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Efflux Pump Inhibitor Carbonyl Cyanide 3-Chlorophenylhydrazone (CCCP) Effect on the Minimum Inhibitory Concentration of Ciprofloxacin in *Acinetobacter baumannii* Strains

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Abstract

Background: One of the most important pathogens with increasing mortality rate in hospitalized patients is *Acinetobacter baumannii*.
Objectives: Therefore, this study aimed at detecting multidrug-resistance and the role of efflux pumps in Ciprofloxacin resistance of *A. baumannii* strains isolated from burn patients.
Methods: Eighty *A. baumannii* strains collected from 240 wound samples were isolated from burn patients hospitalized in the burn unit of Shahid Motahari hospital. Susceptibility to antibiotics was tested by disc diffusion and microdilution methods. Activity of the efflux pump system was assessed using the efflux pump inhibitor, Carbonyl Cyanide 3-Chlorophenylhydrazone (CCCP). Polymerase Chain Reaction (PCR) and sequencing were done for detecting the *AdeABC* genes.
Results: Overall, 100% of *A. baumannii* isolates were resistant to ceftriaxone, ceftazidime, cefepime, cefotaxime, meropenem, ciprofloxacin, co-trimoxazole, piperacilin, piperacillin/tazobactam and imipenem; 72 (90%) to amikacin, 72 (90%) to gentamicin, and 0 (0.0%) to colistin The high increase of the susceptibility to ciprofloxacine in the strains in the presence of the efflux pump inhibitor was remarkable. In 25% of isolates, CCCP decreased the minimum inhibitory concentrations (MIC) by 4 to 64 folds. In 80 (100%) of the isolates, *adeb*, *adeA*, and *adeC* genes were detected.
Conclusions: The results indicated the role of efflux pump in antibiotic resistance in *A. baumannii* isolates. Furthermore, *A. baumannii* strains could resist antibiotics by the efflux mechanism.

Keywords: Antibiotic Resistance, Burn, Efflux Pump Inhibitor, CCCP, *Ade*ABC

1. Background

Burns are damage to the skin, caused by different sources (1). Burn infection is uncertain thus it delays curing, continues scarring, and may cause sepsis, bacteraemia or multiple-organ dysfunction syndrome (1). Bacteria are important pathogens of burn wounds. After 48 to 72 hours of damage, these bacteria produce biofilms (2). The source of these organisms, include gut and respiratory flora, patient's own skin, and contact with contaminated health care environments and workers. One of these clinically important MDR bacteria, prevalent in burn wounds, is *A. baumannii* (3). This bacteria is a non-fermentative gram-negative Coccobacillus species, responsible for nosocomial infections worldwide, such as bacteremia, pneumonia and infections of the urinary tract, bloodstream, and skin and soft tissue (3). *Acinetobacter baumannii* is a new threat to the burn patient population. It is commonly considered difficult to cure for many virulence factors and antimicrobial resistance genes (4). These virulence factor that contribute to its pathogenesis, include motility, adherence, biofilm formation, and iron acquisition. Also, membrane polysaccharide components, the outer membrane protein OmpA, penicillin-binding proteins, phospholipases, and outer membrane vesicles, have also been identified (5). These bacteria are usually resistant to a large range of antimicrobials, thus are problematic and difficult to eradicate. Resistance can be intrinsic, i.e. low membrane permeability and cephalosporinase, or acquired, i.e. transition of foreign genetic elements that render overexpression of efflux pump and can confer to the host multi-drug resistance (MDR) (6). In gram-negative bacteria, there are matchless pumps of resistance-nodulation-cell division (RND) superfamily. So

far 3 RND systems have been identified, including *AdeABC*, *AdeIJK* and *AdeFGH*, that confer multi drug resistance in *A. baumannii*. The first pump, named *AdeABC*, causes resistance to tetracyclines, fluoroquinolones, chloramphenicol, aminoglycosides, and trimethoprim, and decreases susceptibility to tigecycline. *AdeABC* and *AdeFGH* render an essential role in acquired resistance. Expression of each pump is regulated by several mechanisms (6). Nowadays, the universal appearance of multi-drug resistant (MDR) bacteria has resulted in much difficulty for hospitalized patients, particularly burn patients at the intensive care unit (ICU) (6, 7).

