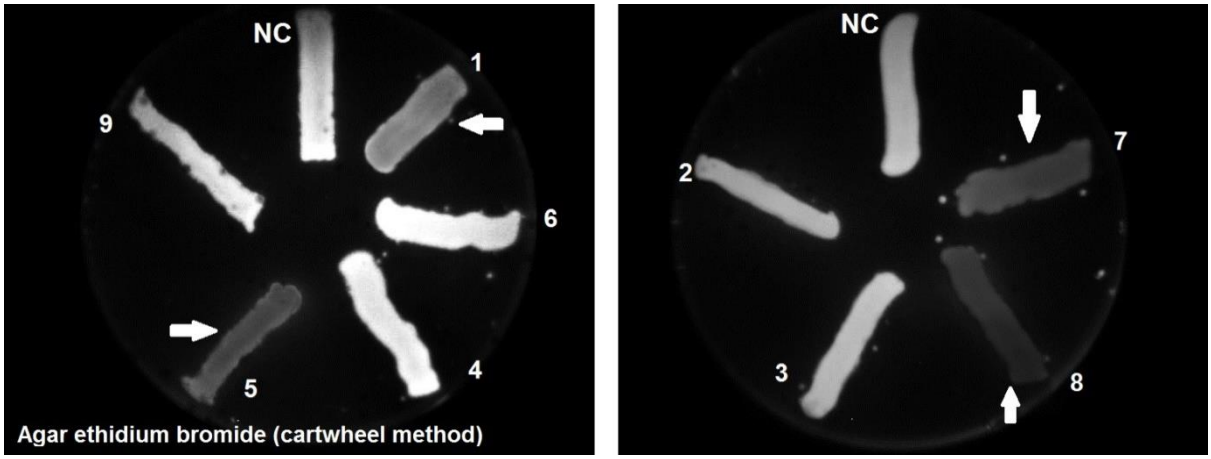

Fig. 3. Ethidium Bromide-agar Cartwheel method. Isolates No. 1, 5, 7, and 8 showed high activity in the AdeABC pump, removing ethidium bromide from bacteria and reducing fluorescence.

In our study, the disk diffusion results demonstrated that all the nine *A. baumannii* isolates were resistant to all tested antibiotics. Carbapenem is generally used as a promising antibiotic in *A. baumannii* infections among the studied antibiotics. However, resistance to this antibiotic is increasing worldwide [17]. On the other hand, using unconventional antibiotics such as fluoroquinolones and tetracycline is another strategy against this bacterium. However, the data on their therapeutic efficacy is limited [18]. The results of the antibiogram assay showed that all isolates of *A. baumannii* were MDR. Consistent with our results, the MDR *A. baumannii* rate range from 84% to 100% in different parts of Iran [19].

The AdeABC efflux pump uses an electrochemical proton gradient to release antimicrobials to the environment outside the cytoplasmic membrane. In this study, CCCP, which is one of the inhibitory compounds of efflux pump, was used to investigate the role of this pump in reducing resistance to tetracycline and ciprofloxacin. The role of this inhibitor has already been confirmed in various studies [20, 21]. For example, a study by Moazzen *et al.* (2018) showed that in 25% of *A. baumannii* isolates, CCCP reduced the minimum inhibitory concentration (MIC) by 4 to 64-fold [21]. The significant change in the pattern of resistance of *A. baumannii* isolates to tetracycline antibiotic in the presence of CCCP inhibitor and conversion of resistant form to sensitive and semi-resistant indicates the active

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presence of efflux pumps in creating resistance in these isolates.

Based on the results of disk diffusion in the presence of CCCP, we concluded that, in terms of resistance to the ciprofloxacin, efflux pumps do not play an active role and possibly other mechanisms such as a mutation in the quinolone resistance-determining region (QRDR) of gyrA and parC are involved in this type of resistance [22, 23].

Our PCR detected *adeA*, *adeB*, and *adeC* genes in all nine isolates (100%). In the last ten years, the frequency of these genes has increased year by year from 53% to 97%. This increase in the frequency of AdeABC efflux pumps showed the increasing role of these pumps in resistance to various antibiotics, especially tetracycline, carbapenems, and aminoglycosides [24, 25]. In a study by Yoon *et al.* (2013), the *adeB* gene was detected in 13 out of 14 MDR *A. baumannii* isolates, and a significant overexpression of the *adeB* gene was observed in 10 isolates [26]. Similarly, Yang *et al.* (2015) demonstrated that the expression level of the *adeB* gene was significantly increased in MDR *A. baumannii* strains [27], indicating the importance of this efflux pump in the development of antibiotic-resistant strains.

Our investigation of the phenotypic type of AdeABC efflux pump using the AEBCM showed that four out of nine isolates showed a decreased fluorescence due to AdeABC pump activity. In a similar study on *Escherichia coli*, *A. baumannii*, *Enterobacter aerogenes*, and *Salmonella enterica*, the AEBCM identified MDR isolates rapidly and appropriately at various ethidium bromide concentrations [11]. According to these findings, the AEBCM can be deployed as a practical, fast and inexpensive method to investigate the role of efflux pumps such as AdeABC in antibiotic resistance. Since this pump is one of the leading causes of resistance in *A. baumannii* clinical isolates to a wide range of antibiotics, rapid detection of this pump in clinical samples can accelerate the treatment of patients with this bacterium.

Despite the promising results, this study had some limitations, the expression level of *adeA*, *adeB*, and *adeC* genes, rather than their existence, is the factor that identifies their activity. Therefore, RT-qPCR should be performed in future studies. Moreover, evaluating only one efflux pump inhibitor (EPIs) is not valid for establishing a method. Therefore, further investigations using several EPIs for different bacteria are needed.

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CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest associated with this manuscript.

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