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One Health Bulletin

Detection of colistin resistance via four methods among Gram−negative bacteria

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ABSTRACT

Objective: We aimed to find the most accurate and robust method for the detection of colistin resistance among Gram-negative bacteria by comparing the performance of four methods.

Methods: Colistin resistantance was determine by disk diffusion (DD), Etest, colistin broth disk elution (CBDE) and compared with standard broth microdilution (BMD) method.

Results: An exploratory study was conducted in a tertiary hospital in Jaipur, India, from November 2021 to November 2022. Of the 384 isolates tested, 41 (10.7%) were resistant against colistin by BMD and CBDE testing. Compared with BMD method, the categorical agreement of DD and Etest methods were 65.9% and 78.1%, respectively, and the rates of very major error were 34.1% and 21.9%, respectively.

Conclusions: Due to the high frequency of very major error, the use of DD and Etest are not recommended by Clinical laboratory and standards institute (CLSI) for colistin susceptibility testing while CBDE is the recommended method which yields a categorical agreement of 100% with BMD method. The CBDE method can be used in routine laboratories for the detection of colistin resistance.

KEYWORDS: Disk diffusion; Etest; Broth micro dilution method; Colistin broth disk elution; Colistin

1. Introduction

Approximately 700 000 deaths in world are attributed to antimicrobial resistance. This number is supposed to increase to ten million by the year 2050, by mortality attributed to antimicrobial resistance overhanging to outrun worldwide general causes of death[1]. One key technique for preventing antimicrobial resistance is the elimination of commonly used antibiotics. In this circumstances, use of an old forgotten antibiotic colistin has been increasing in the recent past.

Colistin is a cationic polypeptide antibiotic of the polymyxin family that is rapidly bactericidal to Gram-negative bacteria and is generated spontaneously by *Bacillus polymyxa*.

Significance

The emergence of multidrug-resistant Gram-negative isolates creates many challenges in the treatment of nosocomial infections. Limited methods for colistin MIC determination are available to clinical microbiology laboratories. In this study, the accuracy of four methods of detection of colistin resistance were evaluated. Compared with currently used techniques, CBDE is an easy and practical method to perform.

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How to cite this article: Gautam PK, Tomar S. Detection of colistin resistance via four methods among Gram-negative bacteria. One Health Bull 2023; 3; 16. https://www.doi.org/10.4103/2773-0344.385536.

Article history: Received 16 April 2023 Revision 31 July 2023
Accepted 6 August 2023 Available online 18 Septemper 2023

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Bacterial death is caused by interference with the structure and function of the outer cell membranes. Colistin was initially launched in 1952 and was used to treat Gram-negative bacilli infections till the early 1980s. Its systemic usage was halted due to adverse issues such as nephrotoxicity, neuromuscular inhibition, and neurotoxicity[2, 3].

Despite the fact that colistin has been utilised in clinical sectors for a long time, the appropriate susceptibility testing methods for colistin remained unknown until recently. The Clinical and Laboratory Standards Institute (CLSI) and the European Committee on Antimicrobial Susceptibility Testing (EUCAST) combined taskforce both approved broth microdilution (BMD) as the reference technique in 2015[4].

2. Materials and methods

2.1. Study area

This was a one-year hospital-based study conducted at the department of Microbiology, National Institute of Medical Sciences, Jaipur, India, from November 2021 to November 2022.

2.2. Sample processing

Specimens of urine, pus, blood, endotracheal-tube, sputum, swab and fluids obtained from patients were cultured on routine bacteriological media. Biochemical identification and antimicrobial susceptibility testing of the isolates were performed according to the standard laboratory procedure. Gram-negative bateria isolated showing resistance by disk diffusion (DD) method were further analysed by Etest, Colistin broth disk elution method (CBDE) and confirmed by BMD method.

2.2.1. Identification of bacterial isolates

After inoculation on solid media i.e MacConkey agar and Blood agar (HiMedia Laboratory Pvt. Ltd), all plates were incubated at 37 ℃ temperature for 18-20 hours. The next morning, gram staining was performed for morphology identification and standard biochemical test was done for species identification.

2.2.2. Screening by disk diffusion method

The colistin disk difussion test was performed using a 10 µg colistin disk (HiMedia Laboratory Pvt. Ltd) onto the surface of muller-Hinton agar (HiMedia Laboratory Pvt. Ltd) plate that were incubated at 37 ℃ for 18-24 hours. As the antibiotic diffused from the filter paper disk into the agar surface, it inhibited the bacterial growth around the disk. The effectiveness of the antibiotic can be calculated by measuring the size of the zone of inhibition.

