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Antimicrobial susceptibility testing of colistin: Evaluation of standard broth microdilution method for gram-negative bacilli

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Abstract

Background: Colistin is regarded as the last resort for managing infections caused by multidrug-resistant (MDR) Gram-negative bacilli (GNB). The World Health Organization (WHO) includes colistin on its list of critically necessary antimicrobials. Minimum inhibitory concentrations (MICs) are used to monitor the development of colistin resistance. This study aimed to assess the performance of the Broth Microdilution Method (BMD) against routine Kirby-Bauer disk diffusion (KBDD) and automated BD Phoenix for the detection of the in vitro activity of colistin against GNB.

Methods: A cross-sectional study was conducted in the Department of Microbiology, LLRM Medical College, Meerut, Uttar Pradesh, from September 2023 to January 2024. The KBDD method, BMD method, and BD Phoenix (Becton Dickinson, USA) automated system were used to detect colistin susceptibility in 320 GNB isolated from various clinical samples. MIC determined by the BMD method was interpreted according to Clinical Laboratory Standards Institute (CLSI) 2023 guidelines.

Results: In our study, 320 isolates of GNB were identified from patients with a mean age of 45.34 years. A total of 320 isolates [145 (45.31%) *Escherichia coli*, 124 (38.75%) *Klebsiella pneumoniae*, 32 (10.0%) *Pseudomonas aeruginosa*, and 19 (5.93%) *Acinetobacter baumannii* complex] were tested simultaneously with all three methods for colistin susceptibility. The overall resistance to colistin among GNB was found to be 17.18% by the gold standard BMD method, 15.31% by BD Phoenix, and 14.37% by KBDD.

Conclusion: BMD is the most cost-effective, authentic method for routine testing of colistin susceptibility as compared to other methods. The comparative analysis revealed that BMD is superior to other methods in detecting colistin susceptibility, emphasizing its potential role in guiding clinicians in antibiotic therapy decisions.

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Introduction

Multidrug resistance (MDR) among Gram-negative bacilli (GNB) has become a serious public health issue, negatively impacting the clinical outcome of infected individuals. Colistin is an ancient antibiotic that has resurfaced as a last-resort treatment for infections caused by these MDR pathogens. The emergence of colistin resistance (COL-R), whether caused by chromosomal mutations or plasmid-mediated (MCR) mechanisms (Which all result in modifications of the lipopolysaccharides of the outer membrane in GNB), has now been identified in animals, food animal products, and human samples, and it represents a new threat to global public health (1).

For patient management and monitoring of colistin resistance, a reliable, reproducible antimicrobial susceptibility testing (AST) approach is necessary. Since 2016, CLSI and the European Committee on Antimicrobial Susceptibility Testing (EUCAST) have both recommended the Broth Microdilution Method (BMD) for determining colistin MIC. However, reference BMD, which requires freshly produced or frozen antibiotic solutions, is rarely performed in routine clinical laboratories (2).

Other methods available, such as agar dilution and gradient diffusion, are currently not recommended susceptibility testing procedures. A variety of more user-friendly commercial automated AST systems based on the BMD approach are now available (3). Due to difficulties with polymyxin testing, such as poor diffusion of polymyxins into agar, inherent cationic properties of polymyxins, the occurrence of heteroresistance to polymyxins in many species, and the lack of a reliable reference method that may allow reliable comparisons of commercial tests, polymyxin susceptibility testing presents a significant challenge for a clinical laboratory (4).

The introduction of automated systems like BD Phoenix provides the benefits of speed, efficiency, and accuracy, making them suitable for routine susceptibility testing. However, their ability to accurately determine colistin susceptibility, particularly in the context of MDR organisms, requires comprehensive evaluation (5). The purpose of this study was to assess the performance of BMD against BD Phoenix and routine KBDD to detect colistin resistance in GNB. The results of colistin susceptibility were evaluated and compared with the reference BMD method.

