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AdeR-AdeS mutations & overexpression of the AdeABC efflux system in ciprofloxacin-resistant *Acinetobacter baumannii* clinical isolates

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***Background & objectives*: Overexpression of efflux pumps is a cause of acquired resistance to fluoroquinolones in *Acinetobacter baumannii*. The present study was done to investigate the presence and overexpression of AdeABC efflux system and to analyze the sequences of AdeR-AdeS regulatory system in ciprofloxacin-resistant *A. baumannii* isolates.**

***Methods*: Susceptibility of 50 clinical *A. baumannii* isolates to ciprofloxacin, imipenem, ceftazidime, cefepime and gentamicin antimicrobials was evaluated by agar dilution method. Isolates were screened for the evidence of active efflux pump. Isolates were also examined for *adeR-adeS* and *adeB* efflux genes by polymerase chain reaction (PCR). The *adeR* and *adeS* regulatory genes were sequenced to detect amino acid substitutions. Expression of *adeB* was evaluated by quantitative reverse-transcriptase PCR.**

***Results*: There were high rates of resistance to ciprofloxacin (88%), ceftazidime (88%), cefepime (74%) and imipenem (72%) and less resistance rate to gentamicin (64%). Phenotypic assay showed involvement of active efflux in decreased susceptibility to ciprofloxacin among 16 isolates. The 12.27-fold increase and 4.25-fold increase were found in *adeB* expression in ciprofloxacin-full-resistant and ciprofloxacin-intermediate-resistant isolates, respectively. Several effective mutations, including A91V, A136V, L192R, A94V, G103D and G186V, were detected in some domains of AdeR-AdeS regulators in the overexpressed ciprofloxacin-resistant isolates.**

***Interpretation & conclusions*: The results of this study indicated that overexpression of the AdeABC efflux pump was important to reduce susceptibility to ciprofloxacin and cefepime in *A. baumannii* that, in turn, could be triggered by alterations in the AdeR-AdeS two-component system. However, gene expression alone does not seem adequate to explain multidrug resistance phenomenon. These results could help plan improved active efflux pump inhibitors.**

Key words *Acinetobacter baumannii* - *adeB* - *adeR* - *adeS* - ciprofloxacin resistance

One of the Gram-negative pathogens that has acquired epidemiological importance among nosocomial infections is *Acinetobacter baumannii*[1]. This pathogen affected mainly patients with

impaired host defenses in the Intensive Care Unit and burn wards. It has been implicated in a wide range of infections, including bacteraemia, wound infection, ventilator-associated pneumonia (VAP) and meningitis[2]. Particularly, the emergence and distribution of *A. baumannii* isolates with multiple drug-resistance (MDR-AB) or extensively drug-resistance in Iran[3,4] and other parts of the world[5] have become of great concern since we have now rather few treatment options against *A. baumannii* infections. Developed in the 1980s, fluoroquinolones showed potent activity against *Acinetobacter* strains and had even a better effect than the extended-spectrum cephalosporins or aminoglycosides[6]. However, resistance to these antibiotics rapidly arose among in *A. baumannii* clinical isolates as a result of extensive or unnecessary use in different medical settings worldwide[7-9].

The best-known mechanism of resistance to quinolones in *A. baumannii* is spontaneous mutations in the quinolone resistance-determining region (QRDR) of *gyrA* and *parC* genes, encoding DNA gyrase and topoisomerase IV, respectively[8,9]. On the other hand, overexpression of efflux pumps is also a source of acquired resistance to fluoroquinolones in *A. baumannii*. AdeABC is the first and the major discovered efflux system in *A. baumannii* and belongs to the resistance-nodulation-cell division (RND) superfamily transporter[10]. The *adeABC* operon encodes the AdeA membrane fusion protein, the multidrug transporter protein AdeB and the AdeC outer membrane protein. Expression of *adeABC* is closely regulated by the AdeR-AdeS two-component system (TCS)[11]. Constitutive overexpression of the *adeABC* efflux system has been shown to be caused either by the IS_{aba-1} insertion upstream of the *adeABC* operon or by single- or multiple-point mutations in *adeR* and *adeS* genes. In these circumstances, due to decreased intracellular antibiotic concentration, *A. baumannii* becomes resistant to not only fluoroquinolones but also aminoglycosides, tetracyclines, chloramphenicol and β-lactams[11,12].

