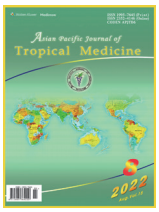

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# Assessment of colistin resistance in Gram negative bacteria from clinical samples in resource-limited settings

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## ABSTRACT

**Objective:** To find a proper method to assess colistin resistance in multidrug resistant Gram negative bacteria (MDR-GNB) on a routine basis in resource limited settings.

**Methods:** Clinical samples were processed. MDR-GNB were identified and were examined for colistin resistance by colistin broth elution method, colistin agar method, and colistin disk elution screening method. Broth microdilution method was used the gold standard.

**Results:** A total of 10 235 clinical samples were processed, in which 857 (8.4%) MDR-GNB were identified. The very significant errors, categorical agreement, major errors, positive predictive values, negative predictive values, specificity and sensitivity of all the phenotypic methods were 5.5%, 0%, 94.4%, 100%, 99.6%, 100% and 94.4%, respectively for the detection of colistin resistance. The colistin elution screening method was cheap and easy to perform with similar results to broth microdilution method.

**Conclusions:** All the evaluation methods for colistin resistance showed similar results. So the laboratories can choose any method for detection of colistin resistance. However, we recommend colistin disk elution screening method because, it is easy and cheap and can be performed in limited resources.

**KEYWORDS:** Multi-drug resistant organism; Colisitin resistance; *mcr* gene; Broth micro dilution; Disk elution method

## 1. Introduction

The colistin (polymyxin) antibiotic was discovered many years ago, but it was never used because of its neurotoxicity and nephrotoxicity. Colistin is now being used as a last-resort treatment for multidrug resistant or carbapenem-resistant Gram negative bacteria[1].

Colistin is a panta-cationic antibiotic that acts on lipopolysaccharide in the cell wall of Gram negative bacteria (GNB) and breaks the outer membrane, which causes cell lysis responsible for cell death[2]. Colistin resistance in Gram negative bacteria develops as a result of a change in target site lipopolysaccharide, commonly due to chromosomal mutations and plasmid and can be transferred from one bacterium to another by plasmid transfer. The *mcr-1* gene is the predominant plasmid identified in colistin resistant bacteria. However, other plasmids from *mcr-2* to *mcr-10* have been identified in colistin resistant bacteria[2,3].

The Enterobacteriaceae family, *Pseudomonas* species and *Acinetobacter* species are identified as resistant to colistin in clinical samples. Intrinsic resistance is also present in several Gram negative bacteria such as *Morganella* species, *Providencia* species, and *Proteus* species[2,4]. However, colistin resistance in anaerobic bacteria is not reported yet[5].

The presence of colistin resistance in forces researchers to develop methods to detect colistin resistance. Several methods have been developed to detect colistin resistance, such as broth microdilution method (BMD), colistin broth disk elution method, CHROM agar

**Significance**

This study compared various methods to detect colistin resistance including sensitivity and specificity of each test. Colistin disk elution screening method is cheaper and reliable, and can be used as an alternative screening method for detection of colistin resistance in resource limited settings.

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COL, ResaPolymyxin NP test, rapid polymyxin NP test, and the colistin agar method[6-8].

The BMD test was recommended by the Clinical and Laboratory Standard Institute (CLSI) and EUCAST (the European Committee on Antimicrobial Susceptibility Testing)[8,9]. However, the test should be performed with colistin sulphate salt with cation-adjusted Mueller Hinton broth without polysorbate-80 containing micro-titreplate[8]. The disc diffusion method could not be used to detect colistin resistance because of the molecule size of colistin. Hence, there is a need for a simple and reliable test to detect colistin resistance in resource limited nations[7,8]. A reliable method and rapid laboratory diagnosis play an important role in preventing morbidity and mortality due to colistin resistance[10]. We are still lagging in the battle against antimicrobial resistance, and existing diagnostic methods require skill, training, and cost. So their performance, cost, and training should be evaluated. We designed this study to evaluate phenotypic methods to detect colistin resistance in GNB isolated from clinical samples with the BMD method.

The aim of the study was to find a reliable, simple, and reasonable test that could be used on a regular basis in a microbiology laboratory with limited resources.

## 2. Materials and methods

### 2.1. Samples

This cross-sectional study was conducted from March 2021 to April 2022 in the Department of Microbiology at the Maharishi Markandeshwar Institute of Medical Science and Research in Mullana, Ambala, India. The clinical samples such as blood, pus, sputum, urine, cerebrospinal fluid were collected and transported to microbiology laboratory. The Institutional Ethical Committee (IEC) granted ethical clearance with latter no. MMIMSR/IEC/1916. However, duplicate samples, Gram positive and intrinsically resistant organisms to colistin were excluded from this study and multidrug resistant Gram negative bacteria (MDR-GNB) were included in this study.

