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Comparative Evaluation of the VITEK 2, Disk Diffusion, Etest, Broth Microdilution, and Agar Dilution Susceptibility Testing Methods for Colistin in Clinical Isolates, Including Heteroresistant *Enterobacter cloacae* and *Acinetobacter baumannii* Strains[▽]

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Increasing antibiotic resistance in gram-negative bacteria has recently renewed interest in colistin as a therapeutic option. The increasing use of colistin necessitates the availability of rapid and reliable methods for colistin susceptibility testing. We compared seven methods of colistin susceptibility testing (disk diffusion, agar dilution on Mueller-Hinton [MH] and Isosensitest agar, Etest on MH and Isosensitest agar, broth microdilution, and VITEK 2) on 102 clinical isolates collected from patient materials during a selective digestive decontamination or selective oral decontamination trial in an intensive-care unit. Disk diffusion is an unreliable method to measure susceptibility to colistin. High error rates and low levels of reproducibility were observed in the disk diffusion test. The colistin Etest, agar dilution, and the VITEK 2 showed a high level of agreement with the broth microdilution reference method. Heteroresistance for colistin was observed in six *Enterobacter cloacae* isolates and in one *Acinetobacter baumannii* isolate. This is the first report of heteroresistance to colistin in *E. cloacae* isolates. Resistance to colistin in these isolates seemed to be induced upon exposure to colistin rather than being caused by stable mutations. Heteroresistant isolates could be detected in the broth microdilution, agar dilution, Etest, or disk diffusion test. The VITEK 2 displayed low sensitivity in the detection of heteroresistant subpopulations of *E. cloacae*. The VITEK 2 colistin susceptibility test can therefore be considered to be a reliable tool to determine susceptibility to colistin in isolates of genera that are known not to exhibit resistant subpopulations. In isolates of genera known to (occasionally) exhibit heteroresistance, an alternative susceptibility testing method capable of detecting heteroresistance should be used.

The polymyxins are a group of polypeptide antibiotics that were first isolated in 1947 from a spore-bearing soil bacillus (*Bacillus polymyxa*). Several chemically different polymyxins (A to E) could be isolated from different strains of this bacillus (19). Only polymyxin B and polymyxin E (colistin) have been used clinically. Systemic use of colistin was restricted, mainly because of reports of serious nephrotoxicity and the emergence of alternative, less toxic antibiotics. Polymyxin B use has continued in the topical treatment of skin, ear, and ocular diseases. Increasing antibiotic resistance in gram-negative bacteria has recently renewed interest in colistin as an intravenous therapeutic option. Colistin is now increasingly being used for life-threatening infections with multidrug-resistant gram-negative bacteria (6, 7, 13, 14). The increasing use of colistin necessitates the availability of rapid and reliable methods for colistin susceptibility testing.

Disk diffusion is a commonly used method for measuring colistin susceptibility. However, evaluation of in vitro susceptibility testing methods for colistin has shown testing errors with various disk diffusion methods compared to MIC-based methods (8, 10, 16, 20). Excellent correlations between the Etest and the broth microdilution and agar dilution tests were demonstrated, suggesting that methods based on MICs, rather than disk diffusion methods, should be used to determine susceptibility to colistin (4, 8, 9, 16, 21). Automated systems performing rapid identification and antimicrobial susceptibility testing are increasingly being used. A recent validation study by Tan and Ng evaluated the performance of the colistin susceptibility test contained in the VITEK 2 automated system compared to agar dilution (22). Based on their data, the VITEK 2 colistin test was considered to be an unreliable method for colistin susceptibility testing (22).

