

Finding An Alternative To BMD- A Comparative Evaluation Of CBDE And Mic E-Strip In North Indian Isolates.

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Abstract

Introduction- Colistin Resistance Is The Turning Point In The Era Of Antimicrobial Resistance. With High Errors In Colistin Disk Diffusion And Paucity Of Resources To Perform Broth Microdilution, Finding Reliable And Accurate Alternative Testing Methods Is The Need Of The Hour.

Aim- To Estimate The Degree Of Agreement And Errors In Phenotypic Colistin Resistance Detection Methods Like Colistin Broth Disc Elution Test (Cbde) And Mic E-Test Gradient Strip, And Their Comparison With Reference Broth Microdilution (Bmd) Method.

Material And Methods- Non Repetitive 150 Clinical Isolates (*E. Coli* And *K. Pneumoniae*) From Admitted Patients Suspected Of Hcai, Were Collected, And Subjected To Standard Microbiology Protocols. Identification Along With Antimicrobial Susceptibility Testing Was Done By Vitek-2 Compact System. Colistin Resistance Testing Was Performed In These Isolates By Bmd, Cbde And Mic E-Test.

Results- In This Study, Cbde Showed An Ea Of 88% With Me Of 12%. E-Strip Showed Higher Ea And Lesser Me, But Showed 4% Vme. Both The Tests Had 100% Specificity While E-Strip Had Higher Sensitivity. Overall Concordance Rate Was 83.33% Between E-Strip And Cbde. Poor Agreement Exists Between E-Strip And Cbde With Kappa=0.149.

Conclusion- For Better Routine Microbiological Reporting Of Colistin Susceptibility Profile, Cbde Can Be Considered As An Alternative To Bmd As It Is Specific And Accurately Identifies The Colistin Resistant Isolates. It Does Not Show Very Major Errors And Shows A High Ppv, Indicating A High Level Of Agreement With Bmd. E- Strip On The Other Hand Does Show High Specificity And Npv But Shows Severe Discrepancies In Mic And Therefore Its Reliability Is Not Yet Agreed Upon. However, It Is Essential To Confirm Mic's Falling In Between 2-4 Mg/ML By Bmd And To Detect Mcr Genes In All Isolates With Mic ≥ 2 Mg/ML.

Keywords- Colistin; E-Test Gradient; Colistin Broth Disc Elution (Cbde); Colistin Resistance; Epidemiological Cut-Off Value (Ecv)

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I. Introduction

The increasing prevalence of antibiotic resistance is a threat to human health, especially vulnerable populations in the hospital, leading to increased healthcare costs and morbidity. Over the last few decades, (CR-Kp), *Acinetobacter baumannii* (CR-Ab) and *Esch. coli* has been isolated from hospitals and long-term facility care. According to a study, there were approximately 47 MDR-GNB cases per 100 ICU admissions in Nepal [1].

Due to the reduced susceptibility of these pathogens to cephalosporins, for empirical therapy, treatment options available are carbapenems, Polymyxins, Tigecycline and Eravacycline. However, the emergence of carbapenem resistant-GNB lead to a silent tsunami of the antibiotic era. Prolonged ICU admissions, longer therapeutic regimes and unavailability of antimicrobials have made Colistin the saviour for treating life threatening infections [2].

It acts by disruption of the divalent bonds within the lipopolysaccharide (LPS) structures causing leakage of contents of the gram-negative bacterial cell and its subsequent death.

Aggarwal et al. were the first to identify Colistin resistance in trauma patients in India [3]. A study by conducted in Vellore detected Colistin resistance in uro-pathogenic *K. pneumoniae* along with its impact on the

treatment regimen [4]. Parallel case reports have been reported from Chennai and Odisha too [5,6]. Kaza et al. in Chandigarh, India found that Colistin resistance among *E. coli* and *K. pneumoniae* lead to higher recurrence of infection, prolonged hospital stays and poor prognosis resulting in higher morbidity [7].

