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Detection of efflux pump genes in multiresistant *Acinetobacter baumannii* ST2 in Iran

ZAHRA MESHKAT[1], HIMEN SALIMIZAND[2,3], YOUSEF AMINI[4,5]*, DAVOOD MANSURY[6], ABOLFAZL RAFATI ZOMORODI[7], ZOLEIKHA AVESTAN[8], AZAD JAMEE[9], JAMAL FALAHI[10], HADI FARSIANI[1] and AZIZOLLAH MOJAHED[11]

[1] Antimicrobial Resistance Research Center, Mashhad University of Medical Sciences, Mashhad, Iran

[2] Liver and Digestive Research Center, Research Institute for Health Development, Kurdistan University of Medical Sciences, Sanandaj, Iran

[3] Department of Microbiology, Faculty of Medicine, Kurdistan University of Medical Sciences, Sanandaj, Iran

[4] Infectious Diseases and Tropical Medicine Research Center, Resistant Tuberculosis Institute, Zahedan University of Medical Sciences, Zahedan, Iran

[5] Department of Microbiology, School of Medicine, Zahedan University of Medical Sciences, Zahedan, Iran

[6] Department of Microbiology, School of Medicine, Isfahan University of Medical Sciences, Isfahan, Iran

[7] Department of Pathobiology, Faculty of Veterinary Medicine, Ferdowsi university of Mashhad, Mashhad, Iran

[8] Emam Khomeini Hospital of Naqadeh, Urmia University of Medical Sciences, Urmia, Iran

[9] Department of Endodontics, Dental Faculty, Urmia University of Medical Sciences, Urmia, Iran

[10] Department of Medicine, Zahedan Medical Branch, Islamic Azad University, Zahedan, Iran

[11] Department of Clinical Psychology, Zahedan University of Medical Sciences, Zahedan, Iran

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ABSTRACT

Acinetobacter baumannii, as a nosocomial pathogen has become a worldwide concern in recent years. In the current study, the resistance to tetracyclines and colistin were assessed in the isolates from different provinces of Iran.

During the timeline of this study, a number of 270 isolates of *A. baumannii* were collected from tracheal aspirates, wounds, urine and blood cultures. The minimum inhibitory concentration (MIC) for tetracycline, doxycycline, minocycline, tigecycline and colistin were evaluated. Tetracycline resistance genes were assessed by PCR. The mean expression level of *adeB*, *adeJ* and *adeG* were assessed using semi quantitative Real-Time PCR. The clonal relationship of the isolates was evaluated by the repetitive extragenic palindromic PCR (REP-PCR), International Clonal (IC) Lineage Multiplex PCR and multilocus sequence typing (MLST) (Pasteur scheme) methods.

The MIC by microdilution method showed that 87.5, 51.4, 28, 0.74 and 0% of the isolates were resistant to tetracycline, doxycycline, minocycline, tigecycline and colistin respectively. The prevalence of tetracycline resistance genes was 99.2, 99.2, 98, 86.7, 10, 3.33, 0.37, 0% for *adeB*, *adeJ*, *adeG*, *tetB*, *tetA(39)*, *tetA*, *tetM* and *tetH* in tetracycline-resistant isolates. Moreover, the expression level of *adeB*, *adeJ*, *adeG* genes in tigecycline-nonsusceptible *A. baumannii* (TNAB) strain was higher compared to the tigecycline-susceptible *A. baumannii* (TSAB). A broad genomic diversity was revealed, but ST2 was the most prevalent ST. Our results indicated that tetracycline resistance in Iran is mediated by resistance-nodulation-cell division (RND) and *tetB* efflux pumps.

*Corresponding author.
Tel.: +98 9143887898.
E-mail: yousefamini1363@gmail.com; amini.y@zaums.ac.ir

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KEYWORDS

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INTRODUCTION

The emergence of multidrug-resistant (MDR), extensively-drug resistant (XDR) and pan-drug resistant (PDR) *Acinetobacter baumannii*, as an opportunistic pathogen has become a worldwide concern in recent years. Because of many remarkable features, especially the intrinsic resistance to several antibiotics, *A. baumannii* is introduced as a notorious nosocomial pathogen, which is able to survive for long periods in the environment and hospital setting [1].