2. Objectives

This study aimed at detecting antibiotic resistance and also the effect of efflux pump(s) in ciprofloxacin resistance of *A. baumannii* strains among burn patients, using the efflux pump inhibitor CCCP.

3. Methods

3.1. Bacterial Identification

From April to August 2014, 80 *A. baumannii* were isolated from 240 wound samples of burn patients hospitalized at Shahid Motahari Burn hospital, Tehran, Iran. Wound exudates were collected by swabbing, and immediately transported to the microbiology laboratory. These specimens was isolated, and identification was done according to standard procedures (8). The typical reaction of *A. baumannii* is glucose positive and oxidase negative, and negative to esculin, mannitol, indole, maltose, and H_2S. Samples verified as *A. baumannii* were reserved in Tryptic Soy Broth, containing glycerol (20%) at -70°C (9). The PCR of the *blaOXA* 51-like gene was used to verify *A. baumannii* species (10).

3.2. Antimicrobial Susceptibility Testing

The following antibiotics were used, meropenem (MEM: 10 μg), ceftazidime (CAZ: 30 μg), imipenem (IPM: 10 μg), cefotaxime (CTX: 30 μg), amikacin (AK: 30 μg), ciprofloxacin (CIP: 5 μg), cefepime (FEP: 30 μg), ceftriaxone (CRO: 30), gentamicin (GEN: 10 μg), piperacillin/tazobactam (100/10 μg), and colistin sulfate (10 μg), all purchased from the mast company, Merseyside, UK. The turbidity of each bacterial suspension was adjusted equivalent to a number 0.5 McFarland standard and then inoculated on Muller-Hinton agar (Merck, Germany). The diameter of the inhibition zones was measured and the information was expressed as resistant, intermediate, and susceptible, according to clinical and laboratory standards institute (CLSI) 2015 (9). Escherichia coli strain 25,922 was used for quality control.

3.3. Minimum Inhibitory Concentration (MIC)

All of the strains resistant to ceftazidime (CAZ:30 μg), meropenem (MEM:10 μg), and imipenem (IMP: 10μg), by the disk diffusion test, were re-examined in broth microdilution method based on CLSI, 2015 (9). Escherichia coli ATCC 25922 was used for quality control.

3.4. Treatment of the Efflux Pump Inhibitor

To confirm the mechanism of efflux pump, the efflux pump inhibitor CCCP was supplemented to each of the M-H agar plates containing 0.5 to 128 μg/mL ciprofloxacin. The final concentration of CCCP in the M-H agar was 25 μg/mL (11). The MIC for ciprofloxacin was then measured again (12). The positive standard for the existence of efflux pump in the isolates was reduction of at least 4 folds or more of ciprofloxacin MIC after adding CCCP (12).

3.5. DNA Extraction

Genomic DNA was extracted from pure cultures using High Pure PCR Template Preparation Kit GeNet (Cat. No. K-3000, Korea), according to the manufacturer.

3.6. Detection of AdeABC Efflux Pump Genes by Polymerase Chain Reaction

The *AdeABC* genes in *A. baumannii* were detected by PCR. The primers used are listed in Table 1. The 25-μL PCR mixture included 2 μL of sample DNA, 10 pmol of each primer, 30 Mm of KCL, 1.5 mM of $MgCl_2$, 12 Mm of Tris-HCL, 250 μM of each dNTP, and 2 U of Taq DNA polymerase (Sinaclon CAT.NO:PR901638). Amplification was performed with the following thermal cycling conditions: 6 minutes at 94°C and 36 cycles of amplification consisting of 45 seconds at 94°C, 45 seconds at 50°C to 58°C, and 45 seconds at 72°C, with 5 minutes at 72°C for the final extension. Sequencing was done by the the Bioneer company (Korea).

