

Role of efflux pumps in reduced susceptibility to tigecycline in *Acinetobacter baumannii*

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Abstract

Acinetobacter baumannii is an important human pathogen responsible for a various type of infections. These bacterial strains are generally resistant to numerous antibiotics. Therefore, eradication of such strains is problematic and related to high mortality. We investigated the effect of cyanide 3-chlorophenylhydrazon (CCCP) efflux pump inhibitor in tigecycline-resistant strains of *Acinetobacter baumannii*. In a cross-sectional study, from July until the end of February 2017, eighty isolates of *A. baumannii* were recovered. Antimicrobial susceptibility testing against tigecycline was performed by the disc diffusion method and determination of minimum inhibitory concentration by broth microdilution method, according to Clinical and Laboratory Standards Institute guidelines. Active efflux pumps were detected by CCCP as an efflux pumps inhibitor, and the gene expression of some of the resistance/nodulation/division (RND)-type efflux pumps was measured by semiquantitative RT-PCR (qRT-PCR). Antibiotic susceptibility tests in this study showed that 78 of 80 *A. baumannii* isolates were resistant to tigecycline. The results of phenotypic detection of efflux pumps revealed that 23.07% of tigecycline-resistant *A. baumannii* isolates can contain active efflux pumps. On the basis of conventional PCR, genes coding for *adeF* and *adeJ* were detected in 76 (98%) *A. baumannii* isolates. The results of qRT-PCR showed that the transcript level of the *adeJ* gene increased in 66.6% *A. baumannii* isolates with CCCP-positive tests and was correlated with tigecycline resistance. The results of this study indicate that RND-type efflux pumps appear to play a significant role in the tigecycline resistance of *A. baumannii*.

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Keywords: *Acinetobacter baumannii*, antibiotic resistance, efflux pump, tigecycline, gene expression

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Introduction

Globally, Gram-negative bacteria, especially *Pseudomonas aeruginosa* and *Acinetobacter baumannii*, are important and serious hospital-acquired pathogens responsible for a various type of

infections, including wound and urinary tract infections, pneumonia, bloodstream infections and secondary meningitis, especially in patients in the intensive care unit [1–5]. *A. baumannii* can persist in different areas of the hospital and can also acquire antibiotic resistance genes or develop resistance mechanisms against antibacterial agents [4,6,7]. However, these bacterial strains are at the same time generally resistant to numerous antibiotics [8]. Previous studies have indicated that a high percentage of *A. baumannii* isolates are multidrug resistant (MDR) to several categories of antibiotics including fluoroquinolones, carbapenems, aminoglycosides and tetracyclines [9,10]. Therefore, eradication of such strains is problematic and is related to high mortality [2,11].

The US Food and Drug Administration has approved tigecycline to treat skin infections, complex intra-abdominal infections and community-acquired respiratory infections [12]. Furthermore, tigecycline, minocycline and colistin are among the few remaining antibiotics for the treatment of MDR *A. baumannii* infections, and tigecycline has shown significant activity to MDR *A. baumannii* [13]. However, reports indicate that tigecycline resistance is increasingly common [14,15], although minocycline remains effective in several infections [16]. Tigecycline-resistant *A. baumannii* involves overexpression of chromosomally encoded RND efflux pumps [10,17,18]. These efflux pumps are mainly composed of three parts: fusion protein, cytoplasmic membrane-spanning transport protein and outer membrane protein [9,13,19–21]. Previously published studies have found that overexpression of AdeABC, AdeFGH or AdeJ, as three major RND pumps, contribute to antibiotic resistance in *A. baumannii* clinical isolates [13,22,23]. According to these data [1–22], evaluating the efflux pump effect on tigecycline resistance in *A. baumannii* probably assists with the prevention of this antibiotic resistant.

We therefore investigated the relative gene expression of RND-type efflux pumps' effect on tigecycline resistance in MDR *A. baumannii*.

Materials and Methods

Bacterial isolates and species identification

In a cross-sectional study conducted from July until the end of February 2017, separate clinical isolates of *A. baumannii* were recovered from diverse clinical samples of patients, including both adults and children. Specimens included blood, cerebrospinal fluid, dialysis fluid and shunt. All isolates were identified using conventional biochemical and microbiologic tests, such as growth on MacConkey agar, oxidase, motility, triple sugar iron agar (TSI) and growth at 42°C.

