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Original Article

Emergence of tigecycline-resistant *Klebsiella pneumoniae* ST11 clone in patients without exposure to tigecycline

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Abstract

Introduction: Tigecycline is a unique class of semi-synthetic glycylcyclines developed to treat infections caused by multidrug-resistant *Klebsiella pneumoniae*. In the past decades, eight tigecycline-resistant *Acinetobacter baumannii* isolates have been identified in Tehran and no *Klebsiella pneumoniae* has been reported.

Methodology: To elucidate the mechanism of *K. pneumoniae* efflux pump-mediated resistance, the expression of efflux pump genes (*oqx*A, *oqx*B, *acr*A, *acr*B, *tol*C) and regulators (*acr*R, *ram*A, *mar*A, *sox*S, *rar*A, *rob*) was investigated by real-time RT-PCR. Multilocus sequence typing (MLST) of tigecycline-resistant strains was also performed.

Results: Compared to the tigecycline sensitive strain K32 (negative control), all resistant strains showed higher expression levels of efflux genes and regulatory factors. Three tigecycline-resistant strains (K53, K67, K79) showed higher levels of *rar*A expression (38.1-fold, 41-fold and 24-fold, respectively) and *oqx*B pump gene (48.2-fold, 60-fold and 58-fold, respectively). The increased expression of *acr*B was associated with the expression of *ram*A. However, to the best of our knowledge, studies on the mechanisms of resistance of *K. pneumoniae* strains to tigecycline are limited, especially in developing countries such as Iran.

Conclusions: In the present study, we found that both AcrAB-TolC and OqxAB efflux pumps may play an important role in tigecycline resistance in *K. pneumoniae* isolates. Finally, the emergence of ST11 molecular type of resistant isolates should be monitored in hospitals to identify factors leading to tigecycline resistance.

Key words: Tigecycline; efflux pump genes; *Klebsiella pneumoniae*; real-time RT-PCR; MLST.

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Introduction

Tigecycline, a derivative of minocycline, belongs to a new class of glycylcyclines and has a broad spectrum of activity against most Gram-positive and Gram-negative bacterial pathogens [1,2]. However, in recent years, resistance to tigecycline has emerged in multidrug-resistant (MDR) strains of various pathogens, in particular, *Acinetobacter baumannii*, *Klebsiella pneumoniae* and Enterobacteriaceae. In recent decades, various strains of tigecycline-resistant pathogenic bacteria have been reported from the USA, UK, France, Saudi Arabia, Greece, Spain, Germany and Taiwan [3,4]. Most of them changed from tigecycline-sensitive to tigecycline-resistant during treatment, with the highest reported minimum inhibitory concentration (MIC) of tigecycline being 24 µg/mL. The development of non-sensitivity to tigecycline has also been reported in *A. baumannii* and Iran [5-7]. Tigecycline, which was approved by the US Food and Drug Administration (FDA) in 2005, has not yet been marketed in Iran. However, three tigecycline-resistant strains were found, which were isolated before tigecycline was used. This phenomenon was consistent with the report of Rosenblum et al. who found resistance before the introduction of tigecycline [8]. Veleba M. and colleagues showed that overexpression of the RND-type efflux pump AcrAB and the recently described efflux pump OqxAB is responsible for the reduced susceptibility of *K. pneumoniae* to tigecycline [9]. To elucidate the mechanism of *K. pneumoniae* resistance-mediated efflux pump, the expression of efflux pumps (*oqxA*, *oqxB*, *acrA*, *acrB*, *tolC*) and regulatory genes (*acrR*, *ramA*, *marA*, *soxS*, *rarA*, *rob*) was investigated by real-time RT-PCR [10,11]. The main objective of this study was to investigate the mechanisms present in tigecycline resistant *K. pneumoniae* strains from Iran.

