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Role of Efflux Pump Gene *adeIJK* to Multidrug Resistance in *Acinetobacter baumannii* Clinical Isolates

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Abstract

Background: The emergence of multidrug-resistant *Acinetobacter baumannii* (MDRAB) as a nosocomial pathogen is one of the major public health problems. This study aimed to evaluate the role of an efflux pump gene *adeJ* for the multidrug resistance of *A. baumannii* clinical isolates. *Methods:* Two groups, MDRAB and susceptible *A. baumannii* (SAB), of *A. baumannii* clinical isolates were studied. The SAB group consisted of strains that did not meet the criteria of MDRAB and were susceptible to more categories of antibiotics than MDRAB. Antimicrobial susceptibility results obtained by the VITEKII system were used in data analysis and bacterial group allocation. We performed real-time reverse transcription PCR to determine the relative expression of *adeJ*. We compared the relative expression of *adeJ* in comparison groups by considering two viewpoints: (1) MDRAB and SAB groups, and (2) susceptible and non-susceptible groups for each antibiotic used in this study. *Results:* The mean value of relative expression of *adeJ* of MDRAB and SAB groups was 1.4 and 0.92, respectively, and showed significant differences ($P = 0.002$). The mean values of relative expression of *adeJ* of susceptible and non-susceptible groups to the antibiotics cefepime, ceftazidime, ciprofloxacin, imipenem, meropenem, tigecycline, piperacillin/tazobactam, ticarcillin/clavulanic acid, trimethoprim/sulfamethoxazole, piperacillin, and gentamicin showed statistically significant differences ($P < 0.05$). *Conclusion:* The overexpression of *adeIJK* might contribute to the multi-drug resistance in *A. baumannii* clinical isolates. Further, the overexpression of *adeIJK* might be one of the factors contributing to the resistance to numerous antibiotics.

Keywords

Acinetobacter baumannii
adeJ gene
Efflux
Multidrug resistance

1. Introduction

Acinetobacter spp. are common causes of healthcare-associated infections, primarily nosocomial infections such as aspiration pneumonia and catheter-associated bacteremia, but can also cause soft tissue and urinary tract infections. Among the various strains of *Acinetobacter* spp., *Acinetobacter baumannii* is the predominant nosocomial agent causing the most common infections clinically and epidemiologically [1]. The number of multidrug-resistant *A. baumannii* (MDRAB) strains is gradually increasing worldwide [2,3].

When treating patients infected with MDRAB, the choice of agents available for treatment is challenging due to its exhibition of multidrug resistance. The high frequency of multidrug resistance among *Acinetobacter* spp. can lead to more serious clinical outcomes when ineffective antibiotics are administered early on [4]. Therefore, it is important to understand the different types of antibiotic resistance and to develop an effective response.

Among the various antibiotic resistance mechanisms possessed by *A. baumannii*, the efflux pump is a mechanism by which antibiotics are transported out of the bacterial cell by a carrier located in the cytoplasmic membrane. Overexpression of the efflux pump facilitates the release of antibiotics, resulting in a lower concentration of antibiotics in the bacterium, and thus the development of drug resistance [5].

The RND efflux system, a representative efflux pump implicated in drug resistance in *Acinetobacter* spp. is composed of tripartite complexes. Among the three structures, the most important transporter is a pump that cooperates with the outer membrane channel and the periplasmic adaptor protein (PAP) to promote the active efflux of many antibiotics and chemotherapeutic agents [5,6]. Therefore, the presence of efflux pumps in samples has been investigated by measuring this transporter even when measuring the presence or expression of various efflux pump genes [7,8]. In this study, we evaluated the activity of the AdeIJK efflux pump system by measuring the expression of the *adeJ* pump gene, the transporter gene of the three-part AdeIJK complex.

Among the *Acinetobacter* drug efflux (Ade) RND efflux pump family in *A. baumannii* strains, AdeABC, AdeFGH, and AdeIJK are well-characterized efflux pumps. Among them, the AdeABC and AdeFGH pumps play a major role in acquired homeostatic resistance, while the AdeIJK pump contributes to endogenous resistance [5]. The association of *adeABC*, a representative RND efflux pump gene in *A. baumannii*, with multidrug resistance has been well documented, but *adeIJK* has been less studied compared to *adeABC*. There are reports of drug resistance caused by overexpression of the *adeJ* gene, which encodes the *adeIJK* efflux pump, in reference strains or a small number of mutant strains [1,5,9], but few studies have investigated the association between efflux pump gene expression and antibiotic resistance in large numbers of clinical isolates or Korean clinical isolates.