Resistance to almost all available antibiotics is the main concern about the *A. baumannii* [2]. Due to the emergence of extensively drug-resistant *A. baumannii* (XDRAB) isolates, there are a limited number of medications to select for treating XDRABs. Aminoglycosides, polymyxins and tigecycline are mostly used solely or in combination for treatment of *A. baumannii* [3].

Tetracycline was originally isolated from *streptomyces* species and introduced as a first broad-spectrum class of antibiotics. These antibiotics target the ribosome of bacteria and inhibit the protein synthesis. Other members of this class, including doxycycline and minocycline were developed by semisynthetic processes. Different generation of tetracycline antibiotics has been used to overcome *A. baumannii* resistance problem [4]. However, due to the wide-range usage of tetracyclines in the industry and medicine, resistance to these antimicrobial agents has reported in many countries and resulted in low efficacy for treatment of infectious diseases.

Generally, *tet* genes can confer resistance to tetracycline antibiotics through three mechanisms: i) tetracycline efflux, ii) ribosome protection, and iii) tetracycline modification. Specifically, the main mechanisms of tetracycline and minocycline resistance in *A. baumannii* are efflux pumps and ribosome protection. Among *tet* genes, *tetA*, *tetB*, *tetA(39)* and *tetH* genes have been recognized as efflux pump genes, by which tetracycline and doxycycline are pumped out of *A. baumannii*. Moreover, *tet(M)* has been reported in minocycline resistant *A. baumannii* isolates. This type of resistance is mediated through ribosomal protection mechanism [5].

To overcome multi drug resistant *A. baumannii,* tigecycline and colistin have remained as the only active antibiotics in recent years [6]. Unfortunately, tigecycline-nonsusceptible *A. baumannii* (TNAB) has been reported in many countries, and even a recent study indicated that it is not associated with preferable outcomes than other antimicrobial agents [4]. Tigecycline resistance mechanism has been developed with overexpression of some efflux pumps, such as AdeABC, AdeIJK and AdeFGH [7]. Colistin (polymyxin E) is an effective antibiotic against gram-negative bacteria. Colistin interacts with lipidA component of lipopolysaccharide (LPS) to disorganize gram-negative outer-membrane. Due to colistin toxicity, its prescription has been prohibited, however it has recently been reconsidered after revised regimen [3].

In the current comprehensive study, we aimed at determining the rate of *A. baumannii* resistance to tetracycline, doxycycline, minocycline, tigecycline and colistin in different provinces of Iran during 2014–2015. Epidemiological relationship between all isolates was also considered.

MATERIAL AND METHODS

Hospital setting and bacterial isolates

Between Jan 2014 to Dec 2015, 270 non-duplicate *A. baumannii* isolates were recovered from different hospitals in 7 provinces of Iran, including Tehran (TRN), Khorasan Razavi (two clinics: KHR-A and B), West Azerbaijan (WAZ), Hamadan (HDN), Fars (FRS) and Kerman (KRN). All health centers had more than 400 beds with different units and wards, including intensive care unit (ICU), neonatal intensive care unit (NICU), pediatric intensive care unit (PICU), surgical ward, burn unit, internal medicine and emergency ward. The isolates were collected from tracheal aspirates, wounds, urine and blood cultures. Biochemical reactions were used for detection of isolates. The $bla_{\text{OXA-51}}$-like and *gyrB* Multiplex Polymerase Chain Reaction (PCR) were applied for identification of *A. baumannii* [8, 9].

Antimicrobial susceptibility testing

The minimum inhibitory concentration (MIC) of tetracycline, minocycline, doxycycline, and tigecycline as well as colistin was determined by microdilution method and the results were interpreted according to the Clinical and Laboratory Standards Institute (CLSI) guidelines [10]. The breakpoint for tigecycline was based on the U.S. Food and Drug Administration (FDA) recommendation for tigecycline susceptibility (MIC $\leq$2 as susceptible and $\geq$4 as resistant). Susceptibility profile of other antibiotics including meropenem, imipenem, gentamicin, amikacin, tobramycin, ceftazidime, cefepime, ceftriaxone, aztreonam, ciprofloxacin, levofloxacin, cotrimoxazole, and ampicillin/sulbactam were performed by disk-diffusion method, however, the results published in the proper papers [2, 11]. *Pseudomonas aeruginosa* ATCC 27853 was used as the control strain.

