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Letter to the Editor

Evaluation of colistin and polymyxin B susceptibility testing methods in *Klebsiella pneumoniae* and *Acinetobacter baumannii*

Yamuna Devi Bakthavatchalam[1], Balaji Veeraraghavan[1], Abirami Shankar[1], Bhuvaneswari Thukaram[1], Dhanabhagyam Naveena Krishnan[1]

[1] *Department of Clinical Microbiology, Christian Medical College, Vellore, India*

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Multi-drug resistant (MDR) Gram-negative infections represent a major clinical concern. Polymyxins (colistin and polymyxin B) are frequently used for the treatment of critically ill patients. Susceptibility testing (ST) of polymyxins is challenging. *In vitro* ST of polymyxin is influenced by i) cationic properties of polymyxin and ii) presence of hetero-resistance in MDR pathogens [1,2]. Disc diffusion (DD) method is not reliable, due to poor diffusion of colistin/polymyxin B molecule into medium [3]. Recently, disc diffusion for colistin and polymyxin B for *Pseudomonas aeruginosa* was removed from the CLSI guidelines [4]. The use of reference BMD for polymyxin ST may not be practical in diagnostic clinical microbiology laboratories. In most clinical laboratories, E-test and Vitek 2 has been used as an alternative for minimum inhibitory concentration (MIC) determination. The present study was aimed to evaluate the performance of E-test and Vitek 2 in polymyxin ST compared to the BMD method.

Non-duplicate isolates of *Klebsiella pneumonaie* (n = 121) and *Acinetobacter baumannii* (n = 120) from bloodstream infection collected from January 2013 to June 2016 were included in this study. Identification up to species level was done by using standard microbiological methods [5]. The MIC of colistin and polymyxin B (Sigma-Aldrich, St. Louis, MO, United States) were determined by BMD using cation-adjusted Muller-Hinton broth according to CLSI guidelines [6] and by E-test method (Biomerieux, Marcy L'Etoile, France). Vitek2 system was used to test colistin susceptibility only, as polymyxin B was not available on N281 card panel. The MIC range for each method were as follows; BMD (colistin and polymyxin B): ≤ 0.06 to 2048 µg/mL, E-test (colistin and polymyxin B): ≤ 0.016 to ≥ 256 µg/mL, Vitek2 (colistin only): ≤ 0.5 - to ≥ 16 µg/mL. For each batch of testing, *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853 were used as susceptible control strain, while *mcr*-1 positive *E. coli* (Courtesy: Dr. Olga Perovic, NCID, Johannesburg, South Africa) was used as the internal polymyxin resistant control strain.

The interpretative breakpoints for colistin and polymyxin B for *Enterobacteriaceae* and *A. baumannii* are shown in table 1. All colistin and/or polymyxin B resistant isolates were repeated twice. The E-test MICs were rounded up to the next highest one-fold dilution for comparison of the results with BMD. Essential agreement (EA) was defined as the percentage of MICs within ±1 $\log_2$ dilution of the MIC determined with BMD. The categorical agreement (CA) was defined as the percentage of isolates with MICs of the same categorical of interpretation. Very major error (VME) was defined as an isolate resistant by BMD but susceptible by E-test/Vitek 2 and major error (ME) was defined as an isolate susceptible by BMD but resistant by E-test/Vitek2. The acceptable rates were ≤ 1.5% for VME and < 3% for ME as recommended by CLSI guidelines (M23-A2) [7].

In BMD, *E. coli* ATCC 25922 and *P. aeruginosa* ATCC 27853 MIC results were within the expected ranges between 0.25 – 2 µg/mL and 0.5-4 µg/mL respectively. Similarly, *mcr*-1 positive *E. coli* consistently demonstrated MIC of 4-8 µg/mL by BMD. Overall $MIC_{50/90}$ (BMD) of colistin and polymyxin B

Table 1. Interpretative breakpoints of polymyxin from CLSI and EUCAST guidelines (2017).