The purpose of this study was to determine the extent to which overexpression of the *adeIJK* efflux pump gene contributes to antibiotic resistance in clinical isolates by first comparing the expression of the *adeJ* efflux pump gene between susceptible *A. baumannii* (SAB) and MDRAB groups, which are susceptible to multiple antibiotics, and second, comparing the expression of the *adeJ* efflux pump gene between susceptible and non-susceptible groups for each antibiotic.

2. Materials and methods

2.1. Bacterial strains

A total of 102 strains were studied, including 100 clinical isolates of *A. baumannii* and two reference strains. As reference strains, *A. baumannii* AYE, a multidrug-resistant clinical strain whose sequence information is registered in NCBI, and *A. baumannii* standard strain (ATCC19606) were used [10]. One

hundred clinical isolates of *A. baumannii* were isolated from culture specimens referred to the Microbiology Laboratory, Department of Diagnostic Laboratory, Chosun University Hospital, and were screened using a VITEKII automated analyzer (bioMérieux, Maray l'Etoile, France) to confirm their identification as *A. baumannii* using molecular biological techniques, i.e., *A. baumannii*-specific PCR tests, $bla_{\text{OXA-51-like}}$ PCR, and *gyrB* Multiplex 1 PCR, were performed on a CFX96 Real-Time System (Bio-Rad, Hercules, CA, USA). The $bla_{\text{OXA-51-like}}$ PCR product size was 353 bp, and the *gyrB* Multiplex 1 PCR yielded amplification products of 490 bp and 294 bp in length, indicating that the strains identified as *A. Baumannii*, and was used as the strain for the study (**Table 1**) [11,12].

The antibiotic susceptibility of the strains was measured by VITEKII automated analyZer (bioMérieux, Maray l'Etoile, France), and the strains were grouped according to their susceptibility to the following antibiotics: aztreonam, cefepime, cefotaxime, ceftazidime, tigecycline, ciprofloxacin, gentamicin, imipenem, piperacillin, piperacillin/tazobactam, ticarcillin/clavulanic acid, trimethoprim/sulfamethoxazole, colistin, meropenem, and minocycline were used to classify the group to which each strain belonged.

MDRABs were defined as isolates that were non-susceptible to at least one antibiotic in each category for at least three antibiotic classes according to the criteria of Magiorakos *et al.* [13]. Since the purpose of the study was to compare the MDRAB group with the SAB group, we did not distinguish between MDRAB and XDRAB and analyzed them all in the MDRAB group. The SAB group consists of strains that do not meet the criteria for MDRAB because they are susceptible to more categories of antibiotics than the MDRAB group. The 100 *A. baumannii* clinical isolates consisted of 70 MDRAB and 30 SAB strains.

For each antibiotic, susceptible and non-susceptible groups were classified according to VITEKII results. The proportion of strains in the non-susceptible (NS) group that were found to be intermediate or resistant when tested for susceptibility to each antibiotic in the 100 clinical isolates was 1.0% for colistin, 7.0% for minocycline, 39.0% for tigecycline, 77.0% for cefotaxime, and 99.0% for aztreonam. Gentamicin and meropenem both had a non-susceptibility rate of 71.0%, while ampicillin/sulbactam, cefepime, ceftazidime, ciprofloxacin, imipenem, piperacillin, piperacillin/tazobactam, ticarcillin/clavulanic acid, and trimethoprim/sulfamethoxazole each had a non-susceptibility rate of 72.0%.

The male-to-female sex ratio of isolates was 2.1:1, with more isolates from male patients, and the predominant specimens were sputum (56.0%) and open pus (26.0%), followed by urine (5.0%), whole blood (4.0%), closed pus (1.0%), pleural fluid (1.0%), cerebrospinal fluid (1.0%), and other specimens (6.0%).