DNA extraction and molecular detection of tetracycline resistance determinants

Tetracycline resistance isolates were subjected to detect *adeB*, *adeJ*, *adeG*, *tetA*, *tetB*, *tetA(39)*, *tetH* and *tetM* genes. DNA extraction was performed by boiling.

PCR was performed with previously published and new designed primers (Table 1). For all detection assays, 12.5 µL of the 2X MasterMix (Parstous, Iran), 50 ng of the intact total DNA, 0.4 µM of each primer and adequate dH_2O were added to reach the 25-µL PCR reaction. The cycling protocol

Table 1. Primers used in this study

Primer name	Sequence	Amplicon size (bp)	Genbank accession no. & Reference
tetA-F	ACTGTCGCGCTCGACGCTG	1,047	This study
tetA-R	AGCCGCATAGATCGCCGTGAAGAG		
tetB-F	TTGGTTAGGGGCAAGTTTTG	659	JN247441 [29, 30]
tetB-R	GTAATGGGCCAATAACACCG		
tetM-F	AACAGGTTCGCCAGTGGTAAC	386	This study
tetM-R	ATGGTTGGAATGTGACGGATTGTAA		
tetH-F	TCTGGGTGCTGTTTACACAATATCG	252	This study
tetH-R	CCATACCTCCTGCCGCTAAG		
tetA(39)-F	CTCCTTCTCTATTGTGGCTA	701	EU495993 [2]
tetA(39)-R	CACTAATACCTCTGGACATCA		
adeB-F	TTAACGATAGCGTTGTAACC	541	AF370885 [21]
adeB-R	TGAGCAGACAATGGAATAGT		
adeJ-F	ATTGCACCACCAACCGTAAC	453	AY769962 [21]
adeJ-R	TAGCTGGATCAAGCCAGATA		

was as follows: initial denaturation at 94 °C for 5 min, 35 cycles of 94 °C for 30 s, annealing at 55–63 °C for 45 S and extension at 72 °C for 1 min, ended with 72 °C for 5 min. The PCR products were evaluated on 1.5% agarose gel stained with ethidium bromide and pictured by Gel Documentation device (UVI Tech, England).

Gene expression analysis using semi quantitative real-time PCR

Two tigecycline resistant isolates were grown aerobically in Luria-Bertani (LB) broth in the presence of 1 µgr/mL of tigecycline. Moreover, 7 tigecycline susceptible isolates were cultured, as well. The RNA was extracted by TRI reagent (Sigma, Germany) according to the manufacturer protocol. RNA templates were DNase-treated by DNaseI RNAase free endonuclease enzyme (SinaClon BioScience, Iran).

The concentration of RNA was quantified with a spectrophotometer. The cDNA was generated from total RNA using a commercial kit (Parstous, Iran). The qRT-PCR was performed by the Rotor-Gene 6000 instrument (Corbett Life Science, Valencia, CA) using 2X SYBR® Green Real Time PCR Master Mix (Parstous, Iran) in triplicates. The used primers for *adeG*, *adeJ* and *adeB* Real-time PCR are presented in Table 1. The results were normalized to the amount of 16S rRNA and the fold change and *P* values were calculated [12, 13].

Clonal lineage relatedness

To find out the clonal relationship among different isolates, Repetitive Extragenic Palindromic PCR (REP-PCR), International Clonal (IC) Lineage by Multiplex PCR and Multi-Locus Sequence Typing (MLST) (Pasteur scheme) were performed.

REP-PCR was done to determine the clonal relatedness of isolates according to a method previously designed [14]. The documented pictures were analyzed by GelJ software version 1. The dice similarity method with UPGMA linkage and the dice correlation coefficient with the 1.5% position tolerance were defined to find the clusters [14]. More than 65% of similarity was considered to find the clusters.

A multiplex PCR for detecting international clonal (IC) lineage of isolates was performed. International clones I and II were found according to the band patterns [15].

