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Tetracycline resistance mediated by *tet* efflux pumps in clinical isolates of *Acinetobacter baumannii*

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ABSTRACT

Acinetobacter baumannii is one of the most frequent nosocomial pathogen capable of acquiring resistance to different antimicrobials. The aim of this study was to investigate the activity of tetracycline, doxycycline and minocycline, the prevalence of *tet(A)* and *tet(B)* determinants, and the role of efflux pump in tetracycline resistance among the *A. baumannii* clinical isolates. Susceptibility of 98 *A. baumannii* isolates to tetracyclines was evaluated by disk diffusion method. The presence of active efflux pump was investigated by determination of the minimum inhibitory concentration (MIC) of tetracycline using the carbonyl cyanide 3-chlorophenylhydrazone (CCCP). Polymerase chain reaction (PCR) was performed to investigate the presence of *tet(A)* and *tet(B)* determinants in tetracycline-resistant isolates. The rate of resistance to tetracycline, doxycycline and minocycline was 47.95%, 0%, and 30.61%, respectively. Among the 47 tetracycline-resistant isolates, 29.79% were originated from burned patients and showed MIC ranging from 128-256 µg/mL with both MIC_{50} and MIC_{90} values of 256 µg/mL, while 70.21% were from ventilator-associated pneumonia (VAP) patients and had MIC values ranging from 32-1024 µg/mL, with MIC_{50} and MIC_{90} of 512 µg/mL and 1024 µg/mL, respectively. The *tet(B)* gene was found in 61.7% of tetracycline-resistant isolates, while none of the isolates carried the *tet(A)* gene. CCCP led to 2-128-fold reduction in tetracycline MIC of the tested isolates. The results showed that doxycycline and minocycline are promising agents for the treatment of *A. baumannii* infections. This study has also revealed the role of efflux activity in the resistance to tetracycline of *A. baumannii* isolates. The emergence of resistance to these agents is likely due to the spread of clones presenting with a higher prevalence of resistance determinants.

KEYWORDS: *Acinetobacter baumannii*. Efflux pump. Tet(A), Tet(B). Tetracycline resistance.

INTRODUCTION

Acinetobacter baumannii is an opportunistic microorganism that has emerged as one of the most troublesome pathogens worldwide[1,2]. This pathogen is responsible for severe infections, such as ventilator-associated pneumonia (VAP), bloodstream, urinary tract, and wound infections[3,4]. VAP is the most frequent ICU-acquired infection, occurring in 9 to 24% of patients intubated for longer than 48 h[5]. Even more worrisome, the ability of this microorganism to acquire resistance to multiple antimicrobial agents makes it difficult to treat under certain clinical conditions, especially in critically ill patients. Thus, there are currently only a few antibiotics expected to be effective against the severe forms of *A. baumannii* infections leading

to the increment of mortality rates, as well as health care costs[3].

Due to the continued emergence and spread of *A. baumannii*-resistant strains and a few number of therapeutic options, other antibiotics have been analyzed for use in clinical practice. Tetracyclines, including minocycline and doxycycline, have shown promising clinical and microbiological effectiveness for treating *A. baumannii* infections. The successful use oftetracyclines in combination with other antibiotics has been reported in 71.9% of respiratory infections and 87.5% of blood stream infections[6].

The efflux system, found in many bacterial genera, is responsible for reducing the antibiotic accumulation and is known as an potent mechanism of drug resistance[7,8]. Efflux pump-encoding genes are carried either by genetic elements, e.g., TetA and CmlA systems in resistance to tetracycline and chloramphenicol, respectively, or by a chromosome, being therefore responsible for acquired or intrinsic resistances when they are overexpressed[9]. Similar to other Gram-negative bacteria, the efflux pump is a main mechanism involved in resistance to tetracycline in *A. baumannii*[8]. The most common tetracycline-specific efflux pumps are members of the major transporters facilitator superfamily (MFS)[10]. These systems expel tetracycline molecules from the inside of cells at the expense of a proton. Several Tet efflux pumps from the MFS superfamily leading to resistance to tetracycline have been acquired by clinical isolates of *A. baumannii*[11]. Tet(A) and Tet(B) are the most prevalent, with Tet(A) efflux conferring resistance to tetracycline but not to minocycline or doxycycline and Tet(B) conferring resistance to tetracycline and minocycline but not to tigecycline[12,13]. It has also been found that Tet(A) acts sinergically with the resistance–nodulation–division (RND) superfamily of efflux pumps, such as AdeABC and AdeIJK, serving as an important resistance mechanism of resistance to tigecycline in *A. baumannii*[14]. The *tet(B)* gene was found in at least 50% of tetracycline-resistant *A. baumannii* isolates and *tet(A)* in 14-46%[15,16]. The genetic basis of these determinants remains largely unknown. A partially characterized Tn1721-like transposon containing the *tet(R)* and *tet(A)* genes, encoding, a regulatory protein and a resistance protein[15], respectively, and *tet(B)* is carried by 5 to 9-kb plasmids in the multidrug resistant of *A. baumannii*[17].