This underlined the importance of Colistin resistance testing and reporting for which the accepted gold standard method is conventional broth microdilution (BMD). However, it is not easy to implement it readily in the routine laboratory due to its laborious technique. In the last ten years, many people worked on different phenotypic and genotypic methods of detection of Colistin resistance in common Gram-negative isolates particularly Enterobacterales, for routine detection of Colistin resistance. Ashna et al. in 2019 in North India, found that Colistin resistance can be detected by Rapid Polymyxin Nordmann Poirel (RPNP) test but it was not compared with Broth microdilution (BMD) [8]. Moreover, there have been unreported instances of Colistin resistance among clinical isolates of *K. pneumoniae* (40%), and *E. coli* (3.57%) in our hospital from October 2019 to December 2019 which was detected by the Vitek 2 Compact system and/or RPNP test, reliability of which is uncertain. The aim of this study was to evaluate the Colistin broth disk elution (CBDE) method and MIC E-strip method for Colistin resistance testing and estimate the degree of agreement of these methods with reference BMD in the clinical isolates of *K. pneumoniae* and *E. coli*.

II. Material And Methods

Study design

The study was prospectively executed in G. B. Pant Institute of Postgraduate Medical Education and Research, a tertiary care institute in North India. It was conducted in the department of Microbiology, for one year from January 2021 to December 2021, after obtaining approval from the Institutional Ethical Committee, bearing certificate No. F.1/IEC/MAMC/82/10/2020/No.226 dated 14th January 2021.

Clinical samples

The study took 150 first isolates (*E.coli* or *K.pneumoniae*) identified from a clinical specimen [Blood, pus, cerebrospinal fluid (CSF), body fluids (percutaneous drain fluid, bile and peritoneal fluid), respiratory samples (sputum and Endotracheal (ET) aspirate) and arterial line tip] from adult patients, who were admitted, and suspected of having an infection after seeking an informed consent.

Recurring isolates from the same patient for the same or other clinical sample were disregarded.

Lab Processing

After sample collection, they were transported to the laboratory and processed as per standard microbiological protocols for each sample. Identification and antimicrobial susceptibility testing were accomplished by automated system (Vitek 2 Compact Systems). Colistin resistance detection was simultaneously done by Broth microdilution (BMD), CBDE and MIC E-strip methods [9,10].

Colistin broth disk elution method. The CBDE method was carried out with four 10-ml cation adjusted Mueller-Hinton broth (CA-MHB, Hi-Media) tubes for each isolate, to which 0, 1, 2, and 4 colistin disks (10 µg; HiMedia) were put, making final concentrations of 0 (growth control), 1, 2, and 4 µg/ml, respectively. These were incubated at room temperature for 20 min to allow colistin to elute from the disks. Inocula were prepared by suspending fresh colonies from an overnight 5% sheep blood agar plate in normal saline and adjusting the turbidity to 0.5 McFarland. A 50-µl of this suspension was added to each tube, and the tubes were gently vortexed for a final concentration of 7.5×10^5 CFU/ml, and incubated for 16-24 hours at 35°C in ambient air according to CLSI recommendation [11].

Colistin MIC values were read visually and interpreted using CLSI ECVs (for *Enterobacteriaceae*). Quality control was performed with *E. coli* ATCC 27853 and an *mcr-1*-producing *E. coli* NCTC 13846 (anticipated MICs, 2 to 4 µg/ml).

MIC E- strip method

Isolated colonies from overnight culture on 5% sheep blood agar were picked and mixed with normal saline to adjust to an inoculum of 0.5 McFarland turbidity after which it was lawn streaked on CA-MHA and E-Strip (Colistin EZY MIC STRIP-0.016-256 mcg/mL, HIMEDIA, Mumbai, India) with Colistin concentrations of 0.016 to 256 µg/mL was added on the plate within 15 minutes of streaking and incubated for 16-24 hours at 35-37°C, was interpreted by ellipse shaped zone of growth coinciding at a particular MIC loaded on the strip. Isolates showing MIC's equal to or less than $\leq 2\mu\text{g/ml}$ and $\geq 4\mu\text{g/ml}$ were considered as Intermediate susceptible and resistant respectively according to CLSI guidelines. Controls were used as CBDE method.

