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Major Article

Over expression of AdeABC and AcrAB-TolC efflux systems confers tigecycline resistance in clinical isolates of *Acinetobacter baumannii* and *Klebsiella pneumoniae*

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ABSTRACT

Introduction: Due to the wide use of tigecycline in the treatment of severe infections caused by multidrug-resistant (MDR) bacteria, clinical resistance to tigecycline has increased in recent years. Here, we investigated the relationship between tigecycline resistance and the expression of efflux pumps. **Methods:** Clinical isolates of *Acinetobacter baumannii* and *Klebsiella pneumoniae* were consecutively collected from hospitalized patients in three hospitals. The minimum inhibitory concentration (MIC) of tigecycline was determined using the broth microdilution method. Expression levels of efflux pump genes and regulators were examined by quantitative real-time reverse transcription polymerase chain reaction. The correlations between tigecycline MICs and gene expression levels were analyzed. **Results:** Overall, 1,026 *A. baumannii* and 725 *K. pneumoniae* strains were collected. Most strains were isolated from sputum. The tigecycline resistance rate was 13.4% in *A. baumannii* isolates and 6.5% in *K. pneumoniae* isolates. Overexpression of AdeABC and AcrAB-TolC efflux systems was observed found in clinical tigecycline-resistant isolates. The tigecycline MIC had a linear relationship with the *adeB* expression level in *A. baumannii* isolates, but not with the *acrB* expression level in *K. pneumoniae* isolates. There were significant linear trends in the overexpression of *ramA* as the tigecycline MIC increased in *K. pneumoniae* isolates. **Conclusions:** Tigecycline resistance in *A. baumannii* and *K. pneumoniae* was strongly associated with the overexpression of efflux systems. More studies are needed to elucidate whether there are other regulators that affect the expression of *adeB* in *A. baumannii* and how *ramA* affects the expression of *acrB* in *K. pneumoniae*.

Keywords: Tigecycline. Resistance mechanism. Efflux pump. *Acinetobacter baumannii*. *Klebsiella pneumonia.*

INTRODUCTION

Nosocomial infections caused by multidrug-resistant (MDR) Gram-negative bacteria represent a great threat to public health worldwide. Carbapenems were previously considered the most active agents against MDR Gram-negative pathogens; however, due to the overuse of these drugs, carbapenem-resistant strains have rapidly emerged in the last decade[1]. Most carbapenem-resistant strains are not only resistant to carbapenems but also resistant to at least one agent in most other antimicrobial categories; these strains are designated as extensively drug-resistant (XDR)[2]. Severe infections caused by XDR bacteria are often associated with high treatment failure and mortality rates because of the lack of effective therapeutic options[3].

Tigecycline, a derivative of minocycline, is the first member of the glycylcycline class of antibacterial agents and has been modified to overcome tetracycline resistance[4]. Tigecycline inhibits protein translation and impedes amino acid synthesis by reversibly binding to the 30S subunit of the bacterial ribosome[4]. It has high *in vitro* activity against a broad range of Gram-negative bacteria, such as *Acinetobacter baumannii* (with 19 different pulsotypes) and *Klebsiella pneumoniae*[5]. Thus, tigecycline has attracted much attention in the research community and is considered the last resort to treat infections caused by MDR bacteria. However, due to the increased use of this drug, tigecycline resistance is now rapidly emerging[6]. Various studies have indicated that tigecycline resistance is associated with the overexpression of efflux systems located in the bacterial cell wall, particularly members of the resistance-nodulation-cell division (RND) family, which includes the AdeABC, AdeIJK, and AdeFGH efflux systems in *A. baumannii* and the AcrAB-TolC efflux system in *K. pneumoniae*[6]. However, the exact mechanisms of resistance have not yet been clearly elucidated, and the relationship between the level of expression of efflux pumps and the minimal inhibitory concentration (MIC) of tigecycline has not been established. Moreover, whether clinical isolates with resistance to tigecycline originating from different geographic locations possess similar mechanisms of resistance is still unclear.

