A comparative evaluation of colistin Minimum Inhibitory Concentration determination by reference broth microdilution with other commonly used phenotypic methods in Multidrug-Resistant Gram-negative bacilli

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Summary

Background: over the past decade, the dependency on colistin as a last resort antibiotic has driven the global emergence of colistin resistance among many bacterial species. This study comparatively evaluated the colistin Minimum Inhibitory Concentration (MIC) by various phenotypic methods, including the reference method of Broth Microdilution (BMD), other approved methods of Colistin Broth Disk Elution (CBDE), and Colistin Agar Test (CAT) and widely available method of Epsilometer Test (E-test) among Multidrug Resistant (MDR) Gram-negative bacteria.

Methods: ninety Gram-negative bacterial isolates that were resistant to three or more classes of drugs (MDR) were included in the study. All the MDR isolates were subjected to colistin susceptibility determination by BMD, CBDE, CAT, and E-test.

Results: amongst 1118 samples, 90 (8.05%) samples yielded MDR Gram-negative bacilli. All the MDR Gram-negative isolates were colistin intermediate by all four methods of phenotypic colistin susceptibility. Three *Acinetobacter baumannii* and two *Klebsiella pneumoniae* isolates that had MIC of 2 µg/mL by BMD, displayed MIC of <1 µg/mL by CBDE and CAT. Three isolates (2 *Citrobacter koseri* and 1 *Enterobacter spp.*) showed higher MIC by the E-strip method in comparison to BMD.

Conclusions: our study holds significance, as there is a paucity of data comparing the four phenotypic methods for colistin MIC determination; BMD is the most reliable, gold standard method, but it is labor-intensive and requires technical expertise. In the present study, CBDE and CAT methods showed good concordance with BMD, and are easy to perform with limited logistics. Thus, they can be used as an alternative to BMD. We found that even though the E-test method was less accurate, it can still be used with caution to exclude the possibility of colistin resistance.

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Introduction

In the current era, the world is facing a silent pandemic known as antimicrobial resistance. The continuous, unmonitored, and irrational antimicrobial use in various sectors like in humans, animals, and agriculture is an enraging global health problem that has led to the development and spread of antimicrobial resistance to higher-level antimicrobials [6,25]. The ability of microorganisms to acquire and disseminate various resistance genes led to better adaptability of these pathogens against antimicrobial agents within a few years after their introduction into the market and their subsequent clinical usage. Unfortunately, the speed of development of newer antimicrobials does not correspond with that of the development of antimicrobial resistance in bacterial pathogens [12].

Multidrug-Resistant (MDR) is defined as the non-susceptibility of a pathogen to at least one antimicrobial agent in three or more antimicrobial categories; and the nonsusceptibility of bacteria to at least one agent in all but two or fewer antimicrobial categories (*i.e.*, bacterial isolates remain susceptible to only one or two antimicrobial categories) is known as Extensively Drug Resistant (XDR),

whereas nonsusceptibility to all agents in all antimicrobial categories is known as Pan-Drug Resistant (PDR)[16]. With the increasing prevalence of MDR and XDR strains among Gram-negative bacteria, notably Carbapenemase-Producing Carbapenem-Resistant Enterobacterales (CP-CRE), the effective treatment options become very limited, and clinicians are often forced to use last resort drugs like polymyxins and tigecycline [15,11]. However, tigecycline displays low serum concentrations, and the outcome is not as good as colistin, so clinicians often rely on colistin for the treatment of life-threatening infections [24].

Colistin, a polycationic antibiotic, was discovered in 1947. Colistin acts by disrupting Lipopolysaccharide (LPS) in the outer membrane of the bacteria. It binds to the phosphate groups of the lipid A region of LPS and displaces the divalent cations (Ca^{2+} and Mg^{2+}) from the phosphate groups, resulting in increased permeability of the outer membrane and leakage of intracellular contents and thus leading to bacterial cell death [19,20,8,18]. Colistin regained global interest in recent years as a consequence of the emergence of MDR and XDR pathogens [10,3]. This antimicrobial has good activity against Gram-negative bacteria like most members of Enterobacterales, and gram-negative non-fermenters like *Pseudomonas aeruginosa, Acinetobacter baumannii,* and *Stenotrophomonas maltophilia* [10,3]. Colistin (Polymyxin E) in dual therapy with other carbapenem drugs (meropenem) results in a significant reduction of morbidity and mortality in serious infections caused by MDR and XDR strains.