Methodology

Bacterial strains

One hundred and eighty seven Enterobacteriaceae were isolated from clinical samples from hospitals in Hamadan and Tehran between 2019 and 2020. The

Table 1. The characteristics and sources of the clinical isolates of *Klebisella pneumoniae*.

strain	Isolate date	Source	Hospital-city	Type	MLST Type
K53	2019.07.29	Burns	Shahid Motahari Burn Hospital, Tehran	wound	ST11
K67	2020.1.18	Intensive Care Unit	Shohada-e Tajrish Hospital, Tehran	Pus	ST11
K79	2019.8.4	Intensive Care Unit	Bessat Hospitals, Hamadan	blood	ST11
K32	2019.9.14	Intensive Care Unit	Shahid Motahari Burn Hospital, Tehran	blood	ST893

MLST: Multilocus sequence typing.

strains were identified by API 20E and their susceptibility was assessed by microbial dilution and E-test, while four *K. pneumoniae* strains were selected to study the mechanism of the efflux pump. These were three tigecycline-resistant *K. pneumoniae* isolates and one tigecycline-susceptible *K. pneumoniae* K32, which were used as negative control. *K. pneumoniae* ATCC 11296, and *Escherichia coli* ATCC 25922 were used as reference strains. The strains used in the study are listed in Table 1.

Tigecycline susceptibility testing

Tigecycline susceptibility testing was carried out using three different methods: First, the Kirby-Bauer method was used as a tigecyclinesensitivity test. Subsequently, the minimum inhibitory concentrations (MICs) of tigecyclinewere determined using standard microdilution tests and E-test (BioMerieux Marcy l'Étoile, France) in accordance with the recommendations of the CLSI documents [12] and the manufacturer's instructions. MIC values for the strains were interpreted according to FDA guidelines for tigecycline, with MIC values of ≤ 2 μg/mL and ≥ 8 μg/mL categorized as sensitive and resistant, respectively. *E. coli* ATCC 25922 strain was used for the quality control.

To verify the clonality of the selected isolates, the four isolates were typed using multilocus sequence typing (MLST) method. MLST was performed with seven housekeeping genes (*gapA*, *infB*, *mdh*, *pgi*, *phoE*, *rpoB* and *tonB*) according to the protocol described in the *K. pneumoniae* MLST database. Allele and sequence type (ST) assignments were performed using the *K. pneumoniae* MLST database tools. BioNumerics software version 7.5 (Applied Maths, Kortrijk, Belgium) was used to generate the minimum spanning tree [13], in which the founder ST was defined as the ST with the most single-locus variation. The founder ST was defined as the ST with the largest number of single-locus variations. The types are represented by circles, and the size of the circle indicates the number of strains belonging to each type.

Efflux pump mechanism

The efflux pump inhibitor (EPI) carbonyl cyanide-chlorophenylhydrazone (CCCP; Sigma) was used to investigate efflux pump activity in tigecycline-resistant and tigecycline-sensitive *K. pneumoniae* strains. MIC values of tigecycline in the presence and absence of CCCP (constant concentration 16 μg/mL) were determined by microdilution method. If MIC values decreased by a four-fold or more in the presence of EPI, this was defined as a significant inhibitory effect [14].

Real-time quantitative PCR

The expression levels of the regulatory genes *acrR*, *marA*, *soxS*, *rarA*, *rob* and *ramA*, as well as the efflux pump components *acrA*, *acrB*, *tolC*, *oqxA* and *oqxB* were analysed by RT-PCR. RNA extraction was performed using the Total RNA Prep Kit (BioFACT™, Daejeon, South Korea) and cDNA synthesis was performed using the cDNA Synthesis Kit (BioFACT™,Daejeon, South Korea) according to the manufacturer's instructions. Quantification of cDNAs was performed by real-time PCR amplification with specific primers (Table 2) using the TaqMan one-Step

Table 2. Primers and fluorescent probes used for PCR.

Gene	Forward primer	Reverse primer
ramA	GCATCAACCGCTGCGTATT	GCATCAACCGCTGCGTATT
marA	TAATGACGCCATCACTATCCA	ATGTACTGGCCGAGGGAATG
soxS	TAGTCGCCAGAAAGTCAGGAT	AGAAGGTTTGCTGCGAGACG
rarA	AGCTTAACGCCTCGATCAT	AGCTTAACGCCTCGATCAT
robA	TATTCTATACCACCGCGCTGAC	GTGCCGTAGACGGTCAGGAT
acrA	GCCTATCGCATTGCGGAAG	TTGGCGCTGTCATAGCTGG
acrB	AGCTTAACGCCTCGATCAT	AGCTTAACGCCTCGATCAT
tolC	TACCAGCAGGCACGCATCA	GCTGTCGCGATAGCCATTGT
oqxB	AGCTTAACGCCTCGATCAT	AGCTTAACGCCTCGATCAT
oqxA	CGCAGCTTAACCTCGACTTCA	ACACCGTCTTCTGCGAGACC
acrR	CCTGGCGAGTTATGAGCGTAT	GGTAGCTGCGCATTAACACG
rpoB	GGTAATTCCGAGCTGCAATACG	CGCGCTCGTAGATCACCAG