In the present study, we investigated (i) the activity of tetracycline, doxycycline and minocycline, (ii) the prevalence of *tet(A)* and *tet(B)* efflux determinants, and (iii) the presence of efflux activity among clinical isolates of *A. baumannii*.

MATERIALS AND METHODS

Ethics statement

The present study was approved by the Ethics Committee of the Iran University of Medical Sciences, protocol Nº IR.IUMS.REC 1395.9221133207. The included patients did not directly participate in this study. All experiments were performed on bacteria isolated from clinical specimens of hospitalized patients.

Bacterial identification

This cross-sectional study was performed on *A. baumannii* isolates from patients admitted at the burn unit and intensive care unit (ICU) of two hospitals, Shahid Motahari and Rasoul Akram, in Tehran, Iran, from 2016 to 2017. Each isolate was recovered from a particular patient, and only one sample was taken from each patient. The standard microbiological and biochemical tests, such as Gram staining, oxidase and catalase tests, triple sugar iron (TSI) agar culture, SIM (sulfide, indole, motility) agar culture, and oxidation-fermentation (OF) media culture (Merck, Darmstadt, Germany) were grown at 44 ºC, used in the initial identification of isolates[18], and then, submitted to PCR-sequencing of *bla*OXA-51-like gene to confirm the isolates as *A. baumannii*[4]. This sequencing was deposited in GenBank data library with the accession number MG920243. All isolates were stored in Luria-Bertani broth (Merck Co., Darmstadt, Germany) containing 20% glycerol at -70 °C for further use. *A. baumannii* ATCC® 19606 (American Type Culture Collection, VA, USA) was used as the quality control.

Antimicrobial susceptibility testing

Susceptibility of *A. baumannii* isolates to three tetracyclines (Mast, Merseyside, UK), including tetracycline (30 µg), doxycycline (30 µg) and minocycline (30 µg) was evaluated by the Kirby-Bauer disk diffusion method according to the Clinical and Laboratory Standard Institute recommendations[10,19]. *Pseudomonas aeruginosa* ATCC® 27853 (American Type Culture Collection, VA, USA) was used as the control.

Detection of active efflux phenotypes

The presence of active efflux systems was investigated in tetracycline-resistant isolates. The minimal inhibitory concentration (MIC) of tetracycline was determined by the agar dilution method before and after treatment of the efflux pump inhibitor with carbonylcyanide

3-chlorophenylhydrazone (CCCP) (Sigma-Aldrich, Dorset, United Kingdom)[20,21]. The addition of CCCP to Mueller-Hinton (M-H) agar plates led to increased intracellular concentration of the antibiotic, reducing the MIC in isolates having any active efflux pumps. Briefly, 50 µg/mL of CCCP were added to each M-H agar plates containing 0.5 to 1024 µg/mL of tetracycline. Then, the MIC of tetracycline was determined for all tetracycline-resistant isolates against the *A. baumannii* ATCC 19606® (American Type Culture Collection, VA, USA). M-H agar plates with CCCP without antibiotic were used as controls. The effect of the efflux pump inhibitor was determined by the detection of a 4-fold or higher increment in the susceptibility after treatment with CCCP[21].