2.2.3. Etest

The minimum inhibitory concentration (MIC) was determined by using E-strip method. An E-strip containing different concentration of colistin was placed onto the surface of Muller-Hinton agar plate. As colistin diffused onto the surface of agar, it inhibited the bacterial growth. The lowest concentration of colistin that will inhibit the visible growth of bacteria is defined as MIC. Isolates with MIC $\leqslant$ 2 mg/L were considered sensitive, while isolates with MIC $\geqslant$4 mg/L were considered resistant.

2.2.4. Colistin broth disk elution method (In house modification)

CBDE was performed with four, 10 ml cation-adjusted Mueller Hinton broth tubes per isolate, to which 0, 1, 2, and 4 colistin 10 µg disks were added, generating a final concentration of 0 (growth control), 1, 2 and 4 µg/mL in the tubes. All tubes were incubated at 37 ℃ for 30-45 minutes to elute the colistin from the disks. Prepare the inoculum by suspending pure colonies from blood agar in normal saline and adjusting the turbidity to match 0.5 MaFarland turbidity. MIC values were read after 18-24 hours of incubation at 37 ℃. The result of colistin MIC were interpreted as per CLSI-2022[5].

2.2.5. Broth microdilution method

BMD was performed in accordance with CLSI recommendations. Colistin sulphate powder (19 000 U/mg) was bought from HIMEDIA Pvt. Ltd, Mumbai, India with two-fold drug dilutions in range of 0.125 to 16 µg/ml were prepared in autoclaved distilled water. The cation-adjusted Mueller Hinton Broth (CAMHB) was prepared according to the manufacturer's instructions. A bacterial suspension with a concentration of 5 x 10^5 CFU/ml was prepared and inoculated in a micro-titre plate. Growth and sterility controls were placed in the last two wells of each micro-titre plate row. Bacterial suspension was the sole substance in the growth control well, while CAMHB was the only substance in the sterility control well.

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Finally, the micro-titre plates were then incubated for 16-18 hours at 37 ℃ in incubator. The MIC of colistin was determined as the lowest concentration that inhibited observable bacterial growth. The results were interpreted in accordance with CLSI-2022[5].

3. Results

The study was conducted in the Department of Microbiology from November 2021 to November 2022. A total of 384 samples were received in microbiology samples including urine 173 (45.1%), pus 88 (22.9%), blood 58 (15.1%), endotracheal aspirates 39 (10.2%), and sputum 26 (6.8%).

Out of 384 Gram-negative clinical isolates, the predominant Gram-negative bacteria was *Escherichia coli* (145, 37.8%), followed by *Klebsiella pneumoniae* (131, 34.1%), *Pseudomonas aeruginosa* (65 , 16.9%), *Acinetobacter baumannii* (31, 8.1%), *Enterobacter cloacae* (8, 2.1%) and *Citrobacter frundii* (4, 1.0%) (Figure 1).

Among 384 clinical samples, maximum organism was isolated from intensive care unit (ICU) (154, 40.1%), followed by medicine (74, 19.3%), surgery (37, 9.6%), obstetrics & gynecology (31, 8.1%), pediatrics (22, 5.7%), orthopedic (21, 5.5%), nephrology (14, 3.6%), oncology (12, 3.1%), urology (10, 2.6%) and gastrology (8, 2.1%).

All the clinical isolates were tested for colistin resistance by four phenotypic test. The DD method detected 7.0% (27/384), by Etest 8.3% (32/384) and by CBDE 10.7% (41/384). All resistant isolates were subjected to confirm resistance by BMD (Gold standard method) and MICs were noticed (Table 1).

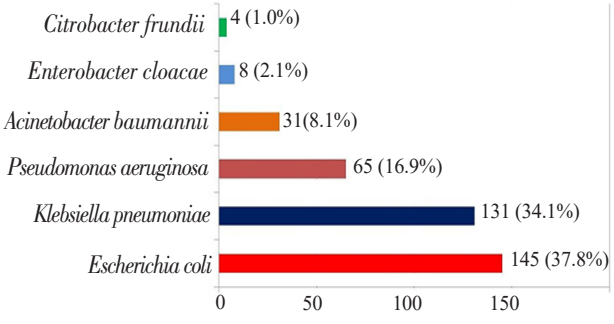

Figure 1. Frequency of Gram-negative cilinical isolates.

Out of 384 isolates, 148 isolates showed colistin MIC of ≤0.5 µg/mL, 134 isolates had MIC of 1 µg/mL, 61 isolates had MIC of 2 µg/mL, 24 isolates had MIC of 4 µg/mL, 6 isolates had MIC of 8 µg/mL, and 11 isolated had MIC of 16 µg/mL. The MICs of all Gram-negative clinical isolates are shown in Table 2.

