WITHDRAWN:
The Role of AdeABC, AdeFGH and AdeIJK Efflux Pumps in Reduced Susceptibility to Tigecycline: Emergence of an Outbreak Associated With an International Clone Variant of Acinetobacter Baumannii Isolated From Burn Patients in Iran

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Abstract

Tigecycline is a new therapeutic agent for treating multiple drug-resistant (MDR) *Acinetobacter baumannii* infections. However, recent reports have suggested the efficacy of tigecycline in fighting *A. baumannii* infections has been reduced. In this study we investigated the role of resistance-nodulation cell division (RND) efflux pumps in reducing the efficacy of tigecycline against clinical isolates of *A. baumannii* collected from hospitalized burn patients in Iran. The susceptibility of 100 isolates to 19 antimicrobial agents was determined using the disc diffusion method, with the exception of colistin and tigecycline, which were assessed using the Etest method. PCR was used to detect the prevalence of genes. Real-time PCR was used to analyze the expression levels of genes encoding RND efflux pumps. Additionally, a three loci dual assay and multiple loci variable number tandem repeat analysis (MLVA) were used to define PCR-based groups (G) and epidemiological studies of isolates, respectively. Colistin (99%), followed by tigecycline (71%) could be effective therapeutic options against *A. baumannii*. Forty-three percent of all isolates belonged to G variants. The high antibiotic resistance rate and over expression of RND efflux pumps were observed in G variants. MLVA identified 44 distinct types with 11 clusters and 29 singleton genotypes. The mean expression levels of *Ade*B in 29 tigecycline non-susceptible *A. baumannii* (TNAB) counterpart 71 tigecycline susceptible *A. baumannii* (TSAB) increased approximately 7- fold. All TNAB and TSAB isolates were shown to express *ade*G at higher level than *ade*B and *ade*J, exceptionally TNAB isolates expressed *ade*B at a greater level than others. Four fold or greater decrease in tigecycline MIC was observed in 20% and 79% of TSAB and TNAB isolates, respectively. We observed an increasing trend in the rate of TNAB isolates and G variants in burn patients. The mechanism of their resistance was mediated by an increase in the expression of *Ade*ABC efflux pump; *Ade*FGH and *Ade*IJK were not observed to have a direct role in tigecycline resistance. Also, this is the first study to report on the tendency of increase to spread of TNAB G variants, as the sub-clone of G1, in Iranian burn center.

Introduction:

Acinetobacter baumannii as an opportunistic pathogen causes a variety of nosocomial infections ranging from pneumonia to serious blood, brain or wound infections in the hospital settings (Dally et al., 2013) and was found to be the second leading cause of nosocomial infection in burn patients in Iran (Farshadzadeh et al., 2015). In recent years, there has been a dramatic increase in the prevalence of *A. baumannii* that likely has been attributed to emergence of antibiotic resistance among these isolates (Moradi et al., 2015; Dally et al., 2013). Antibiotic treatment failure and the emergence of antibiotic resistance among *A. baumannii* isolates are increasing and very depending on the geographic region (Moradi et al., 2015). This increasing antibiotic resistance has been attributed to the overuse of antibiotics among patients infected by *A. baumannii* such as those whom burn infection (Fearshadzadeh et al., 2015; Sharma et al., 2014; Chong et al., 2011). Carbapenems have remained as treatment option for multiple drug-resistant (MDR) infections (Pourhajibagher et al., 2016). However, due to the increased incidence of nosocomial infections caused by carbapenem non-susceptible *A. baumannii*, the use of other therapeutic options including polymyxin and tigecycline has been considered (Pourhajibagher et al., 2016).

Tigecycline has exhibited to have effective *in vitro* activity against XDR *A. baumannii* (Montana et al., 2015) and has been approved for the treatment of complicated skin and intra-abdominal *A. baumannii* infections (Cai et al., 2012). Several studies introduced tigecycline as treatment option for imipenem- and colistin-resistant *A. baumannii*. However, the emergence of tigecycline resistant *A. baumannii* isolates has been reported (Cai et al., 2012; Sarada et al., 2014; Li et al., 2015; Montana et al., 2015; Mavroidi et al., 2015). All *A. baumannii* isolated from Iran were found to be susceptible to tigecycline in 2006, an increasing trend in the tigecycline resistance among *A. baumannii* isolates was found in Iran during 2011 through 2013, from 4% to 13–23%, which is a cause for concern in the medical community (Bahador et al., 2013; Bahador et al., 2014; Farshadzadeh et al., 2015).

Several studies indicated attribution of RND efflux pumps to tigecycline resistance in *A. baumannii*. These pumps included *Ade*ABC, *Ade*FGH, and *Ade*IJK have a wide substrate range, thus could be important in multiple drug resistant (Deng et al., 2014). *Ade*ABC was the first RND efflux pump identified; tigecycline resistance in strains of *A. baumannii* might be due to the upregulation of the *Ade*ABC efflux pump. The expression of this pump is strongly regulated by a two-component *Ade*RS system (Xing et al., 2014; Yoon et al., 2013).

Another known RND efflux pump, *Ade*IJK, is present in all *A. baumannii* strains. Because the overexpression of *Ade*IJK is toxic for *A. baumannii* isolates, its expression is repressed by *Ade*N, a TetR-type regulator (Rosenfeld et al., 2012; Damier-Piolie et al., 2008). *Ade*FGH is another RND efflux pump that has been described in *A. baumannii*. It is responsible for the decreasing susceptibility of *A. baumannii* to tigecycline. A high level of expression of *Ade*FGH due to mutations in *Ade*L, a LysR- type transcriptional regulator, is thought to promote tigecycline resistance (Coyne et al., 2010b). Some studies have shown that only *Ade*ABC and *Ade*IJK are involved in reducing the susceptibility of *A. baumannii* to tigecycline (Rosenfeld et al., 2012; Damier-Piolie et al., 2008), while other studies have revealed the role of *Ade*FGH in tigecycline resistance (Coyne et al., 2010b).

Diverse and complicated mechanisms are involved in tigecycline resistance (Chen et al., 2014). To our knowledge, there are no studies that have reported on the role of the different types of RND efflux pumps involved in tigecycline resistance among clinical isolates of *A. baumannii* in Iran.

The global population structure of *A. baumannii* isolates is changing. As a result, molecular typing is essential for tracing the source and transmission pathways of *A. baumannii*. Three major PCR-based groups (G), I–III, referred to as the international clone (IC) or sequence group (SG), and G variants of *A. baumannii* have been shown to be responsible for several hospital outbreaks globally (Diancourt et al., 2010; Karah et al., 2011a; Karah et al., 2011b; Towner et al., 2008). Several G variants have been observed among hospital-acquired strains of *A. baumannii* in several regions in recent years (Karah et al., 2011a; Karah et al., 2011b; Towner et al., 2008). Various studies have shown 5 to 24% of these variants are resistant to tigecycline (Bahador et al., 2013; Bahador et al., 2014; Farshadzadeh et al., 2015; Karah et al., 2011b; Grosso et al., 2011).

Because the discriminatory power of multiple-loci variable-number tandem-repeat analysis (MLVA) was higher than PCR-based G typing, MLVA enabled the differentiation of unrelated isolates, which were clustered together by PCR-based G analysis in identical G types (Pourcel et al., 2011). Thus, MLVA was used as a reliable molecular fingerprinting technique to identify epidemiological associations among *A. baumannii* isolates. The objectives of this study were to (i)

determine and identify drug susceptibility patterns and the molecular epidemiology of *A. baumannii* isolated from burn patients in Iran; and (ii) to assess the role of the various types of RND efflux pumps involved in tigecycline resistance in *A. baumannii* isolated from burn patients.