Polymerase chain reaction (PCR)

Genomic DNA was extracted by the boiling method. One to three colonies were dissolved in 500 µL of sterile distilled water in microtubes, and then placed in a boiling water bath at 100 °C for 10 min, and immediately centrifuged at 12000×g for 5 min. The supernatant containing total DNA was stored at -80 ºC until further used. The presence of Tet efflux genes, *tet(A)* and *tet(B)*, was examined in tetracycline-resistant *A. baumannii* isolates using the following specific primers16. F, 5'-GCGCGATCTGGTTCACTCG-3' and R, 5'-AGTCGACAGYRGCGCCGGC-3' for *tet(A)*, and F, 5'-TACGTGAATTTATTGCTTCGG-3' and R, 5'-ATACAGCATCCAAAGCGCAC-3' for *tet(B)*. PCR amplification was performed in a total volume of 25 µL containing 12.5 µL of 2×Taq Master Mix RED (Ampliqon, Odense, Denmark) (150 mM Tris-HCl [pH 8.5], 40 mM $(NH_4)_2SO_4$, 4 mM $MgCl_2$, 0.2% Tween® 20, 0.4 mM of each dNTP), 0.05 units/µL Ampliqon Taq DNA polymerase, 1 µL of 10 pmol of each primer, 1 µL (20 ng) of DNA template, and 9.5 µL of sterile distilled water. The thermal cycling protocol for PCR of both *tet(A)* and *tet(B)* genes was as initial denaturation at 94 ºC for 5 min, followed by 30 cycles of denaturation at 94 ºC for 1 min, annealing at 57 ºC for 1 min, and extension at 72 ºC for 1 min, with a final extension at 72 ºC for 5 min. The PCR products were electrophoresed using 1.5% agarose gel with ethidium bromide and photographed under UV light.

Statistical data analysis

All obtained data were recorded, edited and entered using the SPSS statistic software package, version 18.0 (SPSS Inc., Chicago, IL, USA). The differences between categorical variables, including clinical specimen types, antibiotic resistance pattern, and distribution of *tet* genes were compared by the χ^2 (chi-square) test. A *P*-value <0.05 was considered as statistically significant.

RESULTS

Bacterial isolates and susceptibility testing

A total of 98 isolates of *A. baumannii* were obtained from patients, of which 66.36% (65/98) were from wounds and 33.67% (33/98) from respiratory tract infections of burned VAP patients, respectively. Doxycycline was the most active antibiotic tested, followed by minocycline

