Comparative Evaluation of Colistin Susceptibility Testing Methods among Carbapenem-Nonsusceptible *Klebsiella pneumoniae* and *Acinetobacter baumannii* Clinical Isolates

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We compared six colistin susceptibility testing (ST) methods on 61 carbapenem-nonsusceptible *Klebsiella pneumoniae* ($n = 41$) and *Acinetobacter baumannii* ($n = 20$) clinical isolates with provisionally elevated colistin MICs by routine ST. Colistin MICs were determined by broth microdilution (BMD), BMD with 0.002% polysorbate 80 (P80) (BMD-P80), agar dilution (AD), Etest, Vitek2, and MIC test strip (MTS). BMD was used as the reference method for comparison. The EUCAST-recommended susceptible and resistant breakpoints of ≤ 2 and > 2 $\mu\text{g/ml}$, respectively, were applied for both *K. pneumoniae* and *A. baumannii*. The proportions of colistin-resistant strains were 95.1, 77, 96.7, 57.4, 65.6, and 98.4% by BMD, BMD-P80, AD, Etest, MTS, and Vitek2, respectively. The Etest and MTS methods produced excessive rates of very major errors (VMEs) (39.3 and 31.1%, respectively), while BMD-P80 produced 18% VMEs, AD produced 3.3% VMEs, and Vitek2 produced no VMEs. Major errors (MEs) were rather limited by all tested methods. These data show that gradient diffusion methods may lead to inappropriate colistin therapy. Clinical laboratories should consider the use of automated systems, such as Vitek2, or dilution methods for colistin ST.

The increasing occurrence of infections due to multidrug-resistant (MDR) *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacteriaceae* led to the revival of old and neglected antibiotics that may remain active, such as polymyxins (polymyxin B and colistin) (1, 2). Colistin is increasingly being used as a last-resort treatment option for infections caused by MDR organisms (2, 3), particularly carbapenem-resistant (CR) Gram-negative bacteria (4). However, during the last years, increasing colistin resistance emerged worldwide, especially among *Klebsiella pneumoniae* and *A. baumannii* isolates, further limiting treatment options (5–7). In Europe, the evolving colistin resistance is more pronounced in southern countries (notably Greece, Romania, and Italy) (8–10).

Rapid and reliable colistin susceptibility testing (ST) is needed in routine clinical laboratories to allow appropriate therapeutic decision-making. Thus far, few studies have assessed the performance of colistin ST methods, displaying controversial results, and thus, the most accurate one is still challenging (11).

Disk diffusion, commonly used in many clinical laboratories, yielded high error rates compared to MIC-based methods and is considered unreliable for the detection of colistin resistance (12–14). Among commercial methods, gradient diffusion strips are convenient tests for determining colistin MICs, but their performance is not well established. Some studies demonstrated very good correlations between the results of Etest (bioMérieux, Marcy l'Etoile, France) and broth microdilution (BMD) or agar dilution (AD) methods for colistin ST (13–17), while other reports questioned the reliability of Etest (18, 19). Another gradient diffusion test, MIC test strip (MTS) (Liofilchem SRL, Italy), has not been evaluated for colistin ST to the best of our knowledge. Colistin ST using automated methods has been tested in limited studies that tested mainly the performance of the Vitek2 system (bioMérieux) (13, 16, 19).

AD and BMD are widely considered standard methods for

MIC ST (20), which, however, are not convenient for routine clinical laboratories. Additionally, for BMD, technical issues, such as the type or surface pretreatment of microtiter trays, have influenced colistin MICs significantly (3, 21). In particular, colistin displays various levels of adherence to different surfaces used for MIC trays, such as polystyrene, resulting in reduced antibiotic concentrations actually being present under experimental conditions (22). The addition of the surfactant polysorbate 80 (P80) to BMD (BMD-P80) minimized colistin adhesion to BMD panels and thus significantly reduced colistin MICs, affecting mainly bacteria with relatively low MICs (≤ 2 $\mu\text{g/ml}$) (18, 21, 23). Nevertheless, the CLSI does not recommend the use of P80 for colistin ST by BMD (24). In addition, according to recent observations, P80 exhibited a synergistic effect with colistin, perhaps enhancing its interaction with the bacterial cell membrane (J. D. Turnidge, presented at the ESCMID Conference on Reviving Old Antibiotics, Vienna, Austria, 22 to 24 October 2014). Hence, further studies on the accuracy of BMD-P80 in determining the colistin susceptibility of Gram-negative bacteria are needed.