### 2.2. Identification of bacteria and antibacterial sensitivity testing

After direct Gram staining, samples were inoculated on blood agar and MacConkey agar as per standard protocol and incubated at 37 ℃ for 24 h. The automatic system Vitek-2 was used to identify the isolates as per manufacturer's guidelines and the identification was confirmed standard biochemical tests. The antibacterial sensitivity testing of the isolated Gram negative bacteria was performed by Kirby Bauer disc diffusion method as per CLSI-2021[11] and Gram negative bacteria were classified MDR as per US Centers for Disease Control and Prevention (CDC) and European Center for Disease Prevention and Control (ECDC)[12].

### 2.3. Colistin resistance detection by BMD

Colistin resistance was assessed by the BMD method as per the CLSI recommendation. The minimum inhibitory concentration (MIC) ranging from 0.5 μg/mL to 32 μg/mL was prepared by dissolving colistin sulphate salt (HI Media, India) in Cation-Adjusted Muller-Hinton broth (Supplementary Figure 1)[10]. MIC breakpoint for colistin is as follows: ≥4 μg/mL for resistance, ≤2 μg/mL for intermediate. There is no guideline for colistin sensitive interpretation as per CLSI-2021 for Enterobacteriaceae, *Pseudomonas* species, and *Acinetobacter* species[11].

### 2.4. Detection of mcr gene

The heat lysis method was used for extraction, and for one test, 12.5 μL Dream Taq Green PCR Master Mix, 2 μL of primer solution (2 μL of each primer), 2 μL DNA lysate, and 5.5 μL nuclease-free water were added in a PCR tube and were used for each experiment. PCR conditions were as follows: denaturation at 94 ℃ for 15 min, denaturation repeated 25 cycle for 30 sec, annealing at 58 ℃ for 90 sec, elongation at 72 ℃ for 60 sec, and a final cycle of elongation at 72 ℃ for 10 min. The amplified product was electrophoresed on a 1.5% agrose gel stained with ethidium-bromide at 130V and observed under UV rays for *mcr-1* to *mcr-5* carrying isolates (Table 1)[13].

**Table 1.** Primers used for the amplification of target genes.

| Sr. No. | *mcr* genes | Amplicon size (bp) | Primer sequences (5'-3') |
|---|---|---|---|
| 1 | *mcr-1* | 320 | -AGTCCGTTTGTTCTTGTGGC-<br>-AGATCCTTGGTCTCGGCTTG- |
| 2 | *mcr-2* | 700 | -CAAGTGTGTTGGTCGCAGTT-<br>-TCTAGCCCGACAAGCATACC- |
| 3 | *mcr-3* | 900 | -AAATAAAAATTGTTCCGCTTATG-<br>-AATGGAGATCCCCGTTTTT- |
| 4 | *mcr-4* | 1 100 | -TCACTTTCATCACTGCGTTG-<br>-TTGGTCCATGACTACCAATG- |
| 5 | *mcr-5* | 1 644 | -ATGCGGTTGTCTGCATTTATC-<br>-TCATTGTGGTTGTCCTTTTCTG- |

### 2.5. Colistin broth disk elution method

In this method, 4 glass tubes were used, and 10 mL of Cation-adjusted Muller Hinton broth (HI-media) was added in each tube and then the first tube was taken as growth control (no antibiotic disc added). In the second tube, 1 disc of colistin sulphate (10 μg) (Oxiod) was added. In the third tube, 2 discs of colistin sulphate (10 μg) were added and in the fourth tube, 4 discs of colistin sulphate (10 μg) were added and the tubes were incubated at room temperature for 30-45 min to elute the colistin in the medium (Supplementary Figure 2). Colonies from blood agar were used to prepare a 0.5 McFarland solution in the normal saline and after mixing properly, 50 μL inoculum was added to each tube. The test tubes were mixed well and incubated at 37 ℃ for 24 h. The results of colistin MIC were interpreted as per CLSI-2021[11].

### 2.6. Colistin agar test

Colistin agar test was performed with Muller-Hinton agar. The Muller Hinton agar (MHA) was prepared with colistin sulphate salt from 0 to 4 μg/mL concentration in 4 different petri-plates (Supplementary Figure 3). Colonies from blood agar were used to prepare an inoculum of 0.5 McFarland solution in normal saline and the inoculum was diluted 1:10 in saline. MHA was divided into 10 parts and 10 μL diluted solution was streaked onto a specific area and incubated at 37 ℃ for 24 h. Results were interpreted as per CLSI 2021 guidelines[11].

