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ORIGINAL RESEARCH PAPER

Correlation of RND efflux pump expression and AdeRS mutations in tigecycline-resistant *Acinetobacter baumannii* from Thai clinical isolates

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ABSTRACT

Tigecycline-resistant *Acinetobacter baumannii* (TRAB) is increasing in Thailand, complicating antibiotic treatment due to limited antibiotic options. The specific resistance mechanism behind tigecycline resistance is still unclear, necessitating further investigation. We investigated the presence of OXA-type carbapenemases, the antimicrobial susceptibility profile, the inhibitory effect of carbonyl cyanide m-chlorophenylhydrazone (CCCP) on tigecycline susceptibility, the expression levels of RND-type efflux pumps and amino acid substitutions within a two-component regulatory system on 30 Thai clinical isolates. Our investigation revealed that most of (73.3%) TRAB isolates expressed at least one member of the Ade efflux pumps. The *adeB* was most frequently expressed (63.3%), followed by *adeR* (50%), *adeS* (43.3%), *adeJ* (30%) and *adeG* (10%). Overexpression of the AdeABC was associated with increased tigecycline minimum inhibitory concentrations (MICs) and amino acid substitutions within the AdeRS. Notably, isolates harbouring simultaneous mutations in these genes exhibited an increase in the transcription level of the *adeB*. Our findings highlight the significant role of the AdeABC system in tigecycline resistance among Thai clinical TRAB isolates. This is supported by point mutations within the AdeRS and upregulated expression of the *adeB*. These results provide valuable insights for understanding resistance mechanisms and developing novel therapeutic strategies.

KEYWORDS

tigecycline-resistant *Acinetobacter baumannii*, multidrug resistance, RND-type efflux pumps, two-component system

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BACKGROUND

Acinetobacter baumannii is a significant nosocomial pathogen responsible for various healthcare-associated infections, including pneumonia, wound infections, urinary tract infections, and bloodstream infections [1, 2]. This bacterium is resilient in hospital environments, particularly intensive care units (ICUs) [3–5], and it has an alarming ability to acquire multidrug resistance (MDR) mechanisms [4]. Many *A. baumannii* isolates are now multidrug-resistant to three or more antibiotic classes, including fluoroquinolone,

carbapenem, aminoglycoside, and tetracyclines, which were defined as multidrug-resistant *A. baumannii* [6–8].

Tigecycline and colistin are among the few remaining therapeutic options used to combat multidrug-resistant *A. baumannii* infections. Tigecycline, a modified tetracycline, acts by reversibly binding to the 30S ribosomal subunit A site, thereby inhibiting protein translation. The U.S. Food and Drug Administration (FDA) has approved tigecycline for treating skin infections, complex intra-abdominal infections, and community-acquired respiratory infections [9]. However, the emergence of tigecycline-resistant *A. baumannii* (TRAB) strains is a growing concern [10]. Treatment for tigecycline-resistant *A. baumannii* has become more complex as antibiotic options are limited. In different geographies, *A. baumannii* causes tigecycline resistance through various mechanisms, and many resistance mechanisms can even coexist within the same bacterium. More information needs to be provided on the mechanism of tigecycline resistance in *A. baumannii* in Thailand. Screening for resistant mechanisms in local *A. baumannii* isolates will help clinicians for therapeutic purposes.

The complexity of *A. baumannii* tigecycline resistance necessitates further elucidation. Tigecycline resistance in *A. baumannii* is driven by five key mechanisms: 1) overexpression of efflux pumps, 2) altered outer membrane permeability (*plsC*, *abrP*, *gnaA*, and *abuO*), 3) altered tigecycline targets of action (*rpsJ*, *trm*, *rrf*, and *rpoB*), 4) production of tigecycline-inactivating enzymes (TatX), and 5) repair pathways mediating tigecycline resistance after DNA damage (*recA* and *recBCD*) [11]. Notably, overexpression of efflux pumps plays a pivotal role in drug resistance in *A. baumannii*. Efflux pump families linked to tigecycline resistance encompass resistance-nodulation-cell division (RND) efflux pumps (AdeABC, AdeFGH, AdeIJK), multidrug and toxic compound extrusion (MATE) family (AbeM), the ATP-binding cassette (ABC) transporters (MsbA and MacAB-TolC), and the major facilitator superfamily (MFS) efflux pumps (TetA, TetB, TetA(39), and Tet(Y)). Overexpression of resistance-nodulation-cell division (RND) efflux pumps is a curtail mechanism and is the most prevalent efflux pump in TRAB [12]. Three RND-type efflux pump systems, AdeABC, AdeFGH, and AdeIJK, have been identified in *A. baumannii* [13–15]. These chromosomally encoded pumps are subject to strict regulatory control by distinct mechanisms. The AdeRS two-component system regulates AdeABC expression [16], while AdeFGH is controlled by the LysR-type transcriptional regulator AdeL [15]. Finally, the TetR family regulator AdeN governs AdeIJK [17]. Notably, mutations within AdeRS are frequently associated with AdeABC overexpression in multidrug-resistant strains [3, 18–20].

