

Comparison of Disk Diffusion and E-Test with the Reference Method of Microbroth Dilution for Susceptibility Testing of *Acinetobacter baumannii* Isolates to Tetracyclines

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ABSTRACT

Background and Objective: Due to the continuous increase of multidrug-resistant *Acinetobacter baumannii* strains around the world, decision making for an effective treatment of infections caused by this organism depends on the results of antimicrobial susceptibility tests. In this study, the validity of disk diffusion and E-test methods was assessed by their comparison with the reference method of microbroth dilution for three antibiotics of tetracycline, doxycycline and minocycline.

Methods: Total of 68 *A. baumannii* isolates were obtained from patients hospitalized in the burn center of Shahid Motehari Hospital in Tehran, Iran. Susceptibility of the *Acinetobacter* isolates was evaluated using the disk diffusion, E-test and microbroth dilution methods, according to the guidelines of Clinical and Laboratory Standards Institute.

Results: Among the isolates, 82.3% were tetracycline-resistant (with minimum inhibitory concentration 50 (MIC₅₀) and MIC₉₀ of 32 and more than 32 µg/ml, respectively and 41.2% were doxycycline-resistant (with MIC₅₀ and MIC₉₀ of 4 and more than 32 µg/ml, respectively). Minocycline, with resistance of up to 13.3% (MIC₅₀ and MIC₉₀ of 1 and 8 µg/ml, respectively) showed the highest antimicrobial activity against the *A. baumannii* isolates. Antimicrobial susceptibility of bacteria was different depending on the type of methods used. No very major error was observed in any of the methods of susceptibility testing. Overall, the level of major and minor errors in the E-test was lower than the disk diffusion method.

Conclusion: The results of this study indicate that minocycline has notably high antimicrobial activity against *A. baumannii* compared to other antibiotics of the tetracycline group.

Keywords: *Acinetobacter baumannii*; Tetracyclines; Minimum inhibitory concentration.

INTRODUCTION

Acinetobacter baumannii (*A. baumannii*) is an obligate aerobic, gram-negative coccobacillus which has recently become an important nosocomial pathogen, especially in intensive care units (ICUs) and burn centers (1,2,3). Most infections caused by this organism occur in people with underlying diseases and include pneumonia, urinary tract infection, burn wound infection, meningitis, endocarditis, and septicemia (4,5,6). Regarding the clinical importance, *A. baumannii* is considered the second most common non-fermentative bacteria associated with nosocomial infections. It is also responsible for 10% to 43% of mortalities in hospitalized patients in ICU (7).

Tetracyclines are bacteriostatic agents that prevent protein synthesis by inhibiting the binding of aminoacyl-tRNA complex to the ribosome binding site. Although the use of tetracyclines for the treatment of human infections has decreased due to emergence of bacterial resistance, this group of antibiotics, particularly doxycycline and minocycline are still useful antimicrobials against many bacterial infections, including those caused by the *Acinetobacter* species (8,9).

One of the notable features of *A. baumannii* is its intrinsic or acquired resistance to various antibiotics, including aminopenicillins, cephalosporins, cephamycins, aminoglycosides, tetracyclines and fluoroquinolones. Until recently, the carbapenems (imipenem) was considered as highly active antibacterial agent against *A. baumannii* infections. However, nowadays various reports of increased number of carbapenem resistant strains are found worldwide (10). Since the fact that multi-drug resistance among *A. baumannii* strains is continually increasing, the final decision for effective treatment of infections caused by this bacterium in hospitalized patients depends on the results of susceptibility tests (11,12).

The aim of this study was to compare disk agar diffusion and E-test methods with the microbroth dilution reference method for susceptibility testing of *A. baumannii* isolates to three tetracyclines, including tetracycline, doxycycline, and minocycline.

MATERIAL AND METHODS

A total of 68 *A. baumannii* isolates were recovered from burn patients hospitalized in Shahid Motahari Medical and Rehabilitation Center in Tehran, Iran. Bacterial isolates were obtained from patients aged from 2 to 75 years with burn severity of 7% to 92%, who were hospitalized in the medical center for at least one week. Burn wound exudate samples were collected and then transported to the laboratory for microbiological examinations. Initial identification of *A. baumannii* isolates was done by traditional bacteriological and biochemical tests such as, Gram staining, catalase, oxidase, motility, Oxidation-Fermentation (OF), and growth at temperatures of 37 and 44 °C (13).

Polymerase chain reaction (PCR) assay was used as a species confirmatory test to detect the carbapenemase *bla*_{Oxa-51}-like gene, which is intrinsically present in all *A. baumannii* strains (14). Briefly, bacterial genomic DNA was extracted using DNA Purification Kit (Fermentase, Germany). Spectrophotometry was used to evaluate the quality of the DNA by measuring the concentration and relative absorbance of A₂₆₀/A₂₈₀ for each isolate. DNA in each microtube was kept at -80 °C until the PCR assay. The specific primer pairs used for gene amplification are 5'-TAATGCTTTGATCGGCCTTG-3' and 5'-TGGATTGCACTTCATCTTGG-3'. PCR was done according to the following procedure using the Mastercycler gradient (Eppendorf, Germany): Initial denaturation at 94 °C for 5 minutes and then 30 cycles of denaturation at 94 °C for 45 seconds, annealing at 58 °C for 1 minute, extension at 72 °C for 1 minute and final extension at 72 °C for 5 minutes. PCR products were examined for the presence of DNA bands using a UV transilluminator, after electrophoresis on ethidium bromide-containing 1.5% agarose gel.

