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Microbiology Section

Comparative Assessment of Disk Diffusion, E-test and Broth Dilution Methods for Determining Colistin Susceptibility in *Acinetobacter* Species

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ABSTRACT

Introduction: Colistin is the ultimate reserve drug for gram-negative bacilli especially non fermenting bacilli like *Acinetobacter* species and only therapeutic option available against carbapenem resistant bacteria. Clinical and Laboratory Standards Institute (CLSI) recommended broth dilution method for colistin susceptibility testing but this method require expertise and very time consuming and is also not feasible to routinely perform in clinical laboratories. In the background of ever expanding problems of drug resistance coupled with increasing use of colistin, it becomes essential to find a testing method which is faster, accurate and can be used as an alternative to broth dilution method.

Aim: To assess disk diffusion, E-test and broth dilution method to determine colistin susceptibility in *Acinetobacter* species.

Materials and Methods: This cross-sectional observational study was done in the Department of Microbiology, ABVIMS and Dr RML Hospital, New Delhi, India, from November 2016 to March 2018. Various clinical specimens were received from wards and Intensive Care Unit (ICU) and *Acinetobacter* species were isolated and identified following standard protocol. All strains were screened by disc diffusion test for various antibacterial drugs and among them 50 MDR strains were selected. Colistin susceptibility by disc diffusion and Epsilometer test (E-test) were done and compared against the reference broth dilution method following. Minimum Inhibitory Concentration (MIC) of colistin for the isolates were determined using macro broth dilution method using colistin sulphate. Chi-square test was used to investigate distributions of categorical variables.

Results: All 50 strains were tested for colistin susceptibility by disc diffusion method. Among the 50 isolates, 10 isolates (20%) showed resistance to colistin by disc diffusion method while none of the isolate was found resistant by E-test or broth dilution method. High error rate and poor concordance of disc diffusion with reference to broth dilution method rules out the utility of disk diffusion method in clinical laboratories.

Conclusion: A complete agreement between the results of E-test and broth dilution method makes E-test a suitable alternative to broth dilution test in clinical laboratories.

Keywords: Antibiotics, Carbapenem, Polymyxin resistance, Susceptibility

INTRODUCTION

Antibiotics are considered to be one of the most significant events that revolutionised medicine in the 20th century. The discovery of Penicillin by Sir Alexander Fleming in 1928 opened new frontiers of antimicrobial research. Since then, hundreds of antibiotics have been discovered but this breakthrough was associated with unabated consumption of antibiotics. Unfortunately, this unjustified use of antibiotics forced the bacteria to evolve rapidly and express resistance mechanisms to practically every antibiotic in clinical use [1]. The increasing drug resistance is a critical global threat and there is an urgent need for answers that may drive rapid development of newer molecules especially against Multi Drug Resistance (MDR) organisms.

Colistin is also known as polymyxin E and its clinical use was started in 1959. Although, due to its neurotoxic and nephrotoxic side effects there was great reluctance among the medicine experts to use this drug and it was never a choice drug for many years [2] but emerged as an important antimicrobial agent when there was exponential expansion of MDR organisms especially carbapenem resistant enterobacteriaceae and non fermenters like; *Acinetobacter* species [3].

Management of infections caused by MDR *Acinetobacter baumannii (A. baumannii)* has always been an area of concern. In recent times, MDR *Acinetobacter* spp. has emerged as a leading nosocomial pathogen around the world [4]. The genetic potential of MDR *A. baumannii* to carry and transfer diverse antibiotic resistance determinants poses a major threat in hospitals [5,6]. With the reporting of extensive drug resistant and pan drug resistant strains, the situation appears really scary [7,8]. Though carbapenems represent treatment of choice, reporting of resistance to this class of drugs has renewed the interest of clinician in the use of colistin in difficult to treat infections especially caused by *Acinetobacter* and *Pseudomonas* spp. [9,10]. Colistin is one of last line of antibiotic for MDR gram negative bacteria and it appears inevitable that the resistance to colistin would also emerge. Therefore, it becomes obligatory that only laboratory supported use of this drug is permitted and no sanctions for empirical use are allowed [11,12].

It also becomes incumbent on the part of the Microbiologists to follow only standardised protocols for sensitivity testing so that the information generated is precise and accurate.