This study was dedicated to unraveling the role of the RND efflux system in tigecycline resistance. We collected TRAB between 2018 and 2019 from various clinical specimens and investigated the prevalence and expression levels of the three characterized efflux pumps (AdeABC, AdeFGH, and AdeIJK) in TRAB isolates. Furthermore, we explored the potential association between these efflux pump profiles and point mutations within their respective regulatory genes. Our aim was to enhance our comprehension of the mechanisms underlying TRAB and contribute valuable knowledge to developing novel therapeutic strategies for *A. baumannii* infections.

METHODS

Bacterial isolates and ethical statement

Thirty clinical isolates of TRAB were collected between 2018 and 2019 from the Department of Central Laboratory and Blood Bank, Faculty of Medicine in Vajira Hospital of Navamindradhiraj University, Bangkok, Thailand. These isolates originate from various clinical specimens, including blood ($n = 3$), sputum ($n = 22$), body fluid ($n = 1$), urine ($n = 1$), wound ($n = 2$), and catheter tip ($n = 1$) obtained from patients diagnosed with bacterial infections. All isolates were identified using MALDI-TOF MS (Bruker Microflex, Germany). This work was approved by the Ethics Committee of the Faculty of Medicine Vajira Hospital, Navamindradhiraj University (COA009/2561).

Identification of antibiotic-resistance genes

The presence of carbapenemase genes ($bla_{\text{OXA-51}}$-like, $bla_{\text{OXA-23}}$-like, $bla_{\text{OXA-24}}$-like, and $bla_{\text{OXA-58}}$-like genes) was investigated using polymerase chain reaction (PCR) with primers listed in Table S1. The primer sequences were synthesised based on previously published sequences [21–24]. PCR amplification was performed using a T100™ thermal cycler (Bio-Rad, Hercules, CA, USA) following established protocols [21]. Amplified products were separated by electrophoresis on agarose gels stained with ethidium bromide and visualised using a gel documentation system (Bio-Rad, Hercules, CA, USA).

Antimicrobial susceptibility testing

The susceptibility of the isolates to thirteen antimicrobial agents was determined using a BD Phoenix automated susceptibility system, following the guidelines of the Clinical & Laboratory Standards Institute guidelines (CLSI). The agents tested included amikacin (AMK), ampicillin/sulbactam (AMS), cefoperazone/sulbactam (CFP/SUL), cefepime (FEP), ceftazidime (CAZ), ciprofloxacin (CIP), colistin (CL), gentamicin (GEN), imipenem (IPM), levofloxacin (LVX), meropenem (MEM), piperacillin/tazobactam (TZP) and tigecycline (TGC). All antibiotics were from Oxoid™, Basingstoke UK. Multidrug resistance (MDR) was defined as resistance to at least three antimicrobial classes. For tigecycline specifically, susceptibility was also evaluated using the disc diffusion method on Mueller-Hinton agar (Oxoid™, Basingstoke, UK). The broth microdilution method further determined the minimal inhibitory concentration (MIC) of tigecycline. Tigecycline-resistance breakpoints were interpreted according to the European Committee on Antimicrobial Susceptibility testing criteria (susceptible, ≤ 1 mg L^{-1}; and resistant, ≥ 4 mg L^{-1}).

Treatment with an efflux pump inhibitor

The role of efflux pumps in tigecycline resistance was assessed using carbonyl cyanide m-chlorophenylhydrazone (CCCP) (Sigma-Aldrich, St. Louis, MO, USA), a known efflux pump inhibitor. Mueller-Hinton broth (MHB) (Oxoid^{TM}, Basingstoke, UK) containing tigecycline (0.015–128 μg mL^{-1}) was supplemented with and without 25 μM CCCP. All isolates were cultured in MHB containing only 25 μM to evaluate CCCP's independent effect on growth. MIC were determined in triplicate for both CCCP-treated and untreated cultures. A fourfold MIC reduction in the presence of CCCP indicated efflux pump activity.

Mutation analysis of AdeRS

A two-component regulatory system comprising *adeR* and *adeS* genes was amplified by PCR using the primers listed in Table S1. The PCRs for *adeR* and *adeS* were described previously [22]. Sequencing was performed by Bioneer (Daejeon, Korea) and Macrogen (Seoul, Korea), and the results were analyzed using Lasergene DNA Star sequencing software (DNAStar, Madison, WI, USA). Sequence alignments among individual strains were determined using MEGA v.10 to identify mutations within the genes.

