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COMPARISON OF DISC DIFFUSION, AGAR DILUTION, AND BROTH MICRODILUTION METHODS FOR DETECTION OF COLISTIN RESISTANT ENTEROBACTERIACEAE AT MINIA UNIVERSITY HOSPITALS, EGYPT

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Abstract:

Background: With widespread increasing in multidrug resistant Gram-negative bacteria, the use of colistin has increased. Colistin use is associated with high rates of neurotoxicity and nephrotoxicity even at optimal doses. As such, determination of MIC of an infecting organism and categorical interpretation is of significant clinical value. In this study, we compared three different methods for colistin susceptibility testing using a set of Enterobacteriaceae isolates that included colistin-resistant strains.

Methods: The colistin resistance of 372 Enterobacteriaceae isolates collected from different clinical cases were detected by disc diffusion method with determination the MICs by both agar dilution (AD) and broth microdilution (BMD) as the gold standard using the new clinical breakpoints for colistin approved by the Clinical and Laboratory Standards Institute (CLSI). The performance of disc diffusion and AD were evaluated versus reference BMD. Essential agreement (EA), category agreement (CA), very major error (VME), and major error (ME) were calculated for comparison.

Results: Thirty-six of 372 (9.7%) included isolates were found to be resistant to colistin by the reference BMD. The rates of very major errors for AD and disc diffusion were 0.0% and 2.78%, respectively. For the 336 isolates found sensitive by reference BMD, the rates of major errors by AD and disc diffusion were 1.5% and 2.38%, respectively. By AD *Escherichia* and *Klebsiella* spp. showed the highest performance characteristics that met the required standard, but *Citrobacter* spp. met the required standard in EA and VME and *Enterobacter* spp. met the required standard in VME only.

Conclusion: Agar dilution method showed good concordance with BMD especially for *Escherichia* and *Klebsiella* spp. The disk diffusion method can be useful for initial screening in diagnostic laboratories.

Keywords: Colistin; Enterobacteriaceae; Broth microdilution; MIC

Introduction

The ascending increase in antibiotic resistance that emerged in the 1970s among Gram-negative bacteria is becoming a critical global crisis [(1)].

There is now evidence by the WHO and US Centers for Disease Control and Prevention describing a global crisis and an impending catastrophe of a return to the pre-antibiotic era [(2)].

These serious concerns have been catalyzed by the rapid increase in carbapenem-resistant Enterobacteriaceae [(3)].

With the global increase in carbapenem-resistant Enterobacteriaceae, in addition to lack of new antibiotics against gram-negative pathogens, severe infections due to multidrug-resistant bacteria have led to a reevaluation of old antibiotics such as colistin that has gained clinical value as a last-line drug effective against nearly all multidrug-resistant gram-negative bacteria [(4)].

Colistin belongs to the family of polymyxins with broad-spectrum activity against Gram negative bacteria, including most species of the family Enterobacteriaceae. It is a cyclic polycationic peptide which interacts

with the negatively charged lipopolysaccharide in the outer membrane causing its disruption with increase in the outer membrane permeability and subsequently cell death [5, 6].

The increased and inappropriate medical uses of colistin will drive the emergence and dissemination of colistin-resistant and possibly even pan-drug-resistant Enterobacteriaceae [6].

Colistin resistance is most frequently observed in *Escherichia coli*, but is present in various genera, including Klebsiella, Salmonella, Shigella, and Enterobacter [1].

High rates of renal and central nervous system toxicity are associated with colistin use even at optimal doses so, knowledge of an infecting organism's MIC and categorical interpretation is of significant clinical value when using these toxic agents [7].

Disk diffusion method remains the most commonly used technique in clinical microbiology laboratories but not recommended for detecting colistin sensitivity due to poor and slow diffusion of the large colistin molecule through agar that is associated with small zones of growth inhibition and significant assay variation, negating use of this method for susceptibility testing [8].