Table 1. Oligonucleotide primers used in this study

PCR	Gene	Primer	Sequences (5' → 3')	AT
Conventional PCR	*gyrB* Multiplex 1 [11]	Sp4F	CAC GCC GTA AGA GTG CAT TA	60°C
		Sp4R	AAC GGA GCT TGT CAG GGT TA	
		Sp2F	GTT CCT GAT CCG AAA TTC TCG	
	$Bla_{\text{OXA-51-like}}$ [12]	OXA-51-like F	TAA TGC TTT GAT CGG CCT TG	55°C
		OXA-51-like R	TGG ATT GCA CTT CAT CTT GG	
Real-time RT PCR	*rpoB*	rpoB F	CTC ACT ATG GTC GTG TTT GTC	57°C
		rpoB R	CCA AGA AAC CGA AGT CAT TCG	
	adeJ	adeJ F	CAA GTT ATT GCA TTC TAT TCA CCA G	57°C
		adeJ R	GAC CTG TAC CTC ACC AAC AC	

Abbreviation: AT, annealing temperature.

2.2. Extraction of RNA and synthesis of cDNA

The overnight shake culture in Luria-Bertani (LB) liquid medium was inoculated into fresh LB liquid medium to 1% by the same method as above and further shaken for 3 hours. After 3 hours, the turbidity was adjusted to achieve an OD_{600} value of 0.8–0.9. After removing the supernatant, the bacterial suspension was resuspended in 200 μL of phosphate-buffered saline (PBS) and total RNA was extracted using the High Pure RNA Isolation Kit (Roche Diagnostics, Mannheim, Germany) according to the reagent manual. Removal of genomic DNA was performed using an RNA kit with DNase treatment and its concentration was measured immediately after total RNA extraction. RNA of confirmed purity was subjected to cDNA synthesis using the SuperScript VILO cDNA Synthesis Kit (Invitrogen, Carlsbad, CA, USA). For cDNA synthesis, 1 μg of RNA sample was added to 4 μL of SuperScript VILO master mix for RNA synthesis, and distilled water was added to make the total volume of the solution 20 μL. The synthesized cDNA was stored in a freezer at -80°C and then used for the experiment.

2.3. Real-time reverse transcription PCR

To investigate the correlation between the expression of *adeIJK* efflux pump and antibiotic resistance in *A. baumannii* strains, real-time reverse transcription polymerase chain reaction (RT PCR) was performed. Based on the *adeIJK* chains in *A. baumannii* strains, the *adeJ* constructs shown in **Table 1** were constructed. To confirm the suitability of the designed *adeJ* starter sequences, they were aligned with the *adeJ* sequences of all *A. baumannii* clinical isolates in the NCBI to ensure 100% identity, confirming the suitability of the starter sequences (**Table 1**). For real-time RT PCR, 4 μL of synthesized cDNA (200 ng), 1 μL each of anterior and posterior cytoplasm at a concentration of 10 μM, 10 μL of iTaqUniversal SYBR Green Supermix (Bio-Rad), and 4 μL of distilled water were prepared to a total volume of 20 μL, and real-time RT-PCR was performed using a CFX96 Real-Time System (Bio-Rad). The PCR conditions were a pre-denaturation step at 95 °C for 30 min, followed by three cycles of denaturation at 95 °C for 5 s, ligation at 57 °C for 30 s, and extension at 72 °C for 30 s, repeated for 35 cycles. Furthermore, to optimize the experimental conditions and verify the target, melting-curve checks were performed at 5-second intervals of 0.5°C from 65°C to 95°C. The relative expression of the *adeJ* target gene was calculated using the $2^{-\Delta\Delta CT}$ method on a CFX96 Real-Time System (Bio-Rad, USA). In brief, the expression of the *adeJ* target gene was standardized using the *rpoB* housekeeping gene. The standardized *adeJ* gene expression was scaled against the expression of the *A. baumannii* ATCC 19606 strain (assigned an expression level of 1) to calculate relative expression. Analysis of these results was performed using Bio-Rad CFX manager 3.0 software (Bio-Rad) [14].

2.4. Statistical analysis

According to the antibiotic susceptibility results measured by the VITEKII automatic analyzer, the bacterial groups were divided into susceptible and resistant groups for each antibiotic, and the *adeJ* gene expression in each group was compared and evaluated for significant differences by Student's *t*-test. The MDRAB and SAB groups were evaluated in the same way. Data were analyzed using the SPSS program (version 22.0; IBM SPSS Inc., Armonk, NY, USA), and significant differences were considered when $P < 0.05$.