Antimicrobial susceptibility tests

The tigecycline susceptibility test was performed using the disc diffusion method (DDM) on Müller-Hinton agar; the minimum inhibitory concentration (MIC) values (mg/L) of tigecycline were determined by the broth microdilution method. Antibiotic disc and powders were purchased from Mast (Bootle, UK) and MilliporeSigma (St Louis, MO, USA; cat. PZ0021). All experiments and results were interpreted according to Clinical and Laboratory Standards Institute (CLSI) guidelines. Resistance to tigecycline was defined as a MIC of at least 4 mg/L on the basis of CLSI recommendation. *Pseudomonas aeruginosa* ATCC 27853 was used as a control for DDM and MIC tests.

Identification of RND-type efflux pump genes

The existence of genes encoding the efflux pumps AdeF and AdeJ was screened by PCR. The primers used in this study are listed in Table 1. PCR amplification was performed using a 9700 GeneAmp thermocycler (Applied Biosystems, Foster City, CA, USA); PCR conditions have been described previously [23,24]. PCR products were analysed on agarose gels, stained with DNA-safe stain (SinaClon, Tehran, Iran) and visualized on a UV transilluminator. The sequencing of PCR products was performed by ABI 3730X capillary sequencer (Pishgam; Macrogen, Seoul, Korea). Moreover, *A. baumannii* ATCC 19606 was used as the reference strain.

Treatment of efflux pump inhibitor

The activity of the efflux pump system was screened using efflux pump inhibitor carbonyl cyanide 3-chlorophenylhydrazone (CCCP). To explore the existence of the efflux pump mechanism, CCCP was added to each Müller-Hinton agar plate containing 0.5 to 256 µg/mL tigecycline. The final concentration of CCCP in the Müller-Hinton agar was 25 µg/mL, and MIC for tigecycline was determined again. Finally, a fourfold decrease of the MIC with CCCP compared to tigecycline MIC without CCCP showed presence of active efflux pumps.

RNA extraction and complementary DNA synthesis

A. baumannii strains were cultured in brain–heart infusion medium, and total RNA was extracted from exponentially grown bacteria (OD₆₀₀, 1.5–2.0) using the RNeasy Mini Kit (SinaClon) according to the supplier's instructions. RNA samples were treated with 20 U of RQ1 RNase-free DNase (Promega, Madison, WI, USA) to eliminate any genomic DNA carryover and were suspended in 50 µL of diethylpyrocarbonate-treated water (0.1% v/v). The complementary DNA synthesis was performed using the complementary DNA synthesis kit (Bioneer, Daejeon, Korea; cat. K-2041). Synthesis conditions have been described previously [11].

Semiquantitative RT-PCR

The relative expression levels of *adeF* and *adeJ* genes were assessed by qRT-PCR. qRT-PCR reaction was performed using the Power SYBR Green PCR Master Mix (Bioneer) on a Rotor-

TABLE 1. Primers used for detection of efflux pump genes

Target	5'–3'
<i>adeF</i>	Forward: GGTGTCGACCAAGATAAACG Reverse: GTGAATTTGGCATAGGGACG
<i>adeJ</i>	Forward: GCGAATGGACGTATGGTTCT Reverse: CATTGCTTTCATGGCATCAC
16S ribosomal RNA	Forward: AACGGACGACCATCTTTGAGTATT Reverse: CAGTTGTCCATTTCACGCATT

Gene RT-PCR machine (Corbett Research, Sydney, Australia; model RG3000, software version 6). The relative expression of the investigated genes was normalized against the 16S ribosomal RNA housekeeping gene and was calculated on the basis of the $2^{-\Delta\Delta C_t}$ method. *A. baumannii* ATCC 19606 was used as the reference strain. The primer sequences used for qRT-PCR are listed in Table 1.