Real-time quantitative reverse transcription PCR (RT-qPCR)

A. baumannii cultures were grown overnight in Trypticase soy broth (TSB) (Oxoid^{TM}, Basingstoke, UK) at 37 °C with shaking to exponential phase (OD 0.3). Cultures were then incubated for two hours with or without 3 μg mL^{-1} tigecycline. Bacterial cells were harvested by centrifugation (5,000 rpm, 10 min, 4 °C), and RNA was isolated using hot phenol extraction. Following DNase I treatment (RNase-free DNase set, Geneaid, Taiwan) to remove genomic DNA contamination, RNA was purified using an RNA clean-up kit (Geneaid, Taiwan) and eluted in DEPC-treated water (0.1% v/v). RNA quantity and purity were assessed using a Nanodrop-100 spectrophotometer (Nanodrop Technology Inc, Wilmington, DE, USA). The integrity of total RNA was verified by formaldehyde-agarose gel electrophoresis. cDNA synthesis was performed from 1 μg of total RNA using an iScript Reverse Transcription Supermix kit (Bio-Rad, Hercules, CA, USA).

The expression levels of efflux pump genes (*adeB*, *adeG*, and *adeJ*) and their regulators (*adeR* and *adeS*) were quantified by RT-qPCR using Luna Universal qPCR Master Mix on a CFX96 Touch TM Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA). The thermal cycling program consisted of an initial denaturation step (95 °C, 1 min) followed by 40 cycles of denaturation (95 °C, 15 s) and extension (62 °C, 30 s). Primer sequences (Table S1) were designed to amplify specific target genes. mRNA expression was calculated using the difference in Ct values, normalized to the housekeeping gene 16S ribosomal RNA, and expressed as fold-change relative to *A. baumannii* ATCC 19606 (reference strain) based on the $2^{-\Delta\Delta Ct}$ method [25]. Gene with a ≥ 4-fold increase in expression compared to the reference strain were considered overexpressed. Each experiment was duplicated with triplicate technical replicates (n = 6 total).

Statistical analysis

Statistical analyses were performed using STATA software. Data are presented as means ± standard error of the mean (SEM). Statistical significance was evaluated using both a two-sample t-test and a two-sample Wilcoxon rank-sum (Mann–Whitney) test, with $P < 0.05$ considered statistically significant.

Ethics declarations

This work was approved by the Ethics Committee of the Faculty of Medicine Vajira Hospital of Navamindradhiraj University in Bangkok, Thailand (COA 009/2561).

RESULTS

Bacterial strains and susceptibility profile of *A. baumannii* clinical isolates

All thirty *A. baumannii* isolates displayed high-level resistance (100%) to cefepime, ceftazidime, ciprofloxacin, meropenem, and piperacillin-tazobactam. Resistance was also observed towards tigecycline (100%), gentamicin (96.7%), levofloxacin (96.6%), ampicillin/sulbactam (96.2%), and imipenem (92.9%). Susceptibility to cefoperazone/sulbactam (75.9%), amikacin (63.3%), and colistin (14.8%) varied among isolates. Detailed antimicrobial susceptibility data are represented in Table 1 and Fig. S1. Notably, all isolates exhibited a multidrug-resistant phenotype and a tigecycline MIC ≥ 4 μg mL^{-1}, which indicated resistance.

Table 1. Antimicrobial susceptibility profile of 30 Thai clinical isolates of tigecycline-resistant *A. baumannii*

Antibiotics	Resistant %	Intermediate %	Sensitive %
Amikacin (AMK)	63.3	3.3	33.3
Ampicillin/Sulbactam (AMS)	96.2	0	3.8
Cefoperazone/Sulbactam (CFP/SUL)	75.9	17.2	6.9
Cefepime (FEP)	100	0	0
Ceftazidime (CAZ)	100	0	0
Ciprofloxacin (CIP)	100	0	0
Colistin (CL)	14.8	0	85.2
Gentamicin (GEN)	96.7	0	3.3
Imipenem (IPM)	92.9	7.1	0
Levofloxacin (LVX)	96.6	3.4	0
Meropenem (MEM)	100	0	0
Piperacillin/Tazobactam (TZP)	100	0	0
Tigecycline (TGC)	100	0	0

Genotype assessment

All isolates harboured the bla_{OXA-51}-like gene. The bla_{OXA-23}-like was detected in 96.7% (29/30) of strains, while only 13.33% (4/30) possessed the bla_{OXA-58}-like gene. None of the isolates carried the bla_{OXA-24}-like gene. Co-occurrence of bla_{OXA-51}-like and bla_{OXA-23}-like genes was observed in 83.3% (25/30) of isolates. The presence of all three genes (bla_{OXA-51}-like, bla_{OXA-23}-like, and bla_{OXA-58}-like) was identified in 13.3% (4/30) of strains (Table 2).

Activity of the efflux pump system

Next, we evaluated the contribution of efflux pumps to tigecycline resistance by using the efflux pump inhibitor CCCP. The MICs of tigecycline were determined for all isolates in both the presence and absence of 25 μM CCCP (Table 2). A fourfold or greater reduction in the MIC with CCCP compared to the MIC without CCCP is considered indicative of significant efflux pump activity [26]. This finding suggests that efflux pumps contributed to tigecycline resistance in 13.3% (4/30) of the isolates. Additionally, a modest 2- to 4-fold decrease in MIC is observed for 76.67% (23/30) of the isolates when treated with CCCP, potentially indicating a partial role for efflux pumps in these isolates. No reduction in the MIC caused by CCCP was observed in 23.33% (7/30) of the isolates. Notably, our analyses revealed that CCCP lacks intrinsic antimicrobial activity against all bacterial strains (data not shown).