4. Results

4.1. Bacterial Isolates and Antibiotic Resistance

Overall, 80 *A. baumannii* strains were isolated from 240 wound samples of burn patients. Fifteen (25 %) strains were isolated from females and 45 (75 %) from males. The age range of the patients was between 5 and 68 years. The resistance rate of *A. baumannii* isolates to these antibiotics was as follows: 80 (100%) to cefotaxime, ceftriaxone, cefepime, ceftazi-dime, piperacillin/tazobactam, cotrimoxazole, ciprofloxacin, piperacillin, meropenem, and imipenem; 72 (90%) to amikacin; 72 (90%) to gentamicin;

Table 1. Oligonucleotide Primers Used in This Study

Gene	Sequence (5' - 3')	Primer	Product Length, bp	Company	Reference
AdeA	CTCTAGCCGATGTCGCT	*AdeA*-F	510	Bioneer	(13)
	ATACCTGAGGCTCGCCACTG	*AdeA*-R			
Adeb	AAGTATGAATTGATGCTGC	*Adeb*-F	862	Bioneer	(14)
	AACGATTATATTGTTGTGG	*Adeb*-R			
AdeC	TGCGGCAGGTTAGCCCATCG	*AdeC*-F	435	Bioneer	(13)
	GCGGAACAGGATGACCTGCT	*AdeC*-R			

and 0 (0.0%) to colistin (Table 2). The MICs of ceftazidime, imipenem, meropenem, and ciprofloxacin for the *A. baumannii* isolates are presented in Table 3.

Table 2. Antibacterial Susceptibility Testing Results

Antibiotic	Resistance	Intermediate	Sensitive
Gentamicin	72 (90)	2 (2.5)	6 (7.5)
Co-trimoxazole	80 (0)	0 (0)	0 (0)
Amikacin	72 (90)	5 (6.25)	3 (3.75)
Imipenem	80 (0)	0 (0)	0 (0)
Cefotaxime	80 (0)	0 (0)	0 (0)
Cefepime	80 (0)	0 (0)	0 (0)
Meropenem	80 (0)	0 (0)	0 (0)
Ciprofloxacin	80 (0)	0 (0)	0 (0)
Ceftriaxone	80 (0)	0 (0)	0 (0)
Piperacillin/tazobactam	80 (0)	0 (0)	0 (0)
Ceftazidime	80 (0)	0 (0)	0 (0)
Piperacillin	80 (0)	0 (0)	0 (0)
Colistin	0 (0)	3 (3.75)	77 (96.25)

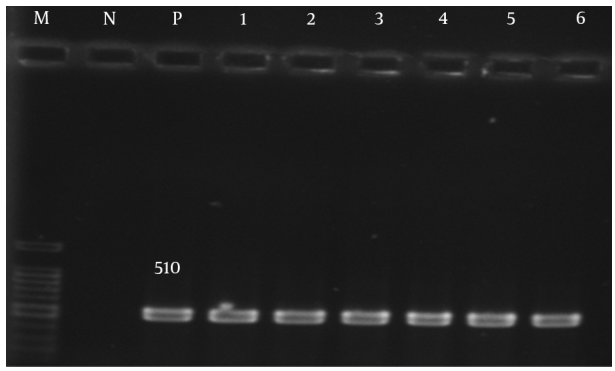
4.2. Role of the Efflux Pump Inhibitor on ciprofloxacin Resistance

To detect the role of the efflux pump in the ciprofloxacin-resistance of the 80 *A. baumannii* isolates, this study assessed the MIC of ciprofloxacin in the presence or absence of the CCCP (25 μg/mL). The results showed that in the presence of the pump inhibitor, MIC of most isolates was reduced in the presence of the efflux pump inhibitor (Table 4). All the *A. baumannii* isolates cultured in MHB, containing the CCCP (25 μg/mL) without ciprofloxacin, showed growth of bacteria, indicating that the CCCP (25 μg/mL) did not have an antibacterial effect itself. These results indicate that drug efflux systems contributed to resistance of ciprofloxacin in the *A. baumannii* isolates in this study.