There was no U.S. Food and Drug Administration (FDA)-cleared tests exist for colistin, as the FDA does not recognize any clinical breakpoints for the polymyxins. The analytical performance of research using only disks and gradient strips has been poor, so the Clinical and Laboratory Standards Institute (CLSI) and the European Committee on Antimicrobial Susceptibility Testing (EUCAST) agree that the only validated test method for the polymyxins is reference broth microdilution (BMD), which is performed in very few clinical laboratories [9].

In 2019, CLSI approved clinical breakpoints of colistin for the Enterobacteriaceae and set intermediate and resistant interpretive categories by ≤2 μg/ml and ≥4 μg/ml respectively with no susceptible category for these drugs [10].

In the present study, we evaluated the accuracy of disk diffusion and agar dilution (AD) methods against BMD as the standard validated method for detection of colistin resistance in Enterobacteriaceae among different clinical isolates in Egypt.

Material and methods

This cross-sectional study was performed during the period between June 2019 and January 2021 in the Medical Microbiology and Immunology Department, Faculty of Medicine, Minia University. All samples in this study were from cultures obtained as part of routine care for hospitalized infected patients. The study followed the Helsinki declarations and was approved by the Ethical committee in the faculty of Medicine, Minia University. Informed consents were obtained from each patient.

Bacterial isolates:

Four hundred fourteen Enterobacteriaceae isolated from unique patients. Samples were obtained from different clinical sources included urine, blood, sputum, and surgical wound of patients admitted at Minia University Hospitals, Egypt. Samples were examined by Gram stain, cultivated on Blood and MacConkey agar (Oxoid, UK), and incubated at 37°C f or 24 hours aerobically. Isolates were identified by standard microbiological techniques (Colonial morphology, Gram stain, oxidase, and the use of several biochemical tests).

The isolates comprised *Escherichia* spp. (n=178), *Klebsiella* spp. (n=171), *Proteus* spp. (n=43), *Citrobacter* spp. (n=16), and *Enterobacter* spp. (n=7) with exclusion of Proteus isolates due to its natural intrinsic resistance to colistin so, the total 372 isolates were included in this study [11].

Bacterial isolates were incubated in tryptone soya broth, preserved in 30% sterile glycerol (Greiner Bio-One, Germany), and stored at -20℃ for further use [12].

Detection of colistin sensitivity:

Disk diffusion method:

disc diffusion susceptibility testing was performed using colistin discs (Oxoid, UK) containing 10μg on Mueller–Hinton agar (Oxoid, UK) plates according to the guidelines of CLSI [13]. The disc zone diameters were interpreted according to criteria of Gales and his colleagues in 2001 as resistant ≤ 11 mm and susceptible ≥ 14 mm [14].

Determination of MIC of colistin in by BMD and AD methods:

BMD panels were prepared in untreated 96-well sterile polystyrene microplates (Evergreen Scientific, Los Angeles, CA). A 1,000- μg/ml stock solution of reagent-grade colistin sulfate (Sigma-Aldrich, St. Louis, MO) was prepared fresh in sterile deionized water. Serial dilutions were made in either Mueller-Hinton broth (Difco, BD Diagnostics, Sparks, MD) for BMD or Mueller-Hinton Agar for AD. Two-fold dilutions of colistin concentrations ranged from 0.5 to 128 μg/ml were tested. Three to 5 isolated colonies of an 18- to 24-h culture grown on blood agar were selected for testing. Standardized organism suspensions prepared

in normal saline and further diluted in sterile water prior to the inoculation. The final concentration of organisms tested by BMD was approximately 3×10^5 to 5×10^5 CFU/ml, and that for AD was 10^4 CFU/spot. Tests were incubated for 16 to 20 h at 35°C in ambient air and were examined visually by two independent observers. [13]

Escherichia coli ATCC25922 was used as quality control and MIC of colistin were measured only when the growth control was acceptable.