Disk diffusion method is the mainstay for determining susceptibility of the organisms to various antimicrobial agents in most of the clinical microbiology laboratories but, inability of this test to detect low level resistance, high error rates and low reproducibility for detecting colistin resistance are well documented [13-15]. CLSI has objections to the use of this method against colistin and has recommended only broth dilution/agar dilution methods for reporting sensitivity to this drug [16]. Broth Microdilution (BMD) is widely used as a method of Minimum Inhibitory Concentration (MIC) estimation in Europe and the USA and current guidelines of CLSI, EUCAST and BSAC also recommend colistin susceptibility testing by estimation of MIC [16,17]. The broth dilution method of

Antimicrobial Susceptibility Testing (AST) is very labour intensive, technically demanding in terms of manpower, technical resources, expertise and unacceptable turnaround time, limiting its utility to research-based endeavors and does not appears to be easily implementable or incorporable in routine laboratory protocols [18]. Since, the first reporting of colistin resistant *Acinetobacter* spp. from Czech Republic in 1999, the number of reports have been increasing all over the world year by year. The highest resistance rate was reported in Asia, followed by Europe [19]. The increasing use of colistin necessitates the availability of rapid and reliable methods for colistin susceptibility testing.

Also, it becomes imperative on the part of the microbiology labs to explore alternate options to permit usage of this drug. In the background of difficulties inherent to broth dilution method, growing importance and urgent need to define an optimal, user-friendly method for susceptibility testing for colistin. Therefore, authors have evaluated currently utilised colistin susceptibility testing methods, i.e., disc diffusion, gradient MIC strip E-test against broth dilution method of antimicrobial susceptibility test for colistin in *Acinetobacter* species.

MATERIALS AND METHODS

This cross-sectional observational study was done in the Department of Microbiology, ABVIMS and Dr RML Hospital, New Delhi, India, from November 2016 to March 2018. Various clinical samples such as pus, body fluids, endotracheal secretions, catheters of patients admitted in wards and ICUs of ABVIMS and Dr RML hospital were received in the laboratory and processed for microbiological workup. The study was initiated after approval from Institutional Ethics Committee of ABVIMS and Dr RML Hospital letter (F.No. TP (MD/MS) (7/2016) /IEC/PGIMER/RMLH 7819/16 dated 05.10.2016). The samples were directly received in the laboratory from wards and ICU for culture and sensitivity test and there was no direct involvement between patients and researcher hence, patient's consent was not necessary to obtain.

Inclusion criteria: Only pure and MDR strains of *Acinetobacter* species isolated from various clinical samples of patients admitted in ICU and wards of ABVIMS and Dr RML Hospital were included for comparative study.

Exclusion criteria: *Acinetobacter* species grown in a mixture, patients on prior colistin therapy and due to institutional ethics, children below 12 years were not included in the study. Sample size of 50 had the statistical sanction of the Institutional review board and Institutional Ethics committee.

Bacterial Strains

A. baumannii strains in the age group above 12 years irrespective of their sex and isolated from different clinical specimens received from wards and ICU [20]. All isolates were identified using standard protocol following Baily and Scott's Diagnostic Microbiology 13th edition [21] and by VITEK automated system of microbial identification (Biomerieux diagnostics). The two strains, *E. coli* ATCC 25922 and *Pseudomonas* aeruginosa ATCC 27853 served as control.

Study Procedure

Colistin susceptibility by three different methods disc diffusion, MIC gradient strips and macro broth dilution method was tested to assess their reproducibility and variability from freshly prepared inoculum by three independent experiments.

All experiments were performed according to the CLSI 2016 guidelines [16]. For all experiments colistin sulfate salt was used (Lot# SLBQ0243V, Sigma-Aldrich, Co, St. Louis, USA). Tubes and plates were incubated in Biological Oxygen Demand (BOD) incubator for 18 hours at 35±2°C followed by visual assessment of turbidity or growth. Results of each experiment were analysed following joint CLSI-EUCAST breakpoints [17] working group for colistin (MIC of 2 µg/mL, susceptible (S), and MIC of 4 µg/mL resistant). Detailed methods are described in specific sub-sections below.

Disk Diffusion Method

The antimicrobial susceptibility of the *Acinetobacter* spp. isolates to colistin was tested by modified Kirby Bauer's disc diffusion method, on cation adjusted Mueller Hinton Agar (MHA). The plates were incubated at 37°C for 18-24 hours and zones of inhibition were recorded as per CLSI 2016 guidelines [16].

E-test method bacterial suspension equivalent to 0.5 McFarland turbidity standard was spread on MHA (Hi-Media), and with the help of a forceps a previously brought to room temperature colistin MIC gradient strip or E strip (colistin E-test® strip (BioMérieux SA, Marcy l'Etoile, France) was applied at the centre of the plate. The MHA plates were incubated for 18-24 hours at 37°C and the MIC was recorded as per manufacturer's instructions.