Methods

Ethical approval and study design

The present cross-sectional study was conducted in the Department of Microbiology at Lala Lajpat Rai Memorial (LLRM) Medical College in Meerut, associated with a tertiary care hospital, over six months (September 2023-February 2024). It was approved by the institutional ethics committee and complied with all regulations vide letter No. SC-1/2023/5328. A total of 320 clinical isolates of common Gram-negative bacteria from various clinical specimens of patients received from different departments, after obtaining informed consent from the patients for routine culture sensitivity testing, were processed in the clinical bacteriology laboratory.

Specimen collection and identification of bacteria

Various clinical specimens were obtained from 320 patients from both the in-patient and out-patient departments of the hospital. The specimens included blood, pus/tissue, body fluids, respiratory specimens, and urine. These isolates comprised a mixed population of immunocompetent, immunosuppressed/immunocompromised, and critically ill patients. Upon collection, all the clinical specimens were

subsequently processed using standard microbiological methods. The clinical samples were streaked onto appropriate agar plates. Confirmation of the identity and antibiotic susceptibility testing (AST) of the isolates was done by automated BD Phoenix M-50.

Antibiotic susceptibility testing

Three distinct methods were used for colistin susceptibility testing: in-house BMD, routine KBDD, and automated BD Phoenix M-50. Colistin testing was done by reporting the MIC values as per the CLSI 2023 standards. The procedures of the methods used are described as follows:

Kirby-Bauer disk diffusion method

This method involves placing an antibiotic disc onto the Mueller-Hinton agar plate inoculated with the test isolates, and the plates were then incubated for 16-18 hours at 37°C. The zone diameters around each disc were measured, and the results were interpreted as "sensitive" or "resistant" according to CLSI 2023 guidelines.

BMD

The reference in-house BMD was performed according to CLSI 2023 guidelines (6,7). Colistin stock solution (5120 g/ml) was made by dissolving 102.4 mg of colistin sulfate powder (Sigma-Aldrich; Potency = 500 µg/mg) in 10 ml of sterile water. Filter-sterilized colistin stock solution was aliquoted in smaller amounts and stored at -60°C. The cation-adjusted Mueller-Hinton broth (CaMHB) was prepared, and a stock solution of colistin was prepared from colistin sulfate salt. The final bacterial inoculum size of 0.5 McFarland was used. The test was done in triplicate in a polystyrene microtitre plate (Corning CLS3585 flat-bottom 96 wells with lid) and incubated for 16 to 20 hours at 35°C ± 2°C and examined visually, and MIC values were noted. For sterility control, physiological saline was added to wells instead of bacterial inoculum. Because the CLSI does not provide clinical breakpoints for colistin for *Enterobacteriaceae*, the European Committee on Antimicrobial Susceptibility Testing (EUCAST) MIC breakpoints were used for interpretation.

Based on the epidemiological cut-off value for *Enterobacteriaceae*, *Klebsiella spp.*, and *Escherichia coli* were considered sensitive if the MIC value was ≤ 2 µg/ml and resistant if the MIC value was ≥ 4 µg/ml. For *Pseudomonas aeruginosa* and *Acinetobacter spp.*, an MIC value of ≤ 2 µg/ml was interpreted as sensitive and an MIC value of ≥ 4 µg/ml was interpreted as resistant (6).

BD phoenix M-50 system

The manufacturer's instructions were followed to determine the colistin susceptibility of the various test isolates. The probable range of MIC for BD Phoenix was ≤ 1 to > 4 µg/ml.

Quality control

For quality control, the following strains were used: *Pseudomonas aeruginosa* (ATCC 27853), *Escherichia coli* (ATCC 25922), *Klebsiella pneumoniae* (ATCC 13883), and *Acinetobacter baumannii* (ATCC 17978).

Reconfirmation of discrepant results

Repeat testing was used to confirm discrepant results between BD Phoenix and the BMD methods.