3. Results

3.1. Comparison of relative expression of *adeJ* between MDRAB and SAB groups

The relative quantification of *adeJ* expression of the studied strains was measured by real-time RT-PCR and the *A. baumannii* standard strain (ATCC19606) was used as a calibrator, and the expression of *A. baumannii*

AYE was 1.06, the mean expression of *adeJ* gene in SAB group was 0.92, and the mean expression of *adeJ* gene in MDRAB group was 1.4. The mean expression of the *adeJ* gene in the SAB and MDRAB groups showed a statistically significant difference ($P = 0.002$) (**Figure 1**).

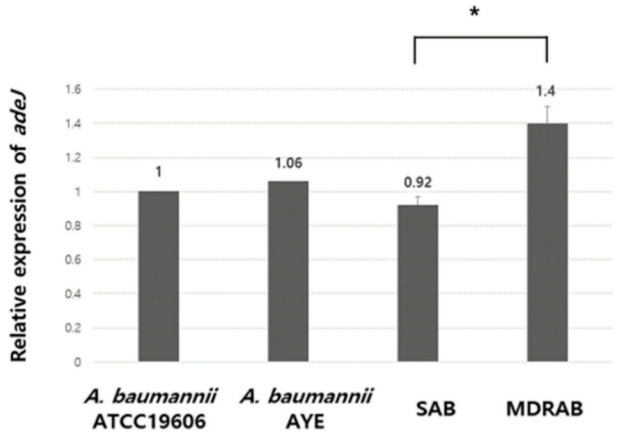

Figure 1. Relative expression of the *adeJ* efflux pump gene determined by real-time reverse transcriptase PCR in *A. baumannii* type strain (ATCC19606), multidrug-resistant *A. baumannii* AYE strain, 70 strains of multidrug-resistant *A. baumannii* (MDRAB), and 30 strains of susceptible *A. baumannii* clinical isolates. The normalized expression of *adeJ* was calibrated with the expression of *A. baumannii* ATCC 19606. The error bars represent the standard error of the mean. *$P < 0.05$.

3.2. Comparison of relative expression of *adeJ* of clinical isolates between the non-susceptible (NS) group and susceptible (S) group to each antibiotic

The studied strains were divided into susceptible and non-susceptible groups according to the susceptibility test results of each antibiotic, and the *adeJ* gene expression of each group was compared, and the gene expression of the non-susceptible group was significantly higher than the susceptible group for most antibiotics except minocycline. In particular, the difference in gene expression of the *adeJ* efflux pump gene between the susceptible and non-susceptible groups for cefepime, ceftazidime, ciprofloxacin, imipenem, meropenem, piperacillin/tazobactam, and ticarcillin/clavulanic acid antibiotics was obvious ($P < 0.001$). Statistically significant differences in *adeJ* gene expression between susceptible and non-susceptible groups were also observed for piperacillin and trimethoprim/sulfamethoxazole ($P = 0.001$), tigecycline ($P = 0.005$), cefotaxime ($P = 0.006$), and gentamicin ($P = 0.019$) antibiotics. However, the number of strains in the non-susceptible group for aztreonam and the susceptible group for colistin was too small to analyze whether there was a statistically significant difference for aztreonam and colistin (**Figure 2**).

4. Discussion

AdeIJK contributes to endogenous resistance to various classes of agents in *A. baumannii* and has been demonstrated in a small number of mutant strains lacking or overexpressing AdeIJK [5,9]. To investigate whether and to what extent this phenomenon also occurs in a large number of clinical isolates, we performed this study on *A. baumannii* isolates from a university hospital in Korea. The results showed that the expression of the *adeJ* efflux pump gene was significantly higher in the MDRAB group than in the SAB group, suggesting that the *adeJ* efflux pump gene contributes to the multidrug resistance of *A. baumannii* in clinical isolates. The expression of the *adeIJK* gene was not significantly higher, which is similar to previous studies [5,9,15]. The finding that the level of *adeIJK* overexpression was lower than that of *adeABC* when tested by transcriptomic microarray and quantitative real-time reverse transcription-PCR (qRT-PCR) was interpreted by Rosenfeld *et al.* as the threshold for host virulence of *adeIJK* is low and the *adeIJK* expression is tightly regulated [15].