Material And Methods:

Bacterial strains

A total of 100 *A. baumannii* isolates were identified from burn patients hospitalized at the Shahid Motahari Burn Hospital in Tehran, Iran, from September 2013 to February 2015. After admission, written informed consent was obtained from the burn patients. The study approved by the Ethics Committee of Tehran University of Medical Sciences (Application No. 92-03-30-23186). The initial identification of bacterial species was performed by using the API 20NE system (bioMérieux, Marcy-l'Etoile, France). Final characterization of collected isolates was done by multiplex PCR using *gyrB*-directed primers (Macrogen, South Korea) according to Higgins et al (Higgins et al., 2010).

Antimicrobial susceptibility testing:

The disc diffusion method was used to determine the susceptibility of *A. baumannii* isolates to several antimicrobial agents according to Clinical and Laboratory Standards Institute (CLSI) guideline (CLSI, 2015). The antimicrobial agents (µg per disc; resistance breakpoint in mm) used in this study included piperacillin (100, ≤17); ticarcillin (75, ≤14); imipenem (10, ≤18); meropenem (10, ≤14); cefotaxime (30, ≤14); ceftazidime (30, ≤14); cefepime (30, ≤14); ceftriaxone (30, ≤13); piperacillin-tazobactam (100 + 10, ≤17); ampicillin-sulbactam (10 + 10, ≤11); gentamicin (10, ≤12); amikacin (30, ≤14); tobramycin (10, ≤12); tetracycline (30, ≤11); minocycline (30, ≤12); ciprofloxacin (5, ≤15); and trimethoprim/sulfamethoxazole (1.25 + 23.75, ≤10) (Mast Diagnostics, Bootle, UK). *Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853 were used as quality control organisms to ensure accuracy of the antimicrobial susceptibility assays, and *E.coli* ATCC 35218 was used as a quality control organism for β-lactam/β-lactamase inhibitor combination (piperacillin-tazobactam, ampicillin-sulbactam) antibiotics.

The MICs of all isolates to colistin and tigecycline (both 0.016–256 µg/ml) were measured using epsilometer test (Etest) strips (Ezy MIC^{TM} strips, Himedia, India) per CLSI guidelines (CLSI, 2015). Colistin MICs were interpreted according to CLSI breakpoint. There is no breakpoint available for tigecycline in the CLSI guidelines; as a proxy, the criteria for the interpretation of MIC values for members of *Enterobacteriaceae* were used following the European Committee on Antimicrobial Susceptibility Testing (EUCAST) (MIC ≤1 µg/ml and MIC ≥2 µg/ml defined as susceptible and resistant, respectively) were used (EUCAST, 2014). *A. baumannii* isolates where characterized as MDR, XDR, or PDR phenotype according to the international expert proposal for Interim standards guidelines (Magiorakos et al., 2012).

Identification of PCR-based G types by multiplex PCR:

G types were determined based on the presence or absence of the alleles of outer membrane protein A (*omp*A), chaperone-subunit usher E (*csu*E), and the intrinsic carbapenemase (bla_{OXA-51}-like) encoding genes in the two simultaneously allele specific multiplex PCR as previously described (Turton et al., 2007). Alleles 1 and 2 of *omp*A, *csu*E, and bla_{OXA-51}-like were detected in multiplex PCR reactions 1 and 2, respectively. All isolates were grouped into one of three major G types (I–III); all other isolates were considered G variants (Turton et al., 2007). All primers are listed in Table 1.

Table 1
List of oligonucleotides used in this study for genes

	Primers		Sequence (5' to 3')	References
Primers used in multiplex PCRs for identification of PCR- based groups	Group1ompA306	Forward	GATGGCGTAAATCGTGGTA	Turton et al., 2007
	Group1and2ompA660	Reverse	CAACTTTAGCGATTTCTGG	
	Group 1 csuE	Forward	CTTTAGCAAACATGACCTACC	
	Group 1 csuE	Reverse	TACACCCGGGTTAATCGT	
	Gp1OXA66F89	Forward	GCGCTTCAAAATCTGATGTA	
	Gp1OXA66R647	Reverse	GCGTATATTTTGTTTCCATTC	
	Group2ompAF378	Forward	GACCTTTCTTATCACAACGA	
	Group1and2ompAR660	Reverse	CAACTTTAGCGATTTCTGG	
	Group2csuE	Forward	GGCGAACATGACCTATTT	
	Group2csuE	Reverse	CTTCATGGCTCGTTGGTT	
	Gp2OXA69F169	Forward	CATCAAGGTCAAACTCAA	
	Gp2OXA69R330	Reverse	TAGCCTTTTTTCCCCATC	
Primers used for MLVA typing	VNTR_3530	Forward	TGCAACCGGTATTCTAGGAAC	Pourcel et al., 2011
		Reverse	CCTTGAACAACATCGATTACTGGA	
	VNTR_3002	Forward	GACTGAAGCAAGACTAAAACGT	
		Reverse	TCTGGGCAGCTTCTTCTTGAGC	
	VNTR_2240	Forward	CCCGCAGTACATCATGGTTC	
		Reverse	AGAACATGTATACGCAACTG	
	VNTR_1988	Forward	GGCAAGGCATGCTCAAGGGCC	
		Reverse	CAGTAGACTGCTGGTTAATGAG	
	VNTR_0826	Forward	TGACTACTGAAACAGTTTTTG	
		Reverse	ATGATTGTACCGAGTAAAAGA	
	VNTR_0845	Forward	AATTTTAATTCCAAATTGCTCC	
		Reverse	ACTTAAAATCGCATTTTTATCA	
	VNTR_2396	Forward	CAAGTCCAATCAACTCATGATG	
		Reverse	CTCCTGTAAGTGCTGTTCAGCC	
	VNTR_3468	Forward	CAGAAGTCACTGCATCTGCAAC	
		Reverse	CGGTTGAAATTTTTTATAATGAAG	
Primers used for PCR and qRT-PCR	*Ade*A	Forward	ATCGCTAACAAAGGCTTGAA	Coyne et al., 2010a
		Reverse	CGCCCCCTCAGCTATAGAA	
	*Ade*B	Forward	CTTGCATTTACGTGTGGTGT	
		Reverse	GCTTTTCTACTGCACCCAAA	
	*Ade*C	Forward	TACACATGCGCATATTGGTG	
		Reverse	CGTAAAATAACTATCCACTCC	
	*Ade*R	Forward	ACTACGATATTGGCGACATT	Rumbo et al., 2013
		Reverse	GCGTCAGATTAAGCAAGATT	
	*Ade*S	Forward	TTGGTTAGCCACTGTTATCT	
		Reverse	AGTGGACGTTAGGTCAAGTT	
	*Ade*F	Forward	GGTGTCGACCAAGATAAACG	Coyne et al., 2010b
		Reverse	GTGAATTTGGCATAGGGACG	
	*Ade*G	Forward	TTCATCTAGCCAAGCAGAAG	

	Primers		Sequence (5′ to 3′)	References
		Reverse	ATGTGGGCTAGCTAACGGC	
	*Ade*H	Forward	CGATCAGCAAATTCAGGCTC	
		Reverse	GCTTGCAATGATTTGGCTGC	
	*Ade*L	Forward	AGGAGTGTGCGTGTGGATC	
		Reverse	GAAATCGGCATCGGTGCTG	
	*Ade*N	Forward	GCTGTTAGGTTGGGGTCGTA	Rumbo et al., 2013
		Reverse	CGTGACCAAAAGTACGAATCA	
	*Ade*I	Forward	CAAATGCAAATGTAGATCTTGG	Coyne et al., 2010a
		Reverse	AAACTGCCTTTACTTAGTTG	
	*Ade*J	Forward	GGTCATTAATATCTTTGGC	
		Reverse	GGTACGAATACCGCTGTCA	
	*Ade*K	Forward	TTGATAGTTACTTGACTGTTC	
		Reverse	GGTTGGTGAACCACTGTATC	
	16sRNA	Forward	TCGCTAGTAATCGCGGATCA	Ruzin et al., 2007
		Reverse	GACGGGCGGTGTGTACAAG	