The breakpoints for colistin developed by various organizations differ (24, 25), further complicating the interpretation of

Received 11 April 2015 Returned for modification 9 May 2015

Accepted 17 May 2015

Accepted manuscript posted online 26 May 2015

Citation Dafopoulou K, Zarkotou O, Dimitroulia E, Hadjichristodoulou C, Gennimata V, Pournaras S, Tsakris A. 2015. Comparative evaluation of colistin susceptibility testing methods among carbapenem-nonsusceptible *Klebsiella pneumoniae* and *Acinetobacter baumannii* clinical isolates. *Antimicrob Agents Chemother* 59:4625–4630. doi:10.1128/AAC.00868-15.

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doi:10.1128/AAC.00868-15

antimicrobial ST results. Additionally, most studies investigating the accuracy of colistin ST methods involved predominantly colistin-susceptible Gram-negative isolates, while they have scarcely been tested on colistin-resistant isolates. In this study, we evaluated various colistin MIC testing methods, such as BMD, BMD-P80, AD, Etest, MTS, and Vitek2, using a collection of carbapenem-nonsusceptible *K. pneumoniae* and *A. baumannii* isolates with provisionally elevated colistin MICs according to routine ST.

(Part of this work was presented at the 54th Annual Inter-science Conference on Antimicrobial Agents and Chemotherapy [ICAAC], Washington, DC, 5 to 9 September 2014 [30].)

MATERIALS AND METHODS

Bacterial isolates. A total of 61 carbapenem-nonsusceptible clinical isolates collected from four tertiary Greek hospitals during 2008 to 2013 were studied. In particular, this collection contained nonduplicate previously characterized KPC, OXA-48, or VIM carbapenemase-producing *K. pneumoniae* ($n = 41$) and OXA-58 or OXA-23 carbapenemase-producing *A. baumannii* ($n = 20$) isolates. The isolates were recovered from patients with bloodstream (42.6%), respiratory tract (16.4%), urinary tract (16.4%), skin and soft tissue (11.5%), and other (13.1%) infections. Colistin was deemed necessary for the treatment of the respective infections; however, the isolates were identified as being colistin nonsusceptible ($\text{MIC} > 2 \mu\text{g/ml}$) by routine laboratory ST, based mainly on the use of automated systems. Due to the existing controversies in colistin ST, to further evaluate the susceptibility results and verify that colistin could not be used for infections caused by these bacteria, the isolates were submitted to the Department of Microbiology at the Medical School of the University of Athens. Prior to use in this study, the isolates had been stored in glycerol stocks at -70°C and subcultured twice before testing.

Susceptibility testing. Colistin MICs were determined by BMD, BMD-P80, AD, Etest, MTS, and Vitek2 methods. Dilution methods were performed according to CLSI procedures (20, 24), using colistin sulfate powder (batch number SLBK0713V; Sigma-Aldrich, St. Louis, MO). Stock solutions of colistin were reconstituted in sterile distilled water, in accordance with the manufacturer's instructions, immediately prior to use. BMD and BMD-P80 MIC determinations (concentration range, 0.125 to 128 $\mu\text{g/ml}$) were conducted by using tissue culture-treated round-bottom polystyrene 96-well trays (Costar 3799; Corning, NY, USA), using a bacterial inoculum of 5×10^5 CFU/ml in cation-adjusted Mueller-Hinton broth (Sigma-Aldrich) without or with the addition of 0.002% P80 (Sigma-Aldrich). For AD, colistin was incorporated into Mueller-Hinton II agar (MHA; Sigma-Aldrich) plates at concentrations of 0.125 to 128 $\mu\text{g/ml}$ and with a final inoculum of 10^4 CFU/spot. Etest and MTS methods, which included the same colistin concentration gradient range (0.016 to 256 $\mu\text{g/ml}$), were performed with MHA plates, according to the manufacturer's instructions. Etest and MTS MICs between 2-fold dilutions were rounded up to the next 2-fold dilution. The Vitek2 AST-EXN8 susceptibility card (bioMérieux), reporting colistin MICs of ≤ 0.5 to $\geq 16 \mu\text{g/ml}$, was employed according to the manufacturer's recommendations. For data analysis, when needed, MICs of $\geq 16 \mu\text{g/ml}$ determined by Vitek2 were considered 16 $\mu\text{g/ml}$. All methods were performed simultaneously with a single inoculum for each strain, with incubation at $35^\circ\text{C} \pm 2^\circ\text{C}$ for 18 to 20 h, and results were reviewed by two independent observers. *Escherichia coli* ATCC 25922 and *P. aeruginosa* ATCC 27853 were used for quality control (24).

