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Investigation of colistin sensitivity via three different methods in *Acinetobacter baumannii* isolates with multiple antibiotic resistance

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Summary
Background: In recent years there has been an increase in life-threatening infections caused by *Acinetobacter baumannii* with multiple antibiotic resistance, which has lead to the use of polymyxins, especially colistin, being reconsidered. The aim of this study was to investigate the colistin sensitivity of *A. baumannii* isolates with multiple antibiotic resistance via different methods, and to evaluate the disk diffusion method for colistin against multi-resistant Acinetobacter isolates, in comparison to the E-test and Phoenix system.
Methods: The study was carried out on 100 strains of *A. baumannii* (colonization or infection) isolated from the microbiological samples of different patients followed in the clinics and intensive care units of Uludağ University Medical School between the years 2004 and 2005. Strains were identified and characterized for their antibiotic sensitivity by Phoenix system (Becton Dickinson, Sparks, MD, USA).
Results: In all studied *A. baumannii* strains, susceptibility to colistin was determined to be 100% with the disk diffusion, E-test, and broth microdilution methods. Results of the E-test and broth microdilution method, which are accepted as reference methods, were found to be 100% consistent with the results of the disk diffusion tests; no very major or major error was identified upon comparison of the tests. The sensitivity and the positive predictive value of the disk diffusion method were found to be 100%.
Conclusions: Colistin resistance in *A. baumannii* was not detected in our region, and disk diffusion method results are in accordance with those of E-test and broth microdilution methods.

Introduction

Colistin, which is synthesized naturally by *Bacillus polymyxa*, is a cationic polypeptide antibiotic of the polymyxin family that is rapidly bactericidal to Gram-negative bacteria. The

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action of colistin is by a detergent-like mechanism, interfering with the structure and function of the outer cytoplasmic membrane of bacteria, resulting in bacterial death.[1,2]

Colistin was first introduced in 1952 and was used until the early 1980s for the treatment of infections caused by Gram-negative bacilli. Its systemic use was discontinued because of problems with toxicity, such as nephrotoxicity, neuromuscular blockade, and neurotoxicity.[3,4]

Problems in permeability of antibiotics due to the external membrane structure and the presence of efflux pumps in Gram-negative bacteria with multiple antibiotic resistance, the lack of novel antibiotic groups discovered in recent years, and especially the increase in the rate of life-threatening infections caused by *Acinetobacter spp* with multiple antibiotic resistance among patients treated in intensive care units (ICUs), have lead to the use of polymyxins, especially colistin, being reconsidered, and the performing of antibiotic sensitivity tests in this group.[5]

Multidrug-resistant Gram-negative bacilli, mainly *Acinetobacter baumannii*, are encountered in our hospital. The aim of this study was to investigate the sensitivity to colistin of *A. baumannii* isolates with multiple antibiotic resistance; these isolates were obtained from patients treated in the clinics and ICUs of our university hospital via different methods.

Methods

This study was carried out on 100 strains of *A. baumannii* (colonization or infection) isolated from the microbiological samples of different patients followed in the clinics and ICUs of Uludağ University Medical School between the years 2004 and 2005. Strains were identified and characterized for their antibiotic sensitivity by Phoenix system (Becton Dickinson, Sparks, MD, USA).

Antibiotic sensitivity tests were performed according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI, Wayne, PA, USA). BBL-labeled 10-μg disks were used for the disk diffusion method. For the determination of minimum inhibitory concentration (MIC) values, the E-test (AB Biodisk, Solna, Sweden) was performed in addition to the values obtained in the Phoenix system.

For the E-test method, the bacterial suspension, which was calibrated to 0.5-McFarland opacity, was cultivated onto Mueller—Hinton agar in accordance with the manufacturer's recommendations, after which the E-test colistin strip (ranging from 0.06 to 1.024 μg/ml) was positioned. MIC values were determined following 16—20 h of incubation at 35 °C. Colistin sensitivity in the Phoenix system was evaluated by the broth microdilution method (ranging from 0.5 to 2 μg/ml). MIC values that inhibited 50% and 90% of strains were accepted as MIC_{50} and MIC_{90}, respectively.

In the disk diffusion method for the evaluation of susceptibility, ≥11 mm and ≤8 mm were accepted as sensitive and resistant, respectively (NCCLS 1981).[6] In the E-test and broth microdilution methods, ≤2 mg/l and ≥4 mg/l were accepted as sensitive and resistant, respectively (CLSI 2005).[7]

Errors were ranked as follows: very major error, if the result of the reference method (E-test and broth microdilution) was resistant, while that of the disk diffusion test was sensitive (false-susceptible result) and major error, if the result of the reference method was sensitive, while that of the disk diffusion test was resistant (false-resistant result). The usefulness of the disk diffusion test method (sensitivity, specificity, and positive and negative predictive values) for the detection of colistin resistance in clinical isolates of *A. baumannii* was also evaluated. *Escherichia coli* strain ATCC 25922 was used as the control.