Single nucleotide polymorphisms (SNPs) are the most abundant form of genetic variations in closely related microbial strains or isolates. DNA sequencing studies detecting polymorphisms and comparing distinct drug-susceptible and drug-resistant strains improve our understanding of the evolutionary mechanisms of bacteria at the genomic levels and facilitate the development of next-generation antimicrobial agents. In this study, analysis of *adeB* gene expression was performed to evaluate the correlation between the active AdeABC efflux system and ciprofloxacin resistance in *A. baumannii* clinical isolates. In addition, sequencing analysis of the AdeR-AdeS TCS was performed to explore the role of mutations in the regulators to overexpression of AdeABC.

Material & Methods

Bacterial isolates: *A. baumannii* clinical isolates included in this study were obtained from the Motahari Burn and Reconstruction Center, a subset of Iran University of Medical Sciences (IUMS), in Tehran during 2012-2013. Bacterial isolates were initially identified by conventional biochemical methods in the department of Microbiology, School of Medicine, IUMS, Tehran, Iran, and then, species identification was performed by the polymerase chain reaction (PCR) amplification of the intrinsic bla_{OXA-51}-like carbapenemase gene and sequencing[13].

In vitro susceptibility testing: Minimum inhibitory concentration (MIC) value of five antibiotics (Mast, Merseyside, UK), including ciprofloxacin (0.015-128 µg/ml), imipenem (0.06-128 µg/ml), ceftazidime (0.25-128 µg/ml), cefepime (0.25-128 µg/ml) and gentamicin (0.06-128 µg/ml) was determined by the agar dilution method based on the recommendations of the Clinical and Laboratory Standards Institute[14]. *Pseudomonas aeruginosa* ATCC 27853 and *Escherichia coli* ATCC 25922 strains were used as controls. For determination of the presence of efflux system, the effect of the efflux inhibitor carbonyl cyanide 3-chlorophenylhydrazone (CCCP) (Sigma-Aldrich, United Kingdom) (final concentration 25 µg/ml) on ciprofloxacin MIC of isolates was tested as described previously[15].

Conventional polymerase chain reaction (PCR) and sequence analysis: Analysis of the *adeR-adeS* locus was performed by PCR and sequencing. The primers specific for the genes encoding the transporter protein *adeB* and TCS comprising *adeS* and *adeR* are listed in Table I. DNA from the prepared isolates of *A. baumannii* was extracted using Genomic DNA Purification Kit (Fermentas; Vilnius, Lithuania). Amplification reactions of three genes were performed with the parameters described previously[16,17]. The PCR products were analyzed by an ABI 3730XL DNA Analyzer (Applied Biosystems Inc., USA). The obtained sequences results were examined by using the NCBI BLAST program (*http://www.ncbi.nlm.*

Table I. Oligonucleotide primers used for various genes

Gene	Primer sequence (5'→3')	Amplicon size (bp)	Reference
adeR	F: ATGTTTGATCATTCTTTTTCTTTTG R: TTAATTAACATTTGAAATATG	686	16
adeS	F: ATGAAAAGTAAGTTAGGAATTAGTAAG R: TTAGTTATTCATAGAAATTTTTATG	1074	16
adeB (qualitative)	F: TTAACGATAGCGTTGTAACC R: TGAGCAGACAATGGAATAGT	541	17
adeB (quantitative)	F: AACGGACGACCATCTTTGAGTATT R: CAGTTGTTCCATTTCACGCATT	84	16
16S rRNA	F: CAGCTCGTGTCGTGAGATGT R: CGTAAGGGCCATGATGACTT	151	16

nih.gov/BLAST/). Multiple-sequence alignment of the deduced peptide sequences was performed using the ClustalW2 software program at the European Bioinformatics Institute website (*http://www.ebi.ac.uk*).