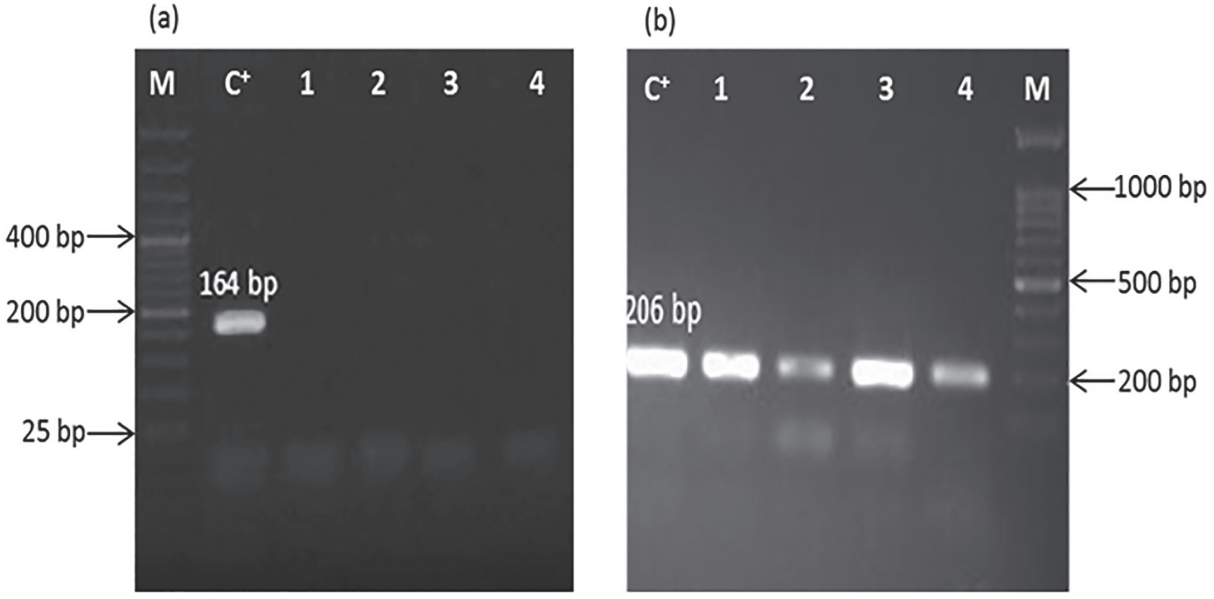

Figure 1 - PCR amplification of *tet(A)* (a) and *tet(B)* (b) genes in selected isolates of *A. baumannii*. M = Molecular weight marker Lane; C+ = Positive control, Lanes 1-4: PCR products of the corresponding gene.

and tetracycline, with susceptibility rates of 96.93% (95/98), 71.57% (68/98), and 43.87% (43/98), respectively (Table 1). Among the 47 tetracycline-resistant isolates, 29.79% (14/47) were from burned patients and showed MIC values ranging from 128-256 µg/mL with both, MIC_{50} and MIC_{90} of 256 µg/mL, while 70.21% (33/47) were from VAP patients and had MIC ranges ranging from 32-1024 µg/mL, with MIC_{50} and MIC_{90} of 512 µg/mL and 1024 µg/mL, respectively. Except for doxycycline, resistance rates for both, tetracycline and minocycline, were significantly higher in VAP isolates than in isolates from burns (100% and 87.87% versus 21.53% and 1.53% of resistance against tetracycline and minocycline, respectively) (p<0.0001) (Table 1).

Active efflux phenotypes

The MIC of tetracycline in *A. baumannii* tetracycline-resistant isolates was tested in the presence of CCCP. In the absence of CCCP, MICs ranged from 128-256 µg/mL and 32-1024 µg/mL, respectively, in isolates from burns and VAP (Table 2). In the presence of CCCP, all tested isolates sshowed tetracycline MIC ranging from 2-128 µg/mL, with a 2-128-fold reduction compared to when CCCP was not added. Furthermore, all bacteria grew well in M-H agar plates with CCCP but without tetracycline (as control), indicating that 50 µg/mL CCCP had no intrinsic antibacterial activity.

Detection of *tet* efflux genes

PCR results were positive for the *tet(B)* gene in 61.7% (29/47) of tetracycline-resistant isolates, while none of the isolates carried the *tet(A)* gene. In addition, 38.3% of *A. baumannii* tested isolates (18/47) had no genes studied. The prevalence of *tet(B)* gene in burned and VAP isolates was 7.14% (1/14) and 84.84% (28/33), respectively (p<0.0001).

DISCUSSION

Due to the increased rate of *A. baumannii* resistance to most antimicrobial agents, evaluating the antimicrobial susceptibility of "old" antibiotics that are not used as first line drugs in clinical practice is of interest. This study showed adequate *in vitro* activity of doxycycline and minocycline in burned patients and only of doxycycline in VAP, showing promising clinical and microbiological effectiveness of tetracyclines either as monotherapy or in combination with other agents for the treatment of *A. baumannii* infections. Similarly, previous epidemiological studies reported high susceptibility rates of *A. baumannii* to doxycycline

Table 1 - Susceptibility pattern of 98 *A. baumannii* isolates against three tetracyclines.

Phenotype	Burned patients isolates (n= 65)			VAP patients isolates (n= 33)		
	No. (%) of susceptibility patterns to			No. (%) of susceptibility patterns to		
	Tetracycline	Doxycycline	Minocycline	Tetracycline	Doxycycline	Minocycline
Susceptible	43 (66.15)	64 (98.46)	64 (98.46)	0	31 (93.93)	4 (12.12)
Intermediate	8 (12.3)	1 (1.53)	0	0	2 (6.06)	0
Resistant	14 (21.53)	0	1 (1.53)	33 (100)	0	29 (87.87)

VAP = Ventilator-associated pneumonia.

Table 2 - Distribution of the tetracycline MICs in 47 tetracycline-resistant *A. baumannii* before and after treatment with CCCP.