Results

A total of 320 GNB isolates from various clinical specimens were obtained and identified. The mean age of the patients was 45.34 years. The study showed male predominance in the sample group, with 187 males (58.43%) and 133 females (41.56%) and a male-to-female ratio of 1.4. The majority of the patients, accounting for 248 (77.5%) cases, were from the outpatient department, and 72 (22.5%) cases were from the various other wards. Analysis of sample type showed that pus was the most common sample, accounting for 76 (23.75%) cases, followed by urine with 59 (18.43%) cases, blood with 48 (15%) cases, sputum with 43 (13.43%) cases, ascitic fluid with 41 (12.81%) cases, tissue biopsy with 25 (7.81%) cases, pleural fluid with 18 (5.62%) cases, and tracheal aspirate with 10 (3.12%) cases, respectively (Figure 1).

Of the 320 Gram-negative bacteria, *Escherichia coli* was the most common isolate in 45.31% of cases, followed by *Klebsiella pneumoniae* in 38.75% of cases, *Pseudomonas aeruginosa* in 10% of cases, and *Acinetobacter baumannii* complex in 5.93% of cases, respectively.

Colistin Resistance: The overall resistance to colistin among GNB was found to be 17.18% (55/320) by the gold standard BMD method, of which 58.18% (32/55) were males and 41.81% (23/55) were females. The resistance to colistin was found to be 14.49% (39/269) in *Enterobacteriaceae* (*Escherichia coli* and *Klebsiella spp.*) and 31.37% (16/51) in non-fermenters (*Acinetobacter spp.* and *Pseudomonas aeruginosa*), respectively. In comparison, BD Phoenix showed overall resistance to colistin among GNB in 49 isolates, i.e., 15.31%. BD Phoenix failed to detect resistance in 6 cases which were shown resistant by the BMD method. KBDD showed resistance to colistin in 46 cases (14.37%) and showed discordant results in 9 cases (Table 1).

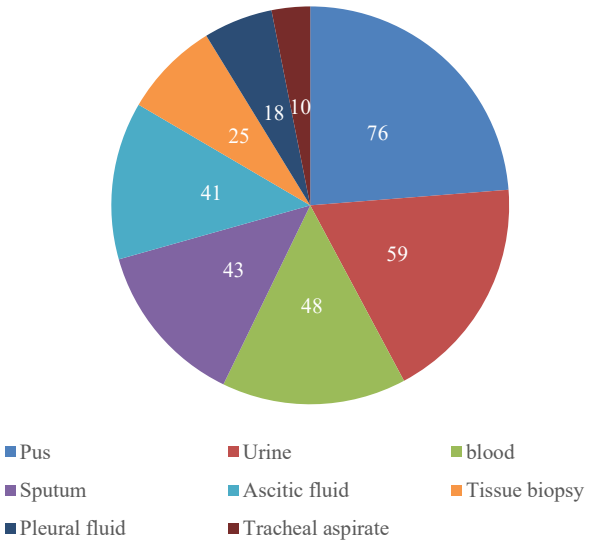

Figure 1. Distribution of different sample types for colistin susceptibility testing

The detailed results of these isolates are presented below (Table 2 and Figure 2).

Escherichia coli

The total number of isolates showing resistance to colistin by BMD, BD Phoenix, and KBDD were 26 (17.93%), 23 (15.86%), and 22 (15.17%), respectively. Out of 26 isolates that were found to be resistant by the BMD method, BD Phoenix detected resistance in only 23 isolates, and the remaining 3 isolates showed discordant results. KBDD detected resistance in only 22 isolates, and the remaining 4 cases failed to detect the resistance.

Klebsiella spp.

The total number of isolates showing resistance to colistin by BMD, BD Phoenix, and KBDD was 13 (10.48%), 13 (10.48%), and 11 (8.87%), respectively. BD Phoenix successfully detected resistance in all 13 isolates that were found to be resistant by the BMD method. KBDD detected resistance in only 11 isolates and failed to detect resistance in two cases.

Pseudomonas aeruginosa

The total number of isolates showing resistance to colistin by BMD, BD Phoenix, and KBDD were 5 (15.62%), 3 (9.37%), and 4 (12.50%), respectively. Out of 5 isolates that were found to be resistant by the BMD method, BD Phoenix and KBDD failed to detect resistance in 2 and 1 isolates, respectively.