Multiple-loci variable-number tandem-repeat analysis:

A. baumannii isolates were genotyped using the MLVA-8 scheme method developed by Pourcel et al. (Pourcel et al., 2011). With this method, large (L)-repeat VNTRs (Abaum_1988, Abaum_2240, Abaum_3002, and Abaum_3530) and small (S)-repeat VNTRs (Abaum_0826, Abaum_0845, Abaum_2396, and Abaum_3468) were amplified using eight paired primers targeting the 5′and 3′ flanking regions of the mentioned VNTR loci in eight separate PCR tests. After agarose gel electrophoresis, the sizes of amplicons were determined using GeneTools, version 4.1 (Syngene, UK). All primers are listed in Table 1. The MLVA-8 scheme profiles in each isolate were identified by the number of repeats estimated at each VNTR locus according to the previous study (Pourcel et al., 2011). Dendrograms were created using Bionumericus version 7.0 (Applied Maths, Sint-Martens-Latem, Belgium). For clustering analysis, the allele strings were entered into Bionumericus as character values. A cut-off of 95% similarity was used to describe clusters (Pourcel et al., 2011).

Detection of the Ade**RND system**:

All isolates were screened for the presence of genes encoding the *ade*ABC (*ade*A, *ade*B, *ade*C), *ade*IJK (*ade*I, *ade*J, *ade*K), and *ade*FGH (*ade*F, *ade*G, *ade*H) efflux pumps by PCR with specific primers as previously designated and listed in Table 1 (Coyne et al., 2010a; Coyne et al., 2010b; Rumbo et al., 2013). PCR was also used to detect *ade*N, *ade*L, and *ade*RS.

Real Time PCR assay:

Because three genes encoding *ade*ABC (*ade*A, *ade*B, and *ade*C), *ade*FGH (*ade*F, *ade*G, and *ade*H), and *ade*IJK (*ade*I, *ade*J, and *ade*K) are co-transcribed (Damier-piolle et al., 2008; Coyne et al., 2010b; Ruzin et al., 2007), this study was performed to verify the expression level of the representative transporter of each efflux pump involved *ade*B, *ade*G, and *ade*J genes in all isolates positive for at least one RND efflux pump type. Total RNA was extracted using the High Pure RNA Isolation Kit (Roche, Germany) per the manufacturer's instructions. The extracted RNA was converted to cDNA using a cDNA synthesis kit (Thermo Scientific, USA). The levels of expression of *ade*B, *ade*J, and *ade*G were measured in triplicate using SYBR Premix Ex Taq II (Takara Bio, Inc. Japan). Real-time PCR was carried out with the following cycle profile: 1 cycle at 95°C for 30 s, followed by 45 cycles at 95°C for 5 s, 63°C for 10 s, and 72°C for 20 s using the ABI Step One^{TM} system (Applied Biosystems, California, United States) with a total of 25 µl reaction mixture containing 200 ng cDNA. Each experiment was performed in triplicate. The specific primers used are listed in Table 1. 16S rRNA was applied as a housekeeping gene and internal control to normalize levels of *ade*B, *ade*J, and *ade*G gene transcripts. The fold changes of the target genes expression levels were calculated by the $2^{-\Delta\Delta CT}$ method using the relative expression software tool (REST) 2009 software (QIAGEN) (Pfaffl et al., 2002). Changes in expression levels of investigated genes were interpreted as significant if the variation was ≥2-fold.

Determination of RND efflux pumps activity:

Activity levels of each RND efflux pump was assessed by measuring the MICs of all isolates when exposed to tigecycline against the efflux pump inhibitor (EPI) and phenyl-arginine-β-naphthylamide (PAβN) (Sigma-Aldrich, Dorset, United Kingdom) as previously (Deng et al., 2014; Hou et al., 2012). PAβN was added to each Muller-Hinton agar plate (Merck, Germany) at a final concentration of 20µg/ml and MIC to tigecycline using Etest before and after exposure to the EPI were recorded. If the MIC values decreased 4- fold or greater in the presence of EPI, this was defined as a significant inhibitory effect.

Statistical analysis:

Data were analyzed using the Student's t-test, Fisher's exact tests, and the chi-square test with the SPSS software package (version 22). Results were considered significant if the *P*-values were less than 0.05.

Results:

Isolate identification and antimicrobial susceptibility profiles:

All 100 isolates identified as *A. baumannii complex* by using the API 20NE system and were confirmed as *A. baumannii* using multiplex PCR gyrB.

Susceptibility profiles of all *A. baumannii* isolates are presented in Tables 2 and 3. The highest susceptibility was found to colistin (99%), followed by tigecycline (71%), minocycline (58%), and tetracycline (46%). Resistance rates against the other studied antimicrobial agents, except for amikacin (81%), imipenem (76%), and tobramicin (70%), were higher than 90%. Among a total of 100 isolates, 64 isolates (64%) exhibited the XDR phenotype. The remaining 36% exhibited the MDR phenotype.

Table 2
The MIC of two antimicrobial agents for the 100 *A. baumannii* isolates as determined by E test

Antimicrobial agents[a]	*A. baumannii* isolates			% of non-susceptibility
	MIC (µg/ml)			
	Range	MIC50	MIC90	
CST	0.016- 2	0.019	1	1
TGC	0.25- 32	1	8	29

[a]Abbreviations: CST, colisitin; TGC, tigecycline.

Table 3
Comparison of the rate of non- susceptibility to antimicrobial agents among TNAB versus TSAB isolates

Isolates (No.)	CLSI Antimicrobial groups[a] (%)																	
	A							B									O	
	IPM[b]	MEM	GEN	TOB	CIP	CAZ	SAM	PIP	TZP	FEP	AMK	SXT	TET	MIN	CTX	CRO	CST	TIC
TNAB[c] (29)	83	93	90	66	93	93	55	97	93	97	76	100	100	100	100	100	3	93
TSAB[d] (71)	73	93	90	72	89	94	56	99	93	100	83	99	35	18	98	98	0	99
Total (100)	76	93	90	70	90	94	56	98	93	99	81	98	54	42	99	99	1	97

[a]Antimicrobial agents are categorized according to CLSI-defined grouping as A, B, and O antimicrobial groups. According to CLSI guideline, considerations in the assignment of agents to Groups A, B, and O include clinical efficacy, prevalence of resistance, minimizing emergence of resistance, cost, FDA clinical indications for usage, and current consensus recommendations for first-choice and alternative drugs. Group A are considered appropriate for inclusion in a routine, primary testing panel, as well as for routine reporting of results for the organism. Group B comprises agents that may warrant primary testing. However, they may be reported only selectively, such as when the organism is resistant to agents of the same class, as in Group A. Group O (other) includes agents that have a clinical indication for the organism, but are generally not candidates for routine testing and reporting in the United States. [b] Abbreviations: AMK, amikacin; CAZ, ceftazidime; CTX, cefotaxime; CRO, ceftriaxone; CIP, ciprofloxacin;CST, colistin; SXT, trimethoprim- sulfamethoxazole; FEP, cefepime; GEN, gentamicin; IPM, imipenem; MEM, meropenem; MIN, minocycline; PIP, piperacillin; SAM, ampicillin-sulbactam; TET, tetracycline; TIC, ticarcillin; TOB, tobramycin; TZP, piperacillin-tazobactam, [c]TNAB, tigecycline non-susceptible *A.baumannii*; [d]TSAB, tigecycline susceptible *A.baumannii*.