Results

One hundred strains of *A. baumannii* were studied, of which 17% were isolated from clinics and 83% were isolated from ICUs (pulmonary diseases, $n = 8$; hematology, $n = 2$; infectious diseases, $n = 2$; general surgery, $n = 1$; thoracic and cardiovascular surgery, $n = 1$; neurosurgery, $n = 1$; orthopedics, $n = 1$; burn, $n = 1$; internal medicine ICU, $n = 3$; general surgery ICU, $n = 13$; thoracic and cardiovascular surgery ICU, $n = 3$; neurology ICU, $n = 7$; neurosurgery ICU, $n = 9$; reanimation ICU, $n = 44$; and trauma ICU, $n = 4$). Of the isolates, 77% were isolated from deep endotracheal aspiration, 18% from blood, 3% from catheters, and 2% from wound samples. The antibiotic susceptibilities of these strains with multiple antibiotic resistance are shown in Table 1.

In all studied *A. baumannii* strains, colistin susceptibility was determined as 100% with the disk diffusion, E-test, and broth microdilution methods. The disk diffusion and E-test results are shown in Table 2.

All *A. baumannii* strains were determined as sensitive to colistin by Phoenix system (MIC_{50} and MIC_{90} values of 0.5 mg/l); the E-test method revealed colistin MIC_{50} and MIC_{90} values of 0.5 mg/l and 2 mg/l, respectively.

Results of the E-test and broth microdilution method, which are accepted as reference methods, were found to be 100% consistent with the results of the disk diffusion tests. The sensitivity and the positive predictive value of the disk diffusion method were found to be 100%.

Discussion

A. baumannii is one of the most important agents causing nosocomial infections, especially in ICUs. Treatment of these infections is difficult due to the multiple antibiotic resistance

Table 1 Antibiotic susceptibilities of *Acinetobacter baumannii* strains tested with the Phoenix system

Antibiotic	Obtained MIC value	% resistant rate ($N = 100$)
Amikacin	>32	100
Aztreonam	>16	100
Cefepime	>16	100
Ceftazidime	>16	100
Ciprofloxacin	>2	100
Gentamicin	>8	100
Imipenem	>8	100
Meropenem	>8	100
Piperacillin	>64	100
Tobramycin	>8	100
Trimethoprim/sulfamethoxazole	>2/38	100

MIC, minimum inhibitory concentration.

Table 2 Comparison of disk diffusion zone diameters and E-test MIC values for colistin susceptibility

Disk diffusion zone diameters	E-test MIC values			
	0.50	1.00	2.00	Total
11 mm	1	5	13	19
12 mm	6	17	1	24
13 mm	50	3	-	53
14 mm	4	-	-	4
Total	61	25	14	100

MIC, minimum inhibitory concentration.

of *A. baumannii* isolates.[8] Colistin usage decreased gradually after the 1980s, due to its nephrotoxic and neurotoxic side effects. However, more recently it has come back into use as an effective antibiotic, especially for the treatment of nosocomial pneumonia and cases of sepsis caused by Gram-negative bacteria with multiple antibiotic resistance.[8,9]

We did not detect colistin resistance in our study. We also found no reports of colistin resistance from other studies in our country.[10–12] However, the use of colistin for serious infections with multidrug-resistant *Acinetobacter spp* has increased and this usage may lead to colistin resistance.[13,14]

Accurate susceptibility test results are crucial for the selection of appropriate antibiotic therapy. There is, however, some controversy surrounding susceptibility tests for colistin in the microbiology laboratory. Different methods have been employed for the investigation of colistin sensitivity.

Although the disk diffusion method is one of the most frequently used techniques in microbiology laboratories, the poor diffusion of colistin to agar in particular, leads to problems in the standardization of sensitivity tests performed with this method. Interpretative criteria for disk susceptibility testing of colistin are not available from the CLSI, and zone size interpretations are made based on the product literature. Gales et al. recommended the use of amended zone diameters for better correlation with reference methods.[5] European guidelines for disk susceptibility testing have been published by the British Society for Antimicrobial Chemotherapy (BSAC), The Société Française de Microbiologie (SFM), and the German Deutsches Institut für Normung (DIN). However, there are differences in these guidelines with regard to media, colistin quantity, and accepted zone diameters in the disk diffusion method, and in MIC breakpoints (Table 3). There are no accepted zone diameters for the disk diffusion method in the guidelines of the European Committee on Antimicrobial Susceptibility Testing (EUCAST) and the CLSI, however there is similarity of MIC breakpoints.[15]

Although agar dilution and broth microdilution methods are frequently recommended for the investigation of colistin susceptibility, there are difficulties in the routine application of these techniques. Conversely, the E-test is a simple alternative method for the susceptibility testing of several microorganisms; however, it is expensive for routine use. Nevertheless, identification and susceptibility tests performed with automated systems are preferred, particularly in hospitals where high levels of routine application are required.