Therefore, in this study, we tested the susceptibility of *A. baumannii* and *K. pneumoniae* isolates from three hospitals

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to tigecycline and investigated the relationships among tigecycline resistance, the expression of efflux pumps, and the functions of efflux pump regulators.

METHODS

Bacterial isolates

Clinical isolates of *A. baumannii* and *K. pneumoniae* were consecutively collected from January 2012 to December 2014 from hospitalized patients in three hospitals in Shandong, China. *Escherichia coli* ATCC25922 was used as the reference strain. All isolated strains were identified using a Vitek 2 Compact System (bioMérieux, Marcyl'Étoile, France). For tigecycline-resistant *A. baumannii* isolates, *rpoB* was amplified by polymerase chain reaction (PCR) and then sequenced to confirm identification of *A. baumannii*.

Tigecycline susceptibility testing

The MICs of tigecycline were determined by the broth microdilution method according to Clinical and Laboratory Standard Institute (CLSI) guidelines[7]. In brief, graded concentrations of antibiotics and bacterial suspensions with a cell density of approximately 3×10^5 colony-forming units (CFU)/mL were prepared with cation-adjusted Mueller-Hinton broth (Becton Dickinson and Co., Franklin Lakes, NJ, USA). One hundred microliters of graded concentrations of antibiotics and 100μL of the bacterial suspension were then added to 96-well Ubottom microplates simultaneously. After sufficient mixing with a vortex mixer, the microplates were incubated for 24h at 37°C in ambient air. All susceptibility tests were repeated three times on different days. Tigecycline MIC results were interpreted according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) clinical breakpoints (< 1.0mg/L was considered susceptible, 2.0mg/L was considered intermediate, and > 2.0mg/L was considered resistant)[8].

Multilocus sequence typing

Multilocus sequence typing (MLST) primers for *A. baumannii* and *K. pneumoniae* and the sequences of seven housekeeping genes were designed according to the Pasteur Institute MLST database (http://bigsdb.web.pasteur.fr/). MLST was carried out as described by Bartual et al.[9]. In brief, internal fragments of seven housekeeping genes (*gltA*, *gyrB*, *gdhB*, *recA*, *cpn60*, *gpi*, and *rpoD*) were amplified, purified, and sequenced. The eBURST algorithm was used to assign sequence types (STs) to clonal complexes (CCs) and define the genetic relatedness of STs with the most stringent definition of the groups by sharing the same alleles with at least 6 of 7 loci

Real-time quantitative reverse transcription PCR

Expression levels of efflux pump genes (*adeB*, *adeJ*, *adeG*, and *adeM* for *A. baumannii*; *acrB* and *OqxB* for *K. pneumoniae*) and regulators (*ramA* and *soxS* for *K. pneumoniae*) were examined by Real-time quantitative reverse transcription-polymerase chain reaction (qRT-PCR). Briefly, overnight bacterial cultures diluted with cation-adjusted Mueller-Hinton broth were grown to log phase at 35°C with vigorous shaking (200rpm). RNase-free DNase (Tiangen, Beijing, China)-treated ribonucleic acid (RNA) was obtained using the Purelink RNA Mini Kit (Ambion, Carlsbad, CA, USA). A Nanodrop 2000C (Thermo, USA) was used to determine the yield and quality of RNA. Total RNA from all isolates was reverse transcribed into complementary DNA (cDNA) using the PrimeScript RT Reagent kit (Tiangen, Beijing, China). Real-time qRT-PCR was performed using a LightCycler 480 II (Roche, Germany) with 40 cycles of 5 s at 95°C, 30 s at 54°C, and 30 s at 72°C, and SYBR Premix Ex Taq (TaKaRa, Dalian, China) was used to quantify the expression of the target gene. All experiments were performed in triplicate, and the average was calculated. The primers used for the aforementioned genes are listed in **Table 1**, as described in other studies[10] [11] [12] [13] [14] [15]. Two multidrug-susceptible strains of *A. baumannii* (AB21) and *K. pneumoniae* (KP18) with a tigecycline MIC of 1mg/L were used as the reference strains for the two microGrams (expression level = 1). Relative expression levels of tested genes were calculated according to the expression of the 16S ribosomal ribonucleic acid (rRNA) housekeeping gene (*A. baumannii*) or ribosomal housekeeping gene *rrsE* (*K. pneumoniae*) using the $2^{-\Delta\Delta CT}$ method.