Of all the colistin resistant isolates, the majority were from ICU (13, 31.7%), followed by medicine (9, 22.0%), orthopaedic (4, 9.8%), pediatrics (3, 7.3%), surgery (3, 7.3%), gastrology (3, 7.3%), urology (2, 4.9%), nephrology (2, 4.9%), and obstetrics & gynecology (1, 2.4%), oncology (1, 2.4%), respectively. The maximum colistin resistant isolates were from urine (20, 48.8%), followed by pus (14, 34.1%), blood (4, 9.8%), endotracheal aspirates (2, 4.9%), and sputum (1, 2.4%), respectively.

The CBDE method showed 100% concordant results for all colistin resistance clinical isolates. The performance of CBDE, Etest and DD methods compared to the BMD method were evaluated on the parameters of major error, very major error, categorical agreement, sensitivity, specificity, positive predictive valves and negative predictive value. The very major error was 34.1% for DD test while no major error was seen for CBDE method.

Categorical agreements of colistin disk elution method was 100%, while for Etest method and DD methods was 78.1% and 65.9% respectively when compared with BMD method.

Compared with BMD, the sensitivity of CBDE method, Etest, and DD method was 100%, 78.1%, and 65.9%, respectively. The specificity and positive predictive value of all phenotypic tests was 100% while negative predictive value of colistin broth elution method, Etest, and DD method was 100%, 97.5%, and 96.2%, respectively (Table 3).

Table 1. Detection of colistin resistance organism by DD, Etest, CBDE and BMD.

S. No	Organism	DD	Etest	CBDE	BMD
1	*Escherichia. Coli*	8	12	15	14
2	*Klebsiella pneumoniae*	11	11	16	15
3	*Pseudomonas aeruginosa*	6	6	8	10
4	*Acinetobacter baumannii*	2	2	2	2
5	*Enterobacter cloaeca*	0	1	0	0
Total		27	32	41	41

DD: Colistin resistantance was determine by disk diffusion; CBDE: colistin broth disk elution; BMD: broth microdilution.

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Table 2. Distribution of MICs of colistin for all clinical isolates (n=384).

Clinical isolates	MICs (μg/mL)					
	0.5	1.0	2.0	4.0	8.0	16.0
Escherichia coli	56	53	22	8	2	4
Klebsiella pneumoniae	52	40	24	9	3	3
Pseudomonas aeruginosa	22	27	6	5	1	4
Acinetobacter baumannii	13	9	7	2	0	0
Citrobacter fruendii	1	3	0	0	0	0
Enterobacter cloacae	4	2	2	0	0	0
Total	148	134	61	24	06	11

Table3. Performance of CBDE, Etest and DD when compared with BMD (n=384).

Parameters	BMD	CBDE	Etest	DD
True positive	41	41	32	27
True negative	343	343	352	357
False positive	0	0	3	0
False negative	0	0	9	14
Major error (%)	0	0	0	0
Very major error (%)	0	0	21.9	34.1
Categorical agreement (%)	100	100	78.1	65.9
Sensitivity (%)	100	100	78.1	65.9
Specificity (%)	100	100	100	100
Positive predictive value (%)	100	100	100	100
Negative predictive value (%)	100	100	97.5	96.2

4. Discussion

Colistin should be kept and used sparingly after antibiotic susceptibility testing and antibiotic stewardship as the last resort medication dut to lack of development of new antibiotics for the treatment of drug-resistant Gram-negative strains.

The emergence of multi-drug resistant Gram-negative isolates in clinical settings, as well as difficulties in colistin susceptibility testing, have resulted in a disastrous situation in treating severe infections. Colistin susceptibility testing is hampered by some issues, such as a lack of agreement on drug resistance breakpoints.

The prevalence of colistin resistant Gram-negative clinical isolates found in this study was 10.7%. Similarly, another study has reported less than 10% colistin resistant globally whereas the quite higher prevalence in India and Philippines with 13.8% and 50% respectively[6]. This variation may be the result of geographic variation, the difference study population, and variation in colistin use.

The distribution of colistin resistance Gram-negative isolates within hospital wards present a shocking picture as most of the colistin isolates were from the ICU, and various studies all around the worlds supports these findings. The use of colistin to treat patients, various surgical procedures, and extended stays in the ICU may all contribute to emergencies[7–9]. The high incidence of these isolates in the ICU and other wards possess a great challenge for their treatment.

The evaluation of colistin resistance is very significant for infection control. In this context, the goal of this study was to assess colistin resistance among Gram-negative clinical isolates using DD, Etest and CBDE, then compared them with BMD which is reference method for colistin susceptibility testing.

The DD method of antimicrobial susceptibility testing has been rather unpopular for colistin. In this study, DD for colistin carried out using Piewngam's criteria[10].

The categorical agreement of DD with BMD was 65.9% in present study with very major of 34.1%. Studies done by various authors' show the categorical agreement ranging from 88.7%-91.2%[11–13].

In the present study, the categorical agreement of Etest found to be 78.1% with very major error of 21.9%. This colistin MIC by Etest was supported by other studies[11–13].