The *adeJ* efflux pump gene is known to contribute to the development of endogenous resistance to β-lactams antibiotics such as ticarcillin, cephalosporins, and aztreonam, as well as fluoroquinolones, tetracyclines, and tigecycline [5]. When the expression of the *adeJ* efflux pump gene was compared for each antibiotic, the expression of the *adeJ* efflux pump gene was

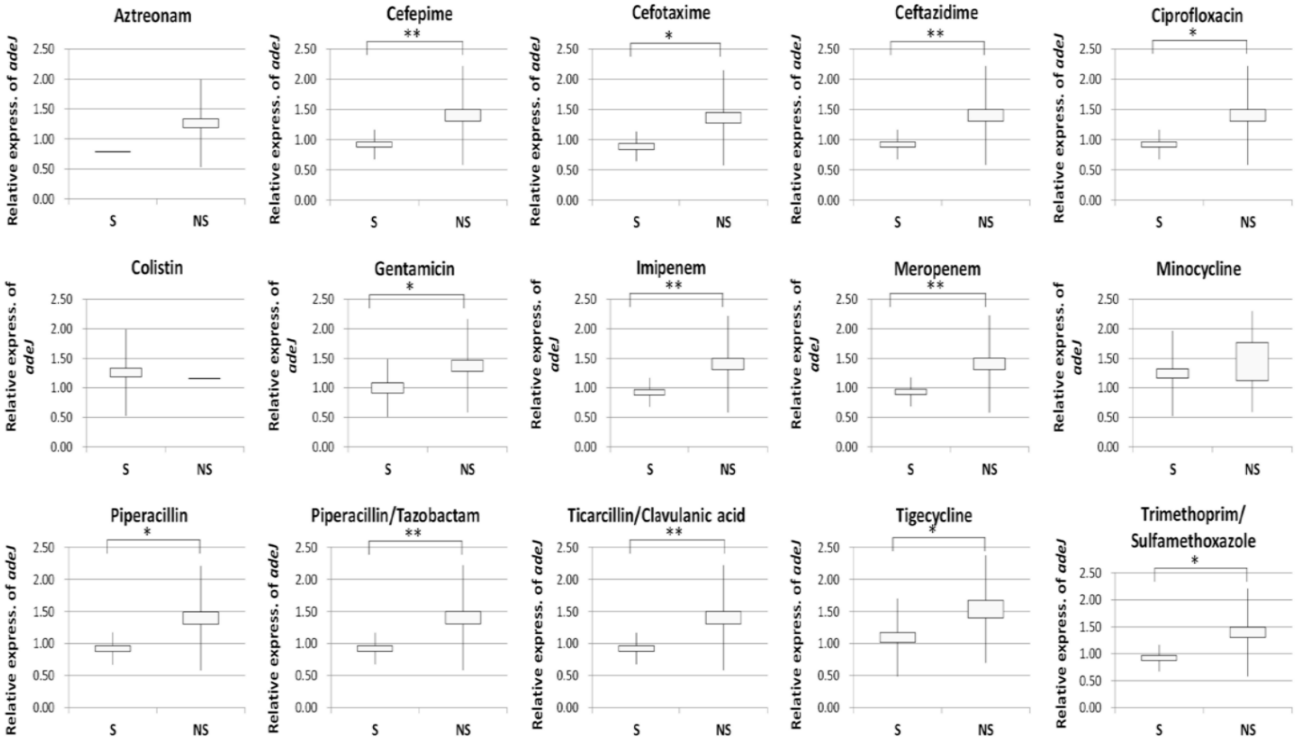

Figure 2. Relationship between *adeJ* pump gene expression and resistance to each antibiotic. Relative expression of the *adeJ* efflux pump gene in the non-susceptible (NS) group was higher than that of the susceptible (S) group to each antibiotic in general. A statistically significant difference was found between S and NS groups to cefepime, cefotaxime, ceftazidime, ciprofloxacin, gentamicin, imipenem, meropenem, piperacillin, piperacillin/tazobactam, ticarcillin/clavulanic acid, tigecycline, and trimethoprim/sulfamethoxazole, respectively. *$P < 0.05$, **$P < 0.001$. The error bars represent the standard error of the mean.