Gene expression analysis of the RND-type efflux pump system

Our study involved quantifying gene expression levels using the RT-qPCR method. Specifically, we focused on the genes encoding the RND efflux system (*adeB*, *adeG*, and *adeJ*) and its regulators (*adeR* and *adeS*). The housekeeping gene 16S rRNA served as a control for normalization, and the tigecycline-susceptible *A. baumannii* strain ATCC 19606 was used as a reference (Table S2). The average relative expression levels of *adeB*, *adeG*, *adeJ*, *adeR*, and *adeS* in all isolates were 16.36, 1.92, 4.65, 7.61, and 4.72, respectively.

Table 2. Effect of CCCP, mutations in AdeR and AdeS amino acid sequences, and expression of the RND-type efflux pump system in 30 Thai clinical isolates of tigecycline-resistant *A. baumannii*

Strains	Sample type	MIC Tigecycline	MIC Tigecycline + CCCP	Fold reduction in MIC with CCCP	*bla* oxa-23⁻ like	*bla* oxa-24⁻ like	*bla* oxa-51 like	*bla* oxa-58 like	Gene overexpression				
									adeB	*adeG*	*adeJ*	*adeR*	*adeS*
G560T	urine	128	64	2	+	–	+	–	+	–	+	+	+
H1074	blood	64	16	4	+	–	+	–	+	–	–	+	–
H1847	blood	128	64	2	+	–	+	–	–	–	–	–	–
H222	blood	64	16	4	+	–	+	–	–	–	–	+	–
M251	sputum	32	32	0	+	–	+	–	+	–	–	–	–
R109	sputum	8	4	2	+	–	+	+	+	–	–	+	+
R171	sputum	8	4	2	+	–	+	–	+	–	–	–	–
R215	sputum	64	64	0	+	–	+	–	+	–	–	–	+
R234	sputum	16	16	0	+	–	+	–	–	–	–	–	–
R270	sputum	64	32	2	+	–	+	–	+	–	+	+	+
R286	sputum	64	32	2	+	–	+	–	+	–	–	–	+
R291	sputum	16	8	2	+	–	+	–	+	–	–	+	+
R297	sputum	128	64	2	+	–	+	+	+	+	+	+	+
R328	sputum	64	16	4	–	–	+	–	+	–	+	+	+
R339	sputum	32	16	2	+	–	+	–	+	–	–	–	–
R380	sputum	16	16	0	+	–	+	+	+	+	+	+	+
R422	sputum	128	64	2	+	–	+	–	+	–	+	–	–
R435	sputum	32	32	0	+	–	+	+	–	–	+	+	+
R465	sputum	64	32	2	+	–	+	–	+	–	–	+	–
R494	sputum	8	4	2	+	–	+	–	+	–	+	+	–
R516	sputum	32	16	2	+	–	+	–	+	–	–	+	+
R64	sputum	64	16	4	+	–	+	–	–	–	–	–	–
R692	sputum	64	64	0	+	–	+	–	–	–	–	–	–
R72	sputum	64	32	2	+	–	+	–	–	–	–	–	–
R83	sputum	128	64	2	+	–	+	–	+	–	–	–	+
S203	wound	16	8	2	+	–	+	–	+	+	+	+	+
S301	sputum	64	32	2	+	–	+	–	–	–	–	+	–
S330	body fluid	32	32	0	+	–	+	–	–	–	–	–	–
S354	wound	32	16	2	+	–	+	–	–	–	–	–	–
S367	catheter tip	64	32	2	+	–	+	–	–	–	–	–	–

Abbreviations: CCCP: carbonyl cyanide 3-chlorophenylhydrazone.

Among the 30 TRAB isolates, 63.33% exhibited 4.05- to 151.51-fold increases in *adeB* expression. Ten percent of the isolates showed 4.09–6.10-fold increases in *adeG* expression, and 30% showed 4.15–13.76-fold increases in *adeJ* expression. Notably, 26.67% and 10% of isolates co-expressed two (AdeABC–AdeIJK) or three (AdeABC, AdeFGH, and AdeIJK) RND systems, respectively. The AdeRS two-component system also exhibited increased expression, with fold changes ranging from 4.39 to 45.70 for *adeR* (50% increase) and 4.04 to 16.16 for *adeS* (43.33% increase). Co-expression of the AdeRS system was observed in 33.33% of TRAB isolates. Notably, overexpression of *adeB*, *adeR*, and *adeS* was detected in a higher proportion of isolates that tested positive for the TGC-CCCP assay (69.57%, 56.52%, and 43.48%, respectively) compared to those that tested negative (Table 2 and Table S2). However, correlation analysis between the efflux pump gene expressions and tigecycline resistance levels did not reveal statistically significant differences (Fig. 1). Isolates with a high tigecycline MIC (≥ 64 μg mL^{-1}) displayed a trend toward increased expression of *adeB*, *adeR*, and *adeS* compared to those with a lower MIC (8–32 μg mL^{-1}). This suggests a potential role for these genes in tigecycline resistance, although further investigation is needed.