Broth microdilution method

BMD was carried out in triplicate using untreated, polystyrene, 96-well plates (U-shaped, Greiner Bio-One, Frickenhausen, Germany) where colistin concentrations were obtained by serial two-fold dilutions on CAMHB (HiMedia, Mumbai, India) reaching a concentration range of 0.25– 8µg/mL. Bacterial suspensions were inoculated to each well to achieve a final concentration of 10⁸ CFU/mL. Growth and media controls were included in each assay. The plates were incubated overnight at 37 °C [12]. The lowest concentration inhibiting bacterial growth was considered the MIC value and the interpretation of these results was done in accordance with CLSI-EUCAST breakpoints for Enterobacteriaceae [13]. Isolates with a MIC ≤ 2 µg/mL were categorized as intermediate susceptible whereas those with a MIC ≥ 4 µg/mL were resistant.

Testing Strategy-

The CBDE and E-strip tests were performed in parallel and BMD was performed later for the isolates. If any discrepant results were apparent between methods for an individual isolate (a >2-doubling-dilution difference), all 2 or 3 methods were repeated from the same subculture, and these results were used for the final analysis.

Quality control for BMD method was performed using *E. coli* ATCC 25922 and *mcr-1* producing *E.coli* NCTC 13846.

Data management and statistical analysis

The data was recorded in MS Excel and in the latest version of SPSS software. The presentation of the Categorical variables was done in the form of percentage (%). BMD was taken as the gold standard. Sensitivity and specificity were compared using McNemar test, chi square test and DeLong et al. test. Inter-rater kappa agreement was used to assess strength of agreement between CBDE and E-Strip. MIC of CBDE and E-Strip were categorized as Essential agreement (EA), Major errors (ME) and very major errors (VME) when the difference of MIC with respect to BMD was ≤1, 2-4 and >4 respectively, with statistical significance(*p-value*<0.005).

III. Results And Observations

This study was conducted in a tertiary care hospital, where admitted patients are at a high risk of developing health-care associated infection (HCAI) and GNB dominate the such scenarios. The prevalence of Colistin resistance was noted as 5.33%. The highest percentage of isolates were from fluids (29%) followed by respiratory samples (25%). Antimicrobial susceptibility profile showed a high resistance towards beta-lactam/beta-lactam inhibitors (81.3%) followed by fluoroquinolones and cephalosporins. Highest susceptibility was seen towards Tigecycline (75%). It was worth noting that, only 12% isolates were sensitive whereas, 65% and 20% isolates were MDR and XDR respectively. As depicted in Table 1, 8 isolates were resistant by the gold standard method whereas 19 and 10 were resistant by CBDE and E-strip respectively.

Table 1: Colistin resistance detection by BMD, CBDE and E-STRIP tests

Detection Method	Clinical isolates (<i>E. coli</i> and <i>K. pneumoniae</i>)	
	Colistin sensitive	Colistin resistant
BMD	142(94.6%)	8(5.33%)
CBDE	131(87.3%)	19(12.6%)
E-STRIP	140(93%)	10(6.6%)

Comparison of CBDE by Broth Microdilution test-

With the objective of comparing the CBDE with BMD, to see the errors of agreement and concordance with the gold standard it was noticed that CBDE showed 100% specificity. The essential agreement with BMD was noted as 88%, however 18 isolates showed ME's as well (Table2a). False resistance (VME) was not seen. Inter-rater kappa agreement with BMD was moderate (0.382).

Table 2a: - CBDE distribution.

CBDE	Frequency	Percentage
Essential agreement	132	88%
Major errors	18	12%
Total	150	100%

Comparison of E-Strip by Broth Microdilution test-

In the evaluation of E-Strip, 90.67% of isolates demonstrated essential agreement, indicating consistent and accurate results. However, 5.33% of isolates were associated with major errors, signifying significant

