ORIGINAL ARTICLE

Comparative Analysis of Colistin Susceptibility Using Micronaut MIC-Strip, Disc Elution, and Broth Microdilution in MDR Gram-Negatives

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SUMMARY

Background: **Rapid, simple, and accurate methods are needed to detect colistin susceptibility in multidrug-resistant Gram-negative isolates. Both EUCAST and CLSI recommend broth microdilution (BMD) for antimicrobial susceptibility testing of colistin, but BMD is rarely used in routine microbiology laboratories. In search of alternative, more practical methods, some commercial kits were propagated, along with CLSI's recommendation of using Colistin disc elution method. In this study, we aimed to compare colistin susceptibility testing both by MMS, which is a commercial BMD product, and by Colistin disc elution method to BMD using multidrug-resistant *Acinetobacter baumanii*, *Pseudomonas aeruginosa*, and *Klebsiella pneumoniae*.**
Methods: **Multidrug-resistant *Acinetobacter baumannii, P. aeruginosa*, and *K. pneumoniae* isolates detected from various clinical samples in Marmara University Pendik Training and Research Hospital´s clinical microbiology laboratory between January 1, 2021, and July 30, 2022, were included in the study. Colistin susceptibilities were determined using the CBDE method, Micronaut MIC Strip colistin assay (MMS), and BMD. The results were compared with BMD as the reference method. Categorical agreement (CA), essential agreement (EA), major error (ME), and very major error (VME) rates were calculated.**
Results: **The study included 185 multidrug-resistant *A. baumannii, P. aeruginosa,* and *K. pneumoniae* isolates from various clinical samples. Out of these isolates, 13 (6.5%) were found to be resistant. The MMS test demonstrated a categorical agreement (CA) of 100% for *A. baumannii* and *P. aeruginosa*, while the CBDE method achieved a CA of 100% only with *A. baumannii*.**
Conclusions: **Both the MMS and CBDE tests are compatible with the BMD method. The CBDE, which does not require specialized equipment or advanced techniques, may be more suitable for laboratories with limited resources. In contrast, laboratories with greater financial means may find the MMS test to be advantageous in providing an accurate minimum inhibitory concentration.**
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INTRODUCTION

Multidrug resistant (MDR) Gram-negative bacterial infections are one of today's major health problems all over the world. The increased incidence of such infections creates a need to search for new antibiotics for successful treatment. Colistin, being effective against multidrug resistant Gram-negatives, is one of the first antibiotics to come to mind. Although colistin was used in 1947 for the first time, it was withdrawn due to its high risk of nephrotoxicity and neurotoxicity. However, in recent years colistin has now resurfaced as the final treatment method against multidrug resistant Gram-negative bacterial infections [1,2]. Colistin disrupts the stability of the outer membrane of Gram-negative bacteria by attaching to the bacteria's lipopolysaccharide and phospholipid layers and through this process leads to the leaking of the cell content [3]. The resistance mechanisms of bacteria against colistin are either related to genetic mutations that affect lipid A synthesis, leading to lipopolysaccharide modifications, or are sometimes mediated by mcr1. Since colistin is currently being used as the ultimate treatment method against MDR Gram-negative infections, it is important to establish colistin resistance; however, colistin antibiotic susceptibility tests are problematic in technical ways. Routine AST's commonly used by microbiology laboratories include automated systems, gradient diffusion tests, and disc diffusion tests. Nevertheless, these tests do not give results with expected quality for colistin. Automated systems are considered unreliable due to their "very major error" (VME) rates. Disk diffusion and gradient tests also lead to a high rate of false susceptible results due to the poor diffusion of colistin molecules into agar. The Clinical and Laboratory Standards Institute (CLSI) and European Committee on Antimicrobial Susceptibility Testing (EUCAST) are two global organizations that establish interpretive criteria for *in vitro* susceptibility data. In 2017, Clinical and Laboratory Standards Institute (CLSI) and European Committee on Antimicrobial Susceptibility Testing (EUCAST) indicated Polymyxin clinical breakpoints and the Method Determining Committee has declared broth microdilution (BMD) method to be the "reference method" according to ISO 20776 [4]. Due to the restrictions implemented by EUCAST and CLSI concerning antimicrobial susceptibility testing (AST) for colistin, routine laboratories are now compelled to incorporate the BMD method into their daily operations. The BMD method, however, is both laborious and impractical to apply for routine clinical microbiology laboratories. In 2020, CLSI approved the colistin agar test and colistin broth disk elution (CBDE) as methods to determine colistin resistance in *Enterobacterales* [5]. Despite these updates, BMD remains the gold standard method to determine exact minimal inhibitory concentration (MIC).

