Antibiotic Disk Elution Method to Determine the Colistin Susceptibility against Enterobacterales and Non-fermenter Bacteria

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ABSTRACT

Colistin minimum inhibitory concentration among Enterobacterales and Non-fermenters was determined using the new susceptibility method, Colistin Broth Disk Elution Method (CBDE), and its sensitivity and specificity. This descriptive cross-sectional study was conducted at Pakistan Railway Hospital, Rawalpindi from October 2020 to August 2021. Gram-negative bacteria were isolated and identified using Gram Stain and standard biochemical profile. Colistin Susceptibility was determined using CBDE and reference methods and then sensitivity and specificity of CBDE with standard reference methods. Essential and Categorical agreements were calculated. A total of 140 Gram-negative isolates were recovered from different specimens. The sensitivity and specificity of CBDE among *Enterobacterales* were 90.90% and 92.07% and for *Pseudomonas aeruginosa 100% and 83.3%* and for *Acinetobacter baumannii* 30% and 50% respectively. CBDE is simple, reliable, and cost effective to determine the colistin susceptibility among *Enterobacterales* and *Pseudomonas aeruginosa* while for *Acinetobacter baumannii*, this procedure is not useful.

Key Words: *Colistin susceptibility testing, CBDE, Enterobacterales, Non-fermenters.*

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Colistin is a cationic polypeptide antibiotic that belongs to the polymyxin class of antibiotics, with hydrophilic and lipophilic properties. Colistin has a particular activity against most members of *Enterobacterales* including *Escherichia coli*, *Klebsiella pneumoniae*, and some non-fermenter bacteria including *Pseudomonas aeruginosa*, *Acinetobacter baumannii* except for intrinsically resistant organisms.[1] Due to toxic effects this drug has been abandoned in the past. However, the emergence of resistant Gram-negative bacteria *i.e*. carbapenemase resistant *Enterobacteriaceae* (CRE) and extended spectrum beta-lactamase (ESBL), and the lack of new antibiotics against such bacteria raised the interest to use abandoned drugs the polymyxins in certain situations. However, the emergence of polymyxin-resistant bacteria is also becoming a clinical concern. To avoid the injudicious use of colistin, a reliable testing method is required.

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CLSI recommended broth microdilution (BMD) for *A.baumannii* and Colistin Agar Test (CAT) for *Enterobacterales* and *P.aeruginosa* for colistin MIC.[1] But BMD/CAT are costly and time--consuming and require trained technical staff to perform these methods.[2] Most clinical laboratories particularly in developing countries observe difficulty in performing such procedures and so cannot provide clinicians with an error-free colistin susceptibility. The aim of the study was to determine an easy, affordable, and reliable method to determine the colistin susceptibility against locally isolated *Enterobacterales* and non-fermenter organisms (*A.baumannii*, *P.aeuroginosa*) and to have experience with local laboratory facilities. The objective of the study was to determine colistin MIC using the new colistin broth disk elution method (CBDE) and to determine the sensitivity and specificity of this method keeping reference methods (BMD for *A.baumannii* and CAT for *Enterobacterales* and *P.aeruginosa*) as standard methods.

The descriptive cross-sectional study was conducted at Pakistan Railway Hospital, Rawalpindi from October 2020 to August 2021, after getting the formal approval from Institutional Ethical Review Committee. A non-probability sampling technique was used. Gram-negative bacteria were isolated from pus, blood, urine, sputum, pleural and peritoneal fluid, HVS *etc*. regardless of age, gender, and type of specimens. The samples were taken from the patient who had already taken colistin and duplicate samples were excluded. The isolates were collected and identified by using Gram stain and other standard biochemical profiles.

Table I: Susceptibility of colistin against Gram-negative isolates (n=140) using reference methods (CAT/BMD) and new method (CBDE).

Organisms	Reference Method (CAT/BMD)		New Method (CBDE)	
	Intermediate susceptible n (%)	Resistant n (%)	Intermediate susceptible n (%)	Resistant n (%)
Escherichia coli (n=79)	70 (88.61)	9 (11.39)	69 (87.34)	10 (12.66)
Klebsiella pneumonia (n=35)	33 (94.29)	2 (5.71)	32 (91.43)	3 (8.57)
Pseudomonas aeruginosa (n=16)	12 (75)	4 (25.0)	12 (75)	4 (25.0)
Acinetobacter baumannii (n=10)	6 (60)	4 (40.0)	9 (90)	1 (10.0)
Total	121 (86.43)	19 (13.57)	122 (87.14)	18 (12.86)

CAT: Colistin agar test, BMD: Broth microdilution method, CBDE: Colistin broth disk elution method. Intermediate susceptible =MIC ≤2, Resistant=MIC ≥4.