Interpretation of results and data analysis. The CLSI provides susceptibility breakpoints for colistin against *A. baumannii* (susceptible, MIC of $\leq 2 \mu\text{g/ml}$; resistant, MIC of $\geq 4 \mu\text{g/ml}$) but not *Enterobacteriaceae* (24). For consistency, EUCAST-recommended breakpoints, which are available for both organisms (susceptible, MIC of $\leq 2 \mu\text{g/ml}$; resistant, MIC of $> 2 \mu\text{g/ml}$) (25) were applied.

Data were analyzed by comparing the results produced by the BMD-P80, AD, Etest, MTS, and Vitek2 methods with those produced by stan-

dard BMD. Essential agreement (EA) was defined as the percentage of MICs within $\pm 1 \log_2$ dilution of the MIC determined by BMD. Categorical agreement (CA) was defined as the percentage of isolates classified in the same susceptibility category by BMD and the method under evaluation. Very major errors (VMEs) denoted a false-susceptible result, and major errors (MEs) denoted a false-resistant result (26). It should be noted that because our collection included mainly colistin-resistant isolates and to avoid overestimation of MEs, for the estimation of errors, the total number of tested isolates was used as the denominator.

Acceptable performance was evaluated according to criteria established by the International Organization for Standardization: $\geq 90\%$ for essential or category agreement and $\leq 3\%$ for VMEs or MEs (27). Statistical analysis was performed by using Student's *t* test, and differences were considered statistically significant at a *P* value of < 0.05 .

RESULTS

Susceptibility to colistin, EA, CA, and errors. The susceptibilities of the study isolates to colistin, $\text{MIC}_{50\text{s}}/\text{MIC}_{90\text{s}}$, EAs, CAs, and errors by each method are presented in Table 1. The colistin MICs per method are shown in Table 2.

In particular, by BMD, 58 isolates (95.1%) were colistin resistant. A trend toward lower colistin MICs was noted for BMD-P80; considerably fewer isolates were colistin resistant (47 isolates; 77%), and the CA with BMD was 82%. AD produced susceptibility results similar to those of BMD, and high rates of CA (91.8%) were observed for both pathogens. Discordant susceptibility rates with serious interpretative errors and unacceptable CA were observed for Etest and MTS (59 and 67.2%, respectively). The *in vitro* activity of colistin was found to be importantly higher by Etest, with only 57.4% of the isolates being classified as resistant; 65.6% of the isolates were classified as resistant by MTS. Vitek2 categorized 98.4% of isolates as colistin resistant, showing excellent CA with BMD (96.7%).

The EA was highest for BMD-P80 (95.1% overall), exceeding the acceptable performance threshold for antimicrobial ST methods. By BMD-P80, 55.7% of the isolates exhibited MICs identical to those determined by BMD, and 39.3% of isolates displayed MICs that were 1 $\log_2$ dilution lower. AD generated rather low EA rates (55.7% overall) and trends toward higher colistin MICs; it produced MICs that were 1, 2, and 3 $\log_2$ dilutions higher than those determined by BMD for 26.2%, 29.5%, and 9.8% of the isolates, respectively. In contrast, Etest produced MICs that were 1, 2, 3, and $> 3 \log_2$ dilutions lower than those determined by BMD for 29.5%, 18%, 19.7%, and 11.5% of the isolates, respectively, resulting in the lowest rate of EA (50.8% overall; 48.8 and 55% for *K. pneumoniae* and *A. baumannii*, respectively). Similarly, MICs obtained by MTS were 1, 2, 3, and $> 3 \log_2$ dilutions lower for 42.6%, 23%, 6.6%, and 4.9% of the isolates, respectively. MTS generated an overall EA rate of 65.6%, with a low EA rate for *K. pneumoniae* isolates (53.7%) but an appropriate EA for *A. baumannii* (EA of 90%). For Vitek2, the EA rate was 78.6% in total; 31.1% of the isolates had MICs equal to those determined by BMD, while 47.5% of the isolates had MICs that were 1 $\log_2$ dilution higher or lower (Table 3).