Quantitative real-time polymerase chain reaction (PCR) of adeB gene: Efflux-positive isolates were assessed for the expression of *adeB* gene. 16S rRNA was used as a housekeeping gene to normalize levels of *adeB* transcripts. Oligonucleotide primer sequences used for *adeB* and 16S rRNA are shown in Table I. Total RNA was initially extracted (Total RNA Purification Kit; Jena Bioscience, Germany) from cultures grown in the mid-log phase of growth in Luria-Bertani broth (Merck, Darmstadt, Germany) and then contaminating DNA was removed by RNase-free DNase I (Fermentas, Thermo Fisher Scientific Inc., Vilnius, Lithuania). The concentrations of RNA in each sample were quantified with a spectrophotometer at 260 and 280 nm. DNAse-treated RNA (2.5 µg) was reverse transcribed into cDNA using the CycleScript RT PreMix Kit (Bioneer, Korea) and 80 pM random hexamer (dN6) (Bioneer, Korea). Reverse-transcriptase PCR (RT-PCR) was performed by using a 2× GreenStar Master Mix Kit (Bioneer, Korea) on a Corbett Rotor-Gene 6000 real-time rotary analyzer (Corbett Life Science, Australia). A typical RT-PCR sample (25 µl) contained 11 µl of PCR Master Mix, 1 µl of cDNA, 0.5 µl of 0.8 µM solutions of both forward and reverse gene-specific primers and 12 µl of double distilled water. Real-time run protocol was the same as previously described[16]. Each sample was run in triplicate. A critical threshold cycle (CT) value was used to represent *adeB* transcripts quantitatively. The ΔCT for *adeB* transcripts was calculated against that for the 16S rRNA gene, and the ΔΔCT was calculated against that for the ciprofloxacin-susceptible strain, *A. baumannii* ATCC 19606. The *adeB* relative expression was calculated by the $2^{-\Delta\Delta CT}$ method.

Statistical analysis: SPSS 18 for Windows (SPSS Inc., Chicago, IL, USA) was used for statistical analysis. Differences in the mean expression level of *adeB* transcripts in the clinical isolate groups against that the *A. baumannii* ATCC 19606 control strain were assessed by independent samples *t* test. Categorical variables, including antibiotic resistance pattern, mutation in the *adeR-adeS* locus and *adeB* expression, were compared using the Chi-square test. The relationship between ciprofloxacin MIC values with the number of *adeR* and *adeS* mutations and with the expression level of *adeB* gene was assessed by calculating Spearman's correlation coefficient.

Results

Bacterial isolates, susceptibility profiles and effects of carbonyl cyanide 3-chlorophenylhydrazone (CCCP): During a nine-month period, a total of 50 single patient isolates of *A. baumannii* were recovered from hospitalized burn patients. Eighteen per cent (9 out of 50) and 82 per cent (41 out of 50) of the bacterial isolates were originated from blood and wound specimen cultures, respectively.

High rates of resistance to ciprofloxacin (88%), ceftazidime (88%), cefepime (74%) and imipenem (72%) were observed. However, isolates showed less resistance to gentamicin (64% resistant). Forty isolates (80%) were classified as MDR-AB based on non-susceptibility to >1 agent in ≥3 antimicrobial

categories[18]. The MIC determinations of all test antimicrobials against *A. baumannii* isolates are shown in Table II. The MIC_{50} and the MIC_{90} values for ceftazidime were both >128 µg/ml, higher than those of other antibiotics tested, while imipenem represents the lowest determinations of MIC_{50} (32 µg/ml) and MIC_{90} (64 µg/ml).

In the phenotypic assay for the detection of efflux phenotype, CCCP reduced MIC of ciprofloxacin from a 4- to >16-fold in 32 per cent (16 out of 50) of *A. baumannii* isolates, suggesting a putative efflux mechanism. All efflux-positive *A. baumannii* isolates in this study were ciprofloxacin-intermediate (MIC=2 µg/ml) and ciprofloxacin-full resistant (MIC=4 to ≥128 µg/ml) (Table II).