Unfortunately, the increased incidence of infections by colistin-resistant pathogens has been reported in the last few years [5,9]. The alterations in the bacterial outer membrane by LPS modification, overexpression of efflux pumps, and over-production of the capsule are some common mechanisms of resistance by pathogens. The pmr A/B, phoP/Q, ramA, crrB, pmrHFIJKLM operon, and arnBCADTEF operon are the recognized genes that play a crucial role in acquired and intrinsic resistance to colistin, these genes act via modification of lipid A of LPS. Mutations of other genes like *acrB, KpnEF, and sapABCDEF* lead to overexpression of the efflux pump [20]. On the other hand, *mcr* genes are responsible for the majority of the plasmid-mediated transferrable mechanism of colistin resistance and act via adding Phosphoethanolamine (pEtN) to lipid A. The expression of the *mcr-1* gene alone can lead to an increase in colistin MIC by four to eight times, without any other mechanism of resistance [20].

The use of colistin warrants caution due to its association with major side effects like nephrotoxicity and neurotoxicity. Therefore, the susceptibility and MIC of this agent should be determined beforehand for dose determination, to curb the side effects and to prevent local emergence of colistin resistance [20]. The widely used methods of antimicrobial susceptibility testing *i.e.,* disk diffusion and gradient strips, are unreliable for colistin as the large and cationic molecules of this drug diffuse poorly in cation-enriched adjusted Mueller-Hinton Agar (MHA). The absence of an established breakpoint for the disk diffusion method also adds to this challenge of susceptibility testing [4]. The most widely followed guidelines, the European Committee on Antibiotic Susceptibility Testing (EUCAST) and the Clinical and Laboratory Standards Institute (CLSI), recommend the use of Broth Microdilution (BMD) test, and provide established breakpoints to assess MIC of colistin in routine laboratories [1,13,17]. Colistin Broth Disk Elution (CBDE) and Colistin Agar Test (CAT) are the other phenotypic methods approved for the determination of colistin susceptibility. Technical expertise and logistics needed for these approved phenotypic methods are often available only at limited healthcare facilities. The utility of easier to perform and widely available Epsilometer Test (E-test) for picking the colistin intermediate strains from resistant strains is still not clearly determined.

Over the past decade, the dependency on colistin as a last resort antibiotic has driven the global emergence of colistin resistance among many bacterial species [3]. Therefore, this study comparatively evaluated the colistin MIC by various phenotypic methods, including reference method of BMD, other approved methods of CBDE and CAT, and widely available method of E-test among MDR Gram-negative bacteria.

Materials and Methods

This was a prospective study conducted in the Department of Microbiology, in a tertiary care hospital in Delhi, over a period of three months (October to December 2022). This study included the bacterial isolates from clinical samples of pus aspirates, body fluids, respiratory & genital samples that were sent for routine diagnostic evaluation to the hospital laboratory. No separate sample was collected for the purpose of this study. Bacterial identification and Antimicrobial Susceptibility Testing (AST) were performed using standard laboratory protocols. Ninety Gram-negative bacterial isolates that were resistant to three or more classes of drugs (MDR) were included in the study [2].

Phenotypic test for colistin resistance

All the MDR isolates were subjected to colistin susceptibility determination by BMD, CBDE, CAT, and E-test.

Colistin Broth Disk Elution (CBDE)

CBDE was performed as per CLSI guidelines by using four sterile glass Mac-Cartney containers (30 mL), each containing 10 mL of Cation-Adjusted Mueller-Hinton Broth (CA-MHB) (HiMedia Laboratories Pvt. Ltd., Mumbai, India) per isolate to which 0, 1, 2, and 4 colistin disks (10mg Colistin Sulphate) (HiMedia) were added to achieve the concentrations of 0 (growth control), 1, 2, and 4 µg/mL, respectively. After vortex and incubation at room temperature for 30 min, a 50-µL of standardized inoculum (0.5 McFarland) was then added and incubated overnight at 35°C. The MIC values were read visually as the lowest concentration that completely inhibited the visible growth. The interpretation was made as per CLSI breakpoints (Table 1) [2]. Quality control was performed with mcr-1-producing *Escherichia coli* with every batch of tests.

Table 1. The current Clinical and Laboratory Standards Institute (CLSI) and European Committee on Antimicrobial Susceptibility Testing (EUCAST) Minimum Inhibitory Concentration (MIC) breakpoints for colistin.