### 2.7. Colistin disk elution screening method

This is an in-house developed screening test. 10 mL of Cations-adjusted Mueller Hinton broth was taken to a glass tube and 2 colistin sulphate discs [10 μg (Oxoid)] were added and incubated at room temperature for 30-45 min to elute antibiotic in medium and after incubation tube was vortexed. The solution was transferred to 4 tubes, 2.5 mL in each tube. Colonies from blood agar were used to prepare an inoculum of 0.5 McFarland solution in normal saline and mixed properly. 13 μL inoculum was added to each of 4 tubes. Tubes were incubated at 37 ℃ for 24 h. The tubes showing turbidity (bacterial growth) were considered as colistin-resistant (Supplementary Figure 4).

### 2.8. Grading criteria

Grading of all the tests was done on the basis of cost, simplicity in performing and training required. A total of 10 laboratory technicians were asked to perform all the four tests and then mean grading was noted. The breakdown of the assigned grades on scale of 1 to 4 are shown in Table 2[8].

**Table 2.** Ease to perform a method with their grading and interpretation.

| Parameter | Grades | Results |
|---|---|---|
| Ease to carry out test | 1 | Laborious to perform |
| | 2 | Hard to perform |
| | 3 | Moderate to perform |
| | 4 | Easy to perform |
| Cost | 1 | Approx. 2 dollar* |
| | 2 | Approx. 1 dollar* |
| | 3 | Below 1 dollar* |
| | 4 | Below 0.5 dollar* |
| Training | 1 | High training required |
| | 2 | Training required |
| | 3 | Average skill required |
| | 4 | Minimum skill required |

*Dollar prices fluctuate, we used an approximation rate, and the rate was set for 2021.

### 2.9. Data analysis

The Microsoft excel was used to record the data and the BMD method was used as a reference method for colistin resistance. The major errors, very major errors, sensitivity, specificity, negative predictive value, positive predictive value, and categorical agreement were used to evaluate the performance of the test as per previous studies[8,14].

## 3. Results

The study was conducted in the Department of Microbiology from March 2021 to April 2022. A total of 10235 samples were received in microbiology laboratory including urine (26.0%), followed by pus (21.6%), blood (16.7%), sputum (18.0%), wound swabs (9.5%), vaginal swabs (2.0%), and 6.6% miscellaneous samples (tissues, ear swabs, pleural fluid, ascitic fluid *etc*.). Out of the total sample, 62.33% were sterile/no growth and 21.46% samples showed growth of Gram negative bacteria and 16.21% showed growth of Gram positive bacteria (Figure 1).

Out of 2196 Gram negative bacteria, predominant Gram negative bacteria was *Escherichia coli* (40.11%), followed by *Klebsiella (K.) pneumonia* (31.46%), *Pseudomonas (P.) aeruginosa* (20.58%), *Acinetobacter (A.) baumannii* (5.01%), *Citrobacter (C.) freundii* (1.50%), *Stenotrophomonas maltophilia* (1.00%) and *Proteus* species (0.77%)

A total of 857 MDR-GNB were isolated. *K. pneumoniae* (45.15%) was the predominant isolate, followed by *Escherichia coli* (29.98%), *P. aeruginosa* (12.83%), *A. baumannii* (10.15%), and *C. freundii* (1.86%). Furthermore, for the detection of colistin resistance, all isolates were subjected to BMD method and their MICs were noted. Out of total 857 MDR-GNB isolates, 668 showed colistin MIC of ≤0.5 μg/mL, 92 isolates had MIC 1 μg/mL, 43 isolates had MIC 2 μg/mL, 41 isolates had MIC 4 μg/mL, 10 isolates had MIC of 8 μg/mL MIC and 3 had MIC of 16 μg/mL. The MICs of all the Gram-negative bacteria are given in Table 3. No *mcr* gene was detected among them.

All the colistin resistant isolates were from In Patient Department patients, among which the maximum organism was isolated from oncology department (23.60%), followed by surgery (18.42%), respiratory (13.50%), and emergency (5.20%) while no colistin resistant organism isolated from Out Patient Department. The maximum colistin resistant organisms were seen from pus 36.4%, followed by urine 31.5%, wound swab 13.57%, sputum 7.8%, blood samples 5.26%, and ascitic fluid 5.26%.