Sequencing of the adeR-adeS regulatory system: All 18 clinical *A. baumannii* isolates, including 16 efflux-positive and two ciprofloxacin-susceptible isolates, were found to carry chromosomal *adeB*, *adeR* and *adeS* genes, simultaneously. The sequencing analysis of the PCR products showed that multiple-point mutations were common in *adeR-adeS* locus. Both ciprofloxacin-susceptible isolates were found to have two mutations each. AB5 had mutations located at the T137A and N115H positions in the AdeR. Isolate AB24 had mutations located in the N115H and N139H positions within the AdeR and AdeS, respectively (Table III). Multiple-sequence alignment of AdeR and AdeS from clinical isolates in comparison to *A. baumannii* ATCC 17978 is shown in Fig. 1. None of the isolates possessed the P116L and T153M substitutions in the AdeR and AdeS, respectively, which were identified to cause altered expression of *adeB*[11]. However, some mutations associated with the *adeABC* overexpression and also SNPs were observed in 87.5 per cent (14 out of 16) of efflux-positive isolates (Table III).

Gene expression analysis of adeB: The 3.29-fold to 19.79- fold increases in the *adeB* expression level were detected in 14 efflux-positive isolates in comparison to ciprofloxacin-susceptible *A. baumannii* control strain. Fig. 2 shows the relative mean±standard deviation expression of *adeB* mRNA transcript as assessed by RT-PCR. Comparing the relative quantification of *adeB* expression in the clinical isolates to those in the ATCC 19606 control strain, 12.27±3.5 and 4.25±0.82 times more *adeB* transcripts were observed in ciprofloxacin-full-resistant and ciprofloxacin-intermediate-resistant *A. baumannii*,

Table II. Susceptibility of clinical *Acinetobacter baumannii* isolates (n=50) to five antibiotics and distribution of minimum inhibitory concentration (MIC) values

Antibiotic	MIC range (µg/ml)	n (%) of isolates with determined MIC (µg/ml)													MIC_{50} (µg/ml)	MIC_{90} (µg/ml)	Antibiotic susceptibility		
		0.015	0.03	0.06	0.125	0.25	0.5	1	2	4	8	16	32	64	128	>128			R, n (%)	I, n (%)	S, n (%)
CAZ	4->128	-	-	-	-	-	-	-	-	3 (6)	1 (2)	2 (4)	-	3 (6)	10 (20)	31 (62)	>128	>128	44 (88)	2 (4)	4 (8)
FEP	4->128	-	-	-	-	-	-	-	-	3 (6)	4 (8)	6 (12)	9 (18)	4 (8)	10 (20)	14 (28)	64	>128	37 (74)	6 (12)	7 (14)
IMP	1-128	-	-	-	-	-	-	1 (2)	3 (6)	6 (12)	4 (8)	6 (12)	9 (18)	18 (36)	3 (6)	-	32	64	36 (72)	10 (20)	4 (8)
CIP	0.5->128	-	-	-	-	-	1 (2)	1 (2)	4 (8)	2 (4)	1 (2)	2 (4)	8 (16)	18 (36)	7 (14)	6 (12)	64	>128	44 (88)	4 (8)	2 (4)
GEM	0.5->128	-	-	-	-	-	1 (2)	-	3 (6)	8 (16)	6 (12)	2 (4)	3 (6)	3 (6)	6 (12)	18 (36)	64	>128	32 (64)	6 (12)	12 (24)

CAZ, ceftazidime; IMP, imipenem; GEN, gentamicin; CIP, ciprofloxacin; FEP, cefepime; R, resistant; S, susceptible; I, intermediate

Table III. Antimicrobial susceptibility profiles, AdeR and AdeS amino acid substitutions and *adeB* gene expression in 18 clinical isolates of *Acinetobacter baumannii*