4.3. Detection of AdeABC Genes

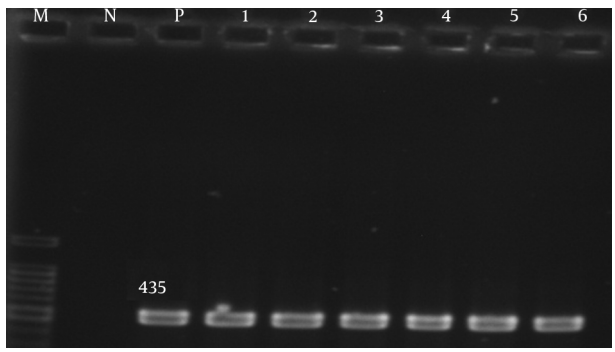
The *adeA*, *adeb*, and *adeC* genes were detected in 80 (100%) of the isolates (Figures 1 - 3).

Figure 1. Polymerase Chain Reaction Results of the *adeA* Gene in Isolates



Lane M, 100 bp ladder; lane N, negative control; lane P, *A. baumannii* ATCC19606 positive control; lane 1 - 6, *adeA* gene positive isolate.

Figure 2. Polymerase Chain Reaction Results of the *adeC* Gene in isolates



Lane M, 100 ladder; lane N, negative control; lane P, *A. baumannii* ATCC19606 positive control; lane 1 -5, *adeC* gene positive isolate.

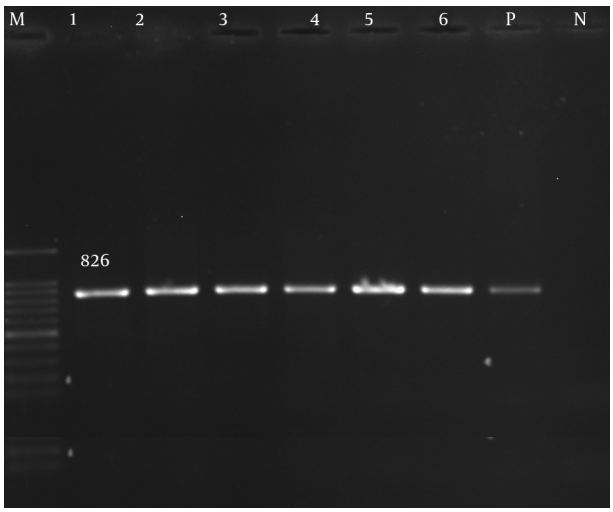
Table 3. Minimum Inhibitory Concentration (MIC) of Imipenem, Meropenem and Ceftazidime in Bacteria

Antibiotic	< 2	4	8	16	32	64	128	256
Imipenem	2 (2.5)	1 (1.25)	0 (0)	4 (5)	21 (26.25)	20 (25)	22 (27.5)	10 (12.5)
Ceftazidime	0(0)	0	1 (1.25)	0 (0)	3 (3.75)	0 (0)	12 (15)	64 (80)
Meropenem	5 (6.25)	1 (1.25)	3 (3.75)	8 (10)	12 (15)	27 (33.75)	20 (25)	4 (5)

Table 4. Effects of Carbonyl Cyanide 3-Chlorophenylhydrazone on Ciprofloxacin Minimum Inhibitory Concentration in Isolates

Isolate (No)	CP	CCCP + CP
1	64	8
1	32	4
3	64	16
2	8	< 2
6	16	< 2
2	16	4
2	64	< 2
1	128	16
2	32	< 2

Figure 3. Polymerase Chain Reaction Results of the *adeb* Gene in the Isolates



Lane M, 100 bp ladder; lane N, negative control; lane P, *A. baumannii* ATCC19606 positive control; lane 1 - 6, *adeb* gene positive isolate.