All MDR-GNB were evaluated for colistin resistance by BMD method (gold standard), colistin broth disk elution method, colistin agar test and colistin disk elution screening test (in-house modification). The BMD method detected 54 (6.30%) colistin resistant organisms, while the colistin broth disk elution method failed to detect 3 organisms, the colistin agar test failed to detect 3 organisms and colistin disk elution screening method failed to detect 2 colistin resistant MDR-GNB.

The colistin broth disk elution method, colistin agar test and

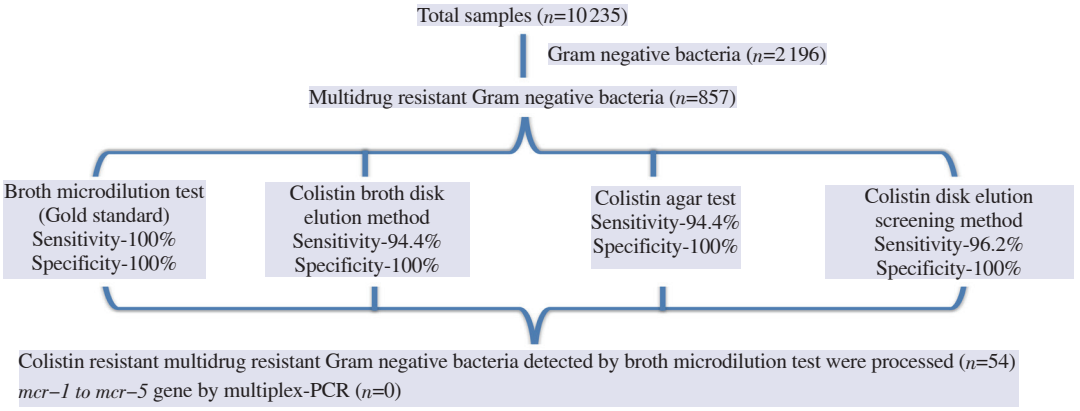


**Figure 1.** A flowchart of this study.

**Table 3.** Frequency of MICs towards colistin in different multidrug resistant Gram negative bacteria isolates (*n*=857).

| MICs of colistin (μg/mL) | *Klebsiella pneumoniae* (*n*=387) | *Escherichia coli* (*n*=257) | *Pseudomonas aeruginosa* (*n*=110) | *Acinetobacter baumannii* (*n*=87) | *Citrobacter freundii* (*n*=16) |
|---|---|---|---|---|---|
| ≤0.5 | 336 (86.82%) | 217 (84.44%) | 63 (57.27%) | 38 (43.68%) | 14 (87.50%) |
| 1 | 17 (4.39%) | 17 (6.61%) | 22 (20.00%) | 36 (41.38%) | 0 (0.00%) |
| 2 | 21 (5.43%) | 12 (4.67%) | 7 (6.36%) | 2 (2.30%) | 1 (6.25%) |
| 4 | 9 (2.33%) | 6 (2.33%) | 17 (15.45%) | 8 (9.20%) | 1 (6.25%) |
| 8 | 3 (0.78%) | 4 (1.56%) | 1 (0.91%) | 2 (2.30%) | 0 (0.00%) |
| ≥16 | 1 (0.26%) | 1 (0.39%) | 0 (0.00%) | 1 (1.15%) | 0 (0.00%) |
| Total | 387 (100%) | 257 (100%) | 110 (100%) | 87 (100%) | 16 (100%) |

MIC: minimum inhibitory concentration.

colistin disk elution screening test showed 100% concordant results for Enterobacterales. In non-Enterobacterales, one isolate of *P. aeruginosa* was not identified by the colistin broth disk elution method, colistin agar test and colistin disk elution screening test, two isolates of *A. baumannii* were not identified by the colistin broth disk elution method and colistin agar test while one *A. baumannii* was not identified by the colistin disk elution screening method (Table 4).

The diagnostic efficacy of colistin broth disk elution method, colistin disk elution screening test and colistin agar test with the BMD method (gold standard) was assessed on the parameters of major error, very major error, categorical agreement, sensitivity, specificity, positive predictive values and negative predictive value. Very major errors was 5.5% for colistin broth disk elution method, 5.5% for colistin agar test and 3.7% for colistin disk elution screen method and no major error was reported (Table 5).

Categorical agreements of colistin broth disk elution method and colistin agar test were 94.4%, while for colistin disk elution screening method was 96.2% when compared with the gold standard. The specificity of all phenotypic tests was 100% while sensitivity was 96.2% for colistin disk elution screening method, 94.4% for colistin broth disk elution method and colistin agar test. Negative predictive value and positive predictive value for all the methods was 99.6% and 100% respectively (Table 5).