Mutation analysis of ade RS for *Acinetobacter baumannii* and acrR for *Klebsiella pneumoniae*

adeR, adeS, and *acrR* were amplified by PCR using the primers listed in **Table 1** and then sequenced to identify mutations within the genes.

Statistical analysis

We assumed that there was a linear relationship between gene expression levels and tigecycline MICs. Hence, we evaluated the association between expression levels and MICs using linear regression analysis with Statistical Package for the Social Sciences (SPSS) Statistics 20.0 software. Statistical significance was established using a conventional significance level of $p < 0.05$.

RESULTS

Tigecycline resistance and multilocus sequence typing

In total, 1,026 isolates of *A. baumannii* (67.9% were carbapenem-resistant strains) and 725 isolates of *K. pneumoniae* (7.7% were carbapenem-resistant strains) were collected. Of the *A. baumannii* strains, 70% were isolated from sputum, 17% were isolated from wounds, and the remaining strains were isolated from urine, blood, cerebrospinal fluid, and other sources. Of the *K. pneumoniae* strains, 42.3% were isolated from sputum, 24% were isolated from blood, 18% were isolated from urine, and the remaining strains were isolated from wounds, cerebrospinal fluid, or other sources. The MIC_{50} and MIC_{90} of tigecycline for *A. baumannii* were 1 and 4mg/L, respectively; the MIC_{50} and MIC_{90} for *K. pneumoniae* were 1 and 2mg/L, respectively. Additionally, 137 *A. baumannii* isolates and 47 *K. pneumoniae* isolates were resistant to tigecycline (MIC ≥ 4mg/L). As shown

TABLE 1- Primer sequences used for this study.

Primer	Product	Sequence (5′–3′)	Usage	Reference
rpoB-F	*rpoB*	GAGTCTAATGGCGGTGGTTC	Strain identification	[10]
rpoB-R		ATTGCTTCATCTGCTGGTTG		
adeB-qPCR-F	*adeB*	AACGGACGACCATCTTTGAGTATT	qRT-PCR	[11]
adeB-qPCR-R		CAGTTGTTCCATTTCACGCATT		
adeJ-qPCR-F	*adeJ*	ATTGCACCACCAACCGTAAC	qRT-PCR	[11]
adeJ-qPCR-R		TAGCTGGATCAAGCCAGATA		
adeG-qPCR-F	*adeG*	TTCATCTAGCCAAGCAGAAG	qRT-PCR	[12]
adeG-qPCR-R		GTGTAGTGCCACTGGTTACT		
adeM-qPCR-F	*adeM*	GTAGGTGTAGGCTTATGGA	qRT-PCR	[13]
adeM-qPCR-R		GTACCGAAGTGACTGAAAT		
acrB-qPCR-F	*acrB*	AAACTTCGCCACTACGTCATA	qRT-PCR	[14]
acrB-qPCR-R		AGCTTAACGCCTCGATCAT		
oqxB-qPCR-F	*OqxB*	CGAAGAAAGACCTCCCTACCC	qRT-PCR	[15]
oqxB-qPCR-R		CGCCGCCAATGAGATACA		
ramA-qPCR-F	*ramA*	GATATCGCTCGCCATGC	qRT-PCR	[14]
ramA-qPCR-R		CTGTGGTTCTCTTTGCGGTAG		
soxS-qPCR-F	*soxS*	TACCTGCAGCGGATGTTC	qRT-PCR	[14]
soxS-qPCR-R		AAGGTTTGCTGCGAGACGTAG		
adeR-F	*adeR*	ATGTTTGATCATTCTTTTTCTTTTG	Mutation detection	[11]
adeR-R		TTAATTAACATTTGAAATATG		
adeS-F	*adeS*	ATGAAAAGTAAGTTAGGAATTAGTAAG	Mutation detection	[11]
adeS-R		TTAGTTATTCATAGAAATTTTTATG		
acrR-F	*acrR*	GCTAAGCTGCCTGAGAGCAT	Mutation detection	[14]
acrR-R		ATGCAAATGCCGGAGAATAC		
16S rRNA-qPCR-F	*16S rRNA*	GACGTACTCGCAGAATAAGC	qRT-PCR	[11]
16S rRNA-qPCR-R		TTAGTCTTGCGACCGTACTC		
rrsE-qPCR-F	*rrsE*	GTCATCATGGCCCTTACGAG	qRT-PCR	[14]
rrsE-qPCR-R		ACTTTATGAGGTCCGCTTGCT		