Table 1. Colistin resistance shown by different methods

Method	Total isolates tested	Colistin resistance, n (%)
BMD (Broth microdilution)	320	55 (17.18)
BD Phoenix (Automation)	320	49 (15.31)
Disc diffusion	320	46 (14.37)

Table 2. Distribution of BD phoenix, disc diffusion and BMD pattern for GNB in isolates of patients

Gram-negative bacteria	BD PHOENIX M 50		Broth microdilution		Disc diffusion	
	N (%)					
	Sensitive	Resistant	Sensitive	Resistant	Sensitive	Resistant
***Escherichia coli* (n=145)**	122 (84.13)	23 (15.86)	119 (82.06	26 (17.93)	123 (84.82)	22 (15.17)
***Klebsiella pneumoniae* (n=124)**	111 (89.51)	13 (10.48)	111 (89.51)	13 (10.48)	113 (91.12)	11 (8.87)
***Pseudomonas aeruginosa* (n=32)**	29 (90.06)	3 (9.37)	27 (84.37)	5 (15.62)	28 (87.50)	4 (12.50)
***Acinetobacter baumannii* complex (n=19)**	9 (47.36)	10 (52.63)	8 (42.10)	11 (57.89)	10 (52.63)	9 (47.36)
Total = 320	271 (84.68)	49 (15.31)	265 (82.81)	55 (17.18)	274 (85.62)	46 (14.37)

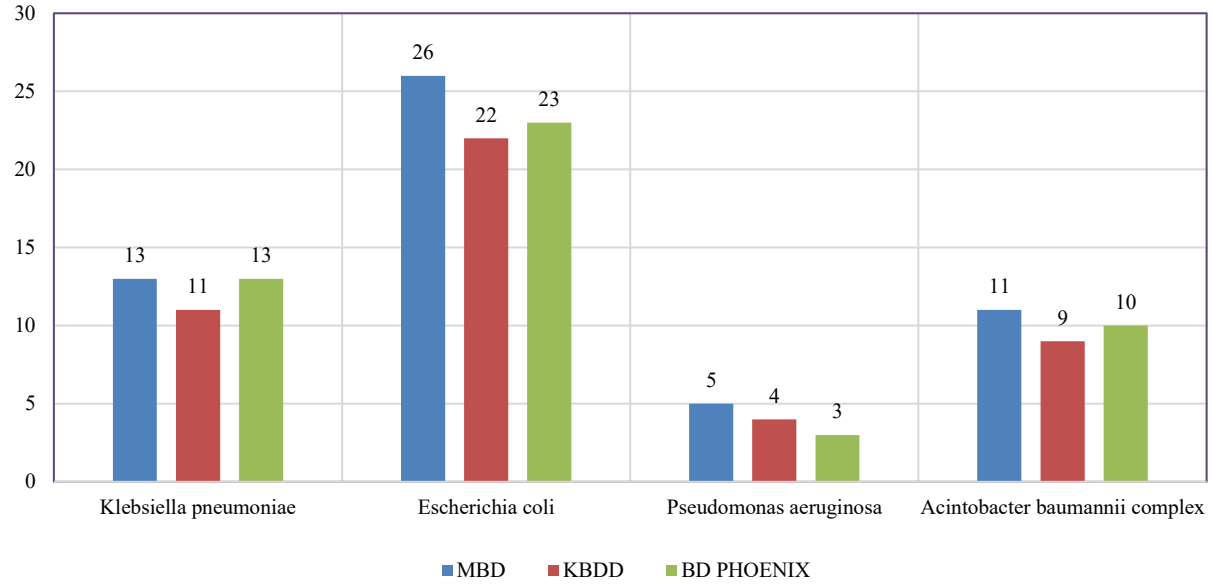

Figure 2. Colistin resistance by different gram-negative bacilli (Abbreviations: MBD: Broth Microdilution method; KBDD: Kirby-Bauer Disk Diffusion)

Acinetobacter baumannii

The total number of isolates showing resistance to colistin by BMD, BD Phoenix, and KBDD was 11 (37.89%), 10 (52.63%), and 9 (47.36%), respectively. Out of 11 isolates that were found to be resistant by the BMD method, BD Phoenix detected resistance in only 10 isolates and failed to detect resistance in 1 strain. KBDD detected resistance in only 9 isolates, and the remaining 2 cases failed to detect the resistance.