The cost of tests, ease to perform, need of training and turnaround time were assessed as per simple grading system. The turnaround time was 24 h for all the methods. The colistin elution screening test was the easiest and cheapest to perform in comparison with other tests. Colistin disk elution screening method requires less training while BMD and other tests require well-trained techniques. According to Table 2, the breakdown of all parameters were assessed on a scale of 1 to 4 (Table 6)[8].

## 4. Discussion

The detection of colistin resistance in clinical samples has become more essential as use of colistin has increased for treatment of MDR-GNB and carbapenem resistant organisms. Due to lack of

**Table 4.** Comparison of different methods to detect colistin resistance in Gram negative bacteria.

| Isolates (*n*=857) | Broth microdilution test (*n*=857), S/R (R%) | Colistin broth disk elution method (*n*=857), S/R (R%) | Colistin agar test (*n*=857), S/R (R%) | Colistin disk elution screening method (*n*=857), S/R (R%) |
|---|---|---|---|---|
| *Klebsiella pneumoniae* (*n*=387) | 374/13 (3.36%) | 374/13 (3.36%) | 374/13 (3.36%) | 374/13 (3.36%) |
| *Escherichia coli* (*n*=257) | 246/11 (4.28%) | 246/11 (4.28%) | 246/11 (4.28%) | 246/11 (4.28%) |
| *Pseudomonas aeruginosa* (*n*=110) | 92/18 (16.36%) | 93/17 (15.45%) | 93/17 (15.45%) | 93/17 (15.45%) |
| *Acinetobacter baumannii* (*n*=87) | 76/11 (12.64%) | 78/9 (10.34%) | 78/9 (10.34%) | 77/10 (11.49%) |
| *Citrobacter freundii* (*n*=16) | 15/1 (6.25%) | 15/1 (6.25%) | 15/1 (6.25%) | 15/1 (6.25%) |
| Total | 803/54 (6.30%) | 806/51 (5.95%) | 806/51 (5.95%) | 805/52 (6.07%) |

S-sensitivity, R-resistant, R%-resistance percentage.

**Table 5.** Diagnostic efficiency/performance of colistin agar test, colistin elution screening test, and colistin broth disk elution method, when BMD considered gold standard ($n$=857).

| Parameters | | BMD | | Colistin broth disk elution method | | Colistin agar test | | Colistin disk elution screening method | |
|---|---|---|---|---|---|---|---|---|---|
| | | + | - | + | - | + | - | + | - |
| BMD | + | 54 (T.P) | 0 (F.N) | 51 (T.P) | 3 (F.N) | 51 (T.P) | 3 (F.N) | 52 (T.P) | 2 (F.N) |
| | - | 0 (F.P) | 803 (T.N) | 0 (F.P) | 806 (T.N) | 0 (F.P) | 806 (T.N) | 0 (F.P) | 805 (T.N) |
| ME (%) | | 0.0 | | 0.0 | | 0.0 | | 0.0 | |
| VME (%) | | 0.0 | | 5.5 | | 5.5 | | 3.7 | |
| CA (%) | | 100.0 | | 94.4 | | 94.4 | | 96.2 | |
| Sensitivity (%) | | 100.0 | | 94.4 | | 94.4 | | 96.2 | |
| Specificity (%) | | 100.0 | | 100.0 | | 100.0 | | 100.0 | |
| PPV (%) | | 100.0 | | 100.0 | | 100.0 | | 100.0 | |
| NPV (%) | | 100.0 | | 99.6 | | 99.6 | | 99.6 | |

BMD-broth microdilution test, T.P-true positive, F.N-false negative, F.P-false positive, T.N-true negative, PPV-positive predictive values, NPV-negative predictive values, CA-categorical agreement, ME-major errors, VME-very significant errors.

**Table 6.** Grading for the test cost, simplicity and training.

| Parameter | BMD (TAT-24 h ) | Colistin broth disk elution method (TAT-24 h) | Colistin agar test (TAT-24 h) | Colistin disk elution screening method (TAT-24 h) |
|---|---|---|---|---|
| Ease to perform the test | 1 | 2 | 1 | 3 |
| Cost | 3 | 3 | 3 | 3 |
| Training | 1 | 2 | 2 | 3 |
| Total grade | 5 | 7 | 6 | 9 |

TAT-turnaround time.

resources and trained laboratory personnel, developing countries require a procedure that is simple to implement, economical and easy to use. Several rapid, non-rapid techniques, disc diffusion tests and *E*-test procedures have been developed and suggested to aid in the detection of colistin resistance, but still we continue to lag in diagnostic performance, necessary skills, sample processing, cost and time[4,6,8].