Broth Dilution Method

The preparation of colistin suspension, inoculum preparation and MIC of the colistin sulphate was determined by means of broth dilution technique in accordance with CLSI 2016 [16]. Colistin stock solutions of concentrations 1024 µg/mL was prepared based on the purity assay provided by the manufacturer sigma using the formula given below for colistin:

$$\text{Volume} = \frac{\text{Weight (mg)} \times \text{Potency (µg/mg)}}{\text{Concentration (µg/mg)}}$$

The medium of reconstitution of antimicrobial powder was sterile distilled water. For MIC testing, the doubling dilution for colistin was taken from 0.125 µg/mL to a maximum 64 µg/mL.

Due to polycationic nature of colistin which shows adherence to plastic surfaces resulting in loss of activity during experiments. Polysorbate 80 (P-80) a commonly used surfactant in BMD panel can synergistically influence colistin concentration in panels and MIC results. A common approach to overcome binding of colistin to plastic is to perform MIC in glass tubes BMD.

Preparation of Antibiotic Suspension

A stock solution of colistin with given potency (Sigma, Germany) at final concentration 1024 mg/L prepared in sterile distilled water following CLSI 2016 [16] guidelines and stored at -20ºC for further use. For MIC determination stock solution was subsequently diluted 1:2 in sterile distilled water up to a concentration of 0.125 µg/mL.

Preparation of Inoculum

Bacterial suspension was prepared in sterile normal saline by transferring 3-4 isolated colonies from 24-hour-old culture grown on non selective medium and turbidity adjusted to 0.5 McFarland turbidity standard equivalents to 1.5×10^8 cfu/mL. The prepared suspension was further diluted 1:150 by transferring 10 µL of inoculum to 15 mL of sterile Mueller Hinton broth to achieve a concentration of 1×10^6 cfu/mL.

Determination of MIC

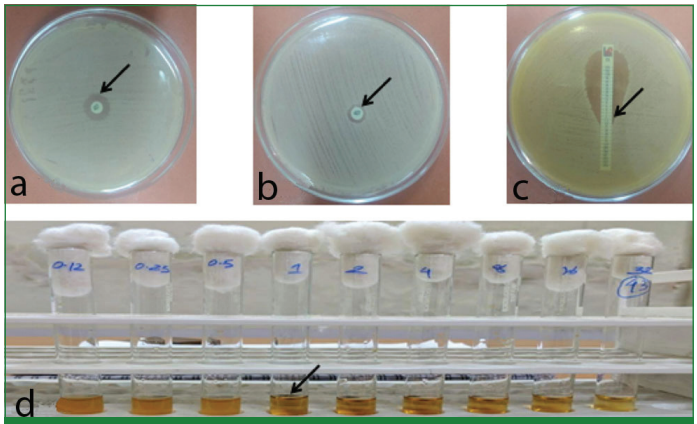
Colistin dilution starting from 128 µg/mL to 0.25 µg was prepared from stock solution in previously labelled sterile glass tubes of 13×100 mm dimension and within 15 minutes, freshly prepared 0.5 mL of diluted bacterial suspension 1×10^6 cfu /mL added to diluted colistin tubes to make colistin dilution 1:64 in first tube and 0.125 µg/mL in last tube. This addition of bacterial inoculum in subsequent tube brings final inoculum concentration to 5×10^5 cfu/mL in each tube. Inoculated tubes incubated at 37ºC for 18-24 hours in BOD incubator. Bacteria inoculated mueller hinton broth tube without colistin served as positive and tube with only broth served as negative control. All tests were performed in triplicates and tubes were observed for bacterial growth with naked eye in bright light by three investigators and result recorded.

STATISTICAL ANALYSIS

The software used for the statistical analysis was Statistical Package for Social Science (SPSS) version 21.0 and Epi-info version 3.0. The categorical variables were summarised as frequencies and percentages. Chi-square test was used to investigate distributions of categorical variables. The p-value <0.05 was taken significant and confidence interval of 95%.

RESULTS

Acinetobacter species isolated from various clinical specimens of patients admitted in ICU and wards of Dr RML Hospital were identified by following standard microbiological techniques and by VITEK automated system of microbial identification. Among the isolates a total of 50 MDR strains of *Acinetobacter baumanii* were investigated for the analysis of antimicrobial sensitivity of colistin by disk diffusion, E-test and broth dilution method [Table/Fig-1]. Results of each method were analysed and compared following joint CLSI-EUCAST breakpoints working group for colistin.