Multi-locus sequence typing (MLST) was carried out based on the Pasteur scheme. The alleles and sequence types (STs) were identified by the MLST database (https://pubmlst.org/abaumannii/). Clonal complexes (CC) were found in goeBURST (v.1.2.1) [16] and the graph was exported. The single locus variant (SLV) and double locus variant (DLV) were considered, where STs differed at a single locus or two loci, respectively. In addition, each CC should include three or more STs.

RESULTS

Isolates, antibacterial susceptibility testing

270 *A. baumannii* isolates were collected from seven hospitals of the selected provinces during the study period. Most of the isolates (76%) were recovered from the patients in ICU, followed by burn unit (16%), surgical ward (5%), internal medicine ward (2%) and emergency ward (1%). They were collected from respiratory discharges (53%), bloodstream (19%), wounds (20%), urine (6%) and cerebrospinal fluid (2%). MIC by microdilution method showed that 87.5% (236/270), 51.4% (138/270), 28% (75/270), 0.74% (2/270) and 0% (0/270) of the isolates were resistant to tetracycline, doxycycline and minocycline, tigecycline, and colistin, respectively (Table 2).

Distribution of tetracycline resistance genes

The resistance genes found in tetracycline-resistant isolates from different regions of Iran, including *tetB*, *adeB*, *adeJ and adeG* with the prevalence of 86.7, 99.2, 99.2% and 98%, respectively (Table 3). All isolates except two, one isolate from the FRS province and the other from WAZ, were

Table 2. Minimum inhibitory concentration of antibiotics and susceptibility testing

Antibiotic	Susceptible (%)	Resistant (%)	MIC_{50}	MIC_{90}
Tetracycline	34 (12.5)	236 (87.5)	256	1,024
Doxycycline	132 (48.6)	138 (51.4)	8	64
Minocycline	195 (72)	75 (28)	2	8
Tigecycline	268	2 (0.74)	0.5	2
Colistin	270	0 (0)	0.25	1

positive for *adeJ* and *adeB*. Moreover, *tetH* was not detected in any isolate. *tetA(39)* was only found in both KHR health centers; however, it showed the higher prevalence rate in the center A compared to the center B (64.7 vs. 11.9%). *tetM* was only detected in one isolate from WAZ province. Furthermore, *tetA* was found in a small number of isolates from KRM 21.6% (8 isolates) and also WAZ and 2.5% (one isolate).

The co-existence of resistance genes, *tetA* and *tetB,* was found in 7 tetracycline resistant isolates (2.6%). On the other hand, 26 isolates (9.6%) were positive for the *tetB* and *tetA(39)* genes. Moreover, co-existence of *tetA* and *tetB* was observed in 3.5% of the doxycycline-resistant and 4.8% of the minocycline-resistant isolates.

Assessment of *adeABC*, *adeIJK* and *adeFGH* expression

Two TNAB as well as seven TSAB isolates were subjected for semi quantitative real-time PCR. The relative range of *adeB*, *adeJ* and *adeG* were variable and depended on the strains. In our study, the real-time PCR results confirmed that the expression level of the three genes in both TNAB strains was increased. Both strains displayed the higher expression levels of *adeB* than the TSAB strains. Strains KHR-A543 and KRN10 showed 267 and 15 folds change of *adeB*, respectively.

The expression level of *adeJ* in TNAB was higher than TSAB strains. KHR-A543 and KRN10 showed 95 and 23 folds, respectively. *adeG* gene indicated the highest expression level. Similar to the previous genes, the strains KHR-A543 and KRN10 demonstrated 689 and 181 folds, respectively.

Typing and genomic diversity

To determine the genomic diversity of *A. baumannii* isolates, REP-PCR, IC lineage and MLST were conducted. REP-PCR of 270 isolates determined 121 unique patterns, which were embedded in 11 clusters. Four strains not belonged to any cluster and were considered as the singleton (Fig. 1). In our study, there was no relationship between resistance rates and clonal diversity. Furthermore, no correspondence was found between the distribution of tetracycline resistance genes and the clonality in the studied health centers. The only noticeable relatedness was the clonal relationship among minocycline-resistant strains in the isolates from Tehran province.