We repeat testing of BMD, AD, and disk diffusion of colistin for all strains displaying resistance to colistin by the reference BMD method. Individual test procedures (agar dilution or disk diffusion) were repeated for reproducibility if they displayed very major or major errors.

Analysis of results

The results from discs diffusions were compared with those obtained by the reference BMD method. A very major error (VME) denoted a false-susceptible result, whereas a major error (ME) denoted a false-resistance result. All other errors were defined as minor errors [15].

VMEs rates were calculated using the number of resistant isolates as the denominator, and MEs rates were calculated using the number of susceptible isolates as the denominator while minor errors rates were calculated using the total number of tested isolates as the denominator [16].

Essential agreement (EA) between AD and BMD was calculated by the percentage of isolates with MICs within 1 doubling dilution from the reference method MIC. Categorical agreement (CA) was calculated by the percentage of isolates with MICs with the same categorical interpretation using all isolates tested as the denominator [16].

CA was calculated using the recently approved CLSI colistin breakpoints (intermediate ≤ 2 μg/ml and resistant ≥ 4 μg/ml). Due to there is no susceptible category, results that were defined as "intermediate" were treated as "susceptible" for the purpose of performance calculations [17].

Unacceptable levels were greater than 1.5% for VME, >3% for ME, >10% for minor errors, and < 90% for CA and EA [16].

Results

In the present study, a total of 372 Enterobacteriaceae isolates were included. They comprise *Escherichia* spp. 47.8% (n=178), *Klebsiella* spp. 46% (n=171), *Citrobacter* spp. 4.3% (n=16) and *Enterobacter* spp. 1.9% (n=7).

Colistin antimicrobial activity

The MICs of quality control strain were all within the expected reference ranges specified by CLSI M100-S30. The activity of colistin against the 372 nonduplicate Enterobacteriaceae strains as tested by BMD is shown in Table 1. Thirty-six isolates (9.7%) were resistant to colistin. Colistin exhibited excellent activity against *Enterobacter* spp. and *Citrobacter* spp. (MIC90 = 1 μg/ml). In contrast, colistin was less active against *Klebsiella* spp. and *Escherichia* spp. (MIC90 = 4 μg/ml).

Comparison of disc diffusion with reference BMD

Figures 1 presents the scattergram of Enterobacteriaceae isolates tested by BMD and disc diffusion methods for colistin. When zone diameters were interpreted according to provisional zone diameter breakpoints (R ≤11 mm and S ≥14 mm), one VME (2.78%), eight ME (2.38%), and four minor errors (1.07%) were observed. This result met the required standard except for VME.

Comparison of agar dilution with reference BMD

Performance characteristics between agar dilution and the reference BMD method among total isolates showed that EA was 95.7% with 98.7 CA, 0% VME, and 1.5% ME. This result met the required standards. For individual genera, *Escherichia* and *Klebsiella* spp. showed the highest performance characteristics that met the required standard; *Citrobacter* spp. met the required standard in EA and VME; *Enterobacter* spp. met the required standard in VME only as shown in table 2 and figure 2.

Discussion

The use of colistin for the treatment of serious infections caused by multidrug-resistant bacilli has increased in many countries. The extensive or inadequate usage of colistin may lead to emergence of colistin resistance among species that usually susceptible [18].

In our study, colistin showed resistance rate 9.7% among total 372 Enterobacteriaceae isolates with higher activity against *Enterobacter* spp. and *Citrobacter* spp. than against *Escherichia* spp. and *Klebsiella* spp. Resistance rates varied from 0% in *Enterobacter* spp. and *Citrobacter* spp. to 10.1 and 10.5% in *Escherichia* spp. and *Klebsiella* spp. respectively. This result agreed with other studies by who found colistin resistance rate between 6.7%, and 12% respectively [19-21]. On the other hand, higher percentage of colistin resistance were reported by 20.8% and 23.1% of *E. coli* isolated from Assiut and Minia University

Hospitals, Egypt, respectively [22]. Lower percentage of colistin resistance were reported by in a rate of 3% and 3.8% in some studies [23, 24].

