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Comparison of Broth Microdilution Method with BD Phoenix, Micro Scan and E-test for Carbapenem-resistant *Enterobacterales*: Colistin Susceptibility Testing

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ABSTRACT

Objective: In the past years, due to the increasing carbapenem resistant *Enterobacterales* (CRE) infection rates, colistin use has been on the rise. Multi-drug resistant Gram-negative bacteria and colistin resistance are increasing simultaneously; therefore, an accurate method for antimicrobial susceptibility testing of colistin is crucial. Clinical and Laboratory Standards Institute and European Committee on Antimicrobial Susceptibility Testing recommend the standard broth microdilution (BMD) method for colistin minimum inhibitory concentration testing. In this study, we aimed to examine the performance of BD Phoenix, MicroScan, and E-tests on CRE isolates on the determination of colistin susceptibility. The existing commercial tests were compared to the reference BMD method.

Methods: One hundred and twenty non-duplicate clinical *Enterobacterales* isolates such as *Klebsiella pneumoniae* (*K. pneumoniae*), *Escherichia coli* (*E. coli*), *Enterobacter cloace* (*E. cloacae*) were collected between August 2017 to June 2018. The BD Phoenix, MicroScan systems, and E-tests were used to test colistin susceptibility. Commercial methods were compared with the reference method BMD.

Results: Colistin susceptibility was evaluated in 120 Gram-negative clinical isolates, including 108 *K. pneumoniae*, 10 *E. coli*, 2 *E. cloacae*, during the study period. Among the isolates, 66 (55%) were susceptible, and 54 (45%) were resistant to colistin, according to BMD. BD Phoenix, MicroScan, and E-test had 90.90%, 95.45%, and 96.96% sensitivity, respectively, when colistin was tested.

Conclusion: In routine clinical practice, the worldwide reference method can hardly be implemented, and commercially available systems are used for the interpretation of colistin susceptibility. Colistin use is increasing for the treatment of multiresistant Gram-negative infections, further and more extensive studies are needed for precise susceptibility testing methods for this compound. We recommend that laboratories use the BMD method at least in selected patient groups in the face of increasing antimicrobial resistance.

Keywords: Colistin susceptibility, carbapenem-resistant *Enterobacterales*, broth microdilution

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INTRODUCTION

Multi-drug resistant organisms are the source of a growing burden with challenging solutions globally. Recently, carbapenem-resistant *Enterobacterales* (CRE) has become a problem worldwide. The treatment of CRE infections is challenging, and the available treatment options are limited (1). In the past years, due to the increasing CRE infection rates, polymyxin use has been on the rise (2,3).

Antimicrobial treatment should be managed carefully by considering the benefits, potential toxicities; therefore, susceptibility testings play an important part in antimicrobial treatment guidance. The European Committee on Antimicrobial Susceptibility Testing (EUCAST) issued the breakpoints of colistin for *Enterobacterales* and reported susceptible and resistant breakpoint of <2 mg/liter and >2 mg/liter, respectively (4). The available methods are limited regarding the performance, reproducibility, and accuracy for the susceptibility testing of colistin (5,6). In 2016, the ISO-20776 standard broth microdilution (BMD) method was recommended for colistin minimum inhibitory concentration (MIC) testing by Clinical and Laboratory Standards Institute (CLSI) and EUCAST (7). Other test techniques such as gradient diffusion, agar dilution, and disk diffusion are not suggested today. Colistin shows a poor diffusion on the agar; therefore, the disk diffusion method has high interpretation error levels, which decreases the reliability (8,9). Reference BMD use is not practical to perform for susceptibility testing due to the individual laboratory burden; also, the production and use of BMD panels are exhausting. There are commercially produced BMD panels as well, but those panels are expensive for most of the hospitals. Up to this day, the accuracy of automated antimicrobial susceptibility methods is not precise.

In this study, we aimed to examine the performance of BD Phoenix (BD Diagnostic Systems, Sparks, MD), MicroScan (Beckman Coulter, CA, USA) and Colistin E-tests (bioMérieux, Marcyl'Etoile, France) on CRE isolates on the determination of colistin susceptibility. The existing tests were compared to the reference BMD method.