RT-PCR master mix (Life Tech, Carlsbad, CA, USA) reagent kit on a Life Tech 7500 Fast Real-time PCR (Applied Biosystems, Foster City, Calif.) system with an initial incubation of 2 minutes at 95 °C and 40 cycles (10 seconds at 95 °C, 30 seconds at 60 °C and 10 seconds at 72 °C). Each sample was processed in triplicate. In each case, a reference gene (*rpoB*) was used to normalize the expression of the target gene for each isolation. Critical threshold cycle (CT) numbers were determined using the sensor system software. The target value was expressed as 2-ΔCT. Expression analysis was performed to measure the relative expression of mRNAs compared to *K. pneumoniae* K32.

Results

The 187 Enterobacteriaceae isolates included *K. pneumoniae* (91 isolates), *Escherichia coli* (84 isolates), *Enterobacter cloacae* (8 isolates) and *Enterobacter aerogenes* (4 isolates). Three isolates of tigecycline-resistant *K. pneumoniae* were obtained. MIC value of tigecycline for all clinically isolated strains of carbapenem-resistant *K. pneumoniae* ranged from 0.5 to 16 μg/mL, MIC_{50} was 4 μg/mL and MIC_{90} was 16 μg/mL. Three clinical isolates of *K. pneumoniae* that had a tigecycline MIC greater than 8 μg/ml (Table 3) were collected. The highest MIC value of tigecycline was 16 μg/ml (K53, K67, K79). In addition, a tigecycline-sensitive common clinical strain K32 type ST11 was identified as the negative control. Thus, three resistant strains and one sensitive strain were included in this study. The sensitivity profiles of the four strains are shown in Table 3.

Molecular typing of the strains

MLST analysis of the four isolates revealed three different sequence types (ST). ST11 was the predominant ST, present in two (66.6%) of the isolates. The other isolate belonged to ST893.

Efflux pump activity

The effect of EPIs on the MIC of tigecycline is shown in Table 3. Two strains (K53, K79) showed a 2-fold decrease in tigecycline MIC from 16 μg/mL to 4 μg/mL and one strain (K67) showed a 2-fold decrease (from 8 μg/mL to 2 μg/mL) in the presence of CCCP (16 μg/mL). No MIC reduction was observed for strain K32 under the same conditions.

Analysis of target pump genes and regulator expression

In the present study, three tigecycline-resistant strains were shown to have efflux pump and pump regulatory genes. The relative x-fold increase in pump genes and pump regulators was quantified after comparison with *K. pneumoniae* K32. We observed higher expression levels of the *rarA* regulator (38.1-fold, 41-fold and 24-fold) and *oqxB* (48.2-fold, 60-fold and 58-fold) in four tigecycline-resistant strains (K53, K67, K79) compared to the K32 strain. These data suggest a correlation between increased expression of *oqxB* and *rarA*. In addition, with the exception of *oqxB*, the amount of *acrB* transcript was higher in the resistant strains compared to the K32 strain. The expression of *acrB* showed an increasing trend together with the expression of *ram*A and *mar*A. Interestingly, the transcript levels of the *soxS* gene, *acrR* gene and *rob* gene seemed to correlate with the expression of *acrB* (data shown in Figures 1 to 10).