Isolate	Antibiotic resistance pattern	MIC (µg/ml) of						Fold reduction in CIP MIC (µg/ml)[a]	Changes in the AdeR	Changes in the AdeS	*adeB* expression level[b]
		CAZ	IMP	GEM	CIP	CIP + CCCP	FEP				
AB5	-	8	1	2	1	1	4	-	T137A, N115H	-	ND
AB24	GEM	4	4	>128	0.5	0.5	4	-	N115H	N139H	ND
AB15	CAZ, IMP, GEM, CIP	128	64	32	8	2	16	4	-	-	1.014
AB31	CAZ, IMP, GEM, FEP, CIP	>128	16	128	4	1	128	4	-	-	1.02
AB1	CAZ, IMP, GEM, FEP	>128	16	>128	2	0.5	128	4	A136V, N138H	V59I, L172P, G186V, D227H	3.29
AB18	CAZ, GEM, FEP	128	2	16	2	0.5	32	4	A91V, A136V	V59I, M129K, L172P, G186V, D227H	4.03
AB40	FEP	4	8	4	2	0.5	32	4	A91V, A136V, V120I	K84E, M129K, G186V	4.45
AB11	CAZ, GEM, FEP	64	4	>128	2	0.25	128	8	A91V, V120I, T137A	K84E, M129K, L172P, G186V	5.26
AB8	CAZ, IMP, GEM, CIP, FEP	128	16	128	128	32	128	4	A91V, F109L, L142I	K84E, L162Y, L172P, V279A	9.22
AB29	CAZ, IMP, CIP, FEP	>128	64	8	128	32	128	4	V120I, A136V, H158L	S55A, D60G, G103D, L105F, V245I	9.68
AB9	CAZ, IMP, GEM, CIP, FEP	>128	64	128	128	32	>128	4	A136V, H158L	S55A, D60G, A94V, G103D, V245I	10.19
AB44	CAZ, IMP, GEM, CIP, FEP	>128	64	>128	128	16	>128	8	A136V, H158L	S55A, D60G, A94V, G103D, V245I	10.95
AB37	CAZ, IMP, CIP, FEP	128	32	8	>128	16	>128	>8	L142I, L192R	A130T, D227H, V245I, V279A, V348I	11
AB22	CAZ, IMP, GEM, CIP, FEP	>128	64	64	>128	16	>128	>8	F109L, L142I, L192R	A130T, D227H, V245I, V279A, V348I	11.8
AB16	CAZ, IMP, GEM, CIP, FEP	128	64	32	>128	16	>128	>8	A91V, L142I	D60G, A94V, L105F, L162Y, V348I	11.45
AB3	CAZ, CIP, FEP	128	8	8	>128	16	>128	>8	V120I, L142I, H158L	D60G, K84E, A94V, G103D, L105F	11.61
AB13	CAZ, IMP, GEM, CIP, FEP	128	32	64	>128	8	>128	>16	V120I, L142I	A94V, G103D, A130T, L172P, V245I, V279A	17.72
AB35	GEM, CIP, FEP	4	4	>128	>128	8	>128	>16	L142I, H158L, L192R	A65G, A94V, G103D, L172P, V245I, V348I	19.79

[a]In the presence of CCCP efflux pump inhibitor; [b]Relative expression compared to that in the reference ATCC 19606 strain. MIC, minimum inhibitory concentration; CAZ, cefatazidime; IMP, imipenem; GEM, gentamicin; CIP, ciprofloxacin; CCCP, carbonyl cyanide 3-chlorophenylhydrazone; FEP, cefepime; ND, not determined

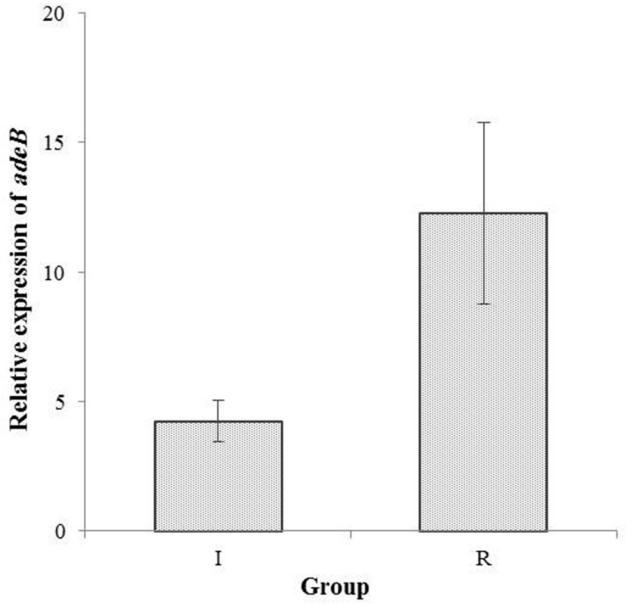

Fig. 1. Expression of *adeB* mRNA transcripts as evaluated by real-time polymerase chain reaction. *adeB* expression in two ciprofloxacin-full resistant (R) and ciprofloxacin-intermediate (I) groups of *Acinetobacter baumannii* was compared relative to that in the reference ATCC 19606 strain. The error bars represent the standard deviation for the average of results from three independent experiments.