In susceptibility testing methods using an agar-based medium, the sizes of the zones of inhibition depend on many variables (e.g., the antimicrobial agent, disk content, and inoculum). One of the most critical variables is the culture medium. From early experiences with the CLSI method, it was clear that different batches of Mueller-Hinton (MH) agar affected the interpretation of susceptibility (17). Significant differences in medium performance were noted for the aminoglycosides, imipenem, and colistin (1). To circumvent this problem, the British Society for Antimicrobial Chemotherapy (BSAC) published a standardized method of disk susceptibility testing

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TABLE 1. Distribution of MIC ranges of isolates in the reference broth microdilution test

Species	Total no.[a] of isolates	No.[a] of isolates with MIC (μg/ml):									
		<0.5	0.5	1.0	2.0	4.0	8.0	16	32	64	>64
E. coli	9	9									
K. pneumoniae	10	3									7
K. oxytoca	4	4									
C. freundii	10	1						5	4		
P. aeruginosa	10			1	5		4				
P. fluorescens	3	1		1							1
A. baumannii	7	4		1	1						1[b]
Acinetobacter spp.	2	1						1			
A. lwoffii	1	1									
S. maltophilia	9						5	4			
E. cloacae	10	1								3	6[b]
Enterobacter spp.	5	4								1	
Klebsiella spp.[c]	10	10									
Enterobacter spp.[c]	9	9									
C. freundii[c]	3	3									

[a] Absolute numbers are shown.
[b] Isolates showing heteroresistance. Heteroresistance was observed as either the presence of skipped wells or trailing end points.
[c] Isolates from intensive-care unit patients not receiving SDD/SOD.

TABLE 2. Percentages of isolates (excluding heteroresistant *E. cloacae* isolates) tested with various susceptibility testing methods showing a difference in $\log_2$ dilutions compared to results of the reference broth microdilution method

Test	% Of isolates showing $\log_2$ dilution difference of:								
	>−3	−3	−2	−1	0	1	2	3	>3
Agar dilution									
MH				4.0	85.4	7.4	3.2		
ISO[a]				18.1	73.4	6.4	2.1		
Etest MH			2.1	15.8	38.9	17.9	20.0	5.3	
Etest ISO	1.0	2.1	1.0	11.6	33.7	18.9	18.9	4.2	6.3
VITEK2			1.1	3.4	71.3	18.4	5.7		

[a] ISO, Isosensitest.

using a medium (Isosensitest agar) with a semidefined composition (2, 15). However, Isosensitest agar from different manufacturers has also been shown to vary considerably (1, 11).

In the present study, we compared seven methods of colistin/polymyxin B susceptibility testing of clinical isolates from intensive-care units where colistin was routinely administered as part of an ongoing trial using selective decontamination of the gastrointestinal tract.

MATERIALS AND METHODS

Bacterial strains. Gram-negative bacteria were isolated from throat swab, sputum, or rectal-swab cultures from patients in an intensive-care unit during a selective digestive decontamination (SDD) or selective oral decontamination (SOD) trial. In brief, patients receiving SDD were treated with intravenous cefotaxime for 4 days. Colistin, tobramycin, and amphotericin B were applied as a daily suspension via a nasogastric tube and applied oropharyngeally using an oral paste. Patients receiving SOD were oropharyngeally treated only with the daily oral paste. A total of 80 bacterial isolates were included: *Escherichia coli* (9 isolates), *Enterobacter cloacae* (10 isolates), *Enterobacter aerogenes* (3 isolates), *Enterobacter asburiae* (1 isolate), *Enterobacter amnigenus* (1 isolate), *Klebsiella pneumoniae* (10 isolates), *Klebsiella oxytoca* (4 isolates), *Citrobacter freundii* (10 isolates), *Pseudomonas fluorescens* (3 isolates), *Acinetobacter baumannii* (7 isolates), *Acinetobacter* spp. (2 isolates), *Acinetobacter lwoffii* (1 isolate), *Stenotrophomonas maltophilia* (9 isolates), and *Pseudomonas aeruginosa* (10 isolates). We also tested 22 gram-negative bacterial strains isolated at a later time in the same intensive-care unit from patients not receiving SDD or SOD. These isolates were *Klebsiella* spp. (10 isolates), *Enterobacter* spp. (9 isolates), and *C. freundii* (3 isolates). The reference strains *E. coli* ATCC 25922 (colistin MIC, 0.25 to 1 μg/ml) and *P. aeruginosa* ATCC 27853 (colistin MIC, 0.25 to 2 μg/ml) were included as quality controls (8).