METHODS

Bacterial isolates: One hundred and twenty non-duplicate clinical *Enterobacterales* isolates such as *Klebsiella pneumoniae* (*K. pneumoniae*), *Escherichia coli* (*E. coli*), *Enterobacter cloace* (*E. cloacae*), that were carbapenem-resistant were collected from a tertiary research and education hospital between August 2017 to June 2018 for this study. The evaluation was performed, prospectively, in the Microbiology Laboratory of Bozyaka Training and Research Hospital, İzmir, Turkey. The isolates were stored at -80 °C in brain heart infusion broth medium with 10% glycerol stocks and subcultured twice before the testing. All isolates were inoculated on 5% Sheep Blood agar (BD Diagnostic Systems, Sparks, MD) and EMB agar (BD Diagnostic Systems, Sparks, MD) from the stock medium before assay. It was incubated at 37 °C for 24 hours. *E. coli* ATCC 25922 and clinical isolate with confirmed MCR-1 positivity were used as the control strain for the drug-susceptibility/resistance.

Antimicrobial powder: Sulfate salts of colistin (Carbosynth, Compton, UK) were dissolved in distilled water, and BMD was performed under the CLSI reference method (10). All the colistin susceptibility tests were performed by following commercial methods and BMD.

BMD: The BMD was carried out in duplicate accordingly the CLSI guidelines that used cation- adjusted Mueller- Hinton broth (Difco™ Becton Dickinson, Sparks, MD). Dilutions were prepared with a MIC range of 0.06 mg/liter to 64 mg/liter in 96-well polystyrene microplates (Citotest, Jiangsu, China) (7). The plates had an incubation time of 18 to 24 hours at 37 °C. The isolates were considered resistant when colistin MIC >2, based on the breakpoints of CLSI and EUCAST (11,12).

Identification and colistin susceptibility testing: The isolates were identified by using BD Phoenix (BD Diagnostic Systems, Sparks, MD) and traditional methods. BD Phoenix and MicroScan automated systems and E-tests were used to determine colistin susceptibility. The manufacturer's instructions were followed while semi-automated systems were used for testing of colistin susceptibility. The E-test method was performed with a colistin strip (bioMérieux SA, Marcyl'Etoile, France) using Mueller-Hinton agar (BD Diagnostic Systems, Sparks, MD) medium in accordance with the manufacturers' recommendations. The probable range of MIC readings for each method were as follows: for BMD, <0.06 to >64 mg/liter; for BD Phoenix, ≤1 to >4 mg/liter; for MicroScan, ≤2 to >4 mg/liter; for E-test, <0.016 to >256 mg/liter. Colistin MIC results were interpreted according to the EUCAST breakpoints (susceptible, ≤2 mg/liter; resistant, >2 mg/liter).

Statistical Analysis

Commercial methods were compared with the reference method BMD. Rates of very major errors (VMEs), major errors (MEs), essential agreement (EA), and categorical agreement (CA) were established.

The CA and EA (EA: MICs within ±1 dilution of reference MICs) were calculated as claimed by the International Organization for Standardization (ISO) standard 20776-2 (13).

The EA was considered when the E-test MIC was ±1log2 when compared with BMD results.

The EA was not determined for BD Phoenix and MicroScan. The MIC ranges were limited to ≤1, 2, 4, ≥4, and ≤2, 4, >4 mg/liter, respectively. CA was defined as the compatibility of MIC results with commercial kits and BMD. Although the reference method result is resistant, VMEs are defined as the sensitivity of the method result tested. Although the reference method result is sensitive, MEs are defined as the resistance of the method result tested (14).

The acceptable performance was accepted following the criteria of the ISO as >90% for essential or category agreement and VME <1.5%, ME <3.0% (14).

J Acad Res Med 2022;12(2):61-5

The study was conducted after it was approved by the University of Health Sciences Turkey, İzmir Bozyaka Training and Research Hospital Ethics Committee (decision no: 02, date: 09.10.2019).

RESULTS

Colistin susceptibility was evaluated in 120 Gram-negative clinical isolates, including 108 *K. pneumoniae*, 10 *E. coli*, 2 *E. cloacae*, during the study period. MICs for *E. coli* ATCC 25922 were examined, the isolates were between 0.25 and 1 mg/liter for all testing methods (7). MICs for mcr-1 positive *K. pneumoniae* were between >2 and 4 mg/liter for all testing methods. The dispersions of colistin MICs determined by BMD and other testing methods for the isolates are presented in Table 1. Among the isolates, 66 (55%) were susceptible, and 54 (45%) were resistant to colistin, according to BMD (Table 2). BD Phoenix, MicroScan, and E-test had 90.90%, 95.45%, and 96.96% sensitivity, respectively, when colistin was tested. The comparison of performance characteristics of different methods with BMD is presented in Table 3. The BD Phoenix system, MicroScan and E-test failed to detect 7, 7, and 13 colistin-resistant *K. pneumoniae* isolates, respectively. The commercial methods and E-test revealed poor performance in species other than *E. coli* and *E. cloacae*.