respectively (P<0.001). All of the 14 isolates with higher levels of *adeB* expression than the reference strain exhibited alterations in *adeR* and *adeS* genes. In terms of drug resistance pattern, 12 of the 16 efflux-positive isolates (75%) showed multidrug resistance, of which two isolates had no *adeB* overexpression.

Nucleotide sequence accession number: A selection of the *adeR* and *adeS* sequences reported in this study has been submitted to NCBI and deposited in the GenBank data library under the following accession numbers: KY000415, KY000416, KY000417, KY000418, KY000419, KY000420, KY000421, KY000422, KY000423, KY000424, KY000425, KY000426 and KY000427.

Discussion

Antimicrobial resistance is the most important challenge in treating infections due to *A. baumannii*[2]. The necessity for effective therapy regimens to control MDR-AB outbreaks in hospital has been well emphasized[4,5]. An alarming trend of increase in MDR-AB resistance towards different classes of antibiotics has been shown in Tehran, Iran. In particular, Bahador *et al*[19] revealed an increase in MIC values to all test antimicrobials among MDR-AB isolates, between 2006 and 2011. Over a period of five years, MIC_{50} for the majority of and MIC_{90} for all antimicrobials tested also rose. Similar to this, in our study also MIC range of all test antibiotics against the majority of isolates was high. Other studies also have shown a significant increase in most antimicrobial agents in the world[20-22]. Generally, our findings were consistent with these studies that showed *A. baumannii* exhibited high-level resistance to all the first-line antibiotics, including anti-pseudomonal cephalosporins (ceftazidime or cefepime), anti-pseudomonal carbapenems (imipenem or meropenem), fluoroquinolones (ciprofloxacin or levofloxacin) and aminoglycosides (gentamicin or amikacin). In such circumstances, it is important to determine antibiotic susceptibility profiles and exert severe infection control measures to overcome nosocomial MDR-AB strains.

The importance of the AdeABC efflux pump in conferring MDR-AB has been discussed[10,23]. In a small-scale study, Higgins *et al*[12] found a 20-fold increase in the *adeB* transcripts of two high-level ciprofloxacin-resistant (both MIC ≥256 µg/ml) and ofloxacin-resistant (both MIC ≥64 µg/ml) isolates. MICs of non-fluoroquinolone antibiotics, including meropenem, were also found to have risen in the both *adeB*-overexpressed strains. In contrast, Bratu *et al*[24] suggested that increased level of *adeB* expression by itself did not have a major role in fluoroquinolone resistance, but other mechanisms must be accounted. Consistent with Higgins *et al*[12], we found a significant correlation between ciprofloxacin resistance and upregulated *adeB*. Fourteen of the 16 efflux-positive isolates overexpressed *adeB*, while 10 and four of 14 hyper-expressing isolates showed full resistance and intermediate resistance to ciprofloxacin, respectively. In addition, it was found that isolates with full resistance to ciprofloxacin had significantly higher *adeB* expression level than intermediate-resistant isolates. Taken together, the results suggested that upregulation of the *adeB* gene might be important as much as mutational mechanism in *gyrA* and/or *parC* genes to decreased susceptibility to fluoroquinolones in *A. baumannii*.