[Table/Fig-1]: Showing colistin susceptibility by disc diffusion, E test and broth dilution method. (a) Arrow indicating colistin sensitive by disc diffusion; (b) Arrow indicating colistin resistant by disc diffusion; (c) Arrow indicating colistin MIC by E test; (d) Arrow indicating colistin MIC by broth dilution assay.

Among the 50 isolates tested, 40 (80%) isolates were sensitive and 10 (20%) found resistant by disc diffusion method, whereas none of the isolate was found resistant by E-test and broth dilution method [Table/Fig-2]. The Categorical Agreement (CA) of E-test and disc diffusion with reference to broth dilition was 100% and 80%, respectively while essential agreement between E test and broth dilution was found 96%. The distribution of the antimicrobial testing was compared between disk diffusion test and broth dilution test using the Chi-square test. Disk diffusion test had significantly (p-value <0.01) lesser chances of testing sensitive compared to broth dilution.

Test method	Isolates tested	Isolates sensitive ≤2 µg/mL	Sensitive percentage	Isolates resistant ≥4 µg/mL	Resistant percentage
Disc diffusion	50	40	80%	10	20%
E-test	50	50	100%	Nil	0%
Broth dilution	50	50	100%	Nil	0%

[Table/Fig-2]: Comparison of antimicrobial sensitivity testing between disk diffusion test, E test and broth dilution test.
Chi-square value=9.000, p-value=0.003*

All the 50 isolates sensitive by the broth dilution method, tested sensitive by E-test method also. The distribution of the antimicrobial testing was compared between E-test and Broth dilution test using the Chi-square test. No significant difference (p-value >0.05) was found in the AST between E-test and Broth dilution method.

Comparison of disk diffusion test with gold standard broth dilution method showed a sensitivity of 80% (95% CI=66.28-89.97%), Positive Predictive Value (PPV) of 100% and accuracy of 80% (95% CI=66.28-89.97%).

Comparison of E-test with gold standard test broth dilution method showed a sensitivity of 100% (95% CI=92.89-100%), PPV of 100% and accuracy of 100% (95% CI=92.89-100%) [Table/Fig-3].

Test method	Sensitivity	Positive predictive value	Accuracy	95% CI*
Disc diffusion test	80%	100%	80%	66.28-89.97%
E-test	100%	100%	100%	92.89-100%

[Table/Fig-3]: Sensitivity, positive predictive value and accuracy of disk diffusion and E-test compared to broth dilution method (Gold standard).
CI*: Confidence interval

DISCUSSION

In the recent past, *Acinetobacter* has shown tendency to develop resistance against multiple antibiotics possibly because of the result of their long exposure to antibiotic producing bacteria in the soil [22]. These drug resistant strains besides causing therapeutic difficulties have a significant capacity for long-term survival in the hospital environment, with corresponding enhanced opportunities for transmission between patients, either via human reservoirs or inanimate materials [23]. Colistin is the last resort for the treatment of MDR *A. baumannii*. Unfortunately, resistance to colistin has been reported all over the world and it is inevitable that resistance to colistin will become more prevalent if it is used suboptimally [24]. There is a relatively little research on colistin resistance in *A. baumannii*. In many cases, colistin or polymyxin B is the only therapeutic option available for MDR *A. baumannii* infection [25]. Of potentially significant clinical concern is the recent observation of heteroresistance to colistin in clinical isolates of multidrug resistance *A. baumannii* against which colistin is believed to be very active on basis of MIC [26]. Even now, there is lack of consensus on breakpoints for colistin susceptibility testing [27]. The disk diffusion test results for colistin are unsatisfactory and hampered by different factors. Colistin poorly diffuse in agar, produce high rate of very major errors and remains unreliable hence refutes usefulness of most commonly used method disc diffusion for susceptibility testing of colistin in clinical microbiology laboratories, which is also stated untrustworthy by several studies [27-29].

Although, other studies have shown concordance between E-test and dilution method. Turlej-Rogacka A et al., found variation of 1 log2 dilution in the result of agar dilution and broth dilution method and based on good concordance between E-test and agar dilution method agar dilution method found superior to broth dilution method due to its high reproducibility and easy to perform protocol [28]. Tan TY and Ng SY also showed 87% agreement in the result of E-test and agar dilution method and recommended E-test as an alternative to broth and agar dilution method [29].