Based on antimicrobial susceptibility testing, 29 tigecycline non-susceptible *A. baumannii* (TNAB) and 71 tigecycline susceptible *A. baumannii* (TSAB) isolates were identified in this study. All TNAB isolates exhibited the XDR phenotype, while 49% of TSAB isolates showed this phenotype and the remaining, 51%, had the MDR phenotype. Among 64% of the isolates exhibited XDR phenotype, 55% were susceptible to tigecycline (XDR-TSAB isolates), while all MDR isolates were tigecycline susceptible. As shown in Table 3, TNAB isolates in comparison with TSAB isolates displayed higher rates of resistance to tetracycline and minocycline (100% for both antibiotics in TNAB isolates versus 35% and 18% for TSAB isolates, respectively; both $p < 0.05$). However, resistance to imipenem was high in both groups (83% for TNAB versus 73% for TSAB; $p = 0.441$) (Table 3).

G types:

Table 4 shows the rates of antibiotic susceptibility among PCR-based groups (G) or IC types. Among all isolates, 37%, 19%, and 1% showed the pattern of G1 (IC II), G2 (IC I), and G3 (IC III), respectively (Figure 1 and Table 4). Based on the variations in combination of PCR amplicons, 43% of isolates exhibited a new combination of amplified products; these isolates has not been defined as G1, G2, and G3. These isolates belonged to six PCR-based groups, namely G6, G7, G9, G15, G16, and G17, and have been discussed in previous studies (Table 5) (Farshadzadeh et al., 2015; Karah et al., 2012). G9 (32%) was the most common G variant in this study.

Table 4
Frequency of antimicrobial non-susceptibility in three major PCR- based group lineages and G variants in 100 *A.baumannii* isolates

% of non-susceptibility to antimicrobial agents[a]																			
Isolates (No.)	**IPM**	**MEM**	**GEN**	**TOB**	**AMK**	**TET**	**MIN**	**TGC**	**CTX**	**CAZ**	**CRO**	**FEP**	**SAM**	**TIC**	**PIP**	**TZP**	**CIP**	**SXT**	**CST**
G[b]1 (37)	70	92	89	78	81	41	30	16	97	92	97	97	41	95	95	92	86	95	0
G2 (19)	79	100	95	68	89	47	26	11	100	100	100	100	74	100	100	100	100	100	5
G3 (1)	100	100	100	100	100	100	0	100	100	100	100	100	0	100	100	100	100	100	0
G variant (43)	79	91	88	63	77	67	60	49	100	93	100	100	63	98	100	91	88	100	0
Total (100)	76	93	90	70	81	54	42	29	99	94	99	99	56	97	98	93	90	98	1

[a]Abbreviations: AMK, amikacin; CAZ, ceftazidime; CTX, cefotaxime; CRO, ceftriaxone; CIP, ciprofloxacin; CST, colistin; SXT, trimethoprim- sulfamethoxazole; FEP, cefepime; GEN, gentamicin; IPM, imipenem; MEM, meropenem; MIN, minocycline; PIP, piperacillin; SAM, ampicillin-sulbactam; TET, tetracycline; TIC, ticarcillin; TGC, tigecycline; TOB, tobramycin; TZP, piperacillin-tazobactam; [b]G, PCR- based groups.

Table 5
Combination of amplicons obtained from two separate multiplex PCRs used to describe 43 *A. baumannii* G variant

Variant type	PCR group 1			PCR group 2		
(No.)	csuE	bla$_{OXA-51}$-like	ompA	csuE	ompA	bla$_{OXA-51}$-like
	702 bp	**559 bp**	**355 bp**	**580 bp**	**343 bp**	**162 bp**
G 6[a] (3)	-	-	-	+	-	-
G 7 (12)	-	-	+	+	-	-
G 9 (14)	-	-	+	+	-	+
G 15 (2)	+	-	-	-	-	+
G 16 (10)	-	+	-	+	-	-
G 17 (2)	+	-	+	-	-	+

[a]G; PCR-based group. Two simultaneous multiplex polymerase chain reactions (PCRs) method based on targeting three genes (*omp*A, *csu*E and *bla*$_{OXA-51}$-like) is a convenient method for rapid assignment of *A. baumannii* isolates into three major PCR-based groups (Gs) corresponding to PCR- based group (G) 1 (IC II), G2 (IC I), and G3 (IC III). Using this scheme, additional groups (G4–G14 and putative G15–G17) have been identified according to new combinations of the PCR amplicons.

Susceptibility rates to tetracycline, minocycline, and tigecycline among two major epidemic lineages G1 and G2 is significantly higher than G variants (P<0.05). G variants were highly resistant to tetracycline (67%), minocycline (60%), and tigecycline (49%); while, these antibiotics were the most effective against isolates belonging to G1 and G2 (Table 4). Most TNAB isolates (69%) belonged to the G variant population, whereas only 31% of TNAB isolates belonged to other clonal lineages (p = 0.002). Thirty-five percent of isolates belonging to G1 (IC II) exhibited the XDR phenotype; while 74 and 81% of isolates within G variant and G2 lineages showed this phenotype, respectively. Indeed the rate of XDR phenotype was significantly lower among isolates belonging to clonal lineage G1 than among those belonging to G variants and clonal lineage G2 (P< 0.01).

MLVA typing:

According to the results of the MLVA-8 scheme typing method, 100 *A. baumannii* isolates were grouped into 44 distinct MLVA types with 11 clusters (named A–K) and 29 single genotypes (Figure 1). All isolates differed in their antibiotic susceptibility profiles. In this study, a lower diversity was recognized in the VNTR loci by using primer pair MLVA-AB_3002 and MLVA-AB_2240 whereas a higher level of diversity was revealed in the VNTR loci by MLVA-AB_2396 and MLVA-AB_3468 (Figure 1).

Forty-five percent of isolates were identified as MLVA type cluster A (n = 24) and F (n = 21). Other clusters including B, C, D, E, G, H, I, J and K were observed to consist of 1- 14 isolates. All isolates within cluster I and K and more than half of isolates within cluster F were G variants, whereas 46% of the G1 (IC II) clones were assigned to the cluster A. As shown in Figure 1, all G variants belonging to G7 were classified in cluster F.

The highest rate of XDR phenotype was found in the clusters G, I and J (100%), followed by the clusters D and C (78%), and K (60%). About 67% of cluster I and 42% of clusters A, B, and C were resistant to tigecycline, and all tigecycline-resistant strains in these clusters showed XDR profiles.

Detection of efflux pump genes:

The distribution of efflux pump genes in all isolates is shown in figures 1 and Table 6. *ade*IJK and *ade*N were detected in all isolates (Table 6). All TNAB isolates harbored all RND efflux pumps and their regulatory systems (*Ade*IJK^{+}-*Ade*N^{+}, *Ade*FGH^{+}-*Ade*L^{+}, *Ade*ABC^{+}-*Ade*RS^{+}), whereas these pumps was found to exist in 86% of TSAB isolates. This combination significantly was high among TNAB in contrast with TSAB isolates (p=0.02). Other combinations were not statistically different between TNAB and TSAB isolates (p>0.1; Table 6). Ninety-three and ninety-four percent of TSAB isolates were positive for the genes encoding *Ade*ABC and *Ade*FGH efflux pumps, respectively (figure 1 and Table 6). Ninety-eight percent of *ade*ABC positive isolates, carried the regulatory system *ade*RS. Of 71 TSAB isolates, 65 isolates (91%) were positive for *ade*ABC and its regulatory system genes (*ade*RS). The presence of *ade*ABC- *ade*RS and *ade*FGH- *ade*L between TNAB and TSAB isolates was not statistically different (p > 0.1).