Genera	CLSI breakpoints (µl/mL)			EUCAST breakpoints (µl/mL)		Method of testing
	S	I	R	S	R	
Enterobacteriaceae	-	≤2	≥4	≤2	>2	CAT, CBDE and BMD
Pseudomonas	-	≤2	≥4	≤4	>4	CAT, CBDE and BMD
Acinetobacter	-	≤2	≥4	≤2	>2	BMD

BMD, Broth Microdilution; CBDE, Colistin Broth Disk Elution; CAT, Colistin Agar Test.

Colistin Agar Test (CAT)

This agar dilution test was performed on MHA (HiMedia) as per CLSI guidelines. MHA with different concentrations of colistin was prepared with xolistin sulfate powder (potency of 19000 IU/mg; Sisco Research Laboratories Pvt. Ltd., Mumbai, India). For the preparation of Primary Stock Solution (PSS) potency of available colistin sulfate powder with reference to pure agent (30,000 IU/mg) was calculated (633.33 IU/µg). To get PSS with the concentration of 1 mg/mL, 10 mg of colistin sulfate was added to 6.33 mL of autoclaved distilled water and stored at -70°C. The different dilutions of colistin (0g/mL, 1 g/mL, 2 g/mL, and 4 g/mL) in 100 mL of molten MHA were prepared from PSS (by using C1V1 = C2 V2 formula) and poured in 90 mm plates. After the solidification of agar, 10 µL of diluted inoculum (1:10 dilution of 0.5 McFarland standardized inoculum) was streaked into each agar plate and incubated overnight at 35°C. Interpretation was done as per CLSI guidelines (Table 1)[2].

Epsilometer Test (E-test)

The MIC of colistin was also determined by E-test using CA-MHA (HiMedia) and MIC gradient strip (Colistin Ezy MIC$_{TM}$ Strip (0.016-256 µg/mL) (HiMedia)) as per manufacturer's instruction. The MIC was considered where ellipsoid or inverted tear drop shaped zone of inhibition intersected the MIC scale on the strip.

Broth Microdilution (BMD)

The test was carried out by using CA-MHB (HiMedia) and Colistin sulfate powder (19000 IU/mg)(Sisco Research Laboratories) in 96 welled round bottom microtiter plates (Corning Inc., Corning, USA). The determination of potency and preparation of primary stock solution was similar to as mentioned above for CAT. The working solution (64 µg/mL) of colistin was prepared from the primary stock solution in a sterile Micro Centrifuge Tube (MCT) by adding 64 µL of primary stock solution to 936 µL of autoclaved CA-MHB. The required concentrations of working solutions (0.25–8 µg/mL) were made by twofold serial dilutions and to achieve 100 µL of volume in each well microtiter plate, 25 µL of drug, 50 µl of MHB, and 25 µL inoculum of concentration (5×10^5 CFU/mL or 1:75 dilution of 0.5 McFarland standardized inoculum) except Column 11 and 12 which were used as growth control and media control respectively. The quality controls were used in every microtiter plate. The lowest concentration of colistin that completely inhibited bacterial growth detected by the unaided eye was considered as MIC and results were interpreted as per CLSI guidelines [2]. The data analysis was done by SPSS ver.2.0 software.

Results

A total of 3818 samples were received over a period of three months. Subsequent to conventional processing of all the samples, antimicrobial susceptibility testing was performed for 1118 samples that yielded pathogens. Among these 1118 samples, 90 samples yielded MDR Gram-negative bacilli (8.05%). A total of 9 antibiotics were used for classifying the organism as MDR. The resistance patterns of all the pathogens isolated are shown in Figure 1. These MDR isolates have been utilized for colistin susceptibility testing by the above-described four methods in our study.

The majority of the MDR bacilli isolated from clinical samples were from Surgery department (52/90), followed by Medicine (15/90) and Orthopaedics (12/90). Percentage of MDR infection was higher in males (56.7%) as compared to females (43.3%). The distribution of MDR Gram negative bacterial isolates among various clinical specimens is shown in Table 2.

The MDR isolates included *Acinetobacter baumannii* (21.1%), *Pseudomonas aeruginosa* (5.5%), *Escherichia coli* (21.1%), *Klebsiella spp.* (27.7%), *Citrobacter spp.* (10%) and *Enterobacter spp.* (14.4%). Twenty-three out of the 90 MDR-bacilli were Carbapenem-Resistant Enterobacterales (25.5%). While 10 out of 90 MDR bacilli were Carbapenem-Resistant *Acinetobacter baumannii* (CRAB).