Susceptibility of all *A. baumannii* isolates to tetracycline, doxycycline, and minocycline was evaluated using disk agar diffusion (30 µg for each antibiotics) (Mast Diagnostics, Merseyside, UK), microbroth dilution (Becton Dickinson, Sparks, MD, USA) and E-test

methods (AB Biodis Solna, Sweden Merseyside, UK), according to the guidelines of Clinical & Laboratory Standards Institute (CLSI) (15,16). Standard strains of *Escherichia coli* (ATCC 25922) and *Pseudomonas aeruginosa* (ATCC 27852) were used as quality controls. Serial dilutions of antibiotics were prepared according to the manufacturer's instruction in the microbroth dilution method: 0.25-32 µg/ml for tetracycline and minocycline, and 0.125-32 µg/ml for doxycycline. Interpretive standards related to MIC of all three antibiotics in two microbroth dilution and E-test methods were as follows: susceptible; MIC ≤ 4 µg/ml, intermediate; MIC = 8 µg/ml, and resistant; MIC ≥ 16 µg/ml. The results of antimicrobial susceptibility were evaluated and recorded after 20 hours of incubation at 35 °C,

according to the CLSI guidelines. Errors related to determination of the antimicrobial susceptibility were categorized as follows: very major error; if the results of the reference microbroth dilution method were resistant, but the results of disk diffusion and E-test were susceptible (false susceptible). Major error; if the results of reference method were susceptible and the results of disk diffusion and E-test were resistant (false resistant). Minor error; if the results of the reference method were susceptible or resistant, but the results of the other two tests were recorded as intermediate.

Data were entered in the SPSS software (Version 11.5; SPSS, Inc., Chicago, IL, USA) and Chi-square test was used to analyze the data. P-value of ≤ 0.05 was considered as statistically significant.

Table 1-MIC distribution of three tetracyclines against 68 *A. baumannii* isolates

Antibiotic	MIC range (µg/mL)	No. (%) of isolates with determined MIC (µg/mL)										MIC ₅₀ (µg/mL)	MIC ₉₀ (µg/mL)
		0.125	0.25	0.5	1	2	4	8	16	32	>32		
Tetracycline	1- ≥32	-	-	-	1 (1.4)	2 (2.9)	5 (7.3)	4 (5.8)	8 (11.7)	15 (22)	33 (48.5)	32	>32
Doxycycline	0.125- ≥32	5 (7.3)	8 (11.7)	6 (8.8)	10 (14.7)	4 (5.8)	2 (2.9)	5 (7.3)	9 (13.2)	9 (13.2)	10 (14.7)	4	>32
Minocycline	0.25- 32	-	6 (8.8)	12 (17.6)	22 (32.3)	12 (17.6)	7 (10.3)	6 (8.8)	2 (2.9)	1 (1.4)	-	1	8

Abbreviations: MIC, Minimum inhibitory concentration.

Table 2- Susceptibility patterns of *A. baumannii* isolates to tetracyclines based on three methods

Antibiotic	No. (%) of isolates		
	Susceptible	Intermediate	Resistant
Tetracycline			
DAD	2 (2.9)	12 (17.6)	54 (79.4)
ET	4 (5.9)	9 (13.2)	55 (80.9)
MBD	8 (11.8)	4 (5.9)	56 (82.3)
Doxycycline			
DAD	27 (39.7)	12 (17.6)	29 (42.6)
ET	30 (44.1)	14 (58.8)	24 (35.3)
MBD	35 (51.5)	5 (7.3)	28 (41.2)
Minocycline			
DAD	23 (33.8)	40 (58.8)	5 (7.3)
ET	47 (69.1)	17 (25)	4 (5.9)
MBD	59 (86.7)	6 (8.8)	3 (4.4)

Abbreviations: ET, E-test; DAD, Disk agar diffusion; BMD, Microbroth dilution.

Table 3- Rates of susceptibility testing errors among 68 *A. baumannii* isolates by antimicrobial agent and testing method

Antibiotic and testing method	% susceptible isolates	% ^a		
		Very major error ^b	Major error ^c	Minor error ^d
Tetracycline				
DAD	2.9	0	11.7	1.4
ET	5.9	0	8.8	1.4
MBD	11.8			
Doxycycline				
DAD	39.7	0	5.9	17.6
ET	44.1	0	4.4	10.3
MBD	51.5			
Minocycline				
DAD	33.8	0	2.9	50
ET	69.1	0	1.4	16.2
MBD	86.7			

Abbreviations: ET, E-test; DAD, Disk agar diffusion; BMD, Microbroth dilution.