Multiplex PCR for IC demonstrated that 43.5% of strains belonged to the IC II and 6.6% to the IC I. The distribution of 16 STs in Iran was approved by MLST technique. STs were 1, 2, 25, 85, 94, 113, 307, 328, 570, 589, 599, 642, 708, 724 and 734. In this study, a novel ST1084 was discovered in Kurdistan province with the allelic profile of 1, 52, 2, 2, 9, 2 and 2 as per *cpn60*, *fusA*, *gltA*, *pyrG*, *recA*, *rplB* and *rpoB*, respectively. The most frequent ST was ST2 found in all health centers (113 out of 270/41.8%), which all were categorized as IC I. Two CCs were found, of which CC2 with a more populated complex, composed of 93 isolates, divided into three STs and contained ST2 as a founder of this CC. The other CC was CC642 with 17 isolates divided into five different STs. All of the sequence alleles were embedded in the goeBURST (v.1.2.1) illustrated in Fig. 2.

DISCUSSION

This study reported the prevalence and resistance mechanisms for tetracycline, doxycycline, minocycline, tigecycline and colistin in 270 strains of *A. baumannii* collected from seven health centers in Iran. The genetic correlation of the strains was also studied.

Our findings were consistent with the reports from Iran and other geographic regions, in which the resistance to tetracycline was common among *A. baumannii* isolates [17, 18]. Doxycycline resistance rate (as a longer-acting tetracycline) was higher than other reports [16, 18]. The results of the present study confirm the results of other studies, such as Maleki et al. and Pei et al. in which minocycline was more effective compared to tetracycline and doxycycline on *A. baumannii* isolates [17, 18–21]. However, this study revealed the increased level of tetracycline, doxycycline and minocycline resistance in comparison to the other studies worldwide [17, 20–26].

Table 3. Distribution of tetracycline resistance determinants in studied medical centers

	tetA	*tetB*	*adeB*	*adeJ*	*tetM*	*tetA(39)*	*tetH*	*tetA & tetB*	*tetB & tetA(39)*
KHR-A (n = 34)	0	33 (97)	34 (100)	34 (100)	0	22 (64.7)	0	0	21 (61.7)
KHR-B (n = 42)	0	39 (93.1)	42 (100)	42 (100)	0	5 (11.9)	0	0	5 (11.9)
KRN (n = 37)	8 (21.6)	24 (64.8)	37 (100)	37 (100)	0	0	0	6 (16.2)	0
FRS (n = 42)	0	39 (92.8)	41 (97.7)	41 (97.7)	0	0	0	0	0
TRN (n = 41)	0	25 (60.9)	41 (100)	41 (100)	0	0	0	0	0
WAZ (n = 40)	1 (2.5)	40 (100)	39 (97.5)	39 (97.5)	1 (2.5)	0	0	1 (2.5)	0
HDN (n = 34)	0	34 (100)	34 (100)	34 (100)	0	0	0	0	0