Organism	Polymyxin	CLSI guidelines MIC (µg/mL)			EUCAST guidelines MIC (µg/mL)		
		S	I	R	S	I	R
Enterobacteriaceae	Colistin	-	-	-	≤ 2	-	> 2
	Polymyxin B	-	-	-	-	-	-
Acinetobacter spp	Colistin	≤ 2	-	≥ 4	≤ 2		> 2
	Polymyxin B	≤ 2	-	≥ 4	-	-	-

CLSI - Clinical & Laboratory Standards Institute (*CLSI*); EUCAST- European Committee on Antimicrobial Susceptibility Testing; S- susceptible; I- intermediate; R- Resistant.

were 1/8 µg/mL (MIC range, 0.25-2048 µg/mL) and 0.5/16 µg/mL (MIC range, 0.06 – 2048 µg/mL) respectively. Notably in *K. pneumonaie*, increased MIC_{90} for colistin (8 µg/mL) and polymyxin B (32 µg/mL) were observed, whereas *A. baumannii* was found with MIC_{90} of 2 µg/mL for each colistin and polymyxin B. Among tested isolates, 85% (79% in *K. pneumonaie* and 91% in *A. baumannii*) of isolates were susceptible to colistin and polymyxin B, while 15% (21% in *K. pneumonaie* and 9% in *A. baumannii*) were resistant to both in BMD. Expectedly, polymyxin resistance (resistance to both colistin and polymyxin) was higher in *K. pneumonaie* than *A. baumannii*.

Of the colistin-susceptible isolates, 20% (n = 20) of *K. pneumonaie* and 15% (n = 15) of *A. baumannii* isolates were found with a borderline MIC of 2 µg/mL. Similarly, 8% (n = 8) and 10% (n = 10) of polymyxin B susceptible *K. pneumonaie* and *A. baumannii* isolates were seen with the MIC of 2 µg/mL respectively. Among *K. pneumonaie*, 14% (MIC 0.5-2 µg/mL) and 12% (MIC 0.25 – 2 µg/mL) of susceptible isolates showed discordant MICs between BMD and E-test for colistin and polymyxin B respectively. However, none of the susceptible *A. baumannii* isolates showed conflicting results. This clearly shows that E-test MICs were significantly lower than by BMD ($p < 0.001$). This clearly demonstrates that colistin/polymyxin B E-test underestimates MIC values and therefore resistance.

The performance of E-test and Vitek 2 in polymyxin ST, compared to BMD is shown in table 2. There were < 90 % of CA and EA for E-test against BMD. Although, Vitek 2 for colistin demonstrated > 90% of CA, overall VME rate was high (11%). Further, a subset analysis of E-test against BMD showed >90% of CA for colistin and polymyxin B in testing *A. baumannii*, but demonstrated with unacceptable VME of 11 % and 28% respectively. *K. pneumoniae* showed two major errors (MEs, 1%) for colistin with E-test. None of the evaluated methods showed ME for polymyxin B.

A large multicentre CANWARD (Canadian Antimicrobial Resistance Alliance) study conducted

Table 2. $MIC_{50/90}$ of colistin and polymyxin B, categorical agreement, essential agreement and types of errors produced by E-test and Vitek2 compared with broth microdilution (BMD) method.

	Polymyxin	Method	$MIC_{50/90}$ (µg/mL)	Susceptible n (%)	Resistant n (%)	Essential agreement (EA) n (%)	Categorical agreement (CA) n (%)	Very major error (VME) n (%)	Major error (ME) n (%)
All isolates (n = 241)	Colistin	BMD	1/8	205 (85)	36 (15)	NA	NA	NA	NA
		E-test	0.38/3	220 (91)	21 (9)	89 (37)	209 (87)	15 (42)	2 (1)
		Vitek2 (n = 76)	NA	44 (58)	32 (42)	56 (74)	72 (95)	4 (11)	0 (0)
	Polymyxin B	BMD	0.5/16	205 (85)	36 (15)	NA	NA	NA	NA
		E-test	0.5/1.5	225 (93)	16 (7)	158 (65)	204 (85)	20 (55)	0 (0)
K. pneumoniae (n = 121)	Colistin	BMD	1/8	96 (79)	25 (21)	NA	NA	NA	NA
		E-test	0.38/4	107 (88)	14 (12)	44 (36)	93 (77)	11 (31)	2
		Vitek2 (n = 45)	NA	22 (49)	23 (51)	31 (69)	42 (93)	3 (8)	0 (0)
	Polymyxin B	BMD	1/32	96 (79)	25 (21)	NA	NA	NA	NA
		E-test	0.5/4	106 (88)	15 (12)	71 (59)	94 (77)	10 (28)	0 (0)
A. baumannii (n = 120)	Colistin	BMD	1/2	109 (91)	11 (9)	NA	NA	NA	NA
		E-test	0.25/0.5	113 (94)	7(6)	45 (38)	116 (97)	4 (11)	0
		Vitek2 (n = 31)	NA	21 (68)	10 (32)	25 (81)	30 (97)	1 (3)	0
	Polymyxin B	BMD	0.5/2	109 (91)	11 (9)	NA	NA	NA	NA
		E-test	0.5/0.75	119 (99)	1 (1)	87 (73)	110 (92)	10 (28)	0 (0)