The categorical agreement of CBDE with BMD was 100% with no very major error. The very major error greater than 3% indicates that DD and Etest are not reliable methods for testing colistin susceptibility and thus could not meet the CLSI recommendation as an alternative method for reference BMD testing. CBDE could be used as an alternative method for routine laboratory colistin susceptibility testing as it is simple, reliable, and cost effective while BMD require high expertise, and training.

5. Conclusions

As the burden of adaptive resistance to colistin grows, it is critical to develop a diagnostic stewardship programme, particularly for countries such as India. The current investigation revealed a high prevalence of colistin resistance among drug resistant individuals suffering from various illnesses. This demonstrates the critical need of rewarming antibiotic-treatment techniques for people. Routine testing of colistin MIC should be done by CBDE method to prevent the increasing use of colistin to treat drug resistant bacterial infections. CBDE method is easy to used and as sensitive as broth micro dilution method.

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Conflict of interest statement

The authors declare that there is no conflict of interest.

Acknowledgements

We would like to thank NIMS University for providing facilities.

Funding

The authors received no extramural funding for the study.

Authors' contributions

Gautam PK and Tomar S conceptualized the study: PK Gautam curated the data: S. Tomar carried out supervision of study

References

[1] The review team. *Antimicrobial resistance: Tackling a crisis for the health and wealth of nations*. [Online] Available at: https://depts.washington.edu/edgh/app-ipc/web/project-resources/AMR-tackling-a-crisis.pdf. [Accessed on 15 March 2023].

[2] Catchpole CR, Andrews JM, Brenwald N, Wise R. A reassessment of the in-vitro activity of colistin sulphomethate sodium. *J Antimicrob Chemother* 1997; **39**(2): 255-260.

[3] Yow EM, Tan E, Shane L, Schonfeld S, Abu-Nassar Hanna. Colistin (colymycin) in resistant bacterial infections. A clinical appraisal. *Arch Intern Med* 1961; **108**(5): 664—670.

[4] European Society of Clinical Microbiology and infectious Diseases. *Recommendations for MIC determination of colistin*. [Online] Available at: https://www.eucast.org/eucast_news/news_singleview?tx_ttnews%5Btt_news%5D=171&cHash=387b192a5e9d82d2e74ae72fdb456602. [Accessed on 15 March 2023].

[5] The Clinical and Laboratory Standards Institute. *Performance Standards for Antimicribial Susceptibility Testing. 32nd ed*. [Online] Available at: https://clsi.org/media/tc4b1paf/m10033_samplepages-1.pdf. [Accessed on 20 April 2022].

[6] Bialvaei AZ, Kafil SH. Colistin, mechanisms and prevalence of resistance. *Curr Med Res Opin* 2015; **31**(4): 707-721.

[7] Trebosc V, Gartenmann S, Tötzl M, Lucchini V, Schellhorn B, Pieren M, et al. Dissecting colistin resistance mechanisms in extensively drug-resistant acinetobacter baumannii clinical isolates. *mBio* 2019; **10**(4): e01083-19.

[8] Janssen AB, van Hout D, Bonten MJM, Willems RJL, van Schaik W. Microevolution of acquired colistin resistance in Enterobacteriaceae from ICU patients receiving selective decontamination of the digestive tract. *J Antimicrob Chemother* 2020; **75**(11): 3135-3143.

[9] Panigrahi K, Pathi BK, Poddar N, Sabat S, Pradhan S, Pattnaik D, et al. Colistin resistance among multi-drug resistant gram-negative bacterial isolates from different clinical samples of ICU patients: Prevalence and clinical outcomes. *Cureus* 2022; **14**(8):e28317.

[10]Piewngam P, Kiratisin P. Comparative assessment of antimicrobial susceptibility testing for tigecycline and colistin against *Acinetobacter baumannii* clinical isolates, including multidrug-resistant isolates. *Int J Antimicrob Agents* 2014; **44**(5): 396-401.

[11]van der Heijden IM, Levin AS, De Pedri EH, Fung L, Rossi F, Duboc G, et al. Comparison of disc diffusion, Etest and broth microdilution for testing susceptibility of carbapenem-resistant *Pseudomonas aeruginosa* to polymyxins. *Ann Clin Microbiol Antimicrob* 2007; **6**: 8. doi: 10.1186/1476-0711-6-8.

[12]Behera B, Mathur P, Das A, Kapil A, Gupta B, Bhoi S, et al. Evaluation of susceptibility testing methods for polymyxin. *Int J Infect Dis* 2010; **14**(7): 596-601.

[13]Gupta P, Sharma R, Vyas A, Tak A. Comparative evaluation of broth micro dilution with E-test, Vitek 2, and disk diffusion for susceptibility testing of colistin on Gram-negative bacteria. *Indian J Med Sci* 2021; **73**(1): 93-98.

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