There was >85% CA between BD Phoenix, MicroScan, E-test, and BMD for all the 120 isolates. Although BD Phoenix, MicroScan, and E-test resulted in close to >85%, in general VME rate was high (10%, 7.5%, and 12.5%, respectively). E-test showed 71.17% EA.

E-tests with colistin showed higher VME rates (12.5%). BD Phoenix had six (5%) MEs, MicroScan had three (2.5%) MEs, and E-test had two (1.7%) MEs for colistin. None of the commercial testing methods for colistin satisfied the CLSI-committed performance standards for commercial AST systems (VME <1.5%, ME <3.0%, CA >90%, EA >90%) (14). Only Micro Scan met the CA and ME performance standards recommended by CLSI for colistin.

DISCUSSION

Multi-resistant *Enterobacterales* can cause severe infections, and colistin is an agent used in the treatment. A false susceptible and false resistant results in this last resort agent should be considered equally serious. Colistin is often among the limited treatment options. Therefore, reliable colistin susceptibility testing should be performed before the use of colistin in clinical practice.

Multi-drug resistant Gram-negative bacteria and colistin resistance are increasing simultaneously; therefore, an accurate method for AST of colistin is crucial. The reference methodology is MIC determination with BMD according to the ISO standard 20776-1 for AST (15).

According to EUCAST experience, it was shown that the correct categorization was difficult, especially in MICs in the range of 2-8 mg/L (https://www.eucast.org/ast_of_bacteria/warnings/). For this reason, according to our study results, MIC should be confirmed with BMD, especially for colistin determined by semi-automated systems between 2-8 mg/liter. The EUCAST uses the BMD method as the reference method according to the latest recommendations of the joint CLSI and EUCAST subcommittee on the polymyxin susceptibility testing and breakpoints (16). The performance of commercial AST systems for colistin is as follows: VME <1.5%, ME <3.0%, EA >90%, and CA >90%, according to CLSI -recommended performance standards (14).

Colistin susceptibility testing studies are limited to Micro Scan. Lee et al. (17) compared the Micro Scan system with agar dilution as a colistin sensitivity test. They found the CA value of 87.3% for Micro Scan. In our study, Micro Scan susceptibility testing for colistin, the rates of VME, ME, and CA were 7.5%, 2.5%, and 90%, respectively.

The BD Phoenix, Micro Scan, and E-test have VMEs of <1.5% rate, which is the recommended value by CLSI (18). In this study, the percentage of VME exceeded the recommendation of CLSI; however, this might be caused by the limited number of isolates. The MEs for all methods (except E-test colistin testing) also exceeded the CLSI recommendation of >3.0% (14).

The use of semi-automated systems for diagnostic purposes has become quite common even in microbiology laboratories of developing countries. It is quite difficult to ensure quality at BMD in small-scale laboratories. In addition, the scarcity of trained technical personnel also requires the use of semi-automated systems (19). For example, it has been reported that VitekVR 2 can be used as a reliable colistin susceptibility test method in studies (20). In addition, another recent study reported that colistin susceptibility testing might not be reliable with a VME rate of 36% (21). Bartoletti et al. (22) found that VME (42%) was common in the colistin susceptibility test performed on semi-automated systems. According to this result, VME was associated with inappropriate antibiotic use and worse outcomes. In another study evaluating six commercial products for colistin susceptibility testing in *Enterobacterales*, the performances of the semi-

Table 1. Colistin MICs distribution determined by BMD and other testing methods for all isolates

MIC detected by BMD (mg/L)	BD Phoenix (n)	Microscan (n)	E-test (n)
≤2 (n=66)	60 (90.9%)	63 (95.45%)	64 (97%)
2-8 (n=13)	8 (61.54%)	7 (53.85%)	3 (23.08%)
16 (n=41)	37 (90.24%)	38 (92.69%)	36 (87.80%)

MIC: minimum inhibitory concentration, BMD: broth microdilution

Table 2. Colistin MIC results of isolates determined by BMD, BD Phoenix, Microscan automated systems and gradient test