As the result of the increasing use of colistin for the treatment of serious infections and the rapid emergence of resistance to this antibiotic in some countries, accurate susceptibility test results are essential [25]. Susceptibility testing for colistin is afflicted by different factors, such as the lack of consensus regarding breakpoints for resistance between the CLSI and the EUCAST; the poor diffusion of colistin in the agar; and the lack of correlation between different methods for the investigation of colistin susceptibility [26]. The disc diffusion method is one of the most frequently used techniques in microbiology laboratories however, resistance to colistin is poorly detected by this method regardless of the criteria used: CA-SFM, BSAC, and Product literature [14]. The objective of our study was to evaluate three methods of colistin susceptibility testing with consideration the BMD to be the reference method. In our study, we evaluated disc diffusion methods using colistin discs 10µg and the zone diameters were interpreted according to criteria of Gales *et al*. Only 2.38% of ME and 1.07% of minor errors were detected but an unacceptable rate of VME 2.78% was found. This result in agreement with other several studies that have found disc diffusion to be an unreliable method to measure susceptibility to colistin with unacceptable high rate of very major errors varied from five to 11% has been reported in these studies [14, 27, 28] So, we recommend using of disk diffusion method for screening for colistin resistance with conformation of the resistance by BMD.

In the current study, we found a good concordance between AD and BMD, with ME rate of 1.5% had a specificity of 98.5%. All resistant isolates by BMD were detected by AD with no VME and 100% sensitivity. This result agreed with many other results that reported AD as a reliable method for colistin MIC determination [17, 25, 29]. So, AD method is suitable for batch testing of large number of strains.

Conclusion

The clinical use of colistin must be based on validated in vitro susceptibility results due to its the potential toxicity. The disk diffusion method remains an unreliable susceptibility testing method for colistin in Enterobacteriaceae and can be used for initial screening. The dilution-based methods such as BMD and AD should be used for testing whenever parenteral use of the colistin is considered in clinical practice. AD method can be used effectively for detection of colistin resistance especially for batch testing of large number of strains.

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Disclosure

There are no conflicts of interest in this work.

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Table 1: MIC distribution by BMD for the 372 strains of Enterobacteriaceae

Organism (no. of isolates)	MIC50 (µg/ml)	MIC90 (µg/ml)	Resistance N (%)	No. of isolates with MIC (µg/ml)									
				≤0.5	1	2	4	8	16	32	64	128	>128
***Escherichia spp.* (n=178)**	1	4	18 (10.1)	84	75	1	5	6	1	0	6	0	0
***Klebsiella spp.* (n=171)**	0.5	4	18 (10.5)	112	38	3	6	8	1	2	0	1	0
***Citrobacter* spp. (n=16)**	0.5	2	0 (0)	9	3	4	0	0	0	0	0	0	0
***Enterobacter* spp. (n=7)**	1	2	0 (0)	3	2	2	0	0	0	0	0	0	0
Total isolates (n=372)	0.5	2	36 (9.7)	208	118	10	11	14	2	2	6	1	0

Abbreviations: MIC, minimum inhibitory concentration; BMD, broth microdilution; MIC50/90, MIC for 50 and 90% of the strains, respectively; N (%), number of isolates and percentage.

Table 2:Comparison of performance characteristics to colistin between agar dilution and BMD method for the four genera of Enterobacteriaceae

Organism	Method	Total	Resistant	Sensitive	EA N (%)	CA N (%)	VME N (%)	ME N (%)
Escherichia spp.	BMD	178	18	160				
	AD		18	160	168 (94.4%)	178 (100%)	0 (0%)	0 (0%)
Klebsiella spp.	BMD	171	18	153				
	AD		19	152	166 (97.1%)	170 (99.4%)	0 (0%)	1 (0.7%)
***Citrobacter* spp.**	BMD	16	0	16				
	AD		2	14	16 (100%)	14 (87.5%)	0 (0%)	2 (12.5%)
***Enterobacter* spp.**	BMD	7	0	7				
	AD		2	5	6 (85.7%)	5 (71.4%)	0 (0%)	2 (28.6%)
Total isolates	BMD	372	36	236				
	AD		41	231	356 (95.7%)	367 (98.7%)	0 (0%)	5 (1.5%)

Abbreviations: EA,Essential agreement; CA, Category agreement; VME, Very major error; ME, Major error; BMD, broth microdilution; AD, agar dilution; N (%), number of isolates and percentage.