Discussion

Tigecycline was created and has attracted a lot of attention because it is used as a treatment of last resort for clinical infections caused by multidrug-resistant *K. pneumoniae*. Three tigecycline-resistant strains were discovered that were resistant to tigecycline before its therapeutic use [15,16]. We hypothesized that this resistance could be indirectly attributed to the use of other antibiotics, such as ciprofloxacin, mediated by the same efflux pump, since tigecycline is a substrate of the nodulation-distribution efflux pump. One possible hypothesis was that these efflux pump systems are widely available in *K. pneumoniae*. If strains are exposed to antibiotics that are substrates for efflux pumps, this could lead to overexpression of these pumps [17-19]. In addition to our report, other reports have described cross-resistance to tigecycline. Hornsey *et al*. and Deng *et al*. reported resistance to antibiotics other than tigecycline. In addition, another study showed that the use of carbapenems can lead to resistance to carbapenems and/or several other antibiotics, including tigecycline [14,19]. These reports confirmed our findings that tigecycline-resistant strains are more likely to be associated with the overexpression of the efflux pump, which causes cross-resistance. It has been reported that antibiotic resistance appears to be mediated in part by active efflux systems [20,21]. More recently, some studies have shown that efflux pump inhibitors (EPIs) can reverse the pattern of resistance by blocking bacterial pumps and preventing efflux of certain antibiotics [22]. To detect whether efflux pumps are overexpressed in resistant *K. pneumoniae* strains, efflux pump inhibitors (EPIs) CCCP were used to assess efflux pump activity.

Table 3. Phenotypic and genotypic characteristics of four tigecycline-resistant *Klebsiella pneumoniae* isolates.

Strain	MIC (mg/L)					Antimicrobial resistance phenotype	Carbapenemase gene(s)	Associated β-lactamases
	IPM	MEM	ETP	TGC	TGC+CCCP			
K53	256	8	8	16	4	CAZ, CTX,IPM, MEM, ETP, FEP, ATM, AMK, GEN, CIP, FOF, CST	$bla_{\text{OXA-48}}$	$bla_{\text{CTX-M-15}}$
K67	256	8	16	8	2	CAZ, CTX, IPM, MEM, ETP, FEP, GEN, ATM, CIP, CST	$bla_{\text{OXA-48}}$	$bla_{\text{CTX-M-15}}$, bla_{TEM}, bla_{SHV}
K79	32	8	16	16	4	CAZ, CTX, IPM, MEM, ETP, FEP, ATM, AMK, GEN, CIP, FOF	$bla_{\text{OXA-48}}$	$bla_{\text{CTX-M-15}}$, bla_{TEM}, bla_{SHV}
K32	16	8	8	2	2	CAZ, CTX, IPM, MEM, ETP, FEP, ATM, AMK, GEN, CIP, CST	$bla_{\text{OXA-48}}$, $bla_{\text{NDM-1}}$	$bla_{\text{CTX-M-15}}$, bla_{TEM}, bla_{SHV}

IPM: imipenem; MEM: meropenem; ETP: ertapenem; AMK: amikacin; GEN: gentamicin; CST: colistin; CAZ: ceftazidime; CTX: cefotaxime; FEP: cefepime; ATM: aztreonam; CIP: ciprofloxacin; FOF: fosfomycin.

Figure 1. Levels of *acrA* gene in tigecycline-resistant *K. pneumoniae* isolates.

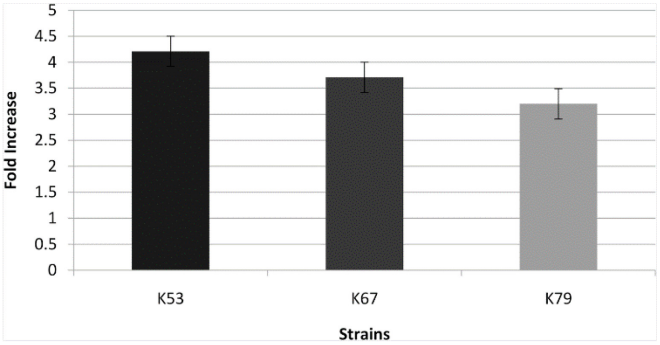


Figure 2. Levels of *acrB* gene in tigecycline-resistant *K. pneumoniae* isolates.

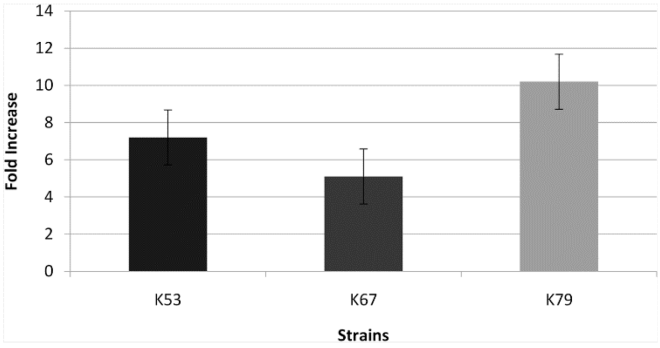


Figure 3. Levels of *acrR* gene in tigecycline-resistant *K. pneumoniae* isolates.