5. Discussion

Infections may cause failure in curing of burns and are responsible for many problems, such as scarring and sepsis, bacteremia or multiple-organ dysfunction syndrome, and may lead to mortality and morbidity in weak patients (15). *Acinetobacter baumannii* has become a new threat to burn patients thus it is mostly successful at colonizing hospital environments. Several reports have confirmed that *A. baumannii* is difficult to cure since it has many of virulence factors and resistance genes that confer antibacterial resistance (5). The detection of *A. baumannii* strains from burn centers is recurrent and has been confirmed by various worldwide studies, including Iran (16). In this study, the resistance rates of the isolates was as follows: 80 (100%) to cefotaxime, ceftazidime, cefepime, ciprofloxacin ceftriaxone, co-trimoxazole, piperacillin, meropenem, piperacillin/tazobactam, and imipenem; 72 (90%) to gentamicin; 72 (90%) to amikacin, and 0 (0.0%) to colistin. In the present study, the best result against the tested isolates was obtained with colistin. Different studies have confirmed that the existence of resistant *A. baumannii* strains is increasing worldwide (16). These researches have also shown the resistance of these strains against B-lactamase, for example carbapenems, third generation cephalosporins and other antibiotic divisions, including aminoglycoside and fluoroquinolones. The other significance of these bacteria is linked to their multi-drug resistance, which limits their treatment. Asadollahi et al. during years 2009 and 2010 showed 100% resistance in *A. baumannii* isolates to ciprofloxacin (17). In this study, all of the isolates showed resistance to ciprofloxacin. However, resistance rate in the UK and China was lower than that of in the current study (50.9% and 61.2 %,) (14, 18). This diversity could be due to differences in the patterns of antibiotic usage and geographic conditions. Mohajeri et al. declared that all of the isolates were resistant to carbapenems (13). Fallah et al. reported that the resistance to colistin was found in two *A. baumannii* strains (1.8%) (16). Gholami et al. reported no resistance to colistin among the *A. baumannii* strains (19). Therefore, colistin could be useful in curing infections that *A. baumannii* cause, mainly in burn patients. Efflux pump systems are a major cause of multi-drug resistance (20). However, the overexpression of these genes among MDR isolates cause multi drug resistance yet the efflux pump alone cannot confer resistance among *A. baumannii* strains. Efflux pump inhibitors reduce this resistance in *A. baumannii* (11). The role of these compounds, for example CCCP, have been examined by some researches. Rajamohan et al. found that addition of CCCP at concentration of 25 μg/mL decreased the MIC

of different biocides from 2 to 12 folds (21). Ardebili et al. found that most isolates (86.1%) became less resistant (2 to 64 folds) to ciprofloxacine in the presence of efflux pump inhibitors (11). On the basis of the results of Lin et al. (22), ciprofloxacin susceptibility of most isolates was increased in the presence of CCCP, by 2 to 8 folds. These results showed that multi drug efflux pumps play important roles in resistance to fluoroquinolone in *A. baumannii* isolated from hospitalized patients. Recently, results have indicated that resistance to antibiotics causes overexpression of this pump, including *AdeABC* (23). In the present study, *adeA*, *adeb*, and *adeC* genes were obtained from 80 (100%) of the isolates. The *AdeABC* efflux pumps were present in 80% (from 53% to 97%) of *A. baumannii* isolates (24). It is obvious from various studies (present and past) that the prevalence of *A. baumannii* strains resisting fluoroquinolones has increased in Iranian hospitalized patients. However, investigations of the current study showed that the drug efflux system has an important effect in resistance to fluoroquinolone in *A. baumannii* isolates and this mechanism is significantly increased among Iranian burn patients. Therefore, endeavors should be made for identification of such resistant bacteria and control of resistance mechanisms and providing better treatment agents against these organisms.

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