Disk diffusion. Disk diffusion testing was performed according to the manufacturer's procedures using both polymyxin B disks (Rosco, Taastrup, Denmark) containing 150 μg polymyxin B and colistin disks (Rosco, Taastrup, Denmark) containing 10 μg colistin. Inocula were prepared by suspending colonies from overnight blood agar plates in sterile saline to the turbidity of a 0.5 McFarland standard. Polymyxin B- or colistin-containing disks were dispensed onto the surfaces of inoculated agar plates and incubated at 35°C for 16 to 18 h. We performed the disk diffusion test using both MH agar (Oxoid, Basingstoke, United Kingdom) and Isosensitest agar (Oxoid, Basingstoke, United Kingdom). Interpretation according to the manufacturer's instructions was possible only for the disk diffusion test on MH agar, since zone diameters were available for this medium only. For polymyxin B, the following zone diameters were used for interpretation (Rosco Diagnostica user's guide for Neo-Sensitabs, 2005/2006): rapidly growing bacteria, ≥20 mm, susceptible, 17 to 19 mm, intermediate, and ≤16 mm, resistant; *Acinetobacter* spp. and *S. maltophilia*, ≥22 mm, susceptible, and ≤21 mm, resistant. The following interpretive criteria were used for colistin (Rosco Diagnostica user's guide for Neo-Sensitabs, 2005/2006): rapidly growing bacteria, ≥13 mm, susceptible, 11 to 12 mm, intermediate, and ≤10 mm, resistant; *Acinetobacter* spp. and *S. maltophilia*, ≥13 mm, susceptible, 11 to 12 mm, intermediate, and ≤10 mm, resistant.

Broth microdilution. Broth microdilution testing was carried out according to CLSI procedures using cation-adjusted Mueller-Hinton broth (BBL-Becton Dickinson, Sparks, MD) (4). Colistin sulfate was obtained from Sigma-Aldrich (St. Louis, MO).

Agar dilution. The agar dilution test was performed on MH agar (Oxoid, Basingstoke, United Kingdom) according to the CLSI procedures (4). Performance on Isosensitest agar (Oxoid, Basingstoke, United Kingdom) was according to the BSAC procedures (2). Colistin sulfate was obtained from Sigma-Aldrich (St. Louis, MO).

Etest. The colistin Etest (AB Biodisk, Solna, Sweden) was performed and interpreted according to the manufacturer's procedures. Both MH agar (Oxoid, Basingstoke, United Kingdom) and Isosensitest agar (Oxoid, Basingstoke, United Kingdom) were used in the testing procedure.

VITEK 2. The VITEK 2 susceptibility card AST-N038 (bioMérieux, Marcy l'Etoile, France) containing a colistin susceptibility test was used according to the manufacturer's instructions. Interpretive breakpoints (MIC ≤ 2 μg/ml, susceptible, and MIC ≥ 4 μg/ml, resistant) were used for the VITEK 2.

RESULTS

All isolates were tested using the above-mentioned methods, and the results were compared to those of broth microdilution, as this was considered the reference method. The colistin MIC measurements for the tested ATCC reference strains were within the published quality control ranges. Table 1 shows the MIC distribution of the tested isolates using the reference broth microdilution test.

In Table 2, the results of the various colistin susceptibility testing methods are compared to those of the broth microdilution reference method. Performing the analysis separately for *Enterobacteriaceae* and *Pseudomonas* species did not reveal significant differences. Table 3 shows a comparison between the broth microdilution reference method and the disk diffusion methods for colistin and polymyxin B.

