Reliability of phenotypic methods for detection of colistin resistance among carbapenem-resistant *Acinetobacter baumannii* clinical isolates from Egypt

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Abstract

Introduction *Acinetobacter baumannii* is a challenging pathogen responsible for serious nosocomial infections. Colistin resistance in carbapenem-resistant *A. baumannii* strains is a critical health problem as it limits the available therapeutic options. The current work aimed to study the reliability of several phenotypic methods for the detection of colistin resistance among carbapenem-resistant *A. baumannii* isolates in Egypt.

Methods A total of 22 carbapenem-resistant *A. baumannii* isolates were recovered. Colistin minimum inhibitory concentrations (MICs) were determined using broth microdilution (BMD) and compared to agar dilution (AD), automated system (VITEK-2) and gradient test (E-test) and were analyzed by statistical methods.

Results Phenotypic testing showed that nine of 22 isolates (40.9%) were colistin-resistant by BMD and seven of them were also resistant by AD, with the categorical agreement (CA) of 72.7% and essential agreement (EA) of 90.9%. Colistin MIC results ranged from 1-8 µg/mL and 1-32 µg/mL by both AD and BMD respectively. Detection of colistin resistance by gradient test and automated system showed high very major error (VME) rates (40.9%) compared to BMD with a lack of CA between them. AD gave moderate agreement with BMD by 90.9% EA, 72.7% CA and only 9.1% VME.

Conclusions In delineating colistin breakpoints BMD followed by AD method are defined as the only reliable phenotypic methods for colistin resistance evaluation. More rapid and reliable tests, other than BMD and AD, are required for the convenient detection of colistin resistance in the routine clinical microbiology laboratory daily workflow.

Keywords Colistin, *Acinetobacter baumannii*, broth microdilution, automated system (VITEK-2), gradient test.

Introduction

Multidrug-resistant (MDR) *Acinetobacter baumannii* related infections are a burden on healthcare facilities owing to limited antibiotic options for treatment.¹ Treatment of MDR *A. baumannii* is often attempted by antibiotic combination therapy including colistin, a member of the polymyxins family, which is a

cationic amphiphilic antimicrobial peptide.² Although colistin has potential side effects, the wide spread of extensive drug resistant bacteria lead to the revived use of colistin as last therapeutic option especially for the treatment of pneumonia and in cases of sepsis caused by bacteria with MDR profile.³ Thus, with the increase of polymyxins usage as last treatment

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options, especially colistin, arises the need for accurate evaluating methods that can be used for routine diagnostic laboratories.^{4,5}

Currently, the available testing methods for colistin include: dilution methods [agar dilution (AD), broth microdilution (BMD), and broth macrodilution], diffusion methods [disk diffusion and gradient diffusion] and automated systems.⁶⁻⁸ The Clinical and Laboratory Standards Institute (CLSI), as well as the European Committee on Antimicrobial Susceptibility Testing (EUCAST) only validated the use of BMD for testing colistin susceptibility. BMD is the only accepted testing method for colistin susceptibility but this was found to be tedious and time-consuming for routine laboratory work.^{9,10} In the last version of CLSI, colistin broth disc elution and colistin agar tests are described as accepted methods for testing colistin sensitivity in Enterobacteriaceae and in *Pseudomonas aeruginosa*. However, these methods gave inaccurate results with *A. baumannii*. Thus BMD is still the only validated method of testing colistin sensitivity in *A. baumannii*.¹¹

In the current study we investigated the reliability of several methods of colistin susceptibility testing to the BMD method on clinical carbapenem-resistant *A. baumannii* (CRAB) isolates from Egypt to detect the most suitable and efficient method which can be used within our daily laboratory work flow.

Methods

Bacterial strains and antimicrobial susceptibility

A total number of 22 non-duplicate CRAB clinical isolates collected from Theodor Bilharz Research Institute (TBRI) hospital during the period 2015-2016 were included in the study. Previously, all strains were identified by API 20E (bioMérieux, Marcy-l'Étoile, France) and VITEK-2 compact system (bioMérieux) and susceptibility to different antimicrobials was performed using antimicrobial susceptibility testing (AST), done primarily by using Kirby Bauer disc diffusion method and VITEK- 2 compact system card N° 222 (bioMérieux).^{1,12}

Colistin susceptibility testing

Colistin MICs were determined by BMD method, AD method, Vitek 2 AST cards N222 and gradient test.

Gradient test was done according to manufacturer's instructions (bioMérieux) using Mueller Hinton agar media (Mast Diagnostics, Bootle, UK).

Dilution methods were done according to the CLSI recommendations¹³ using colistin antibiotic powder (LKT Laboratories, St Paul, MN, USA). Preparations of stock solutions in sterile distilled water was done according to manufacturer immediately before use.