Results

In all, 80 *A. baumannii* strains were collected from July until the end of February 2017. On the basis of DDM and MIC results, 78 (98%) of 80 *A. baumannii* isolates were resistant to tigecycline. To confirm the main role of the efflux pump in the tigecycline-resistant phenotypes in 80 *A. baumannii* isolates, we identified the MIC of tigecycline in the presence of the efflux pump inhibitor CCCP, then compared the MICs with and without CCCP. The results of phenotypic detection of efflux pumps revealed that 23.07% of tigecycline-resistant *A. baumannii* isolates can contain active efflux pumps, with a minimum fourfold decline of the MIC with CCCP in contrast with the MIC of tigecycline without CCCP.

Furthermore, after PCR detection, *adeF* and *adeJ* genes were confirmed to be present in 76 *A. baumannii* isolates (98%). Moreover, the relative expression levels of *adeF* and *adeJ* genes were identified in *A. baumannii* isolates by qRT-PCR. Quantitative analysis indicated that *adeF* and *adeJ* gene expression increased from two times to more than 256 times, and from 32 to more than 256 times compared to ATCC strain, respectively. Moreover, the result of qRT-PCR revealed that seven (38.8%) of 18 and 12 (66.6%) of 18 *A. baumannii* isolates with CCCP-positive test results included an increase in *adeF* and *adeJ* gene expression, respectively. In general, four (22.22%) of 18 *A. baumannii* isolates revealed the highest expression in both efflux pump genes (Table 2).

Discussion

The chromosomal RND-type efflux systems play a significant role in the initiation and progress of clinical antibiotic resistance, bacterial pathogenesis, virulence and biofilm maturation in Gram-negative bacteria, especially *A. baumannii* [18,19]. Furthermore, the overexpression of these efflux pumps has been related to MDR in *A. baumannii* [25]. On the basis of World Health Organization reports, the last few years have seen a dramatic surge in the prevalence of MDR and extensively drug-resistant (XDR) bacteria, thus indicating that antibiotic resistance is a major worldwide health problem [26]. Published studies

reveal that bacteria can acquire antibiotic resistance in the existence of drug efflux pumps; new findings indicate that these systems, by removing various compounds, contribute to bacteria that has had time to acquire resistance to the various classes of antibiotics, including β -lactams, tetracyclines, aminoglycosides, cephalosporins and fluoroquinolones [21,27]. MDR and XDR strains have been recognized as a major concern in different hospital areas, particularly the intensive care unit [18,19,28]. According to US Food and Drug Administration—published guidelines as well as the results of previous studies, a few antibiotics such as tigecycline, minocycline and colistin can be used for the treatment of MDR *A. baumannii* [12,13], but tigecycline resistance is increasingly being reported [16].

The role of RND-type efflux pumps in tigecycline-resistant *A. baumannii* has been extensively studied [7,11,21]. In the current study, 98% of *A. baumannii* isolates were resistant to tigecycline; the susceptibility of isolates to tigecycline was therefore still low. In addition, our finding in the current study also showed that the MIC of 51.25% was considerably reduced by twofold to fourfold when CCCP was added. CCCP reduced MIC by twofold to fourfold in *A. baumannii* strains [29,30]. The results of present study showed that 19 (24.3%) and 13 (16.6%) of 78 tigecycline-resistant *A. baumannii* isolates showed an increase of gene expression in one and both efflux pumps, respectively. Moreover, results of our previously published study have shown that 50% and 70% of tigecycline-resistant *A. baumannii* isolates with CCCP-positive tests included an increase in *adeB* and *abeM* gene expression, respectively. Moreover, results of a published study showed that the AdeABC efflux pump appeared to play an important role in the tigecycline resistance of *Acinetobacter* species [18]. These findings revealed that drug efflux pumps can be involved in resistance to tigecycline in clinical isolates of *A. baumannii* [31]. This study showed that 76 *A. baumannii* isolates (98%) carried *adeF* and *adeJ* genes; these results were similar to a previous study that reported that 90% of 112 *A. baumannii* isolates carried the *adeJ* gene [32].