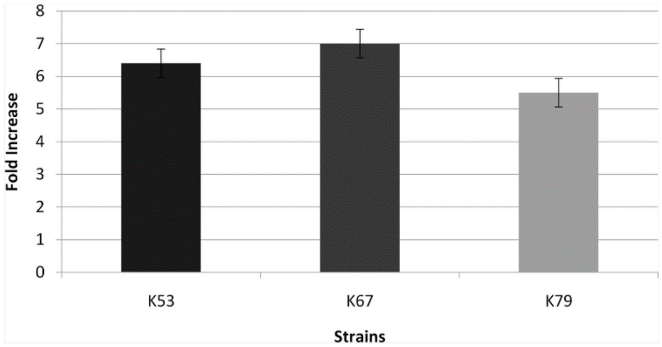


Figure 4. Levels of *tolC* gene in tigecycline-resistant *K. pneumoniae* isolates.

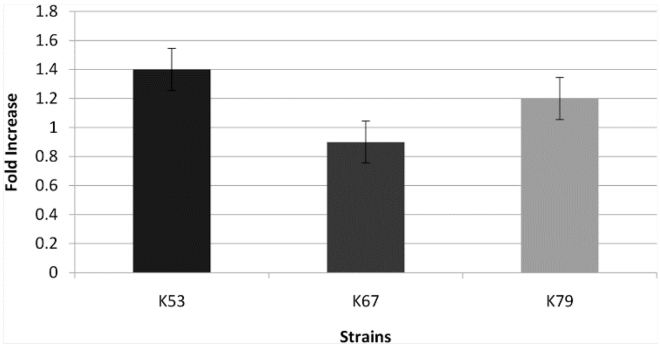


Figure 5. Levels of *oqxA* gene in tigecycline-resistant *K. pneumoniae* isolates.

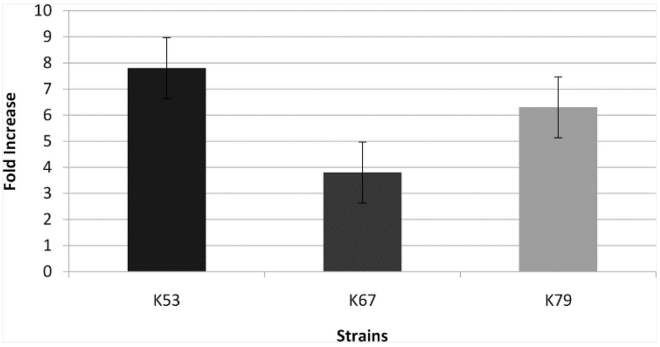


Figure 6. Levels of *oqxB* gene in tigecycline-resistant *K. pneumoniae* isolates.

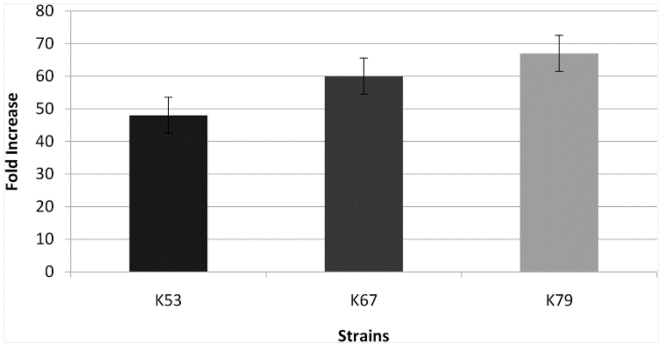


Figure 7. Levels of *rarA* gene in tigecycline-resistant *K. pneumoniae* isolates.

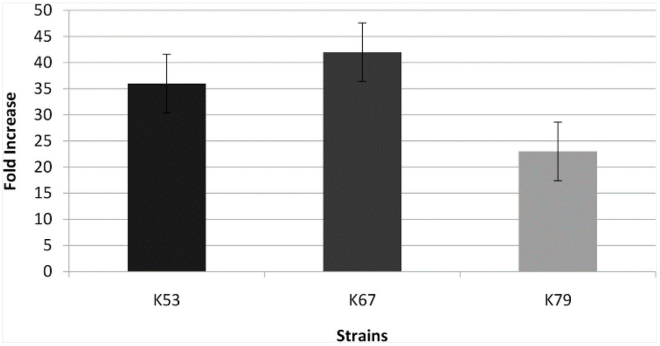


Figure 8. Levels of *marA* gene in tigecycline-resistant *K. pneumoniae* isolates.