qRT-PCR: quantitative real-time reverse transcription polymerase chain reaction; **rRNA:**. ribosomal ribonucleic acid.

in **Figure 1**, the tigecycline resistance rates were 9.8% for *A. baumannii* and 4.9% for *K. pneumoniae* in 2012, but increased to 16.2% for *A. baumannii* and 7.1% for *K. pneumoniae* in 2014. The distributions of tigecycline MICs are shown in **Figure 2**. The MICs of the most resistant strains ranged from 4 to 8mg/L.

Using the eBURST algorithm, 112 of 137 tigecycline-resistant *A. baumannii* isolates were clustered into clonal complex 92, of which 70 isolates belonged to ST92, 26 isolates belonged to ST90, and 16 isolates belonged to ST75. The other 25 isolates belonged to ST91 (16 isolates) and ST20 (nine isolates). Twenty-five of the 47 tigecycline-resistant *K. pneumoniae* isolates belonged to ST11, and the other 22 isolates belonged to diverse STs, including ST15 (seven isolates), ST37 (four isolates), ST30 (three isolates), ST340 (three isolates), ST437 (two isolates), ST392 (two isolates), and ST395 (one isolate).

Relationships between gene expression levels and tigecycline minimal inhibitory concentrations

For *A. baumannii*, the log2-transformed MIC and the log2-transformed *adeB*, *adeJ*, *adeG*, and *adeM* expression levels are plotted in **Figure 3A, B, C** and **D**. Although considerable variability in expression levels was observed at most MICs, a linear relationship was observed between the *adeB* expression levels and tigecycline MICs on the log scale ($r^2 = 0.75$, $p < 0.0001$). Overexpression of *adeJ*, *adeG*, and *adeM* was not observed in most strains, and no linear relationship was observed between the expression levels of these genes and tigecycline MICs.

For *K. pneumoniae*, overexpression of *acrB* and low expression of *soxS* were found in all tigecycline-resistant isolates, while no apparent overexpression of *OqxB* was observed. As shown in **Figure 4A, B** and **C**, no linear relationship was observed between the expression levels of *acrB*, *OqxB*, or *soxS* and tigecycline MICs on the log scale. There were statistically significant linear trends for the overexpression of *ramA* as the tigecycline MIC increased ($r^2 = 0.76$, $p < 0.0001$; **Figure 4D**).

Functional impact of mutations in the ade R, adeS, and acrR genes

Of the 137 tigecycline-resistant *A. baumannii* isolates, 84 harbored wild-type *adeR* and *adeS*, 36 had an IS*Aba1* insertion in *adeS*, 14 had point mutations in *adeR* (Pro116Leu), and three had point mutations in *adeS* (Thr153Met). No mutations were observed in *acrR* of all tested isolates.

DISCUSSION

Tigecycline is one of the few remaining therapeutic options for treating infections caused by MDR or XDR Gram-negative bacteria. However, clinical resistance to tigecycline has been increasingly reported worldwide since 2007[6]. Our study involving three hospitals showed that the tigecycline-resistance rate has been increasing from year to year. Additionally, the resistance rate for MDR *A. baumannii* (12.8%) was higher than that for *K. pneumoniae* (6.2%). This result could be explained by the fact that tigecycline is often used for severe infections caused by *A. baumannii*, particularly MDR strains, in these hospitals. Clonal complex 92 of *A. baumannii* and ST11 of *K. pneumoniae* are the predominant clonal groups among tigecycline-resistant strains, consistent with the outcomes of other studies from China[14] [16] [17] [18].