In our study 100% consistency was determined between the three methods upon analyzing the MIC (E-test and Phoenix system) and disk diffusion zone results. It has been reported that incorrect results may be obtained based on the interpretation criteria of the disk diffusion method, when different methods are used together in a study for the investigation of colistin susceptibility. Rodriguez et al. used two different breakpoints in accordance with the NCCLS 1981 ($\leq$8 mm and $\geq$11 mm) and Jones 2001 ($\leq$11 mm and $\geq$14 mm) for the interpretation of disk diffusion test results. When they compared the agar dilution and disk diffusion methods, they obtained different results for the disk diffusion method with the interpretation criteria of NCCLS 1981 (0.5% minor, 2.2% major, and 4.4% very major error) and Jones 2001 (18.9% minor, 3.8% major, and 0.5% very major error).[16] When we interpreted our data according to the Jones criteria ($\leq$11 mm and $\geq$14 mm), the major error rate increased to 19%, and 77 isolates were found to be in the intermediate zone.

Gales et al. compared the broth microdilution test and the disk diffusion method by using a resistance breakpoint of $\geq$4 mg/l and zone diameter of $\leq$8 mm. They determined 1% minor error and 5% very major error.[5]

Tan and Ng evaluated colistin susceptibility with the agar dilution method (resistance breakpoint of $\geq$4 mg/l) and the disk diffusion method (resistance zone diameter of $\leq$8 mm, susceptible zone diameter of $\geq$11 mm); they showed 100% correlation of results of the agar dilution and disk diffusion methods for colistin susceptibility in 61 *Acinetobacter spp* isolates. They determined that all isolates were susceptible, as we found in our study.[17]

Table 3 MIC and zone breakpoints for *Acinetobacter spp*

Guidelines	Year	MIC breakpoint (mg/l)		Interpretation of zone diameters (mm)				
		S	R	Disk content (μg)	Medium	S	I	R
BSAC	2006	$\leq$4	>4	25	Isosensitest agar	$\geq$15	—	$\leq$14
SFM	2003	$\leq$2	>2	50	Mueller–Hinton agar	$\geq$15	—	<15
NCCLS	1981			10	Mueller–Hinton agar	$\geq$11	—	$\leq$8
CLSI	2008	$\leq$2	$\geq$4					
EUCAST	2008	$\leq$2	>2					

MIC, minimum inhibitory concentration; BSAC, British Society for Antimicrobial Chemotherapy; SFM, Société Française de Microbiologie; NCCLS, National Committee on Clinical Laboratory Standards; CLSI, Clinical and Laboratory Standards Institute; EUCAST, the European Committee on Antimicrobial Susceptibility Testing; S, sensitive; R, resistant; I, intermediate.

Arroya et al. investigated colistin susceptibility by the E-test and broth microdilution (used as the reference method) in 115 clinical isolates of *A. baumannii*. Colistin susceptibility was determined as 80.8% in their study, and they obtained 98.2% correlation between the E-test and broth microdilution methods. In this study, where the colistin susceptibility was determined as 80.8%, no major error was identified during comparison of the tests. A very major error was identified in two isolates, and sensitivity, specificity, and positive and negative predictive values of the E-test were reported as 90.9%, 100%, 100%, and 97.8%, respectively.[14]

We found a 100% categorical agreement between the E-test and disk diffusion test. We detected that no isolates were resistant to colistin in our study. Galani et al. performed antimicrobial susceptibility tests for colistin by using the E-test and disk diffusion method. They used resistance breakpoints of $\geq$4 mg/l according to the CLSI 2007 criteria and zone diameters of $\leq$12 mm for resistance, $\geq$14 mm for susceptibility, and 13 mm for the intermediate category. Among the 226 Acinetobacter isolates, they found nine resistant by E-test. Eight resistant isolates exhibited zone diameters of $\leq$12 mm and only one isolate exhibited a zone diameter of 13 mm in this study. Among the nine isolates displaying a zone diameter of 13 mm, only one isolate was found to be resistant with a MIC of 8 mg/l.[18]

Based on our findings, we are unable to propose that the disk diffusion technique is reliable for colistin susceptibility testing, although different criteria were used.

The reliability of automated susceptibility testing systems like VITEK 2 for the evaluation of colistin susceptibility in *Acinetobacter spp* remains controversial. Lo-Ten-Foe et al. compared the VITEK 2 and broth microdilution methods for colistin susceptibility and found a high level of agreement. They also reported a high level of agreement between the E-test and broth microdilution method. They showed the disk diffusion method to be unreliable in their study.[19]

Tan and Ng compared the E-test and VITEK 2 with agar dilution for colistin susceptibility in 58 *Acinetobacter spp*. They reported one major error for the E-test and no major error for VITEK 2. They showed 100% categorical agreement between the VITEK 2 and agar dilution. All Acinetobacter isolates also were susceptible in their study.[20]

In our study, we found 100% categorical agreement between the Phoenix system and the E-test. This is the first study to compare the Phoenix system and the E-test.

In conclusion, the broth microdilution method and agar dilution are accepted as reference methods for colistin susceptibility testing. However, differences have been reported between the two tests.[5] Based on the studies reported, it would appear that the E-test is a reliable method for colistin susceptibility testing.[14,18–20] However, further studies on automated systems and disk diffusion methods are required. In addition, the standardization of susceptibility tests for colistin is needed.

Conflict of interest: No conflict of interest to declare.

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