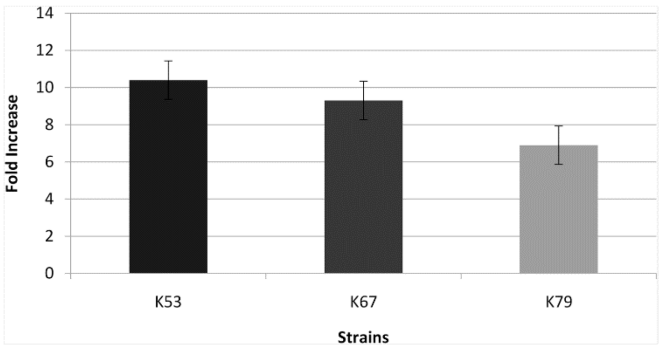


Figure 9. Levels of *soxS* gene in tigecycline-resistant *K. pneumoniae* isolates.

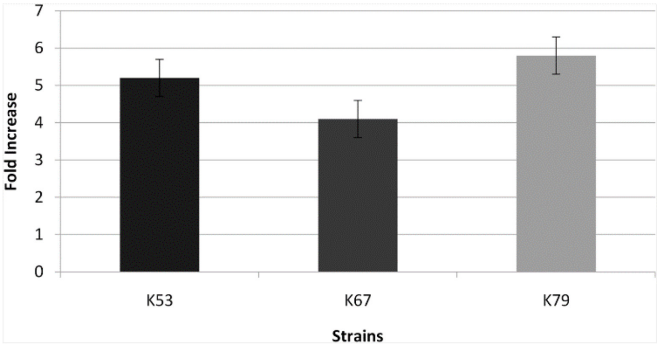
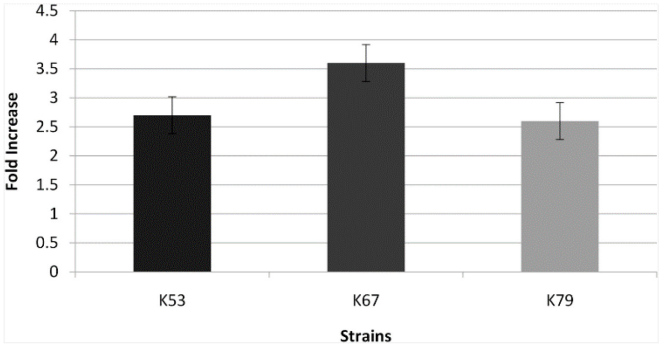


Figure 10. Levels of *rob* gene in tigecycline-resistant *K. pneumoniae* isolates.