Discussion

The literature demonstrates that the rise of carbapenem-resistant *Enterobacteriaceae* (CRE) has become a serious problem. In patients with carbapenem-resistant GNB infections, colistin is the mainstay of treatment, and its usage has increased worldwide, particularly in India, after the emergence of CRE. Nonetheless, there are differences in susceptibility test findings obtained using various methodologies. An accurate method is required to test colistin susceptibility, as there are elevated trends of colistin MICs noted worldwide. In the present study, the performance of the gold standard BMD method was evaluated and compared with automated BD Phoenix M-50 and KBDD for colistin susceptibility testing. The study findings shed light on the scope of antibiotic resistance and the reliability of testing methodologies, providing important insights for clinical practice and antimicrobial stewardship.

The mean age of the patients in our study was 45.34 years. Taneja et al., Aggarwal et al., Arjun et al., and Goel et al. (8-11) reported a similar mean age. In terms of sex distribution, our study included 187 males (58.43%) and 133 females (41.56%), indicating a modest male predominance among the sample group. Kumari et al., Taneja et al., Pragasam et al., Aggarwal et al., and Goel et al. all reported a similar pattern of sex distribution (8-10,12,13). The sample type analysis in our study revealed that pus was the most common sample, which was similar to the studies done by Zaki et al. and Pawar et al. (14,15).

Butta et al. found 19.17% (140/730) resistance to colistin among Gram-negative bacilli using the gold standard BMD technique, which was in concordance with our study (16).

In a study by Bernhardt et al., *Klebsiella pneumoniae* showed higher resistance to colistin when tested by the BMD method (6/10 isolates), and BD Phoenix showed lower resistance to colistin (3/10 isolates), while in our study all the *Klebsiella pneumoniae* showed equal resistance with both methods (17).

In our study, out of 320 Gram-negative isolates tested, 82.81% were sensitive to colistin when tested by the BMD method. Studies by Arjun et al., Ramesh et al., and Behera et al. reported colistin sensitivity of 70.83%, 55.55%, and 94.23%, respectively, among MDR-GNB (11,18,19).

A recent study by Lai CC et al. and Pfennigwerth et al. also showed unreliable colistin MIC results by an automated method (20,21). It is particularly important to test all MDR isolates for colistin susceptibility using the BMD method because false negative and positive results would place the patient on incorrect antimicrobial therapy. In this study, evaluation of both carbapenem-susceptible (non-MDR) and non-susceptible (MDR) bacterial isolates, and colistin susceptibility testing by BMD, was performed concurrently with the BD Phoenix and routine KBDD method on the same day of isolation as a regular colistin susceptibility testing method.

Although performing AST methods such as BMD for clinical testing is technically demanding, laboratories must train the staff to perform BMD and overcome common challenges such as making initial dilutions, multiple skipped wells, contamination, or other quality control issues that are not present in automated systems.

Abbreviations

MICs: Minimum Inhibitory Concentrations; BMD: Broth Microdilution method; GNB: Gram-Negative Bacilli; CLSI: Clinical Laboratory Standards Institute; (COL-R): Colistin Resistance; MCR: Chromosomal Mutations; MDROs: Multidrug-Resistant pathogens; BMD: Broth Microdilution; AST: Antimicrobial Susceptibility Testing; EUCAST: European Committee on Antimicrobial Susceptibility Testing

Conclusion

Compared to disk diffusion and automated methods like BD Phoenix, the BMD method is the most cost-effective and reliable approach for colistin susceptibility testing. Reporting by automated methods is a simple procedure, and the results in terms of BMD are generally acceptable in *Klebsiella spp.*, *Escherichia coli*, and *Acinetobacter baumannii* complex. Automation could be utilized to test colistin susceptibility in low-risk patients. BMD, on the other hand, should be employed in high-risk and immunocompromised patients hospitalized in critical care units. The resistance profiles of Gram-negative organisms in our study highlight the urgent need for novel therapeutic approaches and efficient infection control measures. Strong antimicrobial stewardship initiatives are recommended to counteract the rising rate of resistance.