Previous studies have demonstrated that decreased susceptibility to tigecycline in *A. baumannii* clinical isolates is caused by upregulation of the expression of AdeABC efflux systems[10] [19] [20]. Although spontaneous mutants selected in laboratories have been shown to possess activities of other efflux systems, such as AdeIJK, AdeFGH, and AdeM, which may be associated with decreased susceptibility to tigecycline[12] [21], the extent of the contribution of these efflux systems to tigecycline resistance in a large population of clinical isolates has not been established. Our study found that the overexpression of AdeABC was the prevalent mechanism in tigecycline-resistant *A. baumannii* clinical isolates, and a linear relationship was observed between *adeB* gene expression levels and tigecycline MICs on the log scale. Overexpression of *adeJ*, *adeG*, and *adeM* was not observed in this study, indicating that this three efflux systems may play a relatively minor role in tigecycline resistance.

The expression of AdeABC efflux systems is tightly regulated by a two-component system containing a sensor kinase AdeS and a response regulator AdeR, encoded by the *adeRS* operon, which is located upstream of the *adeABC* operon and transcribed in the opposite direction[22]. Previous studies found that overexpression of the AdeABC system may be stimulated

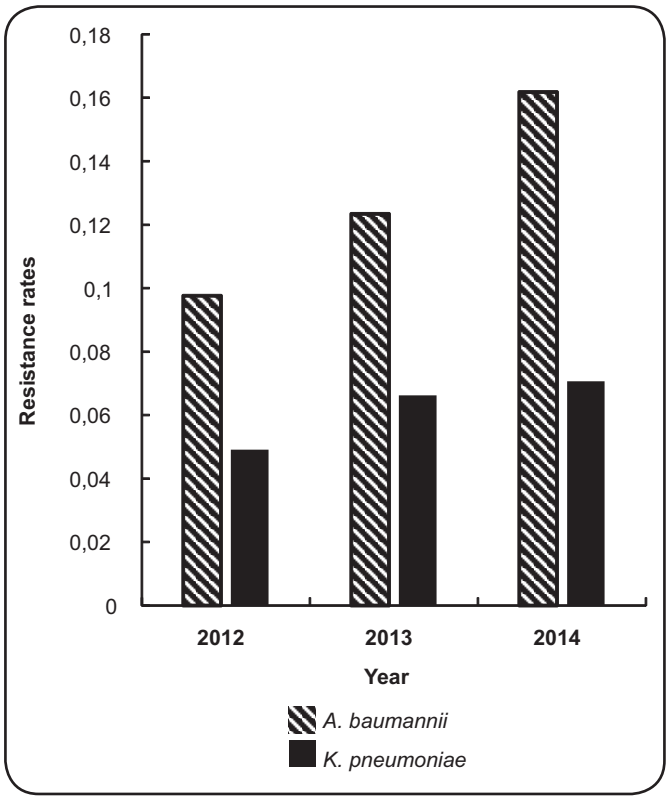

FIGURE 1 - Tigecycline-resistance rates of *A. baumannii* and *K. pneumoniae* clinical isolates collected during different years from 2012 to 2014. ***A.:*** *Acinetobacte*; ***K.:*** *Klebsiella*.