Table 6
Distribution of gene encoding RND efflux pumps in 29 TNAB and 71 TSAB isolates

Gene and genotypes	TNAB[a] (%)	TSAB[b] (%)	*P* value
Total presence of each efflux pump gene in A. baumannii **isolates**			
*Ade*IJK	100	100	1
*Ade*N	100	100	1
*Ade*FGH	100	94	0.2
*Ade*L	100	94	0.2
*Ade*ABC	100	93	0.1
*Ade*RS	100	91	0.1
Combination presence of efflux pump genes in A. baumannii **isolates**			
*Ade*IJK^{+}-*Ade*N^{+}, *Ade*FGH^{+}-*Ade*L^{+}, *Ade*ABC^{+}-*Ade*RS^{+}[c]	100	86	0.02
*Ade*IJK^{+}-*Ade*N^{+}, *Ade*FGH^{+} -*Ade*L^{+}, *Ade*ABC^{-}-*Ade*RS^{-}[d]	0	7	0.1
*Ade*IJK^{+}-*Ade*N^{+}, *Ade*FGH^{-} -*Ade*L^{-}, *Ade*ABC^{+}-*Ade*RS^{+}	0	6	0.2
*Ade*IJK^{+}-*Ade*N^{+}, *Ade*FGH^{+} -*Ade*L^{+}, *Ade*ABC^{+}-*Ade*RS^{-}	0	1	0.7

[a]TNAB, tigecycline non-susceptible *A.baumannii*; [b]TSAB, tigecycline susceptible *A.baumannii*; [c+], isolates positive for genes; [d−], isolates negative for genes.

All MDR isolates were susceptible to tigecycline from which 73% were positive for all RND efflux pump genes. XDR-TSAB isolates really constituted 49 percent of the TSAB isolates and were positive for both *ade*FGH and *ade*ABC.

The prevalence of isolates with all RND efflux pumps (*Ade*IJK^{+}-*Ade*N^{+}, *Ade*FGH^{+}-*Ade*L^{+}, *Ade*ABC^{+}-*Ade*RS^{+}) in the G variant lineage (100%) was higher than that in other G clonal lineages (82%) (p = 0.002).

Analysis of ade**B**, ade**G, and** ade**J expression**:

The expression level of a*de*B was 6.9- fold higher in TNAB than that in TSAB isolates (p = 0.0001), whereas no significant difference in the expression levels of *ade*G and *ade*J were found between TNAB and TSAB isolates (1.16- and 1.18- fold higher than TSAB, respectively, p < 0.05 for both) (figure 2).

As shown in figure 3, All TNAB isolates had XDR profile and exhibited approximately 6- and 8- fold higher expression levels of *ade*ABC than did XDR- and MDR- TSAB isolates (p <0.001 for both). While, expression level of this gene among XDR- TSAB isolates was significantly no different from MDR isolates (p > 0.1). No significant difference in the expression level of *ade*G was found between TNAB, XDR- and MDR- TSAB isolates (p> 0.05). Mean expression level of *adeJ* among TNAB, XDR-and MDR- TSAB isolates was not significantly different (p > 0.1).

The expression level of *ade*G between isolates belonging to G variants was 1.08- fold higher than those belonging to three major epidemic lineages, regardless of tigecycline susceptibility status, while the expression level of the *ade*B was slightly greater than 2 fold in isolates belonging to G variants than other clonal lineages and was statistically different between them (p=0.003). The expression level of *ade*J between isolates belonging to G variant and those belonging to three major epidemic lineages was not significantly different (p = 0.695)

Determination of RND efflux pumps activity:

The effect of PAβN on tigecycline MIC in both TNAB and TSAB isolates is shown in Table 7 and figure 1. As previously mentioned, a 4- fold or greater decrease in tigecycline MIC in the presence of EPI, has defined as a considerable inhibitory effect, <4-fold reduction in the MIC values following exposure to PAβN was considered as a partial change. Tigecycline MIC value decreased ≥ 4-fold in 20% and 79% of TSAB and TNAB isolates, respectively. Indeed the tigecycline MIC following exposure to PAβN in TNAB isolates was significantly affected more than that in TSAB isolates (p= 0.0001). As shown in Table 7, the partial reduction in MIC value after treatment with PAβN was significantly different between TSAB isolates (80%) in comparison with TNAB isolates (21%, p=0.0001).

The partial reduction in MIC value was also significantly higher between XDR-TSAB isolates (35%) following treatment with PAβN than TNAB isolates (p= 0.0001). So, tigecycline MIC of TNAB isolates after exposure with PAβN was significantly reduced in comparison with both TSAB and XDR-TSAB isolates.

Table 7

Reduction in MIC after addition of PAβN[a]

Phenotype	No. (%) of isolates with < 4- fold reduction in MIC		No. (%) of isolates with ≥ 4- fold reduction in MIC		
	TNAB[b] (29)	**TSAB[c] (71)**	**TNAB (29)**	**TSAB (71)**	P **value**
MDR	0	32 (45)	0	4 (5)	N[d]
XDR	6 (21)	25 (35)	23 (79)	10 (14)	0.0001
Total	6 (21)	57 (80)	23 (79)	14 (20)	0.0001

[a]PAβN, phenyl-arginine-β-naphthylamide; [b]TNAB, tigecycline-nonsusceptible *A. baumannii*; [c]TSAB, tigecycline-susceptible *A. baumannii*. [d]N, no statistics are computed.

Discussion:

In recent years, the frequency of the reports of infection by XDR- *A. baumannii* has increased worldwide, similarity several such reports for hospital acquired infections especially those involving burn wound, have recently presented by previous studies in Iran (Yu et al., 2016; Farshadzadeh et al., 2015; Azimi et al., 2015). According to results of our previous and current studies, prevalence rate of XDR isolates in 2006, 2015 and present has been 24, 77 and 64%, respectively, representing a shift towards higher XDR frequency (Bahador et al., 2013; Bahador et al., 2014). As the development of resistance to further antibiotics leads to an increase in failure risk of antibiotic therapy; the ongoing enhancement of XDR prevalence could be one of the most serious challenges in management of *A. baumannii* infected patients (Farshadzadeh et al., 2015; Cai et al., 2012; Bahador et al., 2013; Chen et al., 2014; Giannouli et al., 2010). In our study, resistance rates for imipenem, tetracycline and minocycline were higher than that for colistin and tigecycline. In compared with our previous studies, the rates of resistance to these three antibiotics has had an increasing trend during 2006 to now (Bahador et al., 2013; Bahador et al., 2014; Pourhajibagher et al., 2016). Such an increasing trend has been reported for carbapenems from other countries (Lemos et al., 2014; Hassan et al., 2014; Hammood Hussein et al., 2014; Chang et al., 2015; Niumsup et al., 2009; Kuo et al., 2012). In such condition in which treatment options are limited, tigecycline is commonly used as a mono or combination regimen due to its effective potency against *A. baumannii* infections (Cai et al., 2012). However our previous and current studies have exhibited an increasing trend in the rate of TNAB isolates during recent years similar to that has observed for XDR isolates. No tigecycline non-susceptible isolates were found in Iran in 2006, whereas 8%, 13-23% and 29% of *A. baumannii* isolates were found to be non-susceptible in 2011, 2013 and present study, respectively (Bahador et al., 2013; Bahador et al., 2014). Therefore, it appears that there has been a simultaneous development of XDR and tigicyclin resistance phenotypes over recent years in Iran. The tigecycline resistance rates in different parts of the world such as Israel (78%), Italy (50%), China (40.5%), Taiwan (19.1—45%), India (14.2—57.6%), Turkey (14.3—47%), Singapore (29%), Korea (23.4%), also indicate that tigecycline resistance is a global challenge and cause for concern (Pournaras et al., 2016).