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Ethical statement

The research study has been approved by the institutional ethics committee and is compliant with all necessary regulations. Ref. No. SC-1/2023/5328.

Conflicts of interest

Authors declare no conflicts of interest.

Author contributions

Dr. Naila Begum performed the laboratory work, drafted the paper, and provided critical inputs; Dr. Amit Garg conceived and designed the study and revised the manuscript for important intellectual content; Dr. Karvi Agarwal rechecked the manuscript.

Data availability statement

The authors can make the data available upon a reasonable request.

References

1. El-Sayed Ahmed MA, Zhong LL, Shen C, Yang Y, Doi Y, Tian GB. Colistin and its role in the Era of antibiotic resistance: an extended review (2000-2019). Emerg Microbes Infect. 2020;9(1):868-85. [View at Publisher] [DOI] [PMID] [Google Scholar]
2. Gwozdzinski K, Azarderakhsh S, Imirzalioglu C, Falgenhauer L, Chakraborty T. An improved medium for colistin susceptibility testing.J Clin Microbiol. 2018;56(5):e01950-17. [View at Publisher] [DOI] [PMID] [Google Scholar]
3. Matuschek E, Åhman J, Webster C, Kahlmeter G. Antimicrobial susceptibility testing of colistin-evaluation of seven commercial MIC products against standard broth microdilution for Escherichia coli, Klebsiella pneumoniae, Pseudomonas aeruginosa, and Acinetobacter spp. Clin Microbiol Infect. 2018;24(8):865-70. [View at Publisher] [DOI] [PMID] [Google Scholar]
4. Ezadi F, Ardebili A, Mirnejad R. Antimicrobial susceptibilitytesting for polymyxins: challenges, issues, and recommendations. J Clin Microbiol. 2019;57(4):e01390-18. [View at Publisher] [DOI] [PMID] [Google Scholar]
5. Gales AC, Jones RN, Sader HS. Contemporary activity of colistin and polymyxin B against a worldwide collection of Gram-negative pathogens: results from the SENTRY Antimicrobial Surveillance Program (2006-09). J Antimicrob Chemother. 2011;66(9):2070-4. [View at Publisher] [DOI] [PMID] [Google Scholar]
6. CLSI. Performance Standards for Antimicrobial Susceptibility Testing. 33RD ed. CLSI supplement M100. Wayne, PA: Clinical and Laboratory Standards Institute; 2023. [View at Publisher]
7. CLSI. Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria that grow aerobically. 11th ed. CLSI standard M07. Wayne, PA: Clinical and Laboratory Standards Institute;2018. [View at Publisher]
8. Taneja N, Singh G, Singh M, Sharma M. Emergence of tigecycline & colistin-resistant Acinetobacter baumanii in patients with complicated urinary tract infections in north India. Indian J Med Res. 2011;133(6):681-4. [View at Publisher] [PMID] [Google Scholar]
9. Aggarwal R, Rastogi N, Mathur P, Soni KD, Kumar S, Gupta A, et al. Colistin-resistant Klebsiella pneumoniae in surgical polytrauma intensive care unit of level-1 trauma center: first case series from trauma patients in India. Indian J Crit Care Med. 2018;22(2):103-6. [View at Publisher] [DOI] [PMID] [Google Scholar]
10. Goel G, Hmar L, Sarkar De M, Bhattacharya S, Chandy M. Colistin-Resistant Klebsiella pneumoniae: Report of a Cluster of 24 Cases from a New Oncology Center in Eastern India. Infect Control Hosp Epidemiol. 2014;35(8):1076-7. [View at Publisher] [DOI] [PMID] [Google Scholar]