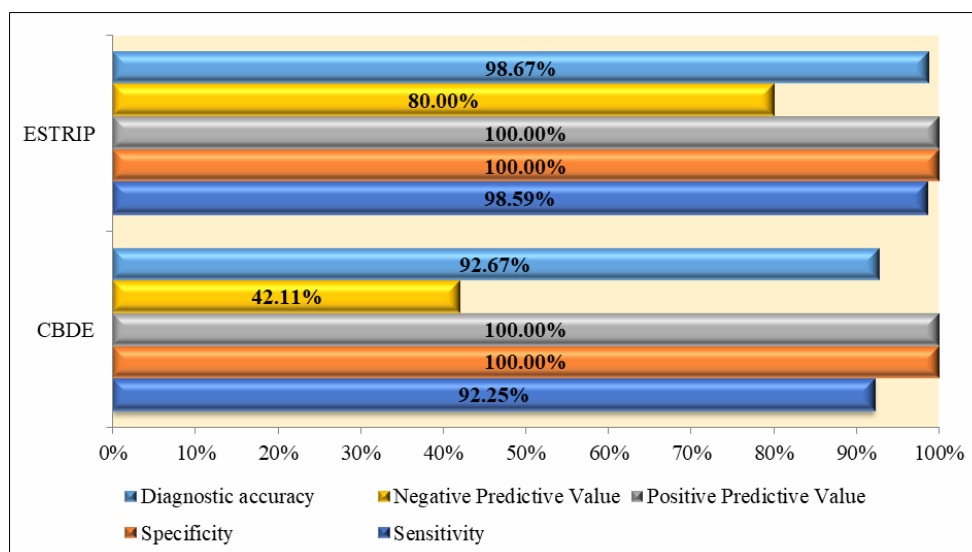
discrepancies or inaccuracies. Additionally, 4.00% of cases were identified with VMEs, indicating critical inaccuracies (false resistance) in the outcomes of E-Strip (Table 2b).

Table 2b: - ESTRIP distribution.

ESTRIP	Frequency	Percentage
Essential agreement	136	90.67%
Major errors	8	5.33%
Very major errors	6	4.00%
Total	150	100%

Comparison of CBDE with E-strip method

Among the isolates (n=150), E-Strip demonstrated higher essential agreement than CBDE indicating more sensitive and equally specific results in comparison to gold standard BMD. Conversely, CBDE showed a lower negative predictive value and diagnostic accuracy (graph1). However, no isolate was falsely reported as resistant as in the case of E-strip where 6 (VME=4.00%) susceptible isolates were reported as resistant. In terms of AUC, which measures the overall diagnostic accuracy, ESTRIP outperformed CBDE with AUC values of 0.99, compared to CBDE's AUC of 0.96. (*p-value*=0.002). Poor agreement exists between ESTRIP and CBDE with kappa 0.149 (*p-value*=0.023). The overall concordance rate was 83.33% between ESTRIP and CBDE. (Table 3)



Graph 1: - Sensitivity, specificity, positive predictive value and negative predictive value of CBDE, ESTRIP for predicting sensitivity after taking BMD as gold standard.

Table 3: - Inter-rater kappa agreement between CBDE and ESTRIP.

CBDE	ESTRIP			Total	<i>p- value</i>	Kappa
	EA (n=136)	ME (n=8)	VME (n=6)			
Essential agreement	123 (82%)	6 (4%)	3 (2%)	132 (88%)	0.023	0.149
Major errors	13 (8.67%)	2 (1.33%)	3 (2%)	18 (12%)		
Total	136 (90.6%)	8 (5.33%)	6 (4%)	150 (100%)		

IV. Discussion

In the present prospective study, the participants were admitted patients (n=150), who were suspected of having HCAI. These patients were infected with either *E. coli* (n=50) or *K. pneumoniae* (n=100). The antibiotic susceptibility profile of these isolates was dominated by high susceptibility to Tigecycline which is a reserve drug according to AWaRe drug classification by WHO and resistant to routinely accessible antimicrobials like cephalosporins, Beta lactams and Fluoroquinolones. The focus of spotlight in the current study is colistin resistance which was 5.3%, detected by the gold standard BMD. Many other studies conducted in the country have published similar findings [14,15]. A study in Eastern India reported overall prevalence of 40% colistin resistance in gram negative isolates from ophthalmic infections like endophthalmitis, keratitis and orbital infections [16].

Colistin as a molecule is heavy and the difficulties with its susceptibility testing is well recognized. Overall, the most accepted method is Broth microdilution which gives the MIC values for each organism tested. These MIC play a significant role in interpreting the therapeutic index of the drug to start an optimal treatment regime. BMD, is known to be a difficult and skilled process. Its chances of high variability in performance and procedure are known. The skip-well phenomenon is a less acknowledged truth associated with it [17].