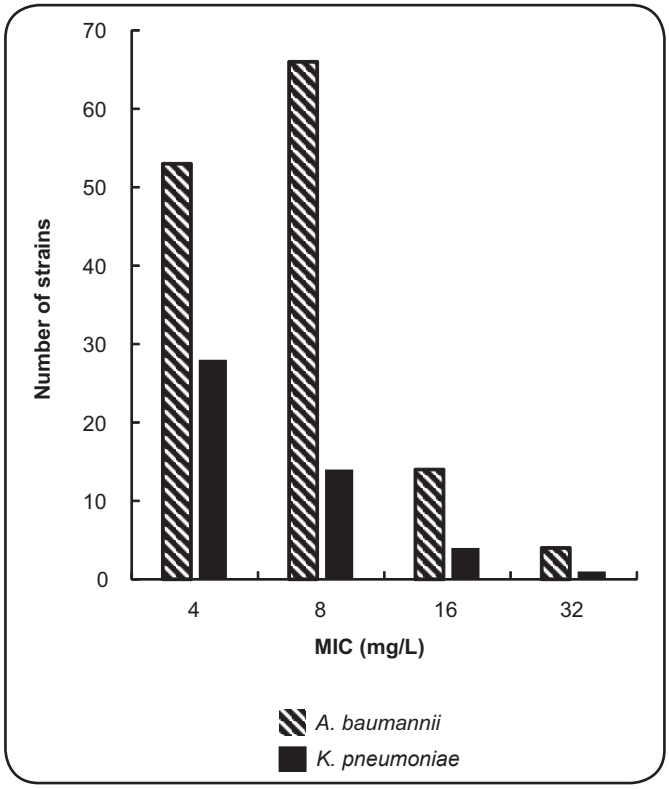

FIGURE 2 - Tigecycline MIC distributions in tigecycline-resistant *A. baumannii* (n = 137) and *K. pneumoniae* (n = 47) clinical isolates. **MIC:** minimal inhibitory concentration; ***A.:*** *Acinetobacte;* ***K.:*** *Klebsiella.*

