EVALUATION OF BROTH DISK ELUTION TEST AND AGAR TEST TO DETERMINE COLISTIN IN VITRO ACTIVITY IN ENTEROBACTERIACEAE

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ABSTRACT:

Background: *Antimicrobial susceptibility testing (AST) for colistin is challenging for clinical laboratories, and its use without prior testing has fostered drug resistance. The CLSI recommended colistin broth disk elution (CBDE) and colistin agar test (CAT) for testing colistin.*

Aim of the work: *The current study aimed to evaluate the accuracy of the CBDE test and CAT compared to the reference broth microdilution (BMD) to determine colistin MICs.*

Materials and methods: *This cross-sectional study was performed on 62 MDR Enterobacteriaceae isolates collected from various clinical samples submitted for routine culture and sensitivity in the Main Microbiology Laboratory, Ain Shams University Hospitals, from July 2022 to January 2023.*

Results: *For CBDE, the sensitivity of the test was 97.3%, the specificity was 100%, the categorical agreement (CA) was 98.4%, the Essential agreement (EA) was 95.1%, one Very major error was observed (VME) 2.7%, and no Major error (ME) 0%. For CAT, a sensitivity of 94.59%, a specificity of 96%, a CA of 95.2%, an EA of 88.7%, two VMEs of 5.4% and one ME of 4% were observed.*

Conclusion: *The CBDE is an accurate, easy, and practical test for identifying colistin MICs therefore it is a reliable method for colistin susceptibility testing. The CAT is handy and can be implemented as a part of routine AST of colistin. CAT is relatively easier to execute than CBDE because up to ten isolates can be inoculated per dilution plate. Our study showed a VME of 5.4% and a Major error of 4% for CAT, requiring further evaluation and studies for this test.*

Keywords: *Colistin broth disk elution, Colistin agar test, MDR Enterobacteriaceae, Colistin, Resistance.*

INTRODUCTION:

Colistin is a cationic polypeptide antibiotic that belongs to the family polymyxin, including polymyxins B and E [(1)]. Worldwide dissemination of multidrug-resistant (MDR) and extremely drug-resistant Gram-negative bacteria, including carbapenemase producing Enterobacteriaceae (CRE), led to reviving colistin as a last therapeutic option [(2)]. It is the final resort for treating MDR Gram-negative bacilli, mainly Escherichia coli (E. coli), Klebsiella pneumoniae (K. pneumoniae), and Pseudomonas aeruginosa. (Ps. aeruginosa) [(1)].

The intensive usage of polymyxin for managing MDR Gram-negative infections provoked the emergence of acquired colistin resistance [(3)].

Different mechanisms result in acquired colistin resistance, such as chromosomal mutations and plasmid-borne colistin resistance [(4)].

In 2017, The Clinical and Laboratory Standards Institute (CLSI) and the European Committee on Antimicrobial Susceptibility Testing (EUCAST) recommended broth microdilution (BMD) as the gold standard for testing colistin [(5)].

However, BMD is inconvenient for routine clinical use as it is tedious and takes a long time to perform [(6)].

On the other hand, the disk diffusion test is an easy, affordable method but unsuitable for colistin susceptibility testing as it yields inaccurate results because of the big-sized colistin poorly diffusing molecules in the media. This gives rise to high error rates [(7)]. So, neither CLSI nor EUCAST recommends the disk and gradient diffusion techniques for testing colistin, leaving microbiology laboratories short of practical methods to diagnose colistin susceptibility [(5)].

The CLSI states the need for further investigations of agar dilution MIC determination because of the growing importance and urgent need to find an ideal and easy method for susceptibility testing for colistin [(8)].

For this purpose, they suggested two methods to enable accurate testing of these agents: colistin broth disk elution (CBDE) and colistin agar test (CAT)[(9)].

Simner et al., described CBDE as a precise, user-friendly, and feasible technique for determining colistin MICs that subdue many of the challenges of colistin. But it needs further studies to be applied [(5)].

AIM OF THE WORK:

The present study aimed to evaluate the accuracy of the colistin broth disk elution (CBDE) test and colistin agar test (CAT) compared to that of broth microdilution (BMD) for identifying colistin MICs.

MATERIALS AND METHODS:

Study design and Study population:

This cross-sectional study was conducted on 62 isolates of MDR *Enterobacteriaceae* collected from different clinical samples submitted for routine culture and sensitivity in the main microbiology laboratory of Ain Shams university hospitals. This work was done in the period between July 2022 to January 2023.

Inclusion criteria:

1. Enterobacteriaceae isolates Identified by conventional microbiological techniques according to CLSI (2022) including colonial morphology, gram stain characteristics and biochemical reactions [(10)].
2. Multi-Drug Resistant Enterobacteriaceae were chosen according to antimicrobial susceptibility testing (AST) by disc diffusion method to many antibiotics according to (CLSI 2022) breakpoints[(10)].