Comparison of agar dilution and broth microdilution. A major difference was found for one *E. cloacae* isolate (MIC of <0.5 μg/ml on MH agar; MIC of >64 μg/ml on Isosensitest agar). This difference was caused by the presence of a relatively resistant subpopulation consisting of 2 to 10 CFU (depending on the colistin concentration in the agar plate) growing on Isosensitest agar and not on MH agar. Minor differences due

TABLE 3. Comparison of disk diffusion testing with the broth microdilution reference method

Species	No. of isolates with reference method MIC (μg/ml)[a] of:		No. of isolates with MH agar disk diffusion result[b] of:					
			Colistin			Polymyxin B		
	≤ 2	≥ 8	S	I	R	S	I	R
E. coli	9		5	4		3	6	
Klebsiella spp.	17	7	10	10	4	13	11	
C. freundii	4	9	5	7	1	11	2	
P. aeruginosa	6	4	10			8	2	
P. fluorescens	2	1	2		1	2	1	
A. baumannii	6	1[c]	2		4 + 1[c]	6		1[c]
Acinetobacter spp.	1	1	1		1	1	1	
A. lwoffii	1		1			1		
S. maltophilia		9	9			5		4
E. cloacae	1	3 + 6[c]	5	1	2 + 2[c]	3		3 + 4[c]
Enterobacter spp.	13	1	11	3		10	4	

[a] For easier comparison, the MICs obtained with the broth microdilution broth reference method have been divided in two categories.
[b] The number of isolates that were sensitive (S), intermediate (I), or resistant (R). Shown are the first measurements with either colistin or polymyxin B.
[c] Heteroresistant isolate.

to relatively resistant subpopulations were also observed for five *E. cloacae* isolates and one *A. baumannii* isolate. However, these resistant subpopulations were observed growing on both MH and Isosensitest agar plates that contained higher concentrations of colistin. Prior passaging of these resistant colonies on sheep blood agar, followed by repetition of the agar dilution test, yielded an identical result. Directly repeating the agar dilution test with these resistant colonies without prior passaging on sheep blood agar demonstrated a completely resistant phenotype (MIC > 64 μg/ml). Comparison of the results of agar dilution testing to those of the broth microdilution method showed high levels of agreement. Differences were found mainly for the heteroresistant *E. cloacae* isolates. MICs measured for the heteroresistant *A. baumannii* isolate agreed completely.

Comparison of VITEK 2 and broth microdilution. Comparison of the VITEK 2 colistin susceptibility test to the broth microdilution reference test showed a high level of agreement, with the exception of the heteroresistant *E. cloacae* isolates, which the VITEK 2 failed to detect. *S. maltophilia* isolates were excluded from the analysis, since the VITEK 2 Advanced Expert System does not interpret the measurements for *S. maltophilia*.

Comparison of Etest and broth microdilution. Comparing the Etest method to the reference broth microdilution method showed relatively high levels of agreement. The Etest on MH agar failed to detect relatively resistant subpopulations of four *E. cloacae* isolates. The resistant subpopulations of the *E. cloacae* isolates that were missed in the Etest on MH agar were detected in the Etest on Isosensitest agar. Here, 2 to 10 colonies were found to grow within the inhibition zone. The Etest on Isosensitest agar seems to be a more sensitive method to detect resistant subpopulations.

Comparison of disk diffusion and broth microdilution methods. Interpretation of the disk diffusion zone diameters according to the manufacturer's procedures was possible only for measurements on MH agar. The results showed a low level of reproducibility. For polymyxin B, an agreement of only 58% was observed between first and second measurements. On MH agar, resistant colonies growing within the polymyxin B inhibition zone were observed for four *E. cloacae* isolates and one *A. baumannii* isolate. Prior passaging of these resistant colonies on sheep blood agar, followed by repetition of the disk diffusion test, showed an identical result. Directly repeating the disk diffusion test with these resistant colonies without prior passaging on sheep blood agar demonstrated complete resistance. For five *E. cloacae* isolates and one *A. baumannii* isolate, resistant colonies growing within the polymyxin B inhibition zone were observed on Isosensitest agar, reflecting the tendency of Isosensitest agar to be a better medium for detecting heteroresistance.