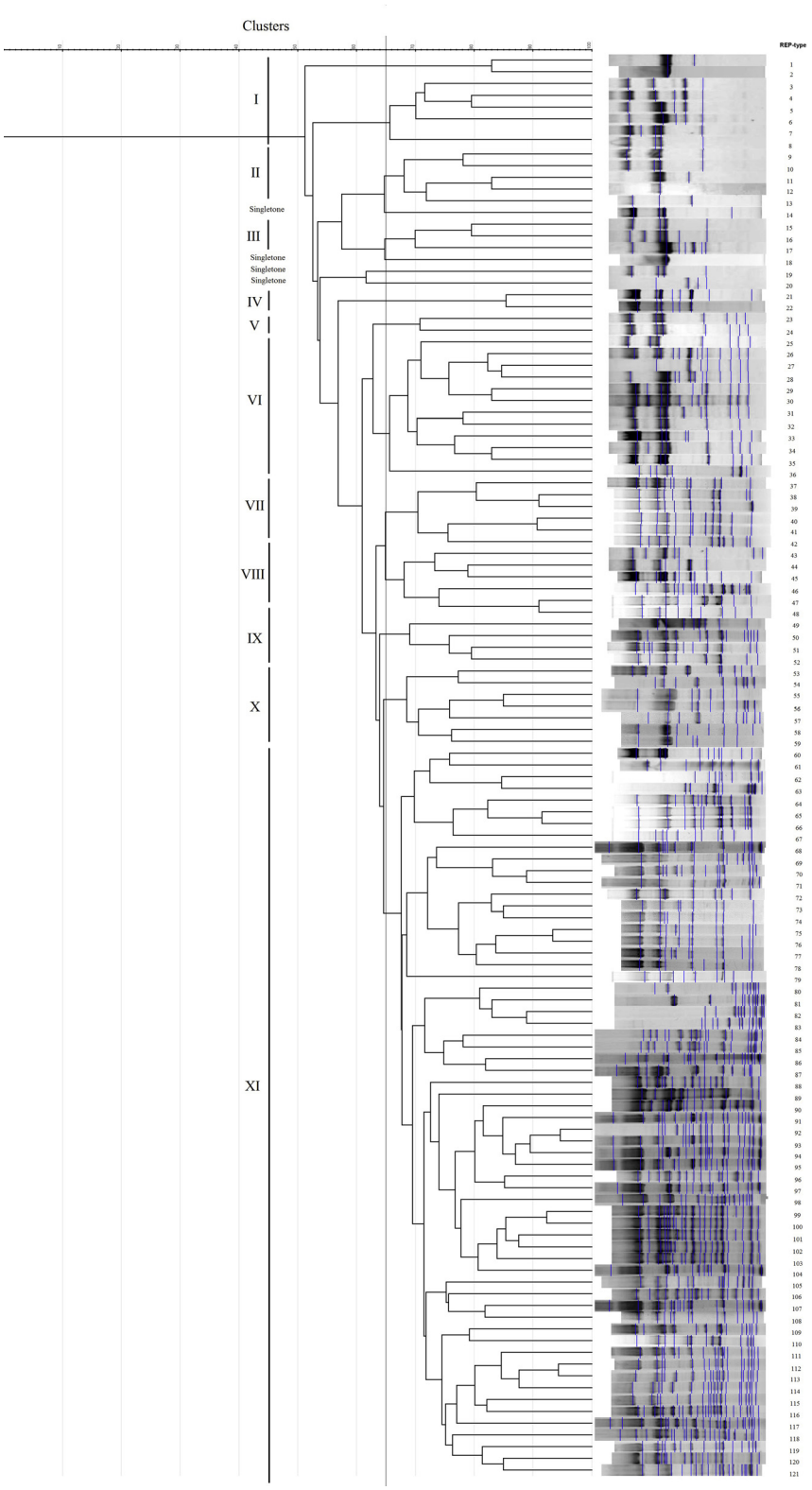

Fig. 1. Dendrogram of 121 unique patterns derived from 270 *A. baumannii* isolates. For each center, REP-PCR was done (data not shown). Unique band patterns of which were picked and subjected to GelJ software again, and the comprehensive illustration presented

Despite alarming rates of tigecycline resistance in the Middle East countries, in our study, only 0.74% of isolates were found tigecycline resistant [27]. This results are not consistent with a recent study results by Owrang et al. [28] in which a high resistance rate to tigecycline reported in Iran. Moreover, no resistance to colistin was observed. The results suggested that tigecycline and colistin are still effective candidates for the treatment of *A. baumannii* infection.

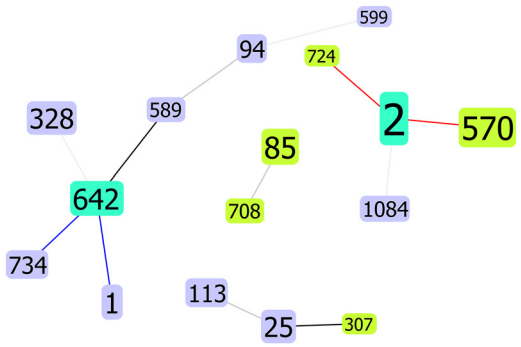

Fig. 2. Linker colors: Black, Link drawn without recourse to variants; Blue, number of SLVs; Green, number of DLVs; Red, number of TLVs; Gray, Links drawn at DLV. *ST nodes colors*: Light green, Group founder; Dark green, Sub-group founder; Light blue, Common node