Amino acid substitution in AdeR and AdeS

To identify mutations potentially associated with tigecycline resistance in *A. baumannii* isolates, the amino acid sequences of their AdeR and AdeS regulators were compared to the reference strain *A. baumannii* ATCC 19606. However, to avoid misinterpreting natural variations (polymorphisms) as mutations, sequences from both *A. baumannii* ATCC 19606 and *A. baumannii* ATCC 17078 reference strains were considered silent polymorphisms, including P241L in AdeR and A153T, P172L, L214F, S263A, A280S, D281Q, F303Y, V331I, N356G, and N357H in AdeS.

Analysis of AdeR sequences revealed point mutations at D93V, V120I, A136V, I175L, F224L and Q225N. The most prevalent mutation was V120I (93.33%), followed by A136V (66.67%), I175L (26.67%), F224L (6.67%), Q225N (6.67%), and D93V (3.33%) (Fig. 2A and Table S3). Four distinct mutation patterns were observed in AdeR: three double mutations (V120I+A136V, V120I+I175L and F224L+Q225N) and one triple mutation (D93V+V120I+I175L), accounting for 66.67%, 23.33%, 6.67% and 3.33% of isolates, respectively. The co-mutations at V120I and A136V mutations was the most frequent in AdeR (Fig. 2A and Table S3).

Amino acid substitutions and deletions were identified in AdeS at positions S8R, E51K, D60A, C70Y, N127K, K132N, T156M, F170I, G186T, R249H, N268H, V279A, N314K, N314D, N314 (deletion), V332M, and V348I. The frequency of each mutation point is shown in Fig. 2B and Table S4. Analysis of AdeS sequences from all 30 isolates revealed 14 distinct mutation patterns, including the wild-type sequence (no mutations), three single mutations (D60A, C70Y, and