Study procedures:

All the tested isolates were subjected to the following:

1. Identification by conventional microbiological techniques as colonial morphology, gram stain characteristics, biochemical reactions.
2. Antimicrobial susceptibility testing according to CLSI, 2022 breakpoints by disc diffusion method [(10)].
3. Antimicrobial susceptibility testing by the reference method Broth microdilution for determination of MIC for colistin according to CLSI, 2022[(10)].
4. Broth disk elution test according to CLSI, 2022[(10)].

5. Colistin agar Test according to CLSI, 2022[10].

Colistin antibiotic susceptibility testing by broth microdilution:

The test was performed according to (CLSI, 2022) [10] using both Cation-adjusted Mueller-Hinton broth (Sigma Aldrich, USA) and Colistin sulphate salt (19000 IU/mg, form powder 100 mg) (Sigma Aldrich, USA). Colistin sulphate salt was utilized to prepare a stock solution and working solution with concentrations of 50 mg/ml and 64 ug/ml, respectively.

Interpretation:

Because the CLSI guidelines lack the polymyxins breakpoints for *Entero-bacteriaceae*, we utilized those of the European Committee on Antimicrobial Susceptibility Testing (EUCAST, 2016) for reference. Enterobacterial isolates with colistin MICs <2 µg/ml were designated susceptible; those with MICs >2 µg/ml were designated resistant [11].

Antibiotic susceptibility testing by colistin broth disk elution (CBDE):

Cation adjusted Mueller-Hinton broth (CAMHB) (Sigma Aldrich, USA) and 10 ug colistin sulfate disks (Oxoid, UK) were used to execute the test according to (CLSI, 2022)[10].

A 0.5 McFarland-adjusted inoculum was prepared. Then, four glass tubes were labelled as Control, 1, 2 and 4. The labelling was done referring to their colistin concentration. Then, ten ml of CAMHB were placed in each tube. Then, 0, 1, 2 and 4 colistin disks were added subsequently to the above-mentioned tubes to reach a final concentration of 0, 1, 2 and 4 µg/ml, respectively. The tubes were gently vortexed with the added disks and then incubated at room temperature for at least 30 minutes but not longer than 60 minutes to allow colistin to elute from the disks. Afterwards, the discs were removed. Then, we dispensed 1ml in each tube (Control, 1, 2 and 4). A 5 µl of the inoculum was added to each tube (final concentration: approximately 7.5 x 105 CFU/ml). A purity plate was inoculated. The purity plate and the tubes were incubated for 16-20 hours at 33-35 ºC.

Interpretation:

- We examined the purity plate to make sure they are pure.
- The growth control tube was examined, which must demonstrate obvious turbidity for the test to be valid.
- The MIC was interpreted as per the breakpoints of the (EUCAST 2016) for reference as above-mentioned in the BMD [11].

Antibiotic susceptibility testing by Colistin Agar test (CAT):

Mueller-Hinton agar (Oxoid, UK) and Colistin sulphate salt (19000 IU/mg, form powder 100 mg) (Sigma Aldrich, USA) were used to execute the test as per (CLSI, 2022). Colistin sulphate salt was utilized to prepare a stock solution and a working solution with concentrations of 50 mg/ml and 40 ug/ml, respectively.

Colistin agar plates with concentrations of 4 µg/ml, 2 µg/ml, and 1 µg/ml were prepared respectively by adding 2ml, 1ml, and 0.5 ml of the 40 µg/ml colistin working solution. Then, sterile molten Mueller-Hinton agar equilibrated to 50ºC was added to reach a final volume of 20ml per a 90x15mm petri dish.

0.5 McFarland adjusted inoculum was prepared by using colonies from non-selective agar plate. Then, it was diluted 1: 10 in saline. Each colistin plate was divided up to 10 parts with a marker to test up to 10 isolates per plate. A sterile cotton swab was dibbed into the 0.5 McFarland suspension of the tested strain. The agar surface area was spotted approximately 20 mm in diameter. Using a 10 µL loop, subculture from the original inoculum to a blood agar plate as a

purity check was done. The plates were incubated for 16 to 20 h at 33-35 °C.

Interpretation:

The test was interpreted as mentioned before in the CBDE. The growth control plate was examined, which must demonstrate confluent growth for the test to be valid. The MIC was read as the lowest colistin agar plate concentration that completely inhibits the growth of the tested isolate.

Statistical analysis:

The collected data were revised, coded, tabulated and introduced to a PC using Statistical package for Social Science (SPSS 25).

For the descriptive statistics, we used the Median and Interquartile range (IQR) for non-parametric numerical data. The frequency and percentage were calculated for non-numerical data. As for the analytical statistics, we applied Fisher's exact test to study the relationship between two qualitative variables when the expected count is less than five in more than 20% of cells. We calculated the NPV, PPV, sensitivity and specificity for the diagnostic performance of the tested methods.