Previous studies confirmed that *tetB* confers resistance to minocycline and doxycycline. Lomovskaya et al. indicated that the absence of *tetB* can infer susceptibility to minocycline, but, we found *tetB* in susceptible strains [29, 31]. Interestingly, *tetB* was identified in all minocycline resistance strains and was associated with high rate of resistance to minocycline. The expression level measurement of *tetB* may provide the answer to this discrepancy. Moreover, it has been suggested that *tetM* may develop resistance to minocycline. Although, in our study, these genes coexisted with *tetB* in one strain (URM42), however they were still susceptible to minocycline and doxycycline (0.03 and 0.25, respectively). More study is necessary to determine the role of *tetM* in minocycline-resistant strains.

tetA(39) transmitted by the horizontal transmission on plasmids and usually has been reported from water samples [31]. Akers et al. and Rumbo et al. introduced *tetA(39)* as an important tetracycline resistant mechanism [30, 32]. Interestingly, our results emphasized that the frequency of *tetA(39)* gene in most of strains (22/27) was associated with the resistance to tetracycline and doxycycline. *tetA(39)* showed epidemic distribution rather than endemic since it was only detected in the strains from KHR, a province located in the Northeast of Iran (Table 3). Most of *tetA(39)* carrying strains were restricted to the KHR-A hospital, however due to the patient transfer between KHR-A and KHR-B health centers, such pattern of transmission can be expected. Another interesting finding of *tetA(39)* was the isolation date of this gene in the studied health centers. *tetA(39)* was detected in the strains that were recovered from 2014 to 2015 in KHR-A hospital, but it only appeared in KHR-B in 2015 isolated strains (data is not shown).

Although resistance-nodulation-cell division (RND)-type efflux pumps seem to be associated with the resistance to tigecycline, the resistance mechanism is not yet fully understood. In the current study, a high prevalence of these genes was observed. Several studies have confirmed that the presence of *adeB*, *adeG* and *adeJ* can increase tetracycline resistance in clinical strains [33–35]. Our study confirmed that the overexpression of *adeB*, *adeG* and *adeJ* is a prevalent mechanism in TNAB strains, which led to the decreased susceptibility to tigecycline. However, no correlation was observed between the expression of efflux pump genes and the MIC of tigecycline. Accordingly, other mechanism may be involved in tigecycline resistance [1].

In this study, the REP-PCR technique was used to evaluate clonal relatedness of antibiotic resistant strains in different provinces of Iran. Based on the REP-PCR results, numerous clusters indicated the genomic diversity of *A. baumannii* strains in Iran. Approximately, 135 strains (50%) were not determined by IC Lineage multiplex PCR method. However, 43.5% of the strains belonged to the widely distributed IC, i.e. IC II. These results are consistent with other previous studies [36–38]. ST2 was the most isolated ST, which was frequently reported in many health centers worldwide and Iran, as well [36, 39–41]. The CC2 (CC92 oxford scheme) with 93 members (34.4%) showed a wide distribution in all health centers, which has also frequently reported from other health centers [40–42].

Since no experimental study to evaluate tetracyclines and colistin has performed in Iran, their antibacterial efficacy is not clearly known. However, according to the high rate of resistant isolates, tetracyclines can be cautiously prescribed for treating *A. baumannii* infections.

CONCLUSION

Our results provided a comprehensive view of resistance to tetracyclines and colistin in *A. baumannii* in Iran. To the best of our knowledge, some of the assessed genes (*tetM* and *tetA(39)*), reported for the first time in Iran. However, clonal distribution of these two genes was not observed, while they appeared local. Moreover, RND efflux pumps especially AdeABC, Ade FGH and AdeIGK seem to play an important role in the reduced tigecycline susceptibility in Iran.

Conflicts of interests: None declared.

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Consent to participate: All authors voluntarily agree to participate in this research study.

Consent for publication: All authors voluntarily agree to publish the paper in International Microbiology journal.

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