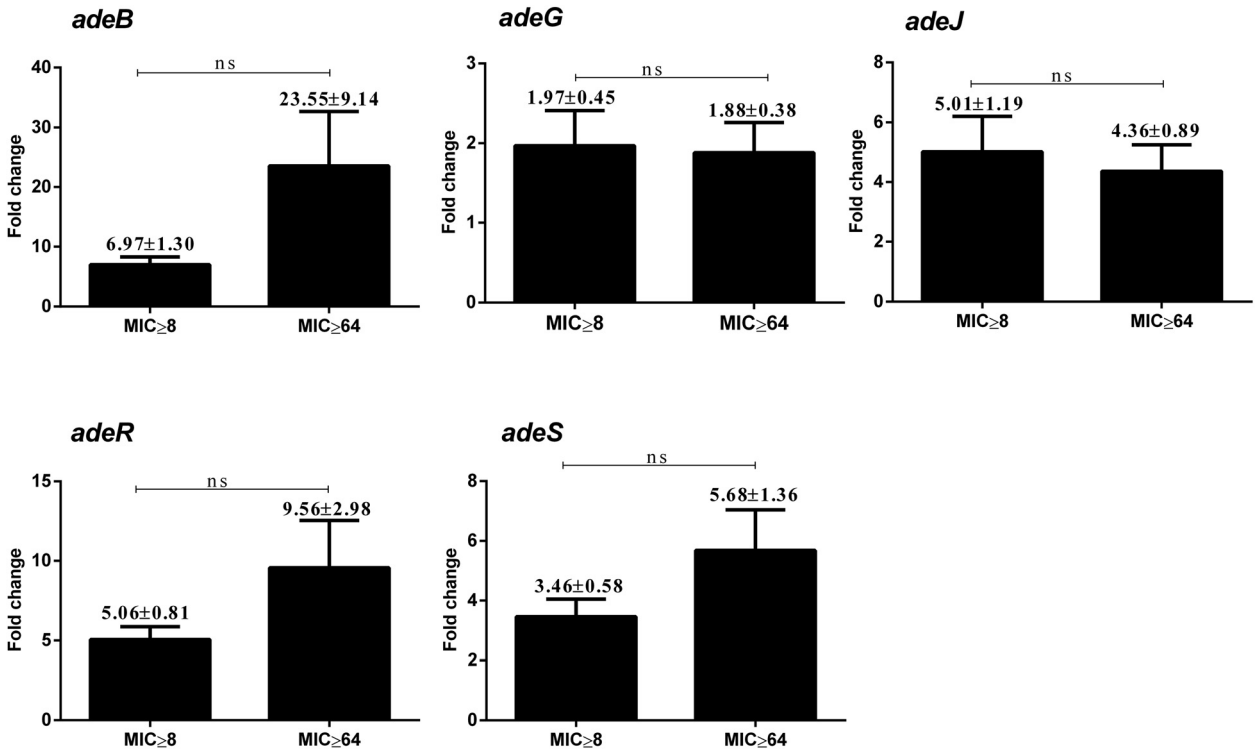

Fig. 1. **Expression level of RND efflux pump genes evaluated in 2 groups of tigecycline MIC at cutoff points of 8–32 μg mL^{-1} and ≥ 64 μg mL^{-1}.** The clinical isolates were divided into two groups by tigecycline's MIC value with a cutoff point of 8–32 μg mL^{-1} and ≥ 64 μg mL^{-1}. The isolates with tigecycline MIC ≥ 64 μg mL^{-1} displayed a trend towards more distinct expression of *adeB*, *adeR*, and *adeS* than that of the isolates with tigecycline MIC 8–32 μg mL^{-1}. The graphs depict the mean ± SEM of three replicates from independent, duplicate experiments. The abbreviation "ns" denotes a statistically significant difference at $P < 0.05$

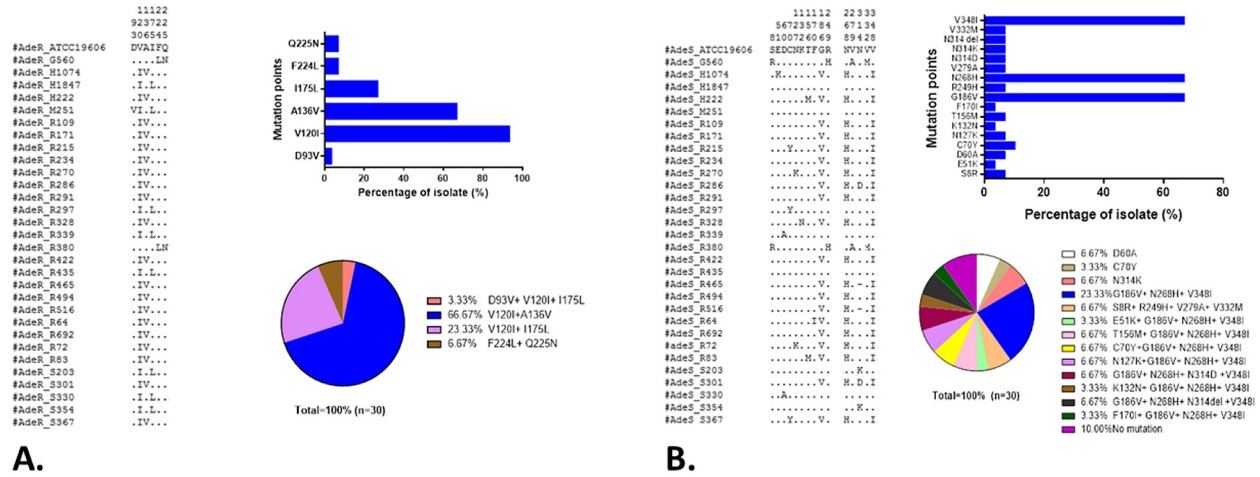

Fig. 2. **Amino acid polymorphic sites, frequencies of each point mutation, and distribution of different mutation patterns in AdeR (A) and AdeS (B) among 30 Thai clinical isolates of tigecycline-resistant *A. baumannii***

N314K), one triple mutation (G186V+N268H+V348I) and nine quadruple mutations (various combination). Among these, the most common patterns were triple mutations (G186V+N268H+V348I; 23.33%), wild type (10%), and single mutations (D60A, C70Y, and N314K; 16.67% combined). The remaining nine patterns were detected in only 3.33%–6.67% of isolates each (Fig. 2B and Table S4). The co-occurrence mutation G186V+N268H+V348I was the most prevalent in AdeS, observed in 66.67% of isolates.

The most frequent mutations in the TRAB isolates were the triple mutation in AdeS (G186V+N268H+V348I) and the double mutation in AdeR (V120I+A136V). Notably, isolates harbouring these mutations displayed increased expression of the *adeB* gene compared to wild-type isolates, as shown in Fig. 3. This finding suggests that mutations in both AdeS (G186V+N268H+V348I) and AdeR (V120I+A136V) may play a role in upregulating the expression of *adeB* considered as an essential efflux pump gene [27].

DISCUSSION

The effective treatment of multidrug-resistant *A. baumannii* infections present a significant challenge in clinical settings. Tigecycline is often the last effective antimicrobial agent for controlling these infections [9]. However, an increasing frequency of TRAB has been reported [10, 28]. All TRAB isolates in this study were multidrug-resistant *A. baumannii* and exhibited a high resistance to multiple antibiotics (Table 1). Notably, all strains harbored carbapenemase genes: 100% $bla_{\text{OXA-51}}$-like, 96.7% $bla_{\text{OXA-23}}$-like, and 13.3% $bla_{\text{OXA-58}}$-like, suggesting a possible association between carbapenem antibiotics use in *A. baumannii* treatment and the emergence of tigecycline resistance.