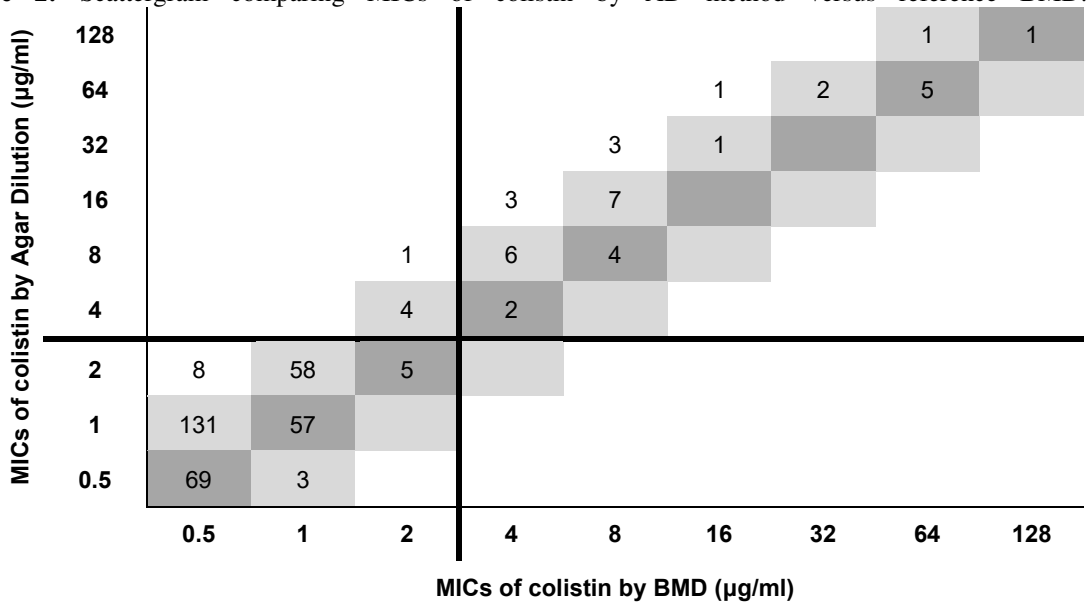
Figure 1: Scattergram comparing results of disc diffusion with reference BMD.

Colistin zone diameter (mm)									
20	16	1	1						
19	64	20							
18	88	72	4						
17	21	8		1					
16	9	1							
15		5	2						
14	9	3							
13	1	2							
12		1							
11			2	2					
10		5	1	8	14	2	2	6	1
	0.5	**1**	**2**	**4**	**8**	**16**	**32**	**64**	**128**

Colistin MIC by BMD (µg/ml)

The area between horizontals solid lines represents the breakpoint values of susceptibility to colistin established by Gales *et al*., (susceptible (S) ≥14 mm, resistant (R) ≤11 mm); The vertical solid line represents the breakpoints for colistin MIC (S ≤ 2 µg/ml and R ≥ 4 µg/ml); MIC, minimum inhibitory concentration; BMD, broth microdilution.

Figure 2: Scattergram comparing MICs of colistin by AD method versus reference BMD.



The vertical and horizontal solid lines represent the breakpoints for colistin MIC (S ≤ 2 µg/ml and R ≥ 4 µg/ml); MIC, minimum inhibitory concentration; BMD, broth microdilution; AD, agar dilution.