AD test was done according to the method mentioned.¹⁴ Briefly, stock solution was used to provide 2-fold dilutions ranging from 0.5 to 32 µg/mL final concentrations, molten Mueller-Hinton agar (Mast Diagnostics) was added to each concentration. After solidification, a bacterial inoculum of 0.5 McFarland was applied on the agar surface by streaking. After incubation for 18 h at 37°C, the minimum concentration that prevented the bacterial growth was recorded as MIC.

BMD: Two-fold serial dilutions of colistin were prepared ranging from 0.5 to 32 µg/mL final concentrations using cation-adjusted Mueller-Hinton broth (BBL-Becton Dickinson, Sparks, MD). Each well of the 96-well polystyrene microtiter plate (Cellstar®, Greiner Bio-One, Kremsmünster, Austria) was inoculated with 100 µL bacterial suspension (0.5 McFarland) and 100 µL of each colistin dilution.¹⁴ After incubation for 18-24 hours at 37°C, the lowest concentration of colistin with no visible growth was recorded as MIC. Interpretation of MIC results was done according to CLSI recommendations.¹⁰

Data analysis

The MIC results obtained by different methods were compared against the MIC of BMD as reference method.¹⁰ The percentage of isolates that had MIC values within one to two-folds dilution of the reference standard was defined as essential agreement (EA), while the percentage of isolates with results in the same category as the reference standard was defined as categorical agreement (CA), taking all isolates

tested as the denominator. When an isolate is resistant by the reference method, but susceptible by the test method denoting false susceptibility, it is defined as Very major error (VME), while defined major error (ME) when an isolate is susceptible by the reference method but resistant by the test method denoting false resistance. Reliability of individual tests in delineating breakpoints was determined according to the following criteria: high if both EA and CA were >90%, moderate if either EA or CA is >90%, low if both EA and CA were <90%, acceptable errors (<2% VME, <5% ME), and poor if the errors were unacceptable, irrespective of EA and CA.¹⁵ Data are expressed as number (%). Comparison between categorical data was performed using Chi square test or Fisher exact test whenever it was appropriate. Agreement between the BMD and different MIC techniques was done using kappa statistic. Statistical Package for Social Sciences (SPSS) computer program (version 19 windows) was used for data analysis (IBM, Armonk, NY, USA). Mode and geometrical means were calculated for each testing method to compare with those of the reference standard methods. Kappa value interpretation: Poor agreement if ≤ 0.20 ; Fair agreement = 0.20 to 0.40; Moderate agreement = 0.40 to 0.60; Good agreement = 0.60 to 0.80 and Very good agreement = 0.80 to 1.00. P value ≤ 0.05 was considered significant.

Results

A total number of 54 *A. baumannii* were isolated during the study period, 32 were CRAB, and 22 of these CRAB isolates were randomly selected and included in the study. Isolates were recovered from respiratory samples (8/22; 36.3%), pus (6/22; 27.3%), urine (6/22; 27.3%), blood (1/22; 4.5%) and central venous catheter (1/22; 4.5%). The intensive care unit (ICU) was the main source of isolates with an isolation rate of 7/22 (31.8 %) followed by the Surgery and Urology units (Table 1). Concerning the antibiotic disc (Mast Diagnostics, UK) diffusion results, interpretation was done according to CLSI recommendation.^{10,12} All of the tested isolates were resistant to ampicillin/sulbactam, piperacillin/tazobactam, ceftazidime, cefotaxime,

ceftriaxone, cefepime, ciprofloxacin, levofloxacin, meropenem, and imipenem. Other resistance percentages were detected, which were 95% to amikacin, 90.09% to both gentamicin and doripenem, 59.09% to tetracycline, and 0% to colistin. Gradient test was done for imipenem, meropenem, and doripenem antibiotics. All isolates showed MIC of ≥ 32 $\mu\text{g/mL}$ which was interpreted as resistant except for one isolate (R11) that showed MIC of 16 $\mu\text{g/mL}$ for doripenem.

The MIC evaluation of colistin was done by using gradient test, automated system, AD and BMD methods (Table 1). Nine of 22 *A. baumannii* isolates (40.9%) were colistin-resistant by BMD reference and seven of them were also resistant by the AD method, with an overall categorical agreement of 72.7% between the BMD and AD (16 of 22 isolates).

By comparing all methods used in the study to detect colistin MICs of *A. baumannii* to the BMD as the reference method, we found that they all produced errors: gradient test and automated system showed nine (40.9%) VMEs with one (4.5%) ME produced by automated system, while AD produced only two VMEs (9.1%) (Table 2). Performance criteria showed that gradient test and automated system gave low reliability results (both CA and EA were less than 90%) and high unacceptable VME rates with lack of agreement between them and BMD. On the contrary, the AD gave moderate agreement with BMD by 90.91% EA, 72.7% CA and only 9.1% VME.