Burn isolates (n= 14)				VAP isolates (n= 33)			
No. (%) of isolate	MIC range (µg/mL) without CCCP	MIC range (µg/mL) with CCCP	MIC fold reduction in the presence of CCCP	No. (%) of isolate	MIC range (µg/mL) without CCCP	MIC range (µg/mL) with CCCP	MIC fold reduction in the presence of CCCP
8 (57.14%)	128-256	32-128	2-4	10 (30.3%)	32-1024	8-512	2-4
4 (28.57%)	256	32	8	22 (66.67%)	512	64	8
1 (7.14%)	128	4	32	1 (3.03%)	256	16	16
1 (7.14%)	128	1	128	NA	NA	NA	NA

MIC = Minimum inhibitory concentration; VAP = Ventilator-associated pneumonia; CCCP = carbonyl cyanide 3-chlorophenylhydrazone; NA = Not applicable.

(100%)[22] and minocycline (56%-94.3%)[23,24]. In contrast, tetracycline exhibited no optimal antibacterial activity for the use in clinical practice. In a study by Adibhesami *et al*.[23] the number of minocycline and doxycycline-susceptible *A. baumannii* isolates was significantly higher than the number of tetracycline-susceptibleones. Maleki *et al*.[25] have also found a resistance rate of 18% to doxycycline and 19% to minocycline against *A. baumannii* isolates, while 80% of isolates showed resistant to tetracycline. Additionally, VAP isolates showed high levels of resistance to tetracycline and high resistance rates to both, tetracycline and minocycline, in comparison with isolates from burned patients. The majority of VAP isolates carried the *tet(B)* gene in comparison with isolates from burned patients. Although the clinical outcomes of patients participating in the present study were not assessed, VAP infections due to *A. baumannii* have been associated with a high mortality rate, prolonged stay in the intensive care unit, and the rapid development of antimicrobial resistance to commonly used antimicrobials[26], indicating the increased risk of more serious infections in critically ill patients.

In addition, our study described *A. baumannii* isolates presenting with the efflux pump phenotype in tetracycline-resistant isolates recovered from burned and VAP patients. The results of the present study showed that the MIC for 43 of 47 tetracycline-resistant isolates (91.48%) was significantly reduced, by 4-16-fold in the presence of the efflux pump inhibitor. Similarly, Ardehali *et al*.[27] found that CCCP reduced considerably the MIC of 51.25% of tigecycline-resistant *A. baumannii* isolates by 2-4-fold. The MICs of isolates resistant to minocycline, doxycycline, and tetracycline in the presence of different efflux pump inhibitors, such as CCCP, phenyl-arginine-β-naphthylamide, 1-(1-naphthylmethyl)-piperazine, reserpine, and verapamil were significantly reduced[28]. Consistent with these findings, our results revealed that active efflux pumps could be involved in the increased rate of resistance to tetracycline in *A. baumannii*.

tet(A) and *tet(B)* determinants conferring efflux phenotypes of resistance to tetracycline have been known in *A. baumannii* isolates[12]. The prevalence of *tet(B)* and *tet(A)* genes have been reported in at least 50% and 14%-46% of tetracycline-resistant *A. baumannii* isolates[9]. Our study showed a high prevalence (61.7%) of *tet(B)* but not of *tet(A)* gene in tetracycline-resistant isolates. Similarly, in two independenpt studies from Iran, Meshkat *et al*.[28] and Mosavat *et al*.[29] detected the *tet(B)* determinant in a significant percentage of *A. baumannii* isolates (100% and 95%, respectively); interestingly, *tet(A)* was not found in any of the isolates. These results were surprisingly different from those reported by other studies[8,25,30]. Importantly, the fact that there was neither *tet(B)* nor *tet(A)* in some isolates in this study indicates that additional genetic determinants other than *tet* genes may play a role in the expression of resistance to tetracycline in some of these strains[27,31-33].

In conclusion, doxycycline presented with a good activity and minocycline with a moderate activity, being promising drugs for the effective treatment of *A. baumannii* infections. In addition, this study revealed that resistance to tetracyclines in the studied isolates is mediated by active efflux pumps. Since tetracyclines are not routinely used to treat *Acinetobacter* infections, the presence of tetracycline-resistant strains is likely due to the spread of clones presenting with a higher prevalence of resistance determinants. Hygienic surveillance programs and stringent infection control strategies are needed to prevent further dissemination.

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CONFLICT OF INTERESTS

None to declare.

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