Several studies have reported that efflux pump systems were the mechanism for reduced tigecycline susceptibility in *A. baumannii* [15, 29, 30]. We employed CCCP to assess efflux pump activity. The addition of CCCP significantly reduced tigecycline resistance (2- to 4-fold) in 76.6% of the isolates, indicating the involvement of the drug efflux pumps in tigecycline resistance development within *A. baumannii*. We further investigated the expression levels of the RND efflux pump system in tigecycline-resistant clinical isolates. Our finding corroborated previous observations, demonstrating that *adeB* gene overexpression is a prevalent mechanism (63%) of tigecycline resistance in *A. baumannii* [3, 18–20]. Nearly 70% of the CCCP-affected strains displayed an upregulated *adeB* gene.

Moreover, the overexpression level of *adeB* had a trend toward being related to the tigecycline MIC (Fig. 1). Conversely, high expression levels of *adeJ*, *adeG*, *adeR*, and *adeS* genes did not appear to influence tigecycline susceptibility reduction and were, therefore, not associated with tigecycline resistance. Consequently, the upregulation of the AdeABC efflux pump emerged as the sole factor demonstrably influencing tigecycline resistance in *A. baumannii*.

The AdeRS two-component system governs the regulation of AdeABC [16]. Previous studies have reported inconsistencies in the association between AdeRS mutations and AdeABC overexpression in TRAB [6, 18, 27, 31]. In this study, AdeR and AdeS exhibited mutations in multiple residues. AdeR mutation sites included D93V, V120I, and A136V in the receiver domain and I175L, F224L, and Q225N in the DNA-binding domain. Notably, this study found that the co-mutations of V120I and A136V occurred most frequently. The mutation sites of AdeS were dispersed across the transmembrane domain (E51K and D60A), catalytic domain (C70Y), HAMP domain (N127K and K132N) and DHp domain (T156M, F170I, G186V, R249H, N268H, V279A, N314K, V332M, and V348I). Several of these mutations have been previously reported in clinical isolates with varying levels of *adeB* expression [6, 18, 20, 27, 32, 33]. Interestingly, G186V and V348I emerged as predominant mutations in this study. A prior investigation linked the G186V mutation in AdeS to efflux pump overexpression [34]. This mutation likely induces conformational changes within the AdeS DHp domain, leading to AdeABC efflux