Discussion

The global emergence of colistin resistance has demanded the use of rapid and accurate methods of detection. Moreover, hetero-resistance to colistin is a widely underestimated common phenomenon, which required specific methods.^{7,16} Therefore, in the last years many researchers tried to study different testing methods to detect colistin-resistance and also to compare their accuracy and error percentage.^{6,8} Over the past five years, besides the automated platforms, new screening and culture-based diagnostics methods used for detecting *mcr-1*-

Table 1. Epidemiological data, colistin susceptibility and MIC values of 22 *A. baumannii* clinical isolates using different methods

Isolate	Sex of patient	Hospital ward	Type of specimen	MIC values of colistin (µg/mL) (interpretation)			
				Automated system	Gradient test	AD	BMD
R2	F	ICU	Resp.Tube	≤0.5 (S)	0.047 (S)	4 (R)	4 (R)
R7	M	ICU	Resp.Tube	≤0.5 (S)	0.032 (S)	1 (S)	1 (S)
R9	M	Surgery	Sputum	≤0.5 (S)	0.047 (S)	1 (S)	1 (S)
R11	F	ICU	Sputum	≤0.5 (S)	0.023 (S)	2 (S)	32 (R)
R13	F	Surgery	Pus	≤0.5 (S)	0.064 (S)	8 (R)	16 (R)
R14	F	ICU	Sputum	1 (S)	0.047 (S)	8 (R)	8 (R)
R18	M	Outpatient	Pus	2 (S)	0.047 (S)	2 (S)	8 (R)
R24	M	Surgery	Sputum	≤0.5 (S)	0.094 (S)	4 (R)	2 (S)
R28	F	ICU	Sputum	≤0.5 (S)	0.094 (S)	4 (R)	1 (S)
R31	F	ICU	Sputum	≤0.5 (S)	0.125 (S)	2 (S)	2 (S)
R34	M	Urosurgery	Blood	≤0.5 (S)	0.094 (S)	4 (R)	1 (S)
R36	M	Internal Medicine	Pus	1 (S)	0.047 (S)	1 (S)	2 (S)
R41	F	Nephrology	Urine	≤0.5 (S)	0.094 (S)	4 (R)	8 (R)
R45	M	HGE	Pus	≤0.5 (S)	0.094 (S)	4 (R)	32 (R)
R51	M	Surgery	Urine	≤0.5 (S)	0.094 (S)	4 (R)	2 (S)
R58	M	ICU	Urine	1 (S)	0.094 (S)	4 (R)	8 (R)
R71	M	Urosurgery	Urine	≤0.5 (S)	0.064 (S)	2 (S)	2 (S)
R73	M	Urosurgery	Urine	2 (S)	0.125 (S)	1 (S)	2 (S)
R74	F	Surgery	Pus	2 (S)	0.094 (S)	2 (S)	2 (S)
R76	M	Urosurgery	Urine	2 (S)	0.094 (S)	2 (S)	2 (S)
R77	M	Surgery	Pus	4 (R)	0.094 (S)	2 (S)	2 (S)
R80	F	Nephrology	CVL	≤0.5 (S)	0.125 (S)	4 (R)	8 (R)

CVL – central venous line; HGE – hepatogastroenterology department; ICU – intensive care unit; MIC – minimum inhibitory concentration; R – resistant; S – sensitive.

positive and polymyxin-resistant Gram-negative bacteria were studied, depending on their sensitivity, specificity, their relative cost, time and relative skill required.¹⁷⁻¹⁹ Some novel manual diagnostics tests are: MIC Test strip® (MTM) (Liofilchem), MICRONAUT MIC-Strip® (MERLIN Diagnostika), Commercial Rapid Polymyxin NP (RPNP), SensiTest™Colistin (Liofilchem), Zeta potential (±EDTA) alteration and others. Although these methods showed good specificity and sensitivity and required a low relative skills to be performed, they are still expensive to be used in our country in the routine work.¹⁸ RPNP is cheap, but it was found to be a less-accurate alternative to BMD.²⁰ Indeed

these newly described methods have good outcomes, however still under evaluation compared with the conventional BMD which is the one procedure that validates any novel technique.^{21,22} In the last version of CLSI, colistin broth dilution and colistin agar tests are described as accepted methods for testing colistin sensitivity in *Enterobacteriaceae* and in *Pseudomonas aeruginosa*, whereas these methods did not solve the problem with *A. baumannii*, as per CLSI, yielding inaccurate results¹¹