Testing on MH agar showed resistant colonies within the colistin inhibition zone in two *E. cloacae* isolates and one *A. baumannii* isolate. Resistant colonies growing within the colistin inhibition zone were found for six *E. cloacae* isolates and one *A. baumannii* isolate using disk diffusion testing on Isosensitest agar, again reflecting the tendency of Isosensitest agar to be a better medium for detecting heteroresistance.

To compare the disk diffusion test results with those of the broth microdilution reference test, the MICs obtained with the broth microdilution reference test were divided into two categories (≤2 μg/ml and ≥8 μg/ml). If MICs of ≤2 μg/ml are considered sensitive and MICs of ≥8 μg/ml resistant, low levels of agreement were found (Table 2).

DISCUSSION

Infections caused by multi drug-resistant gram-negative bacteria are increasing worldwide. The increasing resistance to many antibiotics limits a lot of therapeutic options and has led to an increase in the use of intravenous colistin (6, 7, 13, 14). Therefore, reliable methods to test susceptibility to colistin are needed in order to predict the clinical response adequately. Breakpoints for colistin resistance are available for the BSAC testing procedures (MIC ≤ 4 μg/ml, susceptible, and MIC ≥ 8 μg/ml, resistant). Other interpretive breakpoints exist. The Société Française de Microbiologie provides different breakpoints (MIC ≤ 2 μg/ml, susceptible, and MIC ≥ 4 μg/ml, resistant) (18). The U.S. CLSI provides interpretive breakpoints for *P. aeruginosa* (5) and *Acinetobacter* spp. (MIC ≤ 2 μg/ml, susceptible, and MIC ≥ 4 μg/ml, resistant) (4). At present, it is still unclear which breakpoints are most appropriate. The currently available breakpoints for colistin are based on colistin sulfate. However, for clinical intravenous applications, colistin methanesulfonate is used.

The objective of our study was to evaluate seven methods of colistin susceptibility testing. We considered the broth microdilution method to be the reference method, as was done previously (3). The CLSI standard testing procedures are firmly established and have been used in many studies. The broth microdilution test was able to detect the heteroresistant isolates. Agar dilution testing using either MH agar or Isosensitest agar was performed. We have also used BSAC testing procedures with semidefined Isosensitest agar, as this has been advocated by some authors (1, 2). Agar dilution methods using either MH agar or Isosensitest agar showed highly concordant results. We found no significant differences in the performance of either of these test media. Easier detection of resistant subpopulations of *E. cloacae* isolates in our study was an advantage of using the Isosensitest agar. For one *E. cloacae* isolate, the resistant colonies found on Isosensitest agar were not detected using agar dilution testing on MH agar. This reflects the seemingly inherent quality of Isosensitest agar to be more sensitive in the detection of resistant subpopulations.

Polymyxins diffuse poorly in agar, resulting in relatively small zones of inhibition. This complicates the differentiation between susceptible and resistant isolates. Several studies have found disk diffusion to be an unreliable method to measure susceptibility to colistin (8, 16, 20). We have also found high error rates, as well as a low level of reproducibility between subsequent measurements for the same isolate. Both polymyxin B- and colistin-containing disks were used in our study. Since there is complete cross-resistance between colistin and polymyxin B, testing either colistin or polymyxin B is not expected to make a difference. Polymyxin B was used in this study, as well, because we routinely test for polymyxin B sensitivity in our laboratory in clinical situations possibly requiring topical application of polymyxin B. Comparison to the reference broth microdilution method was omitted because it was not clear which breakpoints would be appropriate to use.