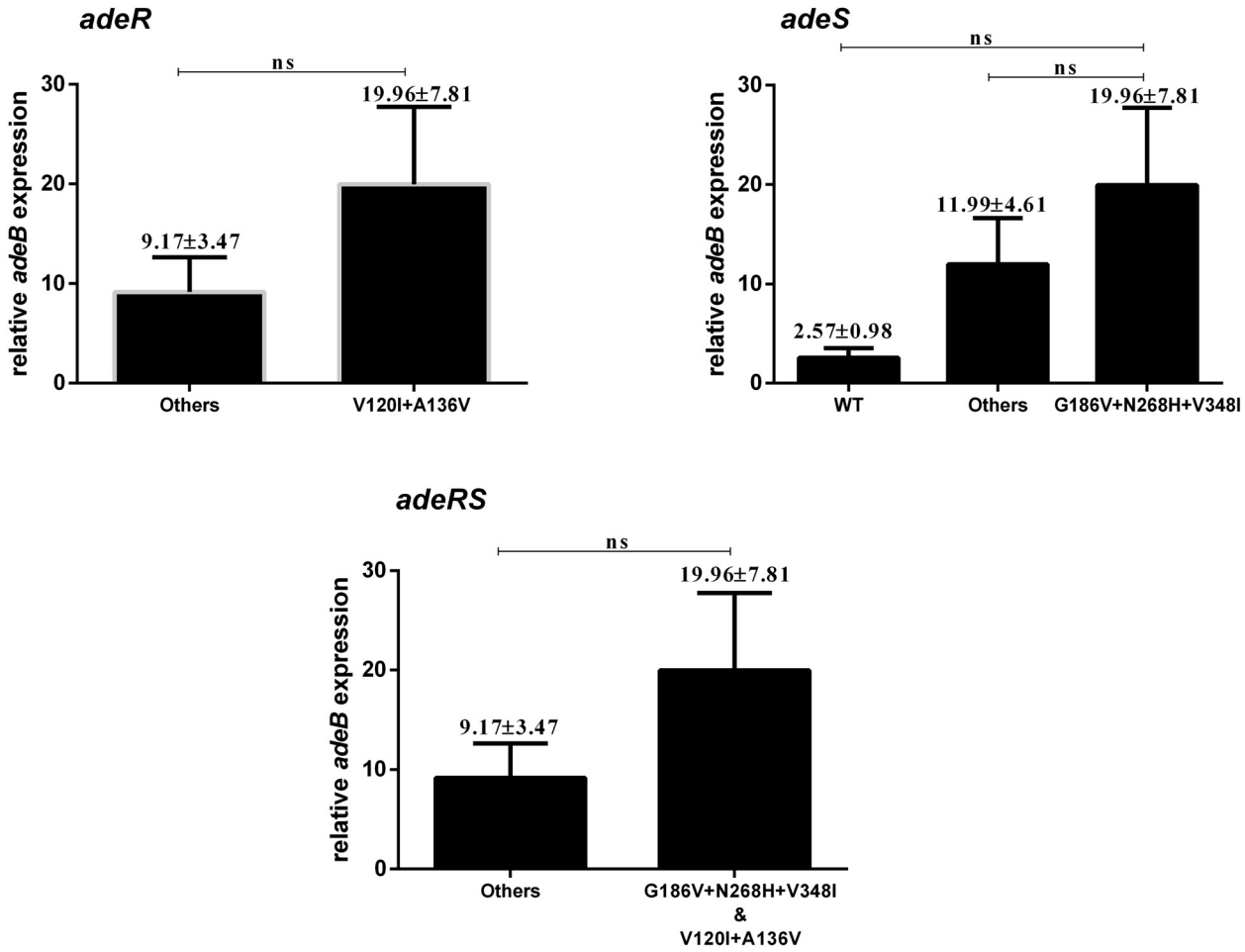

Fig. 3. **Correlation between AdeABC efflux pump gene expression and point mutation pattern in AdeR, AdeS, and combination of *adeRS* genes.** *adeB* expression in the isolates carried V120I and A136V of AdeR and G186V+N268H+ V348I of *adeS* were compared to other mutation patterns. The graphs show the mean ± SEM of two independent experiments, each with three replicates. The results are statistically significant as "ns" indicates no significant different at $P < 0.05$

pump overexpression [20]. Our findings suggest that the double mutation (V120I and A136V) in AdeR and the triple mutation (G186V, N268H, and V348I) in AdeS represent mutational hotspots associated with *ade*B gene overexpression and tigecycline resistance (Fig. 3). Although the RND efflux pump is one of the significant factors contributing to tigecycline resistance, it is not the only mechanism responsible for tigecycline resistance in these strains. Other efflux pump families can be involved in resistance to tigecycline such as ABC transporters (MsbA and MacAB-TolC) [35, 36], and MFS efflux pumps (TetA, TetB, TetA(39), and Tet(Y)) [37–39]. The MFS efflux pump family through TetA synergizes with AdeABC and AdeIJK and stimulates tigecycline-resistant strains [40].

SUMMARY

Previous studies extensively documented the role of RND efflux pumps in TRAB [3, 6, 12, 29]. This study investigated the contribution of RND efflux pumps in tigecycline-resistant *A. baumannii* clinical isolates from the Faculty of Medicine Vajira Hospital, Bangkok, Thailand. We evaluated the impact of the efflux pump inhibitor CCCP on tigecycline resistance. In the presence of CCCP, tigecycline susceptibility increased 2- to 4-fold in 76.6% of *A. baumannii* isolates. This finding suggests that efflux pumps play a significant role in tigecycline resistance for most isolates. Furthermore, the TRAB isolates demonstrated an increased expression of AdeABC (63%), AdeFGH (6.67%), AdeIJK (30%), and AdeRS (20%). These findings support the involvement of multiple RND efflux pumps in tigecycline resistance. Finally, nearly 70% of TRAB isolates that demonstrated increased susceptibility to tigecycline in the presence of CCCP also showed elevated AdeABC efflux pump activity. This finding further strengthens the link between AdeABC efflux pumps and tigecycline resistance in *A. baumannii*.

CONCLUSIONS

The results of this study point towards a significant role for the AdeABC efflux pump system in reducing tigecycline

susceptibility in *A. baumannii*. Our findings suggest that upregulation of AdeABC expression, potentially mediated by point mutations in AdeR or AdeS, contributes to tigecycline resistance. Specifically, mutations like G186V+N268H+V348I in AdeS and V120I+A136V in AdeR may be associated with increased AdeABC transcriptional levels.

Future studies will involve constructing mutants harboring these specific substitutions to definitively establish their role in tigecycline resistance. Elucidating these mechanisms will provide valuable insights into resistance development in *A. baumannii*, ultimately aiding the development of improved treatment strategies and control measures for multidrug-resistant strains.

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ABBREVIATIONS

TRAB	tigecycline-resistant *Acinetobacter baumannii*
RND	resistance-nodulation-cell division
CCCP	carbonyl cyanide m-chlorophenylhydrazone
MIC	minimum inhibitory concentrations
TGC	tigecycline
MDR	multidrug resistance
AMK	amikacin
AMS	ampicillin/sulbactam
CFP/SUL	cefoperazone/sulbactam
FEP	cefepime
CAZ	ceftazidime
CIP	ciprofloxacin
CL	colistin
GEN	gentamicin
IPM	imipenem
LVX	levofloxacin
MEM	meropenem
TZP	piperacillin/tazobactam
TGC	tigecycline
MHB	Mueller-Hinton broth
TSB	Trypticase soy broth
cDNA	complementary DNA
qRT-PCR	quantitative reverse transcription polymerase chain reaction

SUPPLEMENTARY DATA

Supplementary data to this article can be found online at https://doi.org/10.1556/1886.2024.00070.

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