In terms of errors (Table 1 and Fig. 1), high rates of VMEs (18%) and no MEs were detected for BMD-P80, while AD exhibited 3.3% VMEs and 4.9% MEs. The Etest yielded the highest rates of VMEs overall (39.3%) and limited MEs (1.6%); MTS produced high rates of VMEs (31.1%) and also limited MEs (1.6%). No VMEs were observed for Vitek2; it yielded 3.3% MEs.

TABLE 1 Colistin susceptibilities of isolates and MIC₅₀s/MIC₉₀s determined by the ST methods and EA, CA, and types of errors produced by BMD-P80, AD, Etest, MTS, and Vitek2 compared to BMD

Method and isolate group	No. (%) of isolates		MIC (μg/ml)		No. (%) of isolates with:			
	Susceptible	Resistant	50%	90%	EA	CA	VME	ME
BMD								
All isolates	3 (4.9)	58 (95.1)	8	32				
<i>K. pneumoniae</i>	1 (2.4)	40 (97.6)	8	32				
<i>A. baumannii</i>	2 (10)	18 (90)	4	32				
BMD-P80								
All isolates	14 (23)	47 (77)	4	16	58 (95.1)	50 (82)	11 (18)	0 (0)
<i>K. pneumoniae</i>	6 (14.6)	35 (85.4)	8	32	39 (95.1)	36 (87.8)	5 (12.2)	0 (0)
<i>A. baumannii</i>	8 (40)	12 (60)	4	16	19 (95)	14 (70)	6 (30)	0 (0)
AD								
All isolates	2 (3.3)	59 (96.7)	16	64	34 (55.7)	56 (91.8)	2 (3.3)	3 (4.9)
<i>K. pneumoniae</i>	0 (0)	41 (100)	16	64	23 (56.1)	40 (97.6)	0 (0)	1 (2.4)
<i>A. baumannii</i>	2 (10)	18 (90)	8	128	11 (55)	16 (80)	2 (10)	2 (10)
Etest								
All isolates	26 (42.6)	35 (57.4)	4	8	31 (50.8)	36 (59)	24 (39.3)	1 (1.6)
<i>K. pneumoniae</i>	17 (41.5)	24 (58.5)	4	8	20 (48.8)	23 (56.1)	17 (41.5)	1 (2.4)
<i>A. baumannii</i>	9 (45)	11 (55)	4	8	11 (55)	13 (65)	7 (35)	0 (0)
MTS								
All isolates	21 (34.4)	40 (65.6)	4	4	40 (65.6)	41 (67.2)	19 (31.1)	1 (1.6)
<i>K. pneumoniae</i>	14 (34.1)	27 (65.9)	4	4	22 (53.7)	26 (63.4)	14 (34.1)	1 (2.4)
<i>A. baumannii</i>	7 (35)	13 (65)	4	8	18 (90)	15 (75)	5 (25)	0 (0)
Vitek2								
All isolates	1 (1.6)	60 (98.4)	8	≥16	48 (78.6)	59 (96.7)	0 (0)	2 (3.3)
<i>K. pneumoniae</i>	1 (2.4)	40 (97.6)	≥16	≥16	31 (75.6)	41 (100)	0 (0)	0 (0)
<i>A. baumannii</i>	0 (0)	20 (100)	4	≥16	17 (85)	18 (90)	0 (0)	2 (10)

DISCUSSION

Colistin therapy is commonly necessary for the treatment of serious infections caused by CR Gram-negative bacteria (4). Trends toward elevated colistin MICs have been noted worldwide (5, 8), underlining the importance of accurate colistin susceptibility results. Also, suggestions for the optimal methods to be used for colistin ST have not been formulated by the CLSI. In the present study, we evaluated the performances of six colistin ST methods

against carbapenem-nonsusceptible *K. pneumoniae* and *A. baumannii* clinical isolates with provisionally elevated colistin MICs.