The G1 (IC II) has been found to the most predominant *A. baumannii* clonal lineage worldwide (Hojabri et al., 2014; Higgins et al., 2010b), whereas our analysis revealed that the G variant is the most prevalent clonal lineage in Motahari burn center in Iran. All of the 6 variant PCR based-groups (G variants) in the present study, namely G6, G7, G9, and G15—G17, have been documented previously (Farshadzadeh et al., 2015; Karah et al., 2012). According to our previous and present reports the G variants rates have been 4%, 16%, 45% and 43% in 2006, 2011, 2015 and present study, respectively (Bahador et al., 2014; Farshadzadeh et al., 2015). The rates of tigecycline resistant phenotype among G variant isolates were 22%, 24% and 45% in 2013, 2015 and present study, respectively (Bahador et al., 2013; Farshadzadeh et al., 2015). These results showed an increasing trend in G variant frequency during mentioned years. As prevalence rates of G variants have increased, the rising rates of XDR and tigecycline resistance phenotype among this clonal lineage have been also increased; thus the extended antibiotics resistance phenotypes may be the most probable explanation for the increasing trends in G variant prevalence over time. Accompanying increase in the rising rates of XDR and tigecycline resistance phenotypes in G variants can provide a supportive evidence that underlying mechanisms of these phenotypes may share some overlapping components with each other.

Our prior research has revealed that G variants tend to form strong biofilms (Farshadzadeh et al., 2015). The resistance of G variants in recent years and tendency of G variants to produce a biofilm are responsible for the spread of these bacteria. The based on our findings, the rate of antibiotic resistance of G2 (IC I) strains was higher than G1 (IC II) strains, whereas the frequency of G2 isolates was lower than G1. Since our previous study revealed higher biofilm-forming capacity of G1 than G2 (Farshadzadeh et al., 2015), therefore higher dissemination of G1 than G2 was seen with respect to higher biofilm- forming capacity but not to higher rate of antibiotic resistance. On the basis of these results, it is might that biofilm-forming capacity contributes more to dissemination of *A. baumannii* than rate of antibiotic resistance.

Since the discriminatory power of MLVA is higher than PCR-based G typing, MLVA enabled the differentiation of unrelated isolates, which were clustered together by PCR-based G analysis in identical G types (Pourcel et al., 2011), MLVA typing was performed. Comparison of the prevalence rate of each MLVA types in this study with its corresponding prevalence rate in our previous study did not show any significant difference (Farshadzadeh et al., 2015). Evaluation of MLVA typing revealed that isolates which were clustered together by PCR-based G analysis in identical G types may be assign as different MLVA types, thus the results of G clonal lineage typing may not reliably predict MLVA results and vice versa.

In present study all TNAB and TSAB isolates were seen to harbor and express at least one of the RND efflux pumps, and their corresponding regulators, including *Ade*ABC- *Ade*RS, *Ade*FGH- *Ade*L, and *Ade*IJK- *Ade*N. The *Ade*IJK efflux pump appeared to play a general, non-specific, role in reducing susceptibility

of isolates to tigecycline, because both TNAB and TSAB isolates expressed this pump. In addition, the lower expression level of *Ade*IJK efflux pump among TNAB and TSAB isolates compared to that of *Ade*ABC and *Ade*FGH, may be evidence to support an intrinsic role for this pump in *A. baumannii*. The expression level of *Ade*IJK has also been reported as lower than *Ade*ABC in other studies (Xing et al., 2014). It was initially thought that overexpression of *Ade*IJK was toxic for the host cell (Xing et al., 2014). However, in low level resistant mutants, overexpressing *Ade*IJK have been obtained on drug gradients of tetracycline or cefotaxime (Coyne et al., 2010a). The *Ade*N system which was observed in all *ade*IJK-positive strains, can regulate the expression of the *Ade*IJK efflux pump (Xing et al., 2014). According to previous reports, the *Ade*IJK efflux pump synergistically with *Ade*ABC and/or *Ade*FGH acts to export toxic compounds and antibiotics (Damier-Piolle et al., 2008; Potron et al., 2015). In our study, although *ade*IJK was found to co-existe with *ade*ABC and *ade*FGH in all TNAB isolates, synergistic activity of *ade*IJK with others in decreasing susceptibility to tigecycline is not enough evidence; thus further studies appears to be needed for confirming such a claim.

In this research, 64 of 100 isolates were seen to have XDR phenotype and all 29 TNAB isolates also were XDR. XDR isolates were found to harbor all three pumps, while some of the MDR isolates did not possess one of the *Ade*ABC or *Ade*FGH efflux pumps. Besides, the higher expression levels of both these pumps were found in XDR, in comparison with MDR isolates.

All TNAB isolates were shown to express *ade*B at a statistically higher levels than did TSAB (XDR and MDR) isolates, while *ade*G expression in TNAB isolates was found to be significantly no different from that TSAB (XDR and MDR) isolates; thus the higher level of *ade*G expression in all isolates may be associated with MDR and XDR phenotype, but not tigecycline non- susceptibility. By contrast, *ade*B expression level among XDR-TSAB was significantly no different from MDR -TSAB and was lower than XDR-TNAB isolates. All these results together suggest that there appears to be an association between tigecycline non-susceptibility and increasing *ade*B expression, but not *ade*G or *ade*J. In agreement with our present study, the results of other studies have shown that the increasing expression of *ade*ABC in TNAB isolates is the most likely mechanism which has led to a reduction in the susceptibility of *A. baumannii* to tigecycline (Peleg et al., 2007; Hornsey et al., 2010; Yoon et al., 2013; Ruzin et al., 2007). By contrast, the results of other study showed that increasing expression of *ade*FGH in TNAB isolates is an additional mechanism for reducing susceptibility of *A. baumannii* to tigecycline (Coyne et al., 2010b).

It is clear that efflux pumps can be targets for new antimicrobial agents. Peptidomimetic compounds such as phenylalanine arginyl β-naphthylamide (PAβN) have been introduced as efflux pump inhibitor (EPI); their mechanism of action is through competitive inhibition with antibiotics on the efflux pump resulting in increased intracellular concentration of antibiotic (Askoura et al., 2011). Accordingly, in this study the role of efflux pumps was analyzed using the efflux pump inhibitor (PAβN). The MIC levels of tigecycline showed ≥ 4 folds reduction in presence of PAβN in TNAB than in TSAB isolates. Since mean expression of *ade*ABC among TNAB was higher than others, these results suggest importance of *Ade*ABC in the increase of tigecycline resistance.