In previous studies, the Etest showed excellent agreement with agar dilution (16) and broth microdilution (3) methods. Comparing the colistin Etest method to broth microdilution methods showed concordant results. The Etest on MH agar showed somewhat better results than the Etest on Isosensitest agar. Resistant subpopulations of four *E. cloacae* isolates were missed using MH agar, again reflecting the higher sensitivity of Isosensitest agar to detect resistant subpopulations.

So far, there has been only one report in the literature about the performance of automated systems, such as the VITEK 2, for colistin susceptibility testing (22). Tan and Ng considered the VITEK 2 colistin susceptibility test to be an unreliable method (22). In contrast, the VITEK 2 colistin susceptibility test performed well in our study. We found a high level of agreement with the reference broth microdilution method. The main disadvantage of the VITEK 2 is its low sensitivity to detect resistant subpopulations of *E. cloacae* isolates. However, the resistant subpopulations of the *A. baumannii* isolates were detected in the VITEK 2, as well as in the other methods for colistin susceptibility testing. The VITEK 2 colistin susceptibility test can therefore be considered to be a reliable tool to determine susceptibility to colistin in isolates that do not exhibit resistant subpopulations. Although the VITEK 2 is an easy-to-use susceptibility testing method in the setting of a routine diagnostic microbiology laboratory, care should be taken in the interpretation of the results for genera in which heteroresistance has been described. For genera in which occasional heteroresistance has been described, an alternative testing method capable of detecting resistant subpopulations should be used.

Resistant colonies, representing a colistin-resistant subpopulation, were observed for six *E. cloacae* isolates and for one *A. baumannii* isolate. Assaying these resistant colonies directly for colistin susceptibility showed them to be completely resistant. Prior passaging of these resistant colonies on sheep blood agar, followed by retesting, showed an identical result, indicating the resistance to be induced upon exposure to colistin rather than being caused by stable mutations. Heteroresistance to colistin in clinical isolates of *A. baumannii* has been described previously (12). The authors suggested that monotherapy with colistin for treatment of infections caused by heteroresistant *A. baumannii* may be problematic. The achieved concentrations of colistin in plasma may be substantially lower than those required to eradicate the more resistant subpopulations of *A. baumannii*. Therefore, care is required in the use of colistin as monotherapy in infections with *A. baumannii*. Our study is the first to report on heteroresistance in *E. cloacae* isolates. We propose to extend the suggestion of Li et al. to heteroresistant variants of *E. cloacae* isolates, as well. As yet, it is not clear whether these colistin-resistant subpopulations are truly clinically significant or merely represent in vitro artifacts. It remains to be investigated whether colistin-resistant subpopulations exist among other bacteria, as well.

We tested bacterial isolates collected from patient materials during an SDD or SOD trial in an intensive-care unit. The results showed relatively high levels of resistance to colistin. This is probably caused by selection of colistin-resistant bacterial isolates. We have also tested isolates from the same intensive-care unit when no SDD or SOD was applied. In these isolates, no colistin resistance was found, indicating a higher level of resistance during the SDD or SOD trial. Whether previous exposure to colistin in the SDD or SOD trial affected the selection of heteroresistant isolates remains to be elucidated.

In conclusion, the disk diffusion method is an unreliable method to measure susceptibility to colistin. The VITEK 2 colistin susceptibility test is a reliable and easy-to-use tool to determine susceptibility to colistin in isolates of genera that are known not to exhibit heteroresistance. For isolates of genera that are known to (occasionally) exhibit heteroresistance, a testing method that is able to detect heteroresistance should be used. The Etest and agar dilution test are also reliable methods to measure colistin susceptibility and have the advantage that they can detect heteroresistant isolates. Heteroresistance was observed in several *E. cloacae* and *A. baumannii* isolates. Isosensitest agar was a better medium to detect heteroresistance than MH agar. Further investigation is needed to determine the clinical significance of these heteroresistant isolates.

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