Much debate on the need to add the surfactant P80 to test systems for polymyxins has recently arisen. Overall, the available evidence for the performance of BMD-P80 in testing of isolates with elevated colistin MICs is currently limited. We observed that BMD-P80 showed the highest EA and a relatively high CA with BMD but produced significantly low MICs ($P < 0.001$) (data not

TABLE 2 Colistin MICs for isolates

Organism (total no. of isolates)	Method	No. of isolates with MIC (μg/ml) of:										Geometric mean MIC
		≤0.5	1	2	4	8	16	32	64	128	>128	
<i>K. pneumoniae</i> (41)	BMD	0	0	1	12	16	6	4	1	0	1	9.2
	BMD-P80	0	0	6	14	12	4	3	1	0	1	7.0
	AD	0	0	0	5	7	9	15	5	0	0	18.3
	Etest	3	6	8	17	4	3	0	0	0	0	2.8
	MTS	1	1	12	25	0	2	0	0	0	0	3.2
	Vitek2	0	0	1	4	10	26	0	0	0	0	11.2
<i>A. baumannii</i> (20)	BMD	0	0	2	8	6	1	1	1	1	0	7.5
	BMD-P80	0	1	7	5	4	2	0	0	1	0	4.6
	AD	0	1	1	4	3	2	6	0	2	1	14.9
	Etest	6	0	3	6	4	1	0	0	0	0	2.1
	MTS	0	0	7	9	2	1	1	0	0	0	4.0
	Vitek2	0	0	0	10	5	5	0	0	0	0	6.7

TABLE 3 Differences in log₂ dilutions of MICs obtained by BMD-P80, AD, Etest, MTS, and Vitek2 compared to those obtained by BMD

Method and isolate group	No. (%) of isolates showing MIC difference (log ₂ dilution) of:						
	>−3	−3	−2	−1	0	1	2
BMD-P80							
All isolates			3 (4.9)	24 (39.3)	34 (55.7)		
<i>K. pneumoniae</i>			2 (4.9)	12 (29.3)	27 (65.9)		
<i>A. baumannii</i>			1 (5)	12 (60)	7 (35)		
AD							
All isolates			3 (4.9)	3 (4.9)	15 (24.6)	16 (26.2)	18 (29.5)
<i>K. pneumoniae</i>			2 (4.9)	2 (4.9)	11 (26.8)	10 (24.4)	11 (26.8)
<i>A. baumannii</i>			1 (5)	1 (5)	4 (20)	6 (30)	7 (35)
Etest							
All isolates	7 (11.5)	12 (19.7)	11 (18)	18 (29.5)	12 (19.7)	1 (1.6)	
<i>K. pneumoniae</i>	4 (9.8)	7 (17.1)	10 (24.4)	12 (29.3)	7 (17.1)	1 (2.4)	
<i>A. baumannii</i>	3 (15)	5 (25)	1 (5)	6 (30)	5 (25)		
MTS							
All isolates	3 (4.9)	4 (6.6)	14 (23)	26 (42.6)	13 (21.3)	1 (1.6)	
<i>K. pneumoniae</i>	2 (4.9)	3 (7.3)	14 (34.1)	15 (36.6)	6 (14.6)	1 (2.4)	
<i>A. baumannii</i>	1 (5)	1 (5)		11 (55)	7 (35)		
Vitek2							
All isolates	1 (1.6)	2 (3.3)	2 (3.3)	11 (18)	19 (31.1)	18 (29.5)	8 (13.1)
<i>K. pneumoniae</i>	1 (2.4)		2 (4.9)	7 (17.1)	11 (26.8)	13 (31.7)	7 (17.1)
<i>A. baumannii</i>		2 (10)		4 (20)	8 (40)	5 (25)	1 (5)

shown); differences in susceptibility rates were modest for *K. pneumoniae* and more pronounced for *A. baumannii*. The most important differences occurred among isolates with BMD MICs of 4 to 8 µg/ml, resulting in 18% VMEs. Slighter, but still significant, decreases in colistin MICs were also shown in a recent study comparing BMD with and BMD without P80 (21). However, in another study comparing BMD with and BMD without P80 using a different type of MIC tray, P80 did not alter MIC values for isolates with colistin MICs of ≥2 µg/ml (28).