11. Arjun R, Gopalakrishnan R, Nambi PS, Kumar DS, Madhumitha R, Ramasubramanian V. A study of 24 patients with colistin-resistant Gram-negative isolates in a tertiary care hospital in South India. Indian J Crit Care Med. 2017;21(5):317-21. [View at Publisher] [DOI] [PMID] [Google Scholar]
12. Pragasam AK, Shankar C, Veeraraghavan B, Biswas I, Nabarro LEB, Inbanathan FY, et al. Molecular mechanisms of colistin resistance in Klebsiella pneumoniae causing bacteremia from India-a first report. Front Microbiol. 2017;7:2135. [View at Publisher] [DOI] [PMID] [Google Scholar]
13. Anita,Kumari R, Saurabh K, Kumar S, Kumari N. Comparative Evaluation of Broth Microdilution with Disc Diffusion and VITEK 2 for Susceptibility Testing of Colistin on Multidrug-Resistant Gram-Negative Bacteria. Cureus. 2023;15(12):e50894. [View at Publisher] [DOI] [PMID] [Google Scholar]
14. Zaki ME, ElKheir NA, Mofreh M. Molecular study of colistin-resistant clinical isolates of Enterobacteriaceae species. J Clin Mol Med. 2018;1(1):1-4. [View at Publisher] [DOI] [Google Scholar]
15. Pawar SK, Karande GS, Shinde RV, Pawar VS. Emergence of colistin-resistant Gram-negative Bacilli, in a tertiary care rural hospital from Western India. Indian J Microbiol Res. 2016;3(3):308-13. [View at Publisher] [DOI] [Google Scholar]
16. Butta H, Mendiratta L, Sardana R, Gilotra K, Hasan S, Kansal S, et al. Detecting In-Vitro Colistin Resistance-A Comparative Study Between Broth Microdilution Versus Vitek-2 For Colistin Susceptibility Testing. APALM. 2020;7(7):A336-340 [View at Publisher] [DOI] [Google Scholar]
17. Bernhardt JS, Tellis RC, Motagi A, PP A. Colistin and Polymyxin B sensitivity by broth microdilution among carbapenem-resistant clinical isolates of Gram-negative bacilli. Biomedicine. 2023;43(6):1789-93. [View at Publisher] [DOI] [Google Scholar]
18. Ramesh N, Prasanth M, Ramkumar S, Suresh M, Tamhankar AJ, Gothandam KM, et al. Colistin susceptibility of gram-negative clinical isolates from Tamil Nadu, India. Asian biomedicine. 2016;10(1):35-9. [View at Publisher] [DOI] [Google Scholar]
19. Behera B, Jena J, Kar P, Mohanty S, Mahapatra A. Deciphering polymyxin B minimum inhibitory concentration from colistin minimum inhibitory concentration and vice versa: An analysis on 156 carbapenem-resistant Enterobacteriaceae isolates. Indian J Med Microbiol. 2018;36(4):587-9. [View at Publisher] [DOI] [PMID] [Google Scholar]

20. Lai C-C, Chen Y-S, Lee N-Y, Tang H-J, Lee S S-J, Lin C-F, et al. Susceptibility rates of clinically important bacteria collected from intensive care units against colistin, carbapenems, and other comparative agents: Results from surveillance of multicenter antimicrobial resistance in Taiwan (SMART). Infect Drug Resist. 2019;12:627-40. [View at Publisher] [DOI] [PMID] [Google Scholar]
21. Pfennigwerth N, Kaminski A, Korte-Berwanger M, Pfeifer Y, Simon M, Werner G, et al. Evaluation of six commercial products for colistin susceptibility testing in Enterobacterales. Clin Microbiol Infect. 2019;25(11):1385-9. [View at Publisher] [DOI] [PMID] [Google Scholar]

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