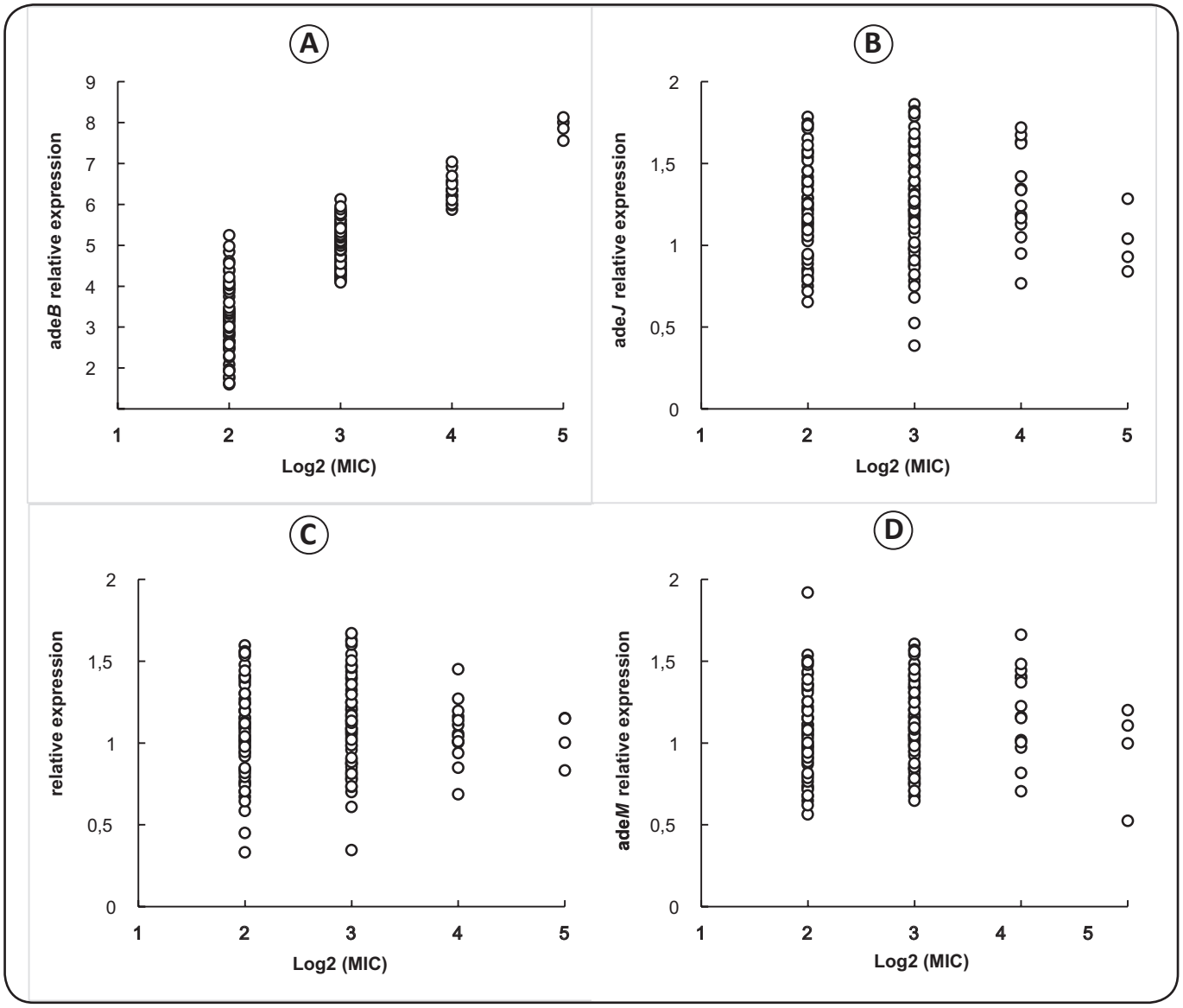

FIGURE 3 - Expression of *adeB*, *adeJ*, *adeG*, and *adeM* versus the minimal inhibitory concentration of tigecycline. The vertical axis shows the log2-transformed geometric mean expression values, whereas the horizontal axis shows the log2-transformed tigecycline MIC values. MIC: minimal inhibitory concentration.

continuously by the mutated AdeRS two-component system[23][24]. In our study, mutations in *adeR* and *adeS* were only observed in 38.7% of strains. A study by Marchand et al. also found that among 13 tigecycline-nonsusceptible *A. baumannii* clinical isolates, all 13 showed increased *adeB* transcription, but none possessed previously known *adeRS* mutations[25]. These results suggested that the overexpression of *adeB* may result from cross-stimulation by other mechanisms as well.

Several studies have shown that tigecycline resistance in *K. pneumoniae* is associated with the overexpression of the *acrAB* operon[26][27]. Additionally, a study by Wang et al. found there was a statistically significant linear trend in the expression of *acrB* as the tigecycline MIC increased[14]. By examining more clinical isolates, we found no linear relationship between the *acrB* expression levels and tigecycline MICs. The overexpression of *acrAB* may have resulted from mutations in its local repressor *acrR* and changes in the expression of global transcriptional regulators of the AraC family, such as *ramA* and *SoxS*[28]. Our results demonstrated that there were no mutations in *acrR* of all tested isolates. However, low expression of *soxS* was observed in resistant isolates, and increased tigecycline MICs were correlated well with the overexpression of *ramA*. Although the overexpression of OqxAB efflux systems may also be also associated with tigecycline resistance[15], no obvious overexpression of *OqxB* was observed in this study.

In conclusion, our study verified that tigecycline resistance in clinical isolates of *A. baumannii* and *K. pneumoniae* was associated with the overexpression of AdeABC and AcrAB-TolC