	S	R
BMD	66 (55%)	54 (45%)
BD Phoenix	73 (60.8%)	47 (39.2%)
Microscan	73 (60.8%)	47 (39.2%)
E-test	79 (65.8%)	41 (34.2%)

MIC: minimum inhibitory concentration, BMD: broth microdilution, S: susceptible, R: resistant

J Acad Res Med 2022;12(2):61-5

Table 3. The comparison of the overall performance characteristics of the different methods with BMD

	CA	VME	ME	Sensitivity	Specificity	Positive predictive value	Negative predictive value
BD Phoenix	101 (85%)	13 (10%)	6 (5%)	90.9%	75.9%	82.2%	87.2%
Microscan	107 (90%)	10 (7.5%)	3 (2.5%)	95.5%	81.5%	86.3%	93.6%
E-test	103 (85.8%)	15 (12.5%)	2 (1.7%)	97%	72.2%	81%	95.1%

BMD: broth microdilution, CA: categorical agreement, VME: very major errors, ME: major error

automated systems Vitek 2 and BD Phoenix were found to be unacceptable (due to the large number of false susceptible results) (23). Colistin heteroresistance is defined as colistin-resistant subpopulations. These subpopulations arise from the colistin-susceptible population under colistin pressure. It can be proved by the presence of skip wells in the BMD (9). A general limitation for semi-automated systems is that they use panels/cards that contain a certain number of colistin concentration, most of the tests has only one or two dilutions up and down from breakpoint for resistance. In our study, the possible range of MIC readings for automated systems was as follows: for BD Phoenix, ≤1 to >4 mg/liter; and for MicroScan, ≤2 to>4 mg/liter. Semi-automated systems commonly exhibit false-sensitivity results, possibly due to the presence of colistin hetero-resistant subpopulations (24). Limited colistin concentrations used in semi-automated systems may not be able to detect colistin heteroresistance due to the presence of skip wells observed in BMD.

Several studies stated that there were methodological difficulties in colistin MIC (25,26). In some studies, different susceptibility testing methods were reviewed (27,28). Recently, Micro Scan Microbiology Systems, Beckman Coulter Inc., conducted an experiment that revealed that the addition of polysorbate-80 caused higher colistin MICs higher (29). BMD without polysorbate-80 supplementation is the current reference method (7).

The E-test allows evaluation of the wider colistin MIC range. However, poor diffusion of the large colistin molecule in the agar causes errors in the interpretation of the results. Colistin E-tests have varying error rates, and the E-test is not an adequate testing method (7,17,30,31). After an E-test, the highest reported rate of VMEs of colistin was 41.5% (17). In our study, the rates of VME, ME, and CA for E-tests were 12.5%, 1.7%, and 85.8%, respectively. EA was particularly poor for E-tests (71.17%) in our study, which could be due to the poor diffusion of colistin molecules causing inhibition of a narrow zone close to the MIC. Colistin disk diffusion testing is also unreliable due topoor diffusion of colistin molecules (18,31,32).

Study Limitations

Our study had some limitations. First of all, the number of species other than *K. pneumoniae* used to determine the compatibility of colistin susceptibility tests was insufficient. In addition, the inability to include Gram-negative non-fermentative rods in the study might be another limitation.

CONCLUSION

This study evaluated automated systems and E-test for colistin MIC determination. According to our results, the reference BMD method should be performed for colistin MIC determination. Colistin MIC should be confirmed with BMD, particularly by using automated systems between 2-8 mg/liter.

The data on the use of colistin in clinical treatment is limited. The subjects related to colistin susceptibility testing methods remain unclear. BMD requires experienced staff, and manual preparation of antibiotic solutions, therefore, is time-consuming and hard to perform in routine laboratories. Nevertheless, BMD is the only test that is recommended by EUCAST and CLSI until further studies are carried out.

In routine clinical practice, the worldwide reference method can hardly be implemented, and commercially available systems are used for the interpretation of colistin susceptibility. Colistin use is increasing for the treatment of multiresistant Gram-negative infections, further and more extensive studies are needed for precise susceptibility testing methods for this compound. We recommend that laboratories use the BMD method at least in selected patient groups in the face of increasing antimicrobial resistance.

Ethics Committee Approval: The study was conducted after it was approved by the University of Health Sciences Turkey, İzmir Bozyaka Training and Research Hospital Ethics Committee (decision no: 02, date: 09.10.2019).

Informed Consent: The study did not require patient consent.

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J Acad Res Med 2022;12(2):61-5

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