Considering BMD as the gold standard method of colistin susceptibility testing, all the MDR isolates were found to be colistin intermediate. Table 3 shows the comparison of susceptibility results

Table 2. Distribution of Multidrug-Resistant (MDR) Gram-negative bacterial isolates among various clinical specimens (n=90).

Specimen	n	*Acinetobacter baumannii*	*Citrobacter spp.*	*Escherichia coli*	*Enterobacter spp.*	*Klebsiella spp.*	*Pseudomonas aeruginosa*
Pus	57	11	7	10	9	16	4
Body fluids	6	2	0	2	2	0	0
Endotracheal	7	2	1	3	0	0	1
High vaginal swab	4	0	0	1	1	2	0
Sputum	3	2	0	0	0	1	0
Tissue	13	2	1	3	1	6	0

Table 3. Comparison of phenotypic assays for different Gram negative MDR isolates (n=90).

Organisms	n	Colistin disk elution		Colsitin agar test		E-test		Microbroth dilution	
		I	R	I	R	I	R	I	R
Acinetobacter baumannii	19	19	0	19	0	19	0	19	0
Citrobacter spp.	9	9	0	9	0	9	0	9	0
Escherichia coli	19	19	0	19	0	19	0	19	0
Enterobacter spp.	13	13	0	13	0	13	0	13	0
Klebsiella spp.	25	25	0	25	0	25	0	25	0
Pseudomonas aeruginosa	5	5	0	5	0	5	0	5	0

*[I – Intermediate (MIC≤2 µg/mL); R- Resistant MIC≥4 µg/mL)].

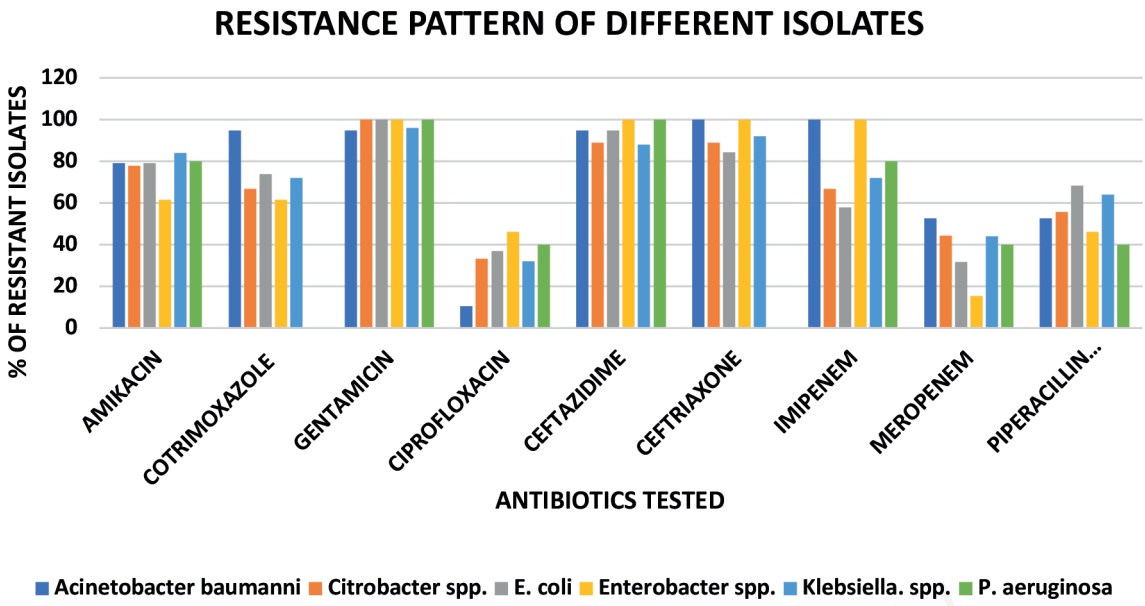

Figure 1. Distribution of resistance to various antibiotics among Gram negative MDR isolates (n=90).

of different Gram-negative MDR isolates by the four phenotypic methods. Though all the isolates were found to be intermediate by all the phenotypic methods, differences in MIC values were observed amongst the different phenotypic tests in various isolates.

Colistin Broth Disk Elution *vs* Micro Broth Dilution

All the isolates were colistin intermediate according to the latest CLSI guidelines, however, 5 isolates (3 *Acinetobacter baumannii* and 2 *Klebsiella pneumoniae*) which had MIC of 2 µg/mL by BMD displayed MIC of <1 µg/mL by CBDE.