^a Unacceptable levels are shown in bold. According to CLSI, they are $\geq 1.5\%$, $\geq 3\%$, and $\geq 10\%$ for very major error, major error, and minor error as, respectively.

^b Very major error, susceptible by DAD or ET when resistant by MBD.

^c Major error, resistant by DAD or ET when susceptible by MBD.

^d Minor error, intermediate by DAD or ET when susceptible or resistant by MBD or susceptible or resistant by DAD or ET when intermediate by MBD.

RESULTS

All 68 bacterial isolates that were identified by routine microbiological and biochemical methods harbored the *bla*_{Oxa-51}-like gene, confirming the *A. baumannii* species. *bla*_{Oxa-51}-like gene amplification in the clinical isolates and *A. baumannii* standard strain (positive control) produced a 353 bp band, while no amplification was observed in *A. lwoffii* strain (negative control).

Among the studied antibiotics, minocycline had the highest antimicrobial activity based on the findings of microbroth dilution method (86.7% of isolates were susceptible with MIC₅₀ and MIC₉₀ values of 1 and 8 µg/ml, respectively). Also, 82.3% and 41.2% of the isolates showed full resistance (MIC > 16µg/ml) to tetracycline and doxycycline, respectively. Moreover, bacterial isolates had MIC₅₀ and MIC₉₀ of 32 and > 32 µg/ml versus 4 and > 32 µg/ml to tetracycline and doxycycline, respectively (Tables 1 and 2).

The accuracy of the disk diffusion and E-test methods was evaluated by comparison of results of 68 *Acinetobacter* isolates tested by these methods to the reference microbroth dilution (Table 3). Susceptibility of isolates to minocycline was varied based on the methods used, ranging from 33.8% by disk diffusion to 86.7% by the microbroth dilution method. There was no very major error in any methods of susceptibility testing. The E-test showed fewer major errors for all three antibiotics (8.8%, 4.4% and 4.1% major errors for

tetracycline, doxycycline and minocycline, respectively), when compared with the disk diffusion method. In addition, minor error was equivalent for tetracycline in E-test and disk diffusion methods (1.4%); while it was fewer for minocycline and doxycycline in the E-test (10.3% and 16.2%, respectively) (Table 3).

In the present study, the level of major error for minocycline in the disk diffusion and E-test methods were acceptable; whereas it was unacceptable for tetracycline and doxycycline. In contrast to our study, Swenson *et al.* reported high level of minor errors (32.1%) by disk diffusion for tetracycline. However, the major errors were the same and, of course, unacceptable according to the CLSI in both studies.

DISCUSSION

Currently, *A. baumannii*, like other members of the "ESCAPE" group (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and *Enterobacter* species) has become a serious challenge in controlling and treatment of infections (17,18). This forces physicians and infectious specialists to use a broad-spectrum antibiotics for treatment, which in turn provides selective pressure allowing the organism to acquire various resistance genes (19,20). Therefore, control and treatment of infections caused by this organism is clearly dependent on laboratory tests that identify or

evaluate antimicrobial resistance. Several methods are used in clinical microbiology laboratories to evaluate the antimicrobial susceptibility of microorganisms. In the present study, the accuracy of disk diffusion and E-test results for three tetracyclines was assessed by comparing to the reference microbroth dilution method. Overall, the accuracy of disk diffusion test for determining minocycline susceptibility was low; so that 52.9% of the isolates were incorrectly considered as nonsusceptible (50% and 2.9% as intermediate- and full resistant, respectively). This likely occurred owing to clustering of the inhibition zone diameter at or near the susceptibility breakpoint for minocycline in many *A. baumannii* strains. By the E-test, 17.6% of isolates were incorrectly reported as minocycline-nonsusceptible (1.4% and 16.2% as intermediate- and full-resistant, respectively), a significant difference and substantial improvement in accurate results over the disk diffusion. Findings obtained from our study is consistent with those of Akers *et al.* in the United States (21), in which 51% versus 18% of *A. baumannii* isolates were wrongly reported as minocycline nonsusceptible by disk diffusion and E-test methods, respectively. In agreement with others (21), our study indicates that minocycline has adequate antimicrobial activity against *A. baumannii*.

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Similarly, in the Maleki *et al.*'s study (22), only 11.8% of isolates were susceptible to tetracycline and high level of resistance was reported to this antibiotic (MIC₅₀ and MIC₉₀ of 32 and ≥ 32 $\mu\text{g/ml}$, respectively). On the other hand, only 19% of the isolates exhibited resistance to minocycline. A key point regarding the assessing the susceptibility of isolates to minocycline is the important difference in accuracy between susceptibility testing methods, as significantly high level of false resistance are observed by the disk agar diffusion. Therefore, the use of the reference microbroth dilution method is recommended to obtain the most accurate susceptibility determination in the cases of *A. baumannii* infections where minocycline is desirable.

CONCLUSION

In order to determine the trend of antimicrobial susceptibility of multidrug resistant (MDR) *A. baumannii* strains, and subsequently to choose an efficient antibiotic regimen for treatment of problematic infections, it is recommended to use the accurate susceptibility testing methods.

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CONFLICTS OF INTEREST

There are no conflicts of interest.

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