In our study, CCCP restored the susceptibility of three strains (K53, K67, K79), indirectly demonstrating that efflux pump overexpression contributed to tigecycline resistance. However, no significant reduction in tigecycline MIC was observed in the other strain (K32), indicating that CCCP was not effective. One possible explanation is that EPIs have different specializations for different efflux pumps and intervene in different ways. It is possible that other EPIs such as PAβN, NMP, reserpine or verapamil could restore the sensitivity of these strains. In addition, results on efflux pump activity (using CCCP) and expression levels of target pump genes and their regulators (isolates K53, K67, K79) indicated that efflux pump overexpression may also mediate resistance to tigecycline by mechanisms other than efflux pump overexpression. Resistance to tigecycline is partly due to active AcrAB-TolC efflux systems [23-25]. Furthermore, the *AcrAB-TolC* pump is activated by various transcriptional regulators such as *ramA*, *marA*, *soxS*, *rob* or *acrR*. These pump regulators play a role in promoting resistance to tigecycline by regulating the *AcrAB-TolC* efflux pump [26-28]. The current study shows that increased MIC of *K. pneumoniae* strains is associated with the overexpression of either *ram*A or *mar*A, demonstrating that *ram*A is not always required for tigecycline resistance and that *mar*A is also a universal activator of the *acrB* transporter (Figure 1). A similar hypothesis has been proposed, and some studies suggest that tigecycline resistance in *K. pneumoniae* must also arise through other pathways [3,23]. One of these studies showed that the recently described *OqxAB* efflux pump may contribute to tigecycline resistance in *K. pneumoniae*, although the chromosomally encoded *rar*A regulator is downstream of the *OqxAB* efflux pump [18]. In our study, the MIC of strains K53 and K79 was 16 µg/mL, and real-time quantitative PCR analyses showed that *rar*A overexpression was observed in association with increased expression of the multidrug-resistant efflux pump *oqxAB*, confirming that *rarA* may be involved in the regulation of *OqxAB* production. The results presented here support the previous report confirming that *rarA* is one of the regulatory pathways that regulate the expression of *oqxB* in *K. pneumoniae* [18,23]. However, for strains K53 and K67, although the expression level of *rarA* was higher than that of K79, the transcriptional level of *oqxB* was higher than *rarA* expression in strains K67 and K79, and the expression level of *rarA* was higher compared to the sensitive strain K32. These data suggest that regulators other than *rarA* may contribute to *oqxB* expression. In addition to the present report, a recent study reports the same results. It showed that no differences in the 5' sequence of *oqxA* were found between the two strains that differed 20-fold in the levels of *oqxB* transcripts. Therefore, we hypothesized that the increased expression of *oqxB* in the two strains does not appear to be due to mutations in a putative promoter, but may be related to differences in other, as yet undetermined regulatory elements in these strains. We assumed that this was the reason why the MIC values of one strain (K32) were lower than those of the other strains (K53, K67 and K79). AcrAB-TolC efflux pumps play an important role in strains with MIC values of 8 µg/mL (K79). In strains with an MIC of 16 µg/mL (K53, K79), both *AcrAB-TolC* and *OqxAB* efflux pumps contributed to tigecycline resistance. Based on the results of the current study, it was clear that genes related to *rar*A and *oqx*B pump were more important in selected tigecycline-resistant *K. pneumoniae* strains. This result was particularly significant in light of previous observations which showed that high levels of resistance to tigecycline in *K. pneumonia* were due to alternative pathways [26]. Regardless of the nature of the pump at work in these strains, we have clearly shown that the efflux pump plays a key role in tigecycline resistance in *K. pneumoniae*. However, the mechanisms responsible for the high *oqxB* expression leading to tigecycline resistance remain unknown and require further investigation. In the present study, MLST was performed for molecular typing. ST11 was an epidemic *K. pneumoniae* clone producing OXA-48 and/or NDM-1 in Iran, and ST893 was the dominant clone in Eastern countries [29].

There were several limitations in the present study, including the small number of *K. pneumoniae* isolates from hospitals that were resistant to tigecycline. However, in the present study, we found differential gene expression of efflux pumps and their regulators in *K. pneumoniae* from ICU and burn patients. In clinical isolates of tigecycline-resistant *K. pneumoniae*, *RamA* plays an important role in the regulated expression of the AcrAB efflux pump, contributing to the reduced sensitivity to tigecycline.

The prevalence of tigecycline-resistant *K. pneumoniae*, particularly the OXA-48-producing clone ST11, has reached extremely serious proportions in Iran in recent years. It is important to identify the internal factors causing the rapid spread of ST11 and to focus on the control and surveillance of *Klebsiella pneumoniae* infections [29,30].

Conclusions

In conclusion, the current study cites the mechanism of tigecycline-resistant *K. pneumoniae* in Iran. Molecular typing results showed that the predominant clone of *K. pneumoniae* strains was ST11. In addition, two new STs, namely ST1414 and ST1415, were identified. Efflux-mediated mechanisms, including high expression of the *AcrAB-TolC* and *OqxAB* efflux pumps, appear to play a key role in tigecycline resistance, while regulators of the *acrR*, *marA*, *soxS*, *rarA*, *rob* and *ramA* pumps individually all contribute to the overexpression of the *AcrAB-TolC* and *OqxAB*efflux pumps. In addition, the *rarA* pump genes and *oqxB* are more important in selected *K. pneumonia* strains with high resistance to tigecycline. Efflux pump inhibitors (EPI)-CCCP were able to reverse the resistance pattern in the majority of *K. pneumoniae* strains. Although tigecycline is a promising antibiotic for the treatment of multidrug-resistant *K. pneumoniae* infections, the emergence of resistance to tigecycline is of great concern.

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