The objective of the current study was to compare colistin broth disk elution test, colistin agar test and colistin disk elution screening method with BMD method in order to provide laboratories, particularly those in resource-limited settings, a simple way to detect colistin resistance. During this study, major errors, very major errors, categorical agreement, sensitivity, specificity, positive and negative predictive values were calculated and grading for required skills, simplicity of application and sample-processing cost were analysed.

The Kirby Bauer disc diffusion method was used to test the antibiotic sensitivity pattern of 2 196 Gram negative bacteria in which 857 (39.02%) MDR-GNB were identified. A study conducted in eastern India by Mohapatra *et al*. and Pattnaik *et al*. found 41.3% and 66.1% MDR-GNB, respectively[15,16], Another study conducted by Agyepong showed higher prevalence of MDR- GNB (81.6%) than the current study[17].

In this study, 857 MDR-GNB were assessed for colistin resistance by BMD method (gold standard) and 54 colistin resistant isolates were identified. *P. aeruginosa* (33.33%) was predominant colistin resistant GNB followed by *K. pneumoniae* (24.07%), *Escherichia coli* (20.37%), *A. baumannii* (20.37%) and *C. freundii* (1.85%) in colistin resistant Gram negative bacteria. A study done by Goli *et al.* identified *P. aeruginosa* as major colistin resistant organisms among Gram negative bacteria and Qadi *et al*. found 41% colistin resisatnce in Enterobacterales[18,19]. In current study, MICs distribution of MDR-GNB towards colistin is given in Table 3.

In this study, the isolation rate of non-lactose fermenting bacteria was low but colistin resistance was high, while in lactose fermenting bacteria, isolation rate was high but colistin resistance was low. In the current study, a total of 6.30% colistin resistant Gram negative bacteria were isolated. The colistin resistance has been emerging in the world[20,21]. The easy and reliable method to detect colistin resistance MDR-GNB is need of hour.

Multiplex PCR was used to screen for *mcr–1* to *mcr–5* genes in 54 colistin-resistant Gram negative bacteria. No *mcr* gene was detected in the current study. The role of *mcr* gene is to spread colistin resistance from one bacterium to another and there is no significant role of mcr gene in phenotypic detection methods for colistin resistance. The absence of *mcr* gene doesn't affect the relevance of this study in term of quality. In India, *mcr–1* gene is the most prevalent gene than other mcr genes. Several investigations have found *mcr–1* gene from north and west India[22–24].

The BMD method is considered as the gold standard to detect colistin resistance. Several methods have been proposed and used, but there is still need of test that can be performed with low cost and low skill in developing countries[6,8].

Colistin broth disc elution and colistin agar test is recommended by CLSI 2021 and is inexpensive, requires little effort. Simner *et al*. suggested colistin broth disc elution method as a replacement of BMD[25]. Colistin broth disc elution method was a reproducible and accurate method that can be used as an alternative of BMD. In the current study, there was 100% concordance in detection of colistin

resistance bacteria belonging to Enterobacterales by all phenotypic method where BMD was considered as gold standard. However, in non Enterobacterales, one isolate of *P. aeruginosa* was not detected by colistin broth disk elution method, colistin disk elution screening test and colistin agar test; one isolate of *A. baumannii* was not detected by the colistin disk elution screening method and 2 isolates were not detected by colistin broth disk elution method and colistin agar method. The non-fermenters still requires a method to improve detection rate. Simner *et al*. and Humphries *et al*. also studied the colistin broth disk elution method and colistin agar method in which they observed that both methods showed similar results[25,26].

Colistin broth disk elution method showed no major error, 5.5% very major error, 94.4% categorical agreement, 94.4% sensitivity, 100% specificity, 100% positive predictive value and 99.6% negative predictive value when compared with BMD (gold standard). Simner *et al*. and Humphries *et al*. also observed similar results[25,26].

The colistin agar test showed no major error, 5.5% very major error, 94.4% categorical agreement, 94.4% sensitivity, 100% specificity, 100% positive predictive value and 99.6% negative predictive value when compared with BMD (gold standard). Approximately similar result was observed by Humphries et al. and Sekyere *et al*[8,26].

The colistin disk elution screening method was a qualitative test. Still there was no major error, 3.7% very major error, 96.2% categorical agreement, 96.2% sensitivity, 100% specificity, 96.2% positive predictive value and 99.6% negative predictive value were noted when compared with BMD (gold standard). This method can be used as a substitute for disc diffusion method in the laboratories of developing countries. The colistin broth disk elution method and colistin agar test was able to provide MICs which were not provided by the colistin disk elution screening method.