Colistin resistance emergence in *A. baumannii* clinical strains has been reported worldwide.²³ In Egypt, it was first reported by Al-agamy et al., in

Table 2. Percentage of errors of colistin susceptibility by different phenotypic methods compared to BMD as reference method

Tested Method	MICs (µg/mL)				Statistical analysis		Error percentages* N (%)			
	MIC breakpoint	MIC range	MIC50	MIC90	Kappa coefficient value	p-value	CA ≥90%	EA ≥90%	ME <5%	VME <2%
Automated system	S ≤ 2; R > 2	0.5-4	0.5 (S)	2 (S)	-0.089	0.394	12/22 (54.5)	6/22 (27.3)	1/22 (4.5)	9/22 (40.9)
Gradient test	S ≤ 2; R > 2	0.023-0.125	0.094 (S)	0.094 (S)	-	-	13/22 (59.1)	0/22 (0)	0 (0)	9/22 (40.9)
Agar Dilution	S ≤ 2; R > 2	1-8	4 (S)	4 (R)	0.455	0.030	16/22 (72.7)	20/22 (90.9)	4/22 (18.2)	2/22 (9.1)

CA – categorical agreement; EA – essential agreement; ME – major error; VME – very major error.

5% (2/40) isolates (of MIC values of 4 and 8 mg/L) by AD using British Society of Antimicrobial Chemotherapy (BSAC) guidelines.²⁴ Recently it was reported in two clinical isolates, with MIC value of 32 mg/L using BMD and current CLSI guidelines.²

To date, the accepted evaluating testing reference method to the polymyxins is the BMD as recommended by CLSI but the literature still includes many studies conducted to evaluate the reliability of testing colistin resistance by more feasible and rapid detection methods. The automated system showed that 100% of the isolates were resistant to meropenem and imipenem.¹² Isolates were also resistant to ceftazidime, cefepime, piperacillin, piperacillin/tazobactam, and ciprofloxacin. Isolates were sensitive to minocycline (45.5%) and amikacin (27.3%). Herein, we assessed three phenotypic methods and compared them to the reference BMD method. Our results showed that nine of 22 isolates (40.9%) were colistin-resistant by BMD and seven of them were resistant by the AD with an overall categorical agreement by the two methods in 16/22 isolates (72.7%). The reliability of phenotypic tests in delineating colistin breakpoints was poor by gradient test and automated system that showed high VMEs of 40.9% for both methods compared to BMD with lack of categorical agreement between them. BMD and AD methods remain to be the only dependable method for detection of colistin susceptibility.

Although BMD method is the only recommended one for the investigation of colistin susceptibility, it is difficult to be

performed in the routine application,^{3,25} as it is time-consuming and requires trained technical staff for preparation and assay assessment.²⁶

Concerning the automated systems, it has been already reported that the Vitek 2 has poor sensitivity.¹⁸ Also, a high rate of false results was stated by using the Phoenix system, suggesting that results obtained with automated systems should be confirmed by standard recommended method.²¹

Although gradient test is a simple alternative method for the susceptibility testing of several bacteria, it is expensive for routine use.³ Colistin gradient test method showed a concordance of 98.2% with BMD, due to the fact that two VMEs were found (1.7%).²⁷ It is reported that gradient test fared poorly for colistin when tested against BMD method with very low essential agreement with broth microdilution in glass-coated plates and polystyrene plates.⁵ In contrast a concordance of 99% was found between the gradient test and BMD, and 97% was found between agar dilution and BMD.²⁸ The reliability and variations of the gradient test results were negotiable and associated with the MIC values. Complete agreement was stated for the strains for which MICs between the range of 0.25 and 1 mg colistin/mL.²⁷ However, there was poor concordance, particularly in extreme dilutions with higher MICs by the gradient test method, as in our study, where the agreement between the gradient test and BMD was 16.5%. Also it is reported that the gradient test and MIC test strip methods produced high rates of VMEs 39.3% and 31.1%, respectively, AD produced 3.3%

VMEs, and automated system produced no VMEs.²⁹

Conclusions

Colistin resistance is an emerging problem in *A. baumannii* isolates in our region. In delineating colistin-resistance, BMD and AD methods are the only reliable phenotypic methods according to our study as both gradient test and automated system showed high VMEs of 40.9% when compared to BMD with a low categorical agreement.

Authors' contributions statement: NSF designed the study. SHM, DG and RMW performed the bacterial culture, laboratory work, interpreted the results, analyzed the data, performed statistical analysis and drafted the manuscript. AAEK and MSS were involved in writing the article and performed statistical analysis. All authors critically revised the manuscript, and approved the final version to be published.

Conflicts of interest: All authors – none to declare.

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