The presence of *ade*RS and *ade*L in TNAB and TSAB isolates containing *ade*ABC and *ade*FGH, respectively, was not statistically different. The higher levels of expression of *ade*B in TNAB and *ade*G in all isolates could stem from other factors other than the presence of the *ade*RS and *ade*L regulatory systems. It was found by several studies that the functional point mutation, or insertion of *IS*Aba1 into the two-component regulatory system *ade*RS, may be related to the overexpression of the *Ade*ABC pump in TNAB strains (Deng et al., 2014; Chen et al., 2014; Ruzin et al., 2007; Yoon et al., 2013). Here we also observed the overexpression of *ade*G in isolates that were positive for *ade*L, which is consistent with the results of past research. *ade*L was found to be located upstream of the *ade*FGH operon, and a mutation in this gene led to the overexpression of *ade*FGH (Coyne et al., 2010b; Xing et al., 2014). One limitation of this study was the lack of sequence analysis in order to identify functional mutations in *Ade*RS and *Ade*L as regulatory systems of *Ade*ABC and *Ade*FGH, respectively.

TNAB and TSAB isolates, despite exhibiting significant differences in expression levels of RND efflux pumps, had no significant difference in resistance to carbapenem which can indicate that other factors in addition to RND efflux pumps might contribute toward carbapenem resistance. In agreement with our results, previous studies suggested that carbapenem hydrolyzing oxacillinases (e.g., OXA-24 and OXA-58) act synergistically with RND efflux pumps to promote carbapenem resistance (Rumbo et al., 2013; Hammoudi et al., 2015; Jia et al., 2015; Azimi et al., 2015). It appears that overexpression of *adeFGH*, which extrudes carbapenems and aminoglycosides, combined with enzymatic resistance to these two classes of drugs, contributes in a more than additive fashion to the level of resistance of the host (Azimi et al., 2015).

As aforementioned, in various regions, of three major G types, G1 has been identified as the most commonly occurring G type, while in our previous and current studies the isolates belonging to G variant were found to be the most frequent G type among *A. baumannii* infections. Our results showed that the resistance rates to tigecycline in the G variants were significantly higher than those in other three major G types. As expression level of *Ade*ABC in the G variants was greater than that in three major G types, the increasing trend in prevalence of G variants over recent years in our region could be explained by increase in ability to express this gene. To our knowledge, there has been no study to completely investigate the phenotypic and genotypic resistance of the three major G types and G variants to tigecycline. However, in numerous prior researches direct association between the increased expression level of efflux pumps and the resistance to carbapenems and tigecycline have been reported (Deng et al., 2014; Hou et al., 2012). Since the resistance of G1 and G variants to tetracycline and minocycline is high, further research into presence and expression of genes involved in tetracycline resistance are needed to clarify the underlying mechanisms of resistance to this antibiotic in our region.

Several studies introduced tigecycline as treatment option due to *in vitro* susceptibility of imipenem- and colistin-resistant *A. baumannii* to this antibiotic (Cai et al., 2012; Sarada et al., 2014). However, the emergence of tigecycline resistant *A. baumannii* isolates has been reported and is on the rise (Li et al., 2015; Montana et al., 2015; Mavroidi et al., 2015). Our findings suggest that there appears to be an association between tigecycline non susceptibility and increasing *Ade*ABC efflux pump expression, but not *Ade*FGH or *Ade*IJK.

In current report, the majority of isolates were shown to present extreme drug resistance phenotype, which is a challenge in treatment of *A. baumannii* infections. Besides, the increasing rise of tigecycline non- susceptible strains has led to a condition in which therapeutic options are limited. Increase of G variant rate, as the most frequent G types, that has accompanied by acquisition tigecycline resistance phenotype and *ade*B overexpression can provide an evidence for effect of this phenotype on *A. baumannii* dissemination, especially burn wound infections. The *Ade*ABC efflux pump appeared to play a potential

role in tigecycline non-susceptibility phenotype, because there was a significant association between *ade*B expression level and this phenotype in TNAB isolates. A greater decrease in tigecycline MIC in the presence of EPI in TNAB isolates can provide another evidence for the role of *ade*ABC efflux pump in decrease susceptibility of *A. baumannii* to tigecycline. It seemed the *Ade*FGH efflux pump did not play a considerable role in tigecycline non-susceptibility, because there apeared no significant difference in the mean *ade*G expression between TNAB and TSAB isolates.

Declarations:

Ethics approval and consent to participate:

This study was approved by the ethics committee of Ahvaz Jundishapur University of Medical Sciences (Ethical code: IR.AJUMS.REC.1400.255) for human participants. All methods were performed in accordance with the relevant guidelines and regulations. All experiments in this study were performed in accordance with ARRIVE guidelines (https://arriveguidelines.org)

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Conflict of Interests:

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Figures

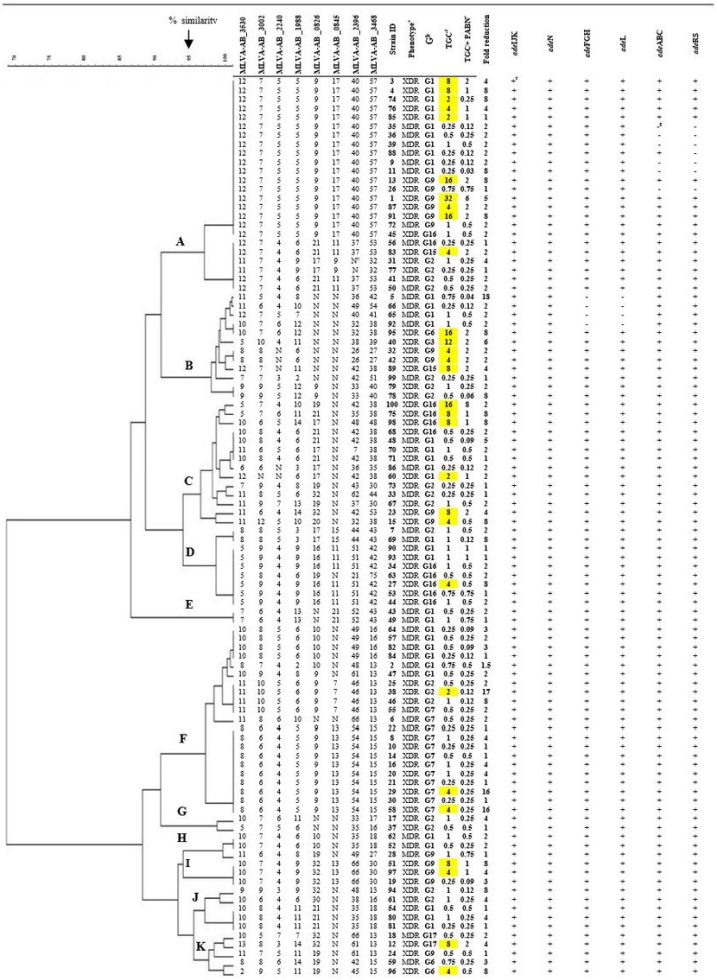

Figure 1

Distribution of efflux pump genes based on MLVA type and international clone type among 100 *Acinetobacter baumannii* isolates