It seems that reduced susceptibility to cefepime should be mediated partly by the AdeABC efflux pump. Bratu *et al*[24] found that in the absence of cephalosporinase activity, AdeABC efflux system was responsible for the reduced susceptibility to cefepime, and in the presence of an effective cephalosporinase, efflux-based mechanisms played a secondary role. Similar to what was observed for ciprofloxacin, our

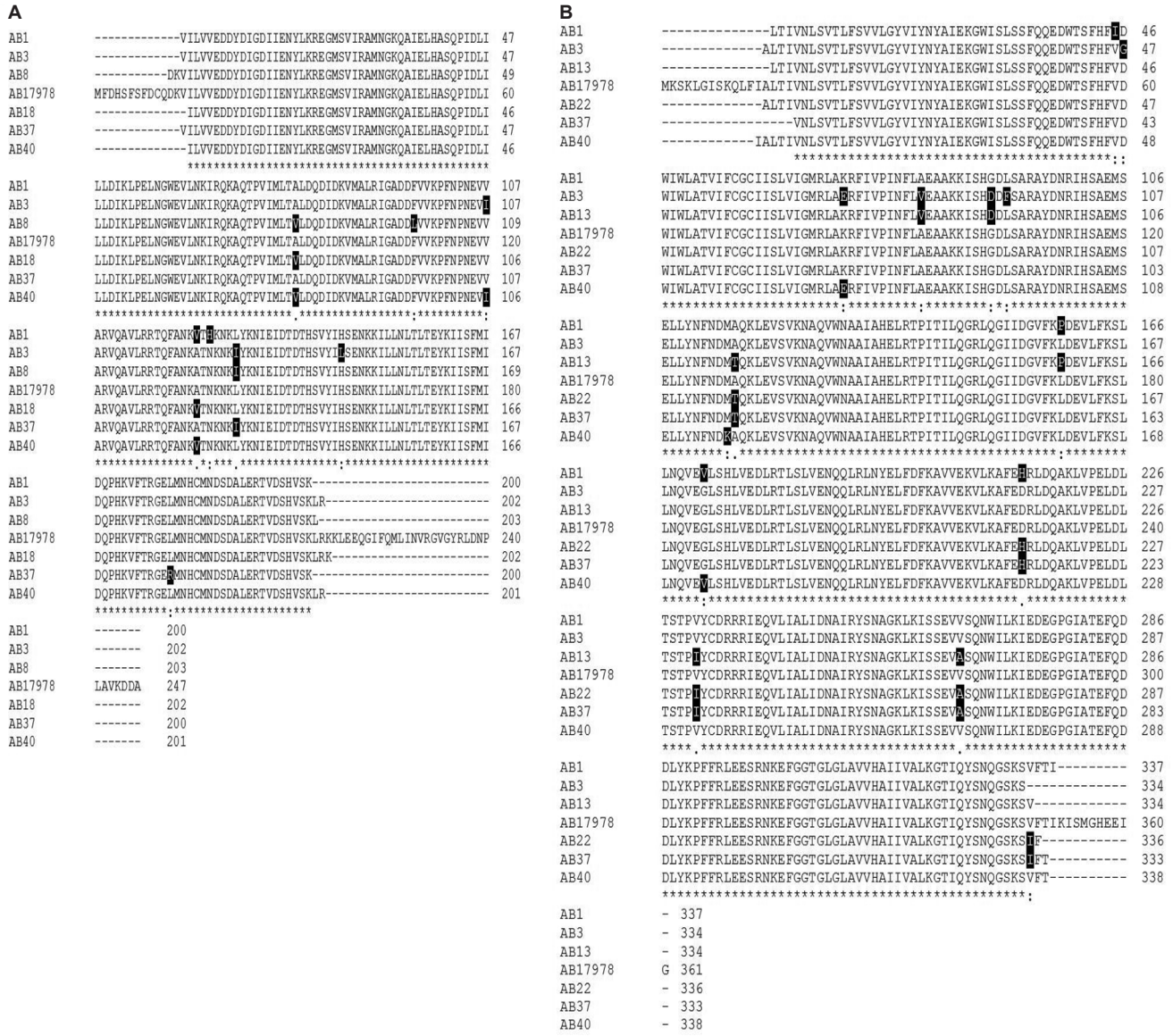

Fig. 2. Multiple-sequence alignment of AdeR (**A**) and AdeS (**B**) from *Acinetobacter baumannii* ATCC 17978 and clinical isolates. Sequence alignment was generated using ClustalW2 software program. The deduced amino acid sequence is designated in a single letter code. Asterisks indicate identical residues, colons indicate strongly similar residues and dots indicate weakly similar residues. Amino acid substitutions in AdeS and AdeR were highlighted in black. Some mutations leading to constitutive expression of AdeABC pump in clinical isolates such as A91V, A136V and L192R in AdeR and A94V, G103D and G186V in AdeS are indicated.