In search of alternative, more practical methods, several companies responded to this challenge, by bringing easy-to-use ASTs for colistin, based on the BMD method with commercial kits. The MICRONAUT MIC-Strip Colistin (MMS) is one of these commercial BMD products for colistin susceptibility testing.

In this study, we evaluated the performance of commercially available MMS and colistin broth disc elution method for the determination of colistin minimum inhibitory concentration (MIC) in comparison to the reference BMD method in multidrug-resistant *A. baumannii*, *P. aeruginosa*, and *K. pneumoniae* isolates.

MATERIALS AND METHODS

Bacterial isolates

The isolates included in our study were selected from the multidrug-resistant *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Klebsiella pneumoniae* isolates detected as pathogens in various clinical samples in Marmara University Pendik Training and Research Hospital´s clinical microbiology laboratory between January 2021 and July 2022. The isolates originated from various anatomical sites such as blood, respiratory samples (sputum, bronchoalveolar lavage), and pleural fluid. Species identification was performed by matrix assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS, BioMerieux, Marcy I'Etoile, France). Only one isolate from each patient was included in the study.

Definition of multidrug-resistant strains

Antibiotic susceptibility testing was performed by disc diffusion method.

Carbapenem resistance of *K. pneumoniae* refers to resistance to any of imipenem, meropenem, or ertapenem. Carbapenem-resistant *P. aeruginosa* was defined as resistance to imipenem or meropenem. Quinolone resistance to *A. baumannii, K. pneumoniae, and P. aeruginosa* was defined as resistance to levofloxacin.

Third generation cephalosporin resistance to *A. baumannii, K. pneumoniae, and P. aeruginosa* was defined as resistance to ceftriaxome or ceftazidime, with the strains susceptible or intermediate to carbapenems (imipenem, meropenem, or ertapenem). *P. aeruginosa* resistant to third-generation cephalosporin was defined as resistance to ceftazidime, with the strains susceptible or intermediate to carbapenems (imipenem, meropenem). *A. baumannii*, *P. aeruginosa*, and *K. pneumoniae* resistant to at least one drug from the cephalosporin, aminoglycoside, quinolone, and carbapenem groups were classified as multidrug-resistant.

Antimicrobial susceptibility testing
Broth microdilution method

Stock solutions were prepared by suspending the active colistin sulfate (Sigma Aldrich, St. Louis, MO, USA) in accordance with the manufacturer's recommendations. The test was performed in cation-adjusted Mueller Hinton broth (CAMHB) medium. Serial dilutions between 0.125 and 64 μg/mL were prepared from the stock solu-

tion and transferred to microdilution plates. After the suspension of all isolates with a turbidity of 0.5 McFarland standard was prepared, and added to the microdilution plates as the final bacterial concentration; 5 x 10^5 CFU/mL, microplates were incubated at 35°C for 16 - 20 hours. The lowest concentration at which there was no growth was determined as the MIC value for colistin. Results were evaluated by EUCAST BMD reading guide, and susceptibilities were interpreted using EUCAST breakpoints (EUCAST 2024). *E. coli* ATCC 25922 or *P. aeruginosa* ATCC 27853 and *E. coli* NTCC 13846 were used as quality control strains in each microplate.

Commercial method; Micronaut MIC Strip (MMS) method

The MMS assay (MERLIN Diagnostika GmbH, Bornheim, Germany) are single isolate strips, containing freeze-dried colistin in 11 two-fold dilutions. Each well contains a different colistin concentration ranging from 0.0625 mg/L to 64 mg/L. The assay was performed according to the manufacturer's instructions. Bacterial suspensions were prepared to 0.5 McFarland turbidity in saline. Out of these; 50 μL were added to the 11 mL of cation-adjusted Mueller Hinton broth. After the mixture was homogenized, 100 μL of the suspension were distributed into the wells and evaluated after 16 - 20 hours of incubation at 35°C. Growth was considered when turbidity was present at the bottom of the well. The tests were considered as valid only if growth was observed in the growth control and if no 'skipped well' occurred (i.e., no growth in a well but growth in a well with a higher colistin concentration).