As far as AD, previous reports have shown results concordant with those of BMD (12, 13). Similarly, in our study, the CA between AD and BMD was acceptable, and errors were relatively limited. However, the EA was low, because AD overall tended to produce MICs that were 1 to 2 log₂ dilutions higher than those determined by BMD, as was also observed previously (12).

Among commercial methods, Etest is convenient and widely applied in clinical laboratories, but concerns have been expressed regarding its suitability for colistin ST (11, 18, 19). Supporting its limitations, in our study, the Etest method generally produced lower MICs than did BMD, resulting in false sensitivity, with rates of VMEs being severalfold beyond the acceptable range for both species; EA and CA rates were also low. Considering that many clinical laboratories currently rely on Etest to determine colistin susceptibility of MDR Gram-negative bacteria, such VMEs could result in inappropriate colistin administration. Previously reported studies, which tested mainly isolates with low MICs, reported higher EA levels and much lower VME rates than those in our study. However, a study including higher numbers of *K. pneumoniae* and *A. baumannii* isolates with increased MICs pointed out considerably lower MICs by Etest compared with BMD-P80 and high VME rates (18), confirming our observations. It should be noted that Etest was recently shown to detect sufficiently colis-

tin-heteroresistant *K. pneumoniae* isolates with PhoPQ alterations; however, our collection did not include isolates with evident heteroresistance (29).

The second gradient diffusion method tested, MTS, also produced lower MICs than those determined by BMD for most isolates, resulting in a shift toward susceptibility. Interestingly, a substantial number of isolates with MTS MICs at the susceptible breakpoint (2 µg/ml) exhibited just 1 or 2 dilutions higher MICs by BMD, thus being interpreted as resistant. Overall, MTS performed better than Etest in terms of EA, CA, and VMEs.

In many clinical laboratories, the main routine antimicrobial ST methods in use are automated systems, such as Vitek2. Previous studies reported that Vitek colistin ST for *A. baumannii* exhibited appropriate performance, using BMD or AD as a reference (13, 16, 19). However, those studies included mainly colistin-susceptible *A. baumannii* isolates. As far as the *Enterobacteriaceae*, controversial findings have been reported about the reliability of Vitek2 colistin ST, implying that it was either unreliable compared with AD (19) or comparable in terms of agreement with BMD (13). Our data suggest that Vitek2 appears to be a useful method for rapid detection of colistin resistance, as it exhibited excellent CA with BMD. It was able to identify all colistin-resistant isolates, with MEs being observed for only two *A. baumannii* isolates.

In conclusion, the findings of the present study showed substantial discordance between the tested methods, with none of the methods under comparison meeting the criteria for acceptable antimicrobial susceptibility test performance. Important shortcomings of gradient diffusion testing methods, which may lead to inappropriate selection of colistin therapy, were probably the most notable observation of this study. Therefore, it is critical for clinical laboratories to be aware of these discrepancies and consider applying a reference method for colistin ST, especially when

FIG 1 Scattergrams showing numbers of isolates ($n = 61$) with colistin MICs determined by BMD-P80 (a), AD (b), Etest (c), MTS (d), and Vitek2 (e) versus BMD as a reference. Solid lines represent the 2015 EUCAST breakpoint for susceptibility (≤ 2 µg/ml). The diagonal line indicates absolute agreement. VMEs are indicated by circles.

colistin administration is deemed necessary. In routine clinical practice in most regions worldwide, where a reference method can hardly be implemented, the interpretation of colistin susceptibility should preferably be based on results of automated systems such as Vitek2.

ACKNOWLEDGMENT

This study was supported by internal funding.

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