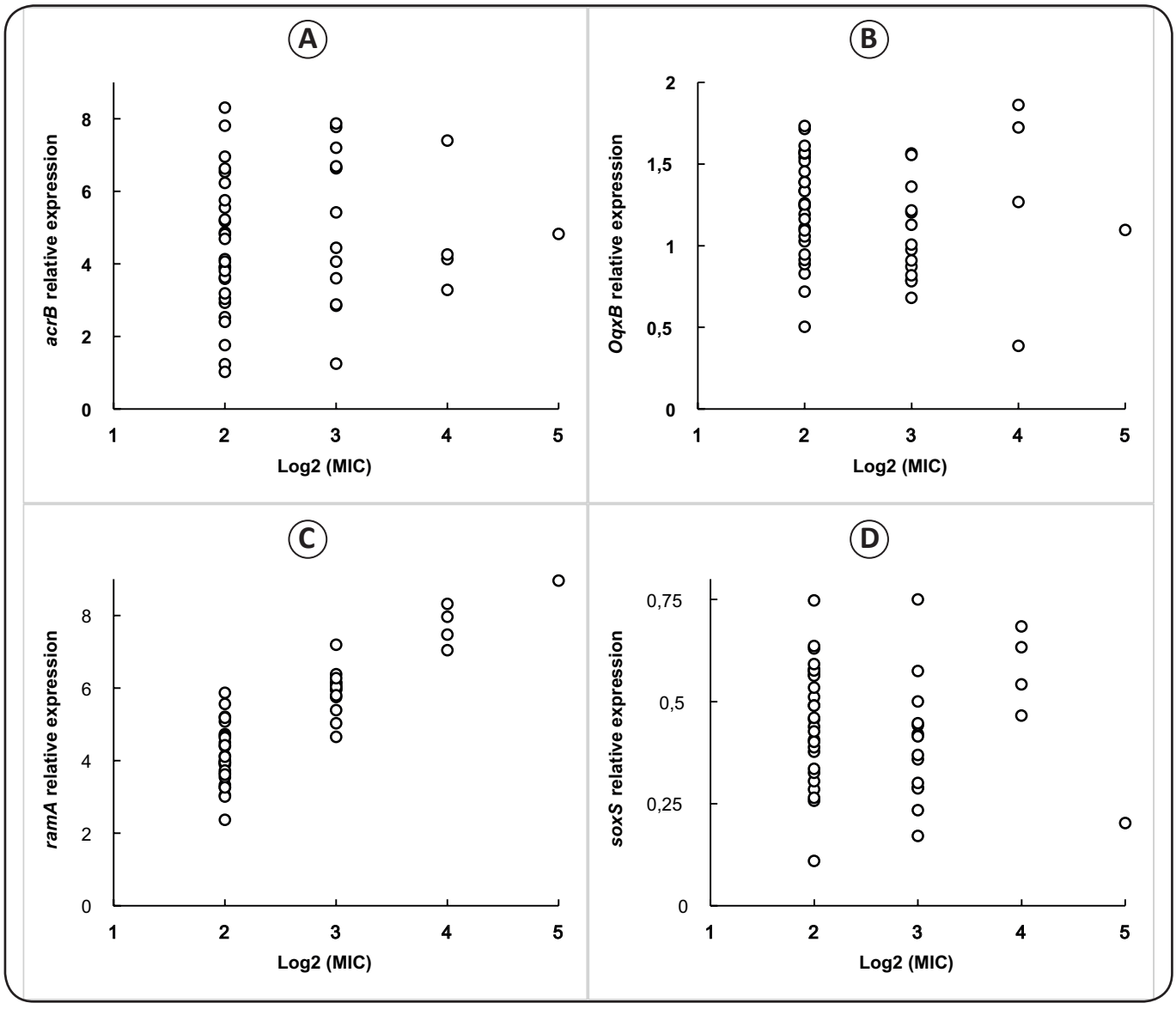

FIGURE 4 - Expression of *acrB*, *OqxB*, *soxS*, and *ramA* versus the minimal inhibitory concentration of tigecycline. The vertical axis shows the log2-transformed geometric mean expression values, whereas the horizontal axis shows the log2-transformed tigecycline MIC values. MIC: minimal inhibitory concentration.

efflux systems. Tigecycline MICs showed a linear relationship with *adeB* expression levels in *A. baumannii* isolates, but not *acrB* expression levels in *K. pneumoniae* isolates. However, significant linear trends were observed for the overexpression of *ramA* as the tigecycline MIC increased in *K. pneumoniae* isolates. Further studies are needed to elucidate whether other regulators also affect the expression of *adeB* in *A. baumannii* and how *ramA* affects the expression of *acrB* in *K. pneumoniae*.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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