Arafa RM et al., exhibited a poor CA of 54% for disc diffusion and 80% for E-test when compared to broth dilution method as a gold standard respectively [30]. The poor performance of disc diffusion and E-test could be due to the poor diffusion of colistin molecules, resulting in a narrow zone of inhibition [30]. Singhal L et al., showed 100% CA between disc diffusion, E-test and broth dilution method while relatively low Essential Agreement (EA) 11.9% between E-test and broth dilution method for colistin was observed and moderate reliability of E-test was found [31]. van der Heijden IM et al., showed very good CA 100% and 79.5% EA between E-test and broth dilution method [32]. While 98.2% CA and 83.4% EA was found between E-test and broth dilution method by Arroyo LA et al., which also support the present study [33]. The subsequent results are shown in [Table/Fig-4].

The disk diffusion method is one of the most frequently used techniques in microbiology laboratories, the poor diffusion of colistin to agar in particular, and unreliable correlation of zone diameters leads to problems in the standardisation of sensitivity tests performed with this method [34]. However, interpretation criteria for disc diffusion susceptibility testing of polymyxins by CLSI was published in 2007 but

Author, year	Country	E test (CA)	E test (EA)	Disc diffusion (CA)
Arafa RM et al., [30] 2021	Egypt	80%	82%	54%
Singhal L et al., [31] 2018	India	100%	11.9%	100%
van der Heijden IM et al., [32] 2007	Brazil	100%	79.5%	100%
Arroyo LA et al., [33] 2005	Spain	98.2%	83.4%	Data N/A

[Table/Fig-4]: Showing Categorical Agreement (CA), essential agreement (EA) of E test and disc diffusion test against broth dilution method [30-33].

due to lack of reliable data on true resistance and minimal research done on this group of antibiotics there is still no consensus regarding the breakpoints for defining resistance to polymyxins [35]. Although, there are different levels of breakpoint for each bacterial species. Yet, the dose and administration frequency of colistin for effective bactericidal activity against MDR bacteria is a major concern and interpretation criteria for in vitro quantitative testing of colistin also differs between nations. Increasing numbers of reports regarding colistin-resistant bacteria indicates a developing threat to future treatment options for diseases caused by gram-negative bacteria. Colistin resistant organisms are reported in various parts of the world, including resistance of *Pseudomonas aeruginosa* in cystic fibrosis from UK [36], carbapenemase-producing *Klebsiella pneumoniae* resistant to colistin [37], *Acinetobacter baumannii*, and polymyxin resistant *Escherichia coli* [38].

Interpretative criteria for disk susceptibility testing of colistin are not available from the CLSI, and zone size interpretations are made based on the product literature (Oxoid, Thermofisher Scientific, Basingstoke, UK). In this study the percentage of colistin sensitivity was 80% and colistin resistance by disk diffusion was 20% which is reproducibly unacceptable and comparable to the study by Gales AC et al., which showed 11% percent of colistin resistance and 5% false susceptible errors for colistin by disk diffusion [39]. The agar dilution and BMD methods showed excellent agreement for testing colistin and polymyxin B. Only three bacterial isolates showed discords (1-log2 dilutions) between the dilution methods, but these differences resulted in very limited one-dilution error.

The CLSI recommends MIC breakpoints of broth dilution as the gold standard reference method for testing of colistin susceptibility. Based on the susceptibility data, CLSI has documented an MIC of ≤2 g/mL as susceptible and an MIC of ≥4 g/mL as resistant for colistin. Although, MIC determination by BMD in polystyrene microplates using cation-adjusted Mueller Hinton broth without additives has been advised by joint CLSI-EUCAST polymyxin working group recently [17,18] and also suggested further study and validation. However, a number of more user-friendly commercial products like E-test and semi-automated devices for colistin MIC have recently become available and widely used at clinical laboratories. Therefore, comparison of different laboratory methods is eventually a necessity of current prevailing scenario of expanding problem of drug resistance to take a final call on choice of therapeutic options.

Limitation(s)

Due to specified time lines for the study, a larger sample size could not be included though size of 50 samples was statistically acceptable. Also, because of resource constraint, molecular work up could not be undertaken.

CONCLUSION(S)

Results of disk diffusion method are erroneous and not trust worthy for making therapeutic decisions because of the unacceptable predictive accuracy for colistin. In contrast, E-test results were comparable to gold standard broth dilution method and found useful for discrimination of colistin resistant and susceptible *Acinetobacter* isolates. Since performing E-test is significantly less demanding on resources and requires less time comparable to broth dilution technique. This appears an acceptable alternative to broth dilution method particularly in laboratory which don't have adequate laboratory resources.

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