In conclusion, unfortunately, with regards to the critical role of drug efflux pumps in the emergence of MDR and XDR strains of bacteria, no inhibitors for drug efflux pumps are available for clinical use [33]. Thus, we suggest that RND-type efflux pumps are interesting targets for inhibition. The relative expression, function and assembly of RND-type efflux pumps can be targeted by numerous strategies, including suppressing efflux pump expression by targeting the regulatory network that controls the expression of efflux pumps, thus altering the molecular design and structures of old antibiotics, disrupting pump assembly by targeting protein–protein interfaces, directly blocking the inner membrane protein and blocking the outer membrane protein [33–36]. Furthermore, the combination therapy of colistin/tigecycline, colistin/

TABLE 2. Synergistic effect of CCCP on tigecycline MIC and expression of RND-type efflux pump in 80 *Acinetobacter baumannii* isolates

Isolate no.	MIC-CCCP (µg/mL)	MIC range (µg/mL) + CCCP	Fold reduction in MIC + CCCP	Presence of <i>adeF</i> and <i>adeJ</i> genes	Gene overexpression	
					<i>adeF</i>	<i>adeJ</i>
1	>64	<0.25	>16	+	—	+
2	>64	>64	—	+	—	—
3	>64	<0.25	>16	+	—	+
4	>64	<0.25	>16	+	+	—
5	>64	>64	—	+	—	—
6	>64	>64	—	+	—	—
7	32	16	2	—	—	—
8	>64	>64	—	+	—	—
9	>64	>64	—	+	—	—
10	>64	>64	—	+	—	—
11	>64	>64	—	+	—	—
12	>64	>64	—	+	—	—
13	2	—	—	—	—	—
14	>64	>64	—	+	—	—
15	>64	>64	—	+	—	—
16	>64	>64	—	+	—	—
17	>64	>64	—	+	—	—
18	>64	>64	—	+	—	—
19	>64	>64	—	+	—	—
20	64	1	12	+	+	+
21	>64	>64	—	+	—	—
22	>64	>64	—	+	—	—
23	>256	>256	—	+	—	—
24	>256	64	>4	+	+	—
25	>256	>256	—	+	—	—
26	>256	>256	—	+	—	—
27	>256	>256	—	+	—	—
28	>256	>256	—	+	—	—
29	>256	>256	—	+	—	—
30	256	256	—	+	—	—
31	256	128	2	+	—	—
32	256	128	2	+	—	—
33	256	128	2	+	—	—
34	>256	>256	—	+	—	—
35	>256	>256	—	+	—	—
36	>256	>256	—	+	—	—
37	256	128	2	+	—	—
38	128	128	—	+	—	—
39	>256	>256	—	+	—	—
40	>256	>256	—	+	—	—
41	>256	>256	—	+	—	—
42	>256	>256	—	+	—	—
43	>256	>256	—	+	—	—
44	>256	>256	—	+	—	—
45	256	128	2	+	—	—
46	>256	>256	—	+	—	—
47	256	64	4	+	+	—
48	256	128	2	+	—	—
49	256	64	4	+	+	—
50	256	256	—	+	—	—
51	256	128	2	+	+	—
52	256	64	4	+	—	—
53	256	256	—	+	—	—
54	32	16	2	+	—	—
55	2	—	—	—	—	—
56	128	64	2	+	—	—
57	64	16	4	+	+	+
58	64	8	6	+	+	+
59	256	256	—	+	+	—
60	256	128	2	+	—	—
61	128	<0.25	18	+	—	—
62	128	64	2	+	—	—
63	128	64	2	+	—	—
64	128	64	2	+	—	—
65	>256	<0.25	>20	+	—	—
66	>256	>256	—	+	—	—
67	32	<0.25	14	—	—	—
68	>256	>256	—	+	—	—
69	>256	<0.25	>20	+	—	+
70	>256	>256	—	+	—	—
71	>256	>256	—	+	—	—
72	>256	>256	—	+	—	—
73	>256	32	>6	+	—	—
74	>256	>256	—	+	—	—
75	32	1	10	+	—	—
76	64	<0.25	16	+	+	—
77	256	125	2	+	+	—
78	256	32	6	+	+	+
79	256	128	2	+	—	—
80	256	256	—	+	—	—

CCCP, cyanide 3-chlorophenylhydrazine; MIC, minimum inhibitory concentration; RND, resistance/nodulation/division.

meropenem and tigecycline/meropenem could be helpful for treatment of tigecycline-resistant *A. baumannii* isolates [28].

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Conflict of Interest

None declared.

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