significantly higher in the non-susceptible group compared to the susceptible group for most of the antibiotics, so *adeJ* efflux pump gene was believed to contribute to the resistance of these various antibiotics. In this study, the expression of the *adeJ* efflux pump gene was significantly higher in the non-susceptible group than in the susceptible group to ticarcillin, ceftazidime, cefepime, meropenem, ciprofloxacin, tigecycline, and trimethoprim, which was in agreement with the results of Yoon *et al.* who suggested that *adeJ* efflux pump gene contributes to resistance to these antibiotics [9]. On the other hand, the antibiotics that showed discrepancies with the results of Yoon *et al.* were imipenem and minocycline. In this study, the *adeJ* efflux pump gene expression was significantly higher in the non-susceptible group to imipenem compared to the susceptible group, but there was no significant difference in the results of Yoon *et al.* [9]. In contrast to the results of Yoon *et al.* who suggested that the *adeJ* efflux pump gene contributes to resistance to minocycline antibiotics [9], in this study there was no significant difference in *adeJ* efflux pump gene expression between the susceptible and non-susceptible groups to minocycline. In addition, *adeJ* efflux pump gene expression was significantly higher in the non-susceptible group than in the susceptible group to gentamicin, which is in contrast to the results of Coyne *et al.* who suggested that aminoglycosides are non-substrates of the *adeJ* efflux pump gene [5]. Fernando *et al.* found that *adeIJK* overexpression was associated with multidrug resistance in *A. baumannii* strains, which was similar to our results, but differed from ours in that they did not find any difference in *adeIJK* expression in the presence of ceftazidime and imipenem [16]. Rumbo *et al.* implicated overexpression of *adeJ* in tigecycline resistance in the *A. baumannii* strain of PFGE-ROC-1-OXA-58 clone,

which was similar to our results, but different from our results for gentamicin and minocycline [17]. We divided the strain groups according to the criteria of the VITEKII automated analyzer for antibiotic resistance, but they divided the strain groups according to arbitrary criteria, which may have caused the difference in results. However, we thought that the study by Rumbo *et al.* showed that the results may differ depending on the characteristics of the strain because they also got different results for strains of different clones studied by the same method [17].

Taken together, our results in a large number of clinical isolates from Korea were similar to several studies in reference strains and mutants, as *adeIJK* efflux pump gene expression was significantly higher in MDRAB strains, suggesting that *adeJ* efflux pump gene overexpression contributes to multidrug resistance in *A. baumannii* clinical isolates. Although there was a significant difference in the expression of the *adeJ* efflux pump gene between the MDRAB and SAB groups, there were also strains with low expression of the *adeJ* efflux pump gene in the MDRAB strains and strains with high expression of the *adeJ* efflux pump gene among the SAB strains. These differences in expression have been observed in other studies and are thought to be due to strain-specific differences [7,17]. It is also known that the genotype of a strain can influence the magnitude of resistance to each antibiotic and that the presence of different antibiotic resistance genes in the genomes of different strains has led to differences in antibiotic resistance among different studies [18].

In this study, there was a significant difference between the quantitative values of the resistant strains and the mean values of the susceptible strains, but the difference was relatively small and varied among the strains, suggesting that overexpression of different efflux pumps or other resistance mechanisms may have contributed to their resistance. *A. baumannii* has multiple mechanisms of antibiotic resistance and the flexibility to easily acquire and disseminate resistance, and the interaction between each mechanism can also increase resistance. It is believed that the interaction between its low permeability to antibiotics and the efflux pump it possesses intrinsically confers endogenous resistance to many classes of antibiotics, limiting the available therapeutic options [4]. The most important mechanism for the acquisition of resistance to carbapenem, a commonly used treatment for *Acinetobacter* spp. infections, is the production of an enzyme (carbapenemase) with hydrolytic capacity for this antimicrobial agent, which is associated with changes in the outer membrane protein (OMP), alterations in the affinity or expression of penicillin-binding protein (PBP), and overexpression of efflux pump genes are also known to contribute to the acquisition of carbapenem resistance [19]. In this study, we only studied the AdeIJK efflux pump and did not test other resistance factors, which limits our ability to determine the interaction between multiple resistance mechanisms or the predominant resistance mechanism. Therefore, it is necessary to investigate the interaction between various resistance mechanisms in multiple clinical isolates in the future.

In summary, our study of multiple *A. baumannii* clinical isolates suggests that overexpression of the AdeIJK efflux pump may be a contributing factor to multidrug resistance in *A. baumannii* clinical isolates and that the AdeIJK efflux pump may be one of the contributing factors to resistance to a variety of commonly used antibiotics in clinical practice.

Disclosure statement

The authors declare no conflict of interest.

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