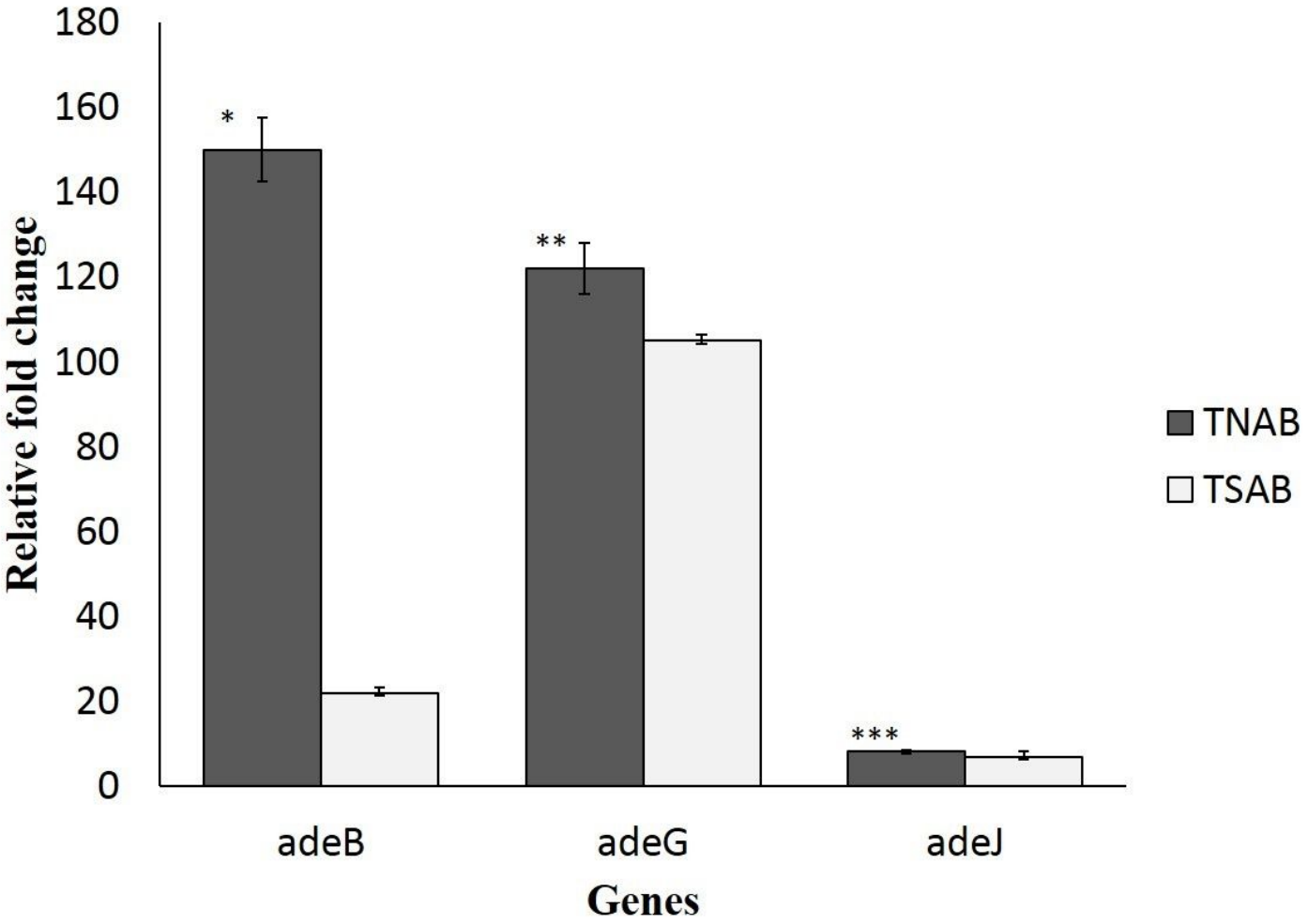

Figure 2

Comparison of mRNA concentration of *ade*B, *ade*G and *ade*J, the main transporter of *Ade*ABC, *Ade*FGH and *Ade*IJK respectively, between TNAB and TSAB isolates by using Real-time RT-PCR. The data for each gene were normalized against those obtained for the 16S rRNA control. The mRNA concentration was calculated as $2^{-\Delta\Delta Ct}$ for each gene, where ΔCT represents the threshold cycle (CT) value of the gene subtracted from the CT value of the 16S rRNA control. Values are mean ± standard devition of the mean [mRNA] for each gene transcript from three replicate experiments. The data were analyzed using Student's t test (two tailed). *, $P < 0.01$; **, $P < 0.05$; ***, $P > 0.1$ significantly different from the control.

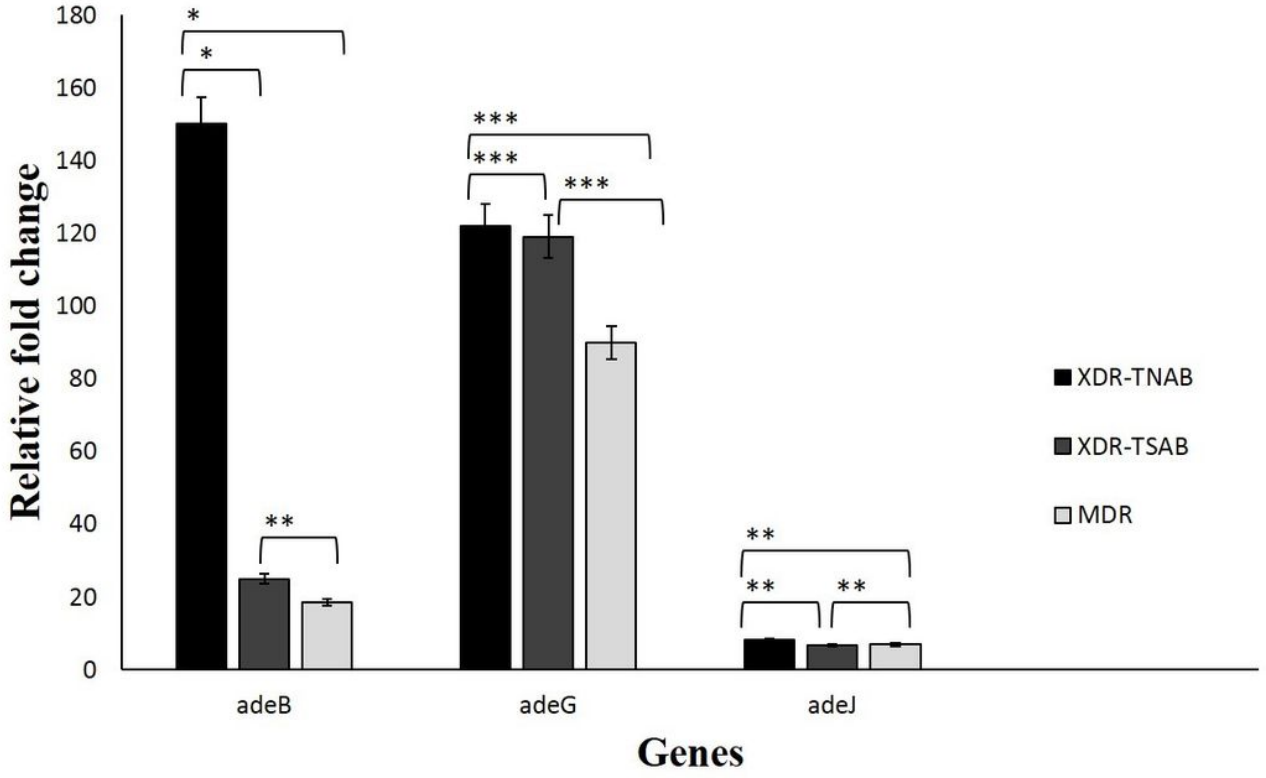

Figure 3

Comparison of mRNA concentration of *ade*B, *ade*G and *ade*J, the main transporter of *Ade*ABC, *Ade*FGH and *Ade*IJK respectively, between XDR-TNAB, XDR-TSAB and MDR isolates by using Real-time RT-PCR. The data for each gene were normalized against those obtained for the 16S rRNA control. The mRNA concentration was calculated as $2^{-\Delta\Delta Ct}$ for each gene, where ΔCT represents the threshold cycle (CT) value of the gene subtracted from the CT value of the 16S rRNA control. Values are mean ± standard devition of the mean [mRNA] for each gene transcript from three replicate experiments. The data were analyzed using Student's t test (two tailed). *, P < 0.001; **, P > 0.1; ***, P >0.05 significantly different from the control.