In the current study, convenience of performing the tests, cost, training requirements, and the reagent requirements per test were examined. This will assist laboratories to select the best test in identifying colistin resistance[8]. The BMD method required high skill, training and reagents while colistin broth disk elution method and colistin agar test was easy to perform and not requiring high skill and training but also providing MICs. The colistin elution method used for screening was easy to perform; with 10 mL of Muller Hinton Broth with 2 discs of colistin sulphate can assess 4 isolates at a single point of time.

However, in the current study, limited numbers of colistin resistant organisms were isolated. There was no *mcr* gene detected in this study and no chromosomal mutation was assessed. There are various other methods like resazurin based rapid method, CHROMagar COL-APSE which should be assessed for the detection of colistin resistance but due to lack of infrastructure and low resource setting, these were not included in current study. The colistin is the last resort of antibiotic and synergism effect of nanoparticle with colistin can be assessed against colistin resistance.

In conclusion, CLSI 2021 endorsed colistin disk elution test and colistin agar test to assess colistin resistance in Gram negative bacteria. We assessed the large number of bacteria in this study for colistin disk elution test, colistin agar test and colistin elution screening test in a resource limited laboratory. Their performance, efficiency, cost and skill were also evaluated. So, according to the requirements, laboratories can choose a phenotypic test to assess colistin susceptibility testing. Colistin broth disc elution method can be used as an alternative of BMD method for a certain level and colistin elution for screening can be used as an alternative for disc diffusion agar test. This method is easy to perform and cheap can be used as a routine test in laboratories with low resources.

## Conflict of interest statement

The authors declare no conflict of interest.

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## Authors' contributions

S.C. and N.K. conceptualized the study; S.C., J.C. and H.K. curated the data; S.C. and J.C. carried out formal analysis; N.K. and A.K.S. carried out supervision of study.

## References

[1] Nation RL, Li J. Colistin in the 21st century. *Curr Opin Infect Dis* 2009; **22**(6): 535-543.

[2] El-Sayed Ahmed MAE, 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): 868-885.

[3] Aghapour Z, Gholizadeh P, Ganbarov K, Bialvaei AZ, Mahmood SS, Tanomand A, et al. Molecular mechanisms related to colistin resistance in Enterobacteriaceae. *Infect Drug Resist* 2019; **12**: 965-975.

[4] Gogry FA, Siddiqui MT, Sultan I, Haq QMR. Current update on intrinsic and acquired colistin resistance mechanisms in bacteria. *Front Med (Lausanne)* 2021; **8**: 677720.

[5] Chauhan S, Garg R, Kaur N, Mittal A, Bala R, Chauhan J, et al. Metronidazole resistant *Bacteroides* species isolated from a tertiary care hospital from North India. *Int J Pharm Qual Assur* 2022; **13**: 62-65.

[6] Bardet L, Rolain JM. Development of new tools to detect colistin-resistance among Enterobacteriaceae strains. *Can J Infect Dis Med Microbiol* 2018; **2018**: 3095249.

[7] Malli E, Florou Z, Tsilipounidaki K, Voulgaridi I, Stefos A, Xitsas S, et al. Evaluation of rapid polymyxin NP test to detect colistin-resistant *Klebsiella pneumoniae* isolated in a tertiary Greek hospital. *J Microbiol Methods* 2018; **153**: 35-39.

[8] Sekyere JO, Sephofane AK, Mbelle NM. Comparative evaluation of CHROMagar COL-APSE, MicroScan walkaway, ComASP colistin, and colistin MAC test in detecting colistin-resistant gram-negative bacteria. *Sci Rep* 2020; **10**(1): 6221.

[9] The European Committee on Antimicrobial Susceptibility Testing (EUCAST). *Recommendations for MIC determination of colistin (polymyxin E) as recommended by the joint CLSI–EUCAST polymyxin breakpoints working group*. 2016. [Online]. Available from: https://www.eucast.org/fileadmin/src/media/PDFs/EUCAST_files/General_documents/Recommendations_for_MIC_determination_of_colistin_March_2016.pdf. [Accessed on 20 July 2022].

[10]Rojas LJ, Salim M, Cober E, Richter SS, Perez F, Salata RA, et al. Colistin resistance in carbapenem-resistant *Klebsiella pneumoniae*: Laboratory detection and impact on mortality. *Clin Infect Dis* 2017; **64**(6): 711-718.