Colistin disks 10µg (Oxoid-UK) and Colistin Sulfate (Alfa Aesar) were used for the following procedures. Broth microdilution method (BMD) & Colistin agar test (CAT) were performed as reported previously.1,3

Cation-adjusted Mueller Hinton Broth (MHB), 10 ml was taken in 4 sterilised glass test tubes each. Colistin sulphate antibiotic disk 10µg were added; 1 disk in tube 1, 2 disks in 2, and 4 disks in tube 4 making the concentration of 1, 2, and 4 µg/ml and tube 0 is considered as growth control. Colistin was eluted at room temperature for 30 minutes. Bacterial suspension turbidity was adjusted according to standard 0.5 McFarland (1.5 X 108 CFU/ml). The suspension (50µl) was added in each test tube. Both test tubes and the purity plate were incubated at 37°C for 16-18 hours. The MIC results were interpreted by visual examination of turbidity in the tubes.2

All analyses were performed using SPSS statistics (Version 21). Descriptive statistics was used for the calculation of qualitative variables (Gram-negative bacilli) frequencies and percentages. For the categorical variable MIC against colistin, the percentage of each category was calculated. MIC determined by each method was evaluated by assessing Essential agreement (EA), Categorical agreement (CA), Very Major Error (VME), Major Error (ME), sensitivity, and specificity for each method. The essential agreement (EA) is the "percentage of isolates with MICs within ± 1 dilution of the reference method (BMD/ colistin agar test)" and the categorical agreement (CA) is the "percentage of isolates with the same category result – susceptible intermediate/resistant – as compared to reference methods. If CA and EA are within ≥90%, the results were acceptable. Major Errors (ME) is defined as the isolates that "were resistant by a new method (CBDE) but susceptible by reference methods and Very Major Errors (VME) is defined as "the isolates were susceptible by the new method and resistant by reference methods". VME and ME of ≤3% defined by ISO standards (ISO 2007) were considered acceptable.4

A total of 140 Gram-negative isolates were identified from 250 different samples. Among them 114(81.4%) were *Enterobacterales* (*E.coli* 57%, *K. pneumoniae* 25%) and non-fermenter bacteria 26 (18.6%) including *A.baumannii* 7% (n=10) and *P. aeruginosa* 11% (n=16).

The susceptibility pattern of all isolates is shown in Table I. Among 114 *Enterobacterales*, CBDE showed 9 categorical disagreement results: One resistant isolate (*E.coli*) according to reference method was intermediate susceptible with CBDE, and 8 intermediate susceptible isolates according to the reference method, were resistant with CBDE. Thus, EA and CA for CBDE were 96.49% and 92.10% respectively for *Enterobacterales* and ME 2.63% and VME 0.8%. Similarly, EA and CA of 100% and 93.75% respectively were observed for *P.aeruginosa* with 0% ME and 0% VME and EA & CA of 40% & 50% respectively were observed for *A.baumannii* with 0% ME and 30% VME. The CBDE method sensitivities for *Enterobacterales, P.aeruginosa,* and *A.baumannii* were 90.9%, 100%, and 30% respectively. Similarly, specificities were 92.1%, 83.3%, and 50% respectively.

In clinical practice, the increased usage of colistin is of major concern and may be the leading cause of the emergence of polymyxins resistant Gram-Negative strains. Under this situation, there is an urgent need of the fast, reliable, and cost effective susceptibility testing to detect the colistin susceptibility to control the unnecessary use of this drug and save it for high-risk patients.

In the present study, EA, CA, ME and VME of CBDE for *Enterobacterales* and EA, CA for the non-fermenter organism was in agreement with the study conducted by Dalmolin *et al.*[4] and Simner *et al.*[2] However, ME, VME and sensitivity and specificity for non-fermenters were in disagreement with these studies.[4] Similarly, Humphries *et al.*[5] and Pasteran *et al.*,[6] revealed satisfactory results for *A.baumannii* in contrast to the present study. This disagreement may be because of some local geographical effects on the organisms and the presence and absence of certain resistant genes in those areas. Nevertheless, our findings for *A.baumannii* conform to the inference deducted by CLSI.[1]

CBDE is proved to be simple, fast, reliable, reproducible, easily performed by using readily available supplies *i.e.*, colistin disks and MHB and there is no need of highly trained technicians to perform this test in limited-resource settings. The limitations of the study include that the study was single centered and the sample size with a small number of non-fermenter bacteria and only two species of *Enterobacterales* resulted in failure to assess colistin susceptibility by CBDE for other species of *Enterobacterales.* The susceptibility patterns of colistin among Gram- negative bacteria are different among different regions across the world that can

justify the disagreements between compared studies. Another reason for variation in different regions, BMD will detect resistance against colistin if the organisms are having chromosomally mediated colistin resistance (mcr-1 gene), but CBDE will not show them as resistant so it may be the reason of ME and VME among *Enterobacterales* when using CBDE.[2]

Colistin Broth Disk Elution test is a simple, practical, and inexpensive colistin susceptibility test and can replace BMD/CAT in routine microbiology laboratories. However, this procedure may not be useful for colistin susceptibility testing against *A.baumannii* and we have to adopt the classical method of BMD.

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