NA- Not Applicable.

during 2007-11, has reported MIC_{90} of 1 µg/mL using BMD for *Klebsiella* sp., and it falls within the susceptibility breakpoint of ≤ 2 µg/mL [8]. In contrast, 3-fold increased MIC_{90} of 8 µg/mL was observed for *K. pneumoniae* in this study. Subsequently, a previous study has reported 13% of increased colistin resistance in *K. pneumoniae* [9]. Substantially, the present study showed 21% of colistin resistance in *K. pneumonaie* and is concordant with the previous study findings. Further, colistin resistance in A. *baumannii* was not significantly increased ranging from 6.1% to 9.3% and concurs with the present study findings [10,11].

In this study, EA and CA of E-test and Vitek2 against BMD were observed to be poor. In *K. pneumonaie*, poor CA of 77% with excessive rates of VME (31% and 28%) was found for colistin and polymyxin B in E-test respectively. Similarly, a study has reported CA of 56%, VME of 41.5% and ME of 2.4 % between BMD and E-test for colistin [12]. Further, Arroyo *et al*. demonstrated 98.2% of CA between BMD and E-test in testing colistin with no ME and borderline VME (1.7%) in *A. baumannii* [13]. In contrast, the present study showed VMEs of 11% for colistin and 28% for polymyxin B in testing *A. baumannii* using E-test. Although, Vitek 2 demonstrated good CA (93-97%), but found with undesirable VME rate (3-11 %). Considerably, this high CA could be true of a relatively small number of isolates tested with Vitek2.

The colistin/polymyxin B MICs determined by E-test were reported with a VME rate of 5% - 32% with agar dilution(AD)/BMD method [14-17]. However, few studies have reported good categorical agreement between BMD and E-test with very less VME (< 1%) and ME (< 1%) [18–21]. This inconsistency could be relatively due to a varying number of colistin/polymyxin B resistant isolates included for evaluation of E-test. Discrepancies in the MICs determined between BMD and E-test depend on the species of organism tested, poor diffusion of polymyxin into medium and different cation concentration in Mueller-Hinton agar (MHA) brands used for MIC determination by E-test. In present study, evaluation of BMD and E-test showed i) Poor concordance with high MICs were seen for *K. pneumoniae* and *A. baumannii* ii) E-test produces high VMEs.

Recently, EUCAST has given a warning for colistin susceptibility testing using E-test [22]. Additionally, the joint CLSI-EUCAST commission recommends BMD as the reference method for colistin/polymyxin B susceptibility testing and doesn't recommend DD, AD or gradient E-test method [23]. Beyond this recommendation, Rogacka *et al*., described that AD was superior than BMD in terms of reproducibility and robustness [24]. However, this observation was controversial to CLSI-EUCAST recommendation and tested for only limited number of strains. Thus, studies including number more number of strains is warranted to accept the reliability of AD in colistin/polymyxin B testing.

In clinical settings, polymyxin has been extensively used to treat MDR gram-negative infections. However, the issues related to colistin/polymyxin B ST remains unresolved. Notably, E-test showed poor CA, EA and associated with higher rate of VMEs. Although, Vitek2 showed > 90% of CA but found with high rate of VMEs. The present study showed poor performance of Vitek 2 and E-test for colistin/polymyxin B susceptibility testing. In the light of this, colistin/polymyxin B ST by E-test and Vitek2 is of great concern can mislead the therapy. Thus, colistin/polymyxin B resistant isolates should be further confirmed by reference BMD method.

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Corresponding author

Dr. Balaji Veeraraghavan
Professor and Head
Department of Clinical Microbiology,8th Floor ASHA building,
Christian Medical College, Ida Scudder Road, Vellore – 632 004,
Tamil Nadu, India
Phone: +91 9442210555
Fax: 0416-2232103
E-mail: vbalaji@cmcvellore.ac.in

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