[11]Clinical and Laboratory Standards Institute. *Performance standards for antimicrobial susceptibility testing: Thirty one informational supplement M100–S31*. CLSI, Wayne, PA, USA; 2021.

[12]Magiorakos AP, Srinivasan A, Carey RB, Carmeli Y, Falagas ME, Giske CG, et al. Multidrug-resistant, extensively drug-resistant and pandrug-resistant bacteria: An international expert proposal for interim standard definitions for acquired resistance. *Clin Microbiol Infect* 2012; **18**(3): 268-281.

[13]Rebelo AR, Bortolaia V, Kjeldgaard JS, Pedersen SK, Leekitcharoenphon P, Hansen IM, et al. Multiplex PCR for detection of plasmid-mediated colistin resistance determinants, *mcr–1, mcr–2, mcr–3, mcr–4* and *mcr–5* for surveillance purposes. *Euro Surveill* 2018; **23**(6): 17-00672. doi: 10.2807/1560-7917.ES.2018.23.6.17-00672.

[14]Dalmolin TV, Mazzetti A, Ávila H, Kranich J, Carneiro GIB, Arend LNVS, et al. Elution methods to evaluate colistin susceptibility of Gram-negative rods. *Diagn Microbiol Infect Dis* 2020; **96**(1): 114910.

[15]Mohapatra DP, Debata NK, Singh SK. Extensively drug-resistant and pandrug-resistant Gram-negative bacteria in a tertiary-care hospital in Eastern India: A 4-year retrospective study. *J Glob Antimicrob Resist* 2018; **15**: 246-249.

[16]Pattnaik D, Panda SS, Singh N, Sahoo S, Mohapatra I, Jena J. Multidrug resistant, extensively drug resistant and pan drug resistant gram negative bacteria at a tertiary care centre in Bhubaneswar. *Int J Community Med Public Health* 2019; **6**: 567-572.

[17]Agyepong N, Govinden U, Owusu-Ofori A, Essack SY. Multidrug-resistant gram-negative bacterial infections in a teaching hospital in Ghana. *Antimicrob Resist Infect Control* 2018; **7**: 37.

[18]Goli HR, Nahaei MR, Ahangarzadeh Rezaee M, Hasani A, Samadi Kafil H, Aghazadeh M. Emergence of colistin resistant *Pseudomonas aeruginosa* at Tabriz hospitals, Iran. *Iran J Microbiol* 2016; **8**(1): 62-69.

[19]Qadi M, Alhato S, Khayyat R, Elmanama AA. Colistin resistance among Enterobacteriaceae isolated from clinical samples in Gaza strip. *Can J Infect Dis Med Microbiol* 2021; **2021**: 6634684.

[20]Peeters P, Ryan K, Karve S, Potter D, Baelen E, Rojas-Farreras S, et al. The impact of initial antibiotic treatment failure: Real-world insights in patients with complicated, health care-associated intra-abdominal infection. *Infect Drug Resist* 2019; **12**: 329-343.

[21]World Health Organization. *Antimicrobial resistance 2020*. Geneva, Switzerland: WHO; 2020.

[22]Singh S, Pathak A, Rahman M, Singh A, Nag S, Sahu C, et al. Genetic characterisation of colistin resistant *Klebsiella pneumoniae* clinical isolates from North India. *Front Cell Infect Microbiol* 2021; **11**: 666030.

[23]Karade S, Sen S, Shergill SPS, Jani K, Shouche Y, Gupta RM. Whole genome sequence of colistin-resistant *Escherichia coli* from western India. *Med J Armed Forces India* 2021; **77**(3): 297-301.

[24]Ghafur A, Shankar C, GnanaSoundari P, Venkatesan M, Mani D, Thirunarayanan MA, et al. Detection of chromosomal and plasmid-mediated mechanisms of colistin resistance in *Escherichia coli* and *Klebsiella pneumoniae* from Indian food samples. *J Glob Antimicrob Resist* 2019; **16**: 48-52.

[25]Simner PJ, Bergman Y, Trejo M, Roberts AA, Marayan R, Tekle T, et al. Two-site evaluation of the colistin broth disk elution test to determine colistin *in vitro* activity against gram-negative bacilli. *J Clin Microbiol* 2019; **57**(2): e01163-18. doi: 10.1128/JCM.01163-18.

[26]Humphries RM, Green DA, Schuetz AN, Bergman Y, Lewis S, Yee R, et al. Multicenter evaluation of colistin broth disk elution and colistin agar test: A report from the clinical and laboratory standards institute. *J Clin Microbiol* 2019; **57**(11): e01269-19.