results indicated a significant association between cefepime resistance and AdeABC expression; increased level of the *adeB* expression was present along with higher resistance level to cefepime (MIC ≥128 µg/ml) in a significant percentage of efflux-positive isolates. However, similar to previous studies[12,25], resistance to three remaining antibiotics, imipenem, ceftazidime and gentamicin, was not significantly associated with the overexpression of *adeB* gene, indicating involvement of mechanisms other than the *adeB* expression to develop multidrug resistance.

AdeR-AdeS is an example of TCS that regulates strongly the expression of *adeABC* efflux pump in response to stimuli[10,26]. In the present study, mutations in the *adeR-adeS* operon were investigated to identify the amino acid substitutions affecting AdeABC expression. No changes were found in *adeR* and *adeS* genes of AB15 and AB31; the isolates had *adeB* expression levels equal to the susceptible reference strain. Considering to the restoration of ciprofloxacin activity with the addition of CCCP, there must be other efflux systems but AdeABC, including AdeIJK,

AdeFGH, AbeM and AbeS, to remove fluoroquinolones from the cell[27].

In AdeR, three SNPs leading to increased expression of AdeABC have been reported: D20N located in the D box of the phosphorylation site[28], the A91V and A136V in the signal receiver domain[25,29] and P116L at the first residue of the helix α5[16]. Of these, polymorphisms A91V or A136V were detected in our nine overexpressed isolates (AB1, AB8, AB9, AB11, AB16, AB18, AB29, AB40 and AB44). Although the exact mechanisms need to be explained, such mutations in signal receiver domain of AdeR may change the interactions between the AdeS and AdeR and likely results in the *adeABC* overexpression. Furthermore, another mutation associated with multidrug resistance, L192R, was seen in AdeR of three *adeB* hyper-expressed isolates (AB22, AB35 and AB37). This mutation in the effector domain could alter protein stability[30].

Several substitutions in AdeS were found to be responsible for *adeABC* overexpression: G30D located in the periplasmic loop[31], the A94V and G103D alterations in the histidin kinase, adenylyl cyclase, methyl-accepting chemotaxis protein and phosphatase (HAMP) linker domain[25], the G186V in the α-helix of the dimerization and histidine phosphotransfer (DHp) domain[32] and T153M in the H box[11]. In the present study, while isolates AB16 and AB29 had polymorphisms A94V and G103D, respectively, there was coexistence of these mutations in five isolates AB3, AB9, AB13, AB35 and AB44. It is thought that such amino acid substitutions in the HAMP domain of AdeS protein disrupt transmembrane signal transduction process and have been suggested to be associated with constitutive phenotypes[33]. In addition, it is speculated that G186V mutation detected in four isolates AB1, AB11, AB18 and AB40 causes conformation changes of the AdeS DHp domain and then leading to activation of AdeS and stimulating the expression of the AdeABC efflux pump via interaction with AdeR. It is noteworthy that coexistence of such alterations in AdeR and AdeS TCS, regardless of whether is associated with other SNPs, may lead to efflux overexpression and affects drug susceptibility, synergistically.

In conclusion, our results suggest that the efflux-based system AdeABC is an important contributor to reduced susceptibility to antibiotics of choice for treatment, including ciprofloxacin and cefepime, in *A. baumannii* isolates. It is possible that the effective replacements together with the accumulation of SNPs in AdeR or AdeS may contribute to increased AdeABC expression and ciprofloxacin resistance, although the detailed effect of these variations on pump expression should be determined by rigorous experiments. Although *adeB* expression affects resistance to antimicrobials, gene expression alone is insufficient to develop multi-resistance, and so, multiple causes must be considered. These results may benefit to design active efflux pump inhibitors.

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***Conflicts of Interest*:** None.

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