Broth disc elution method

CBDE method was performed according to the instructions in CLSI [6]. Four tubes with 10 mL of CAMHB were labeled as growth control (GC), 1 mg/L, 2 mg/L, and 4 mg/L. Following this, 0, 1, 2, and 4 colistin discs (10 μg, Becton, Dickinson and Company Diagnostics, India) were added to each of the bottles, making final concentrations of 0, 1, 2, and 4 mg/L. Bottles were then vortexed for 1 minute and incubated at room temperature for 30 minutes to allow colistin to elute from the discs. Inoculum was prepared from the colonies grown overnight on sheep blood agar in normal saline (according to the 0.5 McFarland standard). Then, 50 μL of inoculum was added to each of the bottles and gently vortexed. After overnight incubation at 35 ± 2°C, growth was detected by turbidity in the tubes. All methods were evaluated according to the EUCAST MIC standards.

Furthermore, quality control (QC) strains *E. coli* ATCC 25922 (colistin susceptible) or *P. aeruginosa* ATCC 27853 (colistin susceptible) and *E. coli* NTCC 13846 (mcr-1 positive) were included.

Statistical analysis

Accuracy, defined as the closeness of the result obtained with the alternative tests to the reference standard, was determined by calculating essential agreement (EA) and categorical agreement (CA).

Categorical agreement (CA) is defined as the susceptibility result of the isolate obtained by the test method being the same as the reference standard method.

Essential agreement (EA) is defined as the agreement of the antibiotic MIC of the tested strain within ± 1 dilution, compared to the reference method [7].

Very major error (VME) and major error (ME) proportions were calculated. Very major error (VME) refers to a strain being found to be resistant by the reference test method, giving a sensitive result with the tested method. Major error (ME) refers to a strain being found to be susceptible by the reference test method, giving a resistant result with the tested method. The results of BMD, which is the reference test, were compared with the results of Micronaut MIC Strip (MERLIN Diagnostika GmbH, Germany) and the CBDE method, and VME and ME ratios were calculated [8].

RESULTS

A total of 185 multidrug-resistant Gram-negative bacteria isolated from respiratory samples (n = 119), blood (n = 57), and pleural fluid specimens (n = 9) were included in the study. Out of these, 40.5% (n = 75) were *P. aeruginosa*, 35.1% (n = 65) were *K. pneumoniae*, and 24.3% (n = 45) were *A. baumannii* isolates. Most of the colistin-resistant isolates were identified from respiratory specimens, blood, and pleural fluid (Table 1). By using BMD as the phenotypic reference method, 13 strains were found to be colistin-resistant. The MIC_{50} value and the MIC_{90} value for *A. baumannii* were both 1 mg/L. The MIC_{50} value for *K. pneumoniae* was 0.5 mg/L and 2 mg/L for MIC_{90}. The MIC_{50} for *P. aeruginosa* was 0.5 mg/L, and the MIC_{90} was 2 mg/L. The distribution of MIC values of all isolates by Micronaut MIC-Strip test and BMD method is given in Figure 1. The categorical agreement of the Micronaut MIC Strip test was found to be 100% for *A. baumannii* and *P. aeruginosa*. Essential agreement was lowest in *K. pneumoniae*, at 87.6%. However, with the CBDE test, categorical agreement was determined to be 100% for *P. aeruginosa* and > 95% for *A. baumannii* and *K. pneumoniae*. With both methods, the MIC of one *K. pneumoniae* isolate was found to be 4 mg/L (resistant), and a dilution difference with the cutoff value was considered a major error. Therefore, 1.5% ME was detected, whereas no VME was recorded (Table 2).

Table 1. Antimicrobial susceptibility of Gram-negative isolates to colistin by specimen type.

Specimens/ Isolates	*A. baumannii*		*P. aeruginosa*		*K. pneumoniae*		Total	
	Colistin		Colistin		Colistin			
	S n (%)	R n (%)	S n (%)	R n (%)	S n (%)	R n (%)	S n (%)	R n (%)
Blood	10 (24.4)	1 (25)	17 (24.3)	1 (20)	27 (44.2)	1 (25)	54 (31.3)	3 (23)
Respiratory specimens	29 (70.7)	2 (50)	48 (68.6)	3 (60)	34 (55.7)	3 (75)	111 (64.5)	8 (61.5)
Pleural fluids	2 (4.9)	1 (25)	5 (7.1)	1 (20)	-	-	7 (4)	2 (15.3)

Table 2. Performance of two colistin susceptibility tests with colistin broth microdilution method.