The study analysed the clinical isolates by subjecting them to both, CBDE and E- Strip testing and then verifying the results with in-house BMD, which brought to the forefront, the fact that CBDE which was recommended by CLSI-EUCAST committee as a test for *P. aeruginosa* and Enterobacterales has a sensitivity of 92.25%, which was supported by 100% specificity of the test, however the power to predict true negatives by CBDE was 42% which was lower than E-strip but was not statistically significant ($p\text{-value}=0.119$). On the other hand, considering E-strip, the results show that it has a higher sensitivity (98.5%) in comparison to BMD. In their pilot study, Das et al. found E-strip to be 100% sensitive but the EA was 63.7% only [17].

The present study showed that CBDE reported 88% EA, and 12% major errors which had 18 isolates showing MIC between 2-4 $\mu\text{g/ml}$. When repeat tested by BMD, out of those 18 isolates, 7 isolates showed MIC = 2 $\mu\text{g/ml}$ and 4 isolates had MIC falling in the range of 0.5-1 $\mu\text{g/ml}$. Conversely, E-strip showed only 5.3% ME, with a higher EA, which could have been because of the broad MIC range (0.016-256 $\mu\text{g/ml}$), which is not possible with CBDE. But, E-strip showed a high percentage of very major errors ($n=6$ isolates) which when repeat tested by BMD, showed that 2 isolates which were reported as having MIC's 16 and 4 $\mu\text{g/ml}$ (resistant) by E-strip had BMD MIC as 2 $\mu\text{g/ml}$ (intermediate susceptible). In a similar study, Goyal et al. also found 3 isolates having higher MICs by E-strip method than by BMD [18]. Pfennigwerth et al. found that the E-test gradient showed an EA of 80.6% comparable to the BMD. The E-test stripper performed almost equally to semi-automated system (Walk Away), but was nonetheless not recommended due to 9.4% false susceptible results, especially with isolates of *E. cloacae* [19]. The performance of the E-test in our study was well in line with previously published results [20,21,22].

Interestingly, though the E-strip had a higher statistical significance in various areas of comparison in this study, the CBDE still had a higher inter-rater kappa agreement with BMD ($K=0.382$), which can be attributed to the fact that CBDE, although showed a variation of ± 2 dilutions in the susceptibility results but did not detect false resistance as in the case of E-Strip. Overall, E-strip showed an over estimation of MIC's, and in some cases, it crossed ± 4 dilutions. The overall concordance rate between CBDE and E-Strip was 83%, which highlights the errors and inaccuracies in the testing of colistin resistance.

As, published and discussed in various studies worldwide, the major mediator of colistin resistance is the mobile colistin resistance gene (*mcr*) acquired by a plasmid, and therefore, the study recognizes the need to evaluate the presence of this gene in resistant isolates, as it is important to study its prevalence in the hospital environment. [23,24]

The detection of colistin resistance is a topic of high concern and intrigues every medical microbiologist globally. The truth that MICs in the 2 to 4 $\mu\text{g/ml}$ range are frequently observed for *mcr*-expressing *Enterobacterales* raises concerns as to whether the Epidemiological Cut-off Value (ECV) should be redefined, as it was established prior to the *mcr* acquired resistance being widely present. We recommend that colistin MICs of $\geq 2\mu\text{g/ml}$ by the CBDE or the E-Strip method be confirmed by rBMD (the results for two isolates from this study had required confirmation), and consideration should be taken to test those with MICs of $\geq 2\mu\text{g/ml}$ for *mcr* genes.

Author Contributions

Conceptualization Poonam Loomba and Bibhabati Mishra.; Data curation Tanvi Aggrawal; Formal analysis Poonam Loomba, Tanvi Aggrawal and Abha Sharma; Resources Poonam Loomba and Abha Sharma; Supervision Poonam Loomba and Bibhabati Mishra; Validation Bibhabati Mishra, writing—original draft preparation Tanvi Aggrawal and Vikas Vijayran; writing—review and editing Poonam Loomba and Bibhabati Mishra. All authors have read and agreed to the published version of the manuscript.

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Transparency declaration

Authors declare no conflict of interest.

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