Colistin Agar test *vs* Micro Broth Dilution

Findings similar to CBDE test were observed with CAT, wherein all but 5 isolates (3 *Acinetobacter baumannii* and 2 *Klebsiella pneumoniae*) had MIC of 2 µg/mL by BMD but had MIC of <1 µg/mL when tested with the CAT.

Colistin E-strip test *vs* Micro Broth Dilution

All the MDR isolates were intermediate to colistin by either of the two methods. Eight samples showed discrepancies in the MICs by the two tests. Three isolates (2 *Citrobacter koseri* and 1 *Enterobacter spp.*) showed higher MIC by E-strip method in comparison to BMD. The same three isolates also had higher MIC by E-strip in comparison to the other two tests, *i.e.*, CBDE and CAT. The remaining 5 isolates (3 *Acinetobacter baumannii* and 2 *Klebsiella pneumoniae*) had higher MIC by BMD in comparison to the E-strip method.

No particular trend was observed between E-test and the rest of the methods, including the reference method of BMD. With the use of E-test, "in-between" values were obtained, MIC values below 1 µg/mL obtained by E-test method were in agreement with the MIC values obtained by the reference method. However, MIC values greater than 1 µg/mL did not correspond with those obtained through the BMD test.

Discussion

Colistin, also known as polymyxin E, has regained its importance in treating multidrug-resistant Gram-negative bacilli in the current times. This antibiotic was introduced more than 50 years back, but was superseded by aminoglycosides and other antibiotics in view of the increased nephrotoxicity displayed by the latter. In the recent scenario, colistin is used as a part of "salvage therapy" for treating MDR organisms. IDSA recommends the use of colistin in treating CRE cystitis, Difficult To Treat (DTR) *Pseudomonas aeruginosa*, and CRAB cystitis [22]. Since colistin is an antibiotic of extreme importance, appropriate susceptibility testing forms the cornerstone of antimicrobial stewardship.

Colistin plays an important role in management of patients presenting with pneumonia, bacteremia, sepsis, Urinary Tract Infections (UTI), *etc*. It particularly plays a significant role in patients with cystic fibrosis who are infected by DTR *Pseudomonas aeruginosa,* wherein this antibiotic is given intranasally to the patient. CLSI-EUCAST jointly recommends BMD as the gold standard method for colistin susceptibility testing. E-strip method has not been mentioned as a standard method of susceptibility testing in either of the guidelines, but we have tested our isolates in order to explore the potential of this test. In the year 2020, CLSI introduced two new techniques for colistin susceptibility testing, namely, CBDE and CAT. Our study holds significance, as there is a paucity of data comparing these four phenotypic methods.

We observed comparable susceptibility patterns in our study by all the four methods *i.e.*, CBDE, CAT, E-strip and the gold standard method BMD. All the 90 MDR isolates were found to be colistin intermediate as per the latest CLSI guidelines. A study by Simner *et al.* demonstrated the essential agreement and categorical agreement between CBDE and BMD to be 98% and 99%, respectively [23].

In our study, though all the samples were colistin intermediate, slight variations were observed in the MIC by different methods. Our findings of slightly higher MIC by E-strip method are in concordance with other studies [7,21,26]. Since there were no discrep-

ancies observed by any of the four phenotypic methods for colistin MIC determination, there were no major or very major errors. However, Kar *et al.,* in their study reported rates of very major error for CAT and E-test to be 11% and 37% respectively in comparison to BMD [14].

Though BMD is considered as the standard reference method for colistin susceptibility, it needs technical expertise and training. The majority of labs lack the skills and will to implement testing of colistin susceptibility through BMD. Therefore, there is rampant use of colistin without its susceptibility testing. Though the newer recommended methods of CBDE and CAT are comparatively easier, their implementation on a wide scale is still a major challenge. These CBDE and CAT methods present accurate, comparatively less labour-intensive viable alternatives to BMD for colistin susceptibility testing. The present study has also assessed the utility of the widely available method of E-strip in comparison to BMD for colistin susceptibility. The MIC values below 1 µg/mL obtained by the E-test method were in agreement with the MIC values obtained by the reference method. However, MIC values greater than 1µg/mL did not correspond with those obtained through the BMD test. The potential role of E-strip method in ruling out the colistin-resistant isolates warrants further elaborate studies involving larger sample sizes and multiple centers.

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