Microorganism		Micronaut-MIC-Strip		CBDE	
	Essential agreement n (%)	Categorical agreement n (%)	Disagreement major error n (%)	Categorical agreement n (%)	Disagreement major error n (%)
A. baumanii (n = 45)	43 (95.6)	45 (100)	-	43 (95.6)	-
P. aeruginosa (n = 75)	67 (89.3)	75 (100)	-	75 (100)	-
K. pneumoniae (n = 65)	57 (87.6)	64 (98.4)	1 (1.5)	62 (95.3)	1 (1.5)

		Broth microdilution method								
	Colistin MIC	0.125 mg/L	0.25 mg/L	0.5 mg/L	1 mg/L	2 mg/L	4 mg/L	8 mg/L	16 mg/L	≥ 32 mg/L
Micronaut MIC-Strip method	0.125 mg/L			3						
	0.25 mg/L			10	1					
	0.5 mg/L		3	47	41	11				
	1 mg/L			15	24	9				
	2 mg/L				3	4				
	4 mg/L					1	3		1	
	8 mg/L									
	16 mg/L								1	
	≥ 32 mg/L							2	1	5

Figure 1. The distribution of MIC values of all isolates by Micronaut MIC-Strip and BMD method.

DISCUSSION

In this study, the colistin susceptibility of 185 carbapenem-resistant Gram-negative bacteria isolated from various clinical samples, including respiratory samples, blood, and pleural fluids, were assessed. The bacterial species included *P. aeruginosa*, *K. pneumoniae,* and *A. baumannii*.

By using BMD as the phenotypic reference method, we identified 13 colistin-resistant strains. The MIC_{50} and MIC_{90} values for *A. baumannii* were both 1 mg/L, indicating a consistent susceptibility across this species. For *K. pneumoniae*, the MIC_{50} was 0.5 mg/L and the MIC_{90} was 2 mg/L, while for *P. aeruginosa*, the MIC_{50} and MIC_{90} were 0.5 mg/L and 2 mg/L, respectively. These MIC values highlight the variability in colistin susceptibility among different species.

The increasing prevalence of infections caused by multidrug-resistant (MDR) Gram-negative bacteria has led to a renewed interest in older antibiotics, such as colistin, as a last resort to treat these infections. Colistin is particularly used against MDR *P. aeruginosa, A. baumannii, K. pneumoniae,* and *E. coli*. It is effective against various bacteria, including *Acinetobacter* spp., *P. aeruginosa*, *Klebsiella* spp., *Enterobacter* spp., and *E. coli* [9]. Colistin resistance rates can vary significantly between regions and countries due to differences in antibiotic usage practices, infection control measures, and prevalence of resistant bacterial strains [10]. In the results of SMART between 2016 - 2019, *K. pneumoniae* and *P. aeruginosa* exhibited low resistance rates to colistin, 6.4% and 11.6%, respectively [11]. A review of the epidemiology of colistin resistance in *A. baumannii* isolates found that Southeast Asian and Eastern Mediterranean countries had higher levels of resistance than other regions of the globe. Although different resistance rates have been reported in various parts of the world, the average is stated to be 11.2% (Germany 0.2%, United Kingdom 2.3%, India 8.2%, China 11.8%, and Lebanon 17.5%) [12]. In a meta-analysis examining the prevalence of colistin resistance in *A. baumannii* strains, it was demonstrated that Iraq and Greece exhibited the highest levels of resistance to colistin, with rates of 19% and 18%, respectively. It has been documented that rates of colistin resistance increased during the Covid-19 pandemic. The authors indicated that colistin resistance rates in *A. baumannii* isolates increased in certain countries, including France, Israel, and the United Arab Emirates [13].

The high resistance rates to colistin prove the crucial need to accurately determine colistin susceptibility in routine clinical microbiology laboratories. However, automated systems, gradient diffusion tests, and disc diffusion tests are unreliable for colistin due to its molecular characteristics. In response, the joint CLSI-EUCAST polymyxin breakpoint working group recommended specific guidelines in 2016 for colistin MIC determination, including the ISO-20776 standard broth microdilution method. Despite its effectiveness, the method is time-consuming and labor-intensive, prompting the development of alternative commercial methods [14]. In this study, the performances of MMS and CBDE tests were evaluated for *A. baumannii, P. aeruginosa,* and *K. pneumoniae* isolates. A categorical agreement of 100% was observed for *A. baumannii, P. aeruginosa,* and a CA of over 98% was observed for *K. pneumoniae* using the MMS test. EA was 87.6, 89.3, and 95.6% for *K. pneumoniae, P. aeruginosa,* and *A. baumannii,* respectively. Previous studies have also shown promising results for alternative methods. For example, Kon et al. reported a sensitivity of 98.5% and a specificity of 99.5% for the MMS test in carbapenem-resistant *A. baumannii* and Enterobacterales [15,16]. Yusuf et al. reported a CA of 96% using the MMS test across 70 clinical isolates [17]. When compared to our results, Matuschek et al. found higher EAs, > 97% for *K. pneumoniae, P. aeruginosa,* and *A. baumannii* isolates, yet lower CAs, 86%, 91%, and 94% for the same isolates, respectively [18]. In this study, the CBDE method demonstrated a CA of 100% for *A. baumannii* and > 95% for *P. aeruginosa* and *K. pneumoniae*. In the literature, various studies have shown that the CBDE method achieves CAs ranging from 94% to 99.5%. For example, Banerjee et al. found a CA of 95% and an EA of 97% for the CBDE method compared to the BMD method [19]. In another comparative study, the CBDE method showed CA rates of 98.6% for Enterobacterales, 99.3% for *P. aeruginosa*, and 93.1% for *Acinetobacter* spp. [5]. Another investigation reported CA, ME, and VME rates of 99.5%, 0%, and 1.11% for CBDE performance with a reference method [20]. Bell et al. found a 100% susceptibility rate using the CBDE method in correlation with molecular genotype results and a specificity of 95.8% [21]. Fenwick et al. reported a positive predictive value of 100% and a negative predictive value of 94.3% for colistin with EDTA Broth Disc Elution (BDE) method [22]. Further studies by Cielo et al. documented a CA of 99.5% with minimal errors using the BDE method compared to the reference method for polymyxin B [20]. Similar to our results, the study by Foldes et al. found 100% CA for the CBDE method when comparing six phenotypic methods with the BMD method for assessing colistin resistance in carbapenem-resistant *Enterobacterales* isolates [23]. In another study of Sharma et al., CBDE method was compared with those of BMD in a total of 125 *A. baumannii* isolates. The EA, CA, sensitivity, and specificity for CBDE were found to be 97.6% (n = 122), 98.4% (n = 123), 100%, and 98.40%, respectively. The percentage of ME was 1.6% (n = 2), and no VME was found [24]. Notable differences in test performance against specific pathogens were observed. While *A. baumannii* and *P. aeruginosa* showed consistent results across both methods, *K. pneumoniae* exhibited greater variability with the CBDE test. This variability could be attributed to intrinsic resistance mechanisms or variations in colistin uptake and activity among these organisms.

In our study we found a CA of > 95% for *K. pneumo-*

niae, P. aeruginosa, and A. baumannii isolates and an ME of 1,5% for *K. pneumoniae* with no VME. According to ISO, an acceptable performance requires CA ≥ 90% and VME and ME ≤ 3%. The FDA requires VME ≤ 1.5%. For our isolates, VME, ME, and CA were acceptable by both standards [25]. The MMS test has the advantage of being FDA-approved and widely validated across multiple laboratories, ensuring consistency and reliability in results. The CBDE test offers advantages in terms of ease of use and cost-effectiveness, making it more accessible for smaller or less-funded laboratories. It does not require specialized equipment or reagents. The CBDE test is less resource-intensive and potentially more adaptable to variable laboratory settings, particularly in resource-limited environments. However, its novelty means that there is less widespread validation compared to CBMD.

In conclusion, while both the CBDE and MMS methods show promise in colistin susceptibility testing, their application may vary depending on laboratory resources and specific bacterial pathogens. Further research and validation are necessary to ensure optimal use in clinical settings.

Ethical Approval:

The study was approved by Marmara University´s Ethics Committee, Clinical Research Ethics Committee (approval date - no. 08.09.2023.1113).

Declaration of Interest:

The authors declare that they have no conflicts of interest.

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