The accuracy of AST, namely, categorical agreement (CA) and essential agreement (EA), were measured and interpreted as per Humphries and his team [12]. The accepted range for both CA and EA is ≥ 90% CA.

CA discrepancies are divided into three types of errors, i.e., minor errors (mEs), major errors (MEs), and very major errors (VMEs). Accepted mE rates are ≤10%. ME and VME rates are less than 3% of the susceptible and resistant isolates. The VMEs should be less than 1.5% of resistant isolates.

Ethical consideration:

All study procedures were as per ethical guidelines of the 1975 Declaration of Helsinki and approved by the ethical committee of Faculty of Medicine, Ain Shams University. (No. FWA 000017585) (FMASU MS 379/2022).

RESULTS:

In this study, 62 well identified *Enterobacteriaceae* isolates (54 isolates of *Klebsiella* (87.1%) and 8 isolates of *E. coli* (12.9%)) were randomly obtained from patient specimens submitted from different departments to the Main Microbiology Laboratory of Ain Shams University Hospitals during the period from July 2022 to January 2023 for routine culture and sensitivity. They were isolated from different sites, pus, blood, wound, urine, sputum, central line and body fluids.

Descriptive statistics:

1. Demographic data:

Regarding the demographic data, we found that the age of the patients enrolled in the current study ranged from 4 days to 78 years old with an inter quartile range (IQR) of 15 (1 - 60). The number of females 29/62 (46.77%) and male patients 33/62 (53.23%). As for the departments, it was noticed that many isolates were collected from Pediatric Intensive Care Unit (PICU) (14/62, 22.58%). Concerning the sample type, it was observed that many of our isolates were recovered from blood cultures (16/62, 25.81%). The tested isolates were *Klebsiella* 54/62 (87.1%) and *E. coli* 8/62 (12.9%). Table (1) summarize the demographic data and characteristics of the 62 tested isolates included in the current study.

Table 1: Demographic data and characteristics of the 62 tested isolates in the current study.

	N= 62
Age (years)	
Median (IQR) Range	15 (1 - 60) 4 days – 78 years
Gender	
Females	29 (46.77%)
Males	33 (53.23%)
Department	
Burn ICU	4 (6.45%)
Chest department	2 (3.23%)
Chest ICU	2 (3.23%)
PICU	14 (22.58%)
Pediatric hospital	6 (9.68%)
Nephrology department	2 (3.23%)
Endocrinology department	1 (1.61%)
Neurosurgery department	1 (1.61%)
Rheumatology department	1 (1.61%)
General surgery department	3 (4.84%)
Internal medicine ICU	12 (19.35%)
Emergency department	1 (1.61%)
Geriatric department	1 (1.61%)
NICU	7 (11.29%)
Surgery ICU	1 (1.61%)
Outpatient	3 (4.84%)
Oncology department	1 (1.61%)
Sample type	
Blood	16 (25.81%)
Sputum	14 (22.58%)
Wound	7 (11.29%)
Urine	15 (24.19%)
Central line	6 (9.68%)
CSF	2 (3.23%)
Pus	2 (3.23%)
Organism	
Klebsiella	54 (87.1%)
E. coli	8 (12.9%)

ICU: Intensive care unit, NICU: Neonatal intensive care unit, PICU: Pediatric intensive care unit, CSF: cerebrospinal fluid

2. Antibiotics given to patients:

Among the 62 patients included in our study, we found that 43 (69.35%) patients received antibiotics. Meropenem (MEM) rated the first among all the used antibiotics where 10/62 (23.26%) patients received it as shown in Table (2).

Table 2: The percentage of antibiotic intake among the 43 studied patients who received antibiotics in the current study.

Antibiotics taken	N (%)
Total patients on antibiotics	43 (69.35%)
Penicillins	7 (16.27%)
Penicillin	1 (2.33%)
Amoxicillin/ Clavulanic acid	1 (2.33%)
Ampicillin/ Sulbactam	3 (6.98%)
Piperacillin tazobactam	2 (4.65%)
Cephalosporin	17 (39.53%)
Ceftriaxone	2 (4.65%)
Cefepime	4 (9.3%)
Ceftazidime	4 (9.3%)
Cefoperazone	5 (11.63%)
Cefotaxime	2 (4.65%)
Carbapenem	12 (27.91%)
Meropenem	10 (23.26%)
Imipenem	2 (4.65%)
Aminoglycosides	8 (18.6%)
Gentamicin	2 (4.65%)
Amikacin	6 (13.95%)
Quinolones	9 (20.93%)
Ciprofloxacin	4 (9.3%)
Levofloxacin	5 (11.63%)
Others	14 (32.55%)
Linezolid	1 (2.33%)
Vancomycin	4 (9.3%)
Clindamycin	4 (9.3%)
Tigecycline	1 (2.33%)
Teicoplanin	1 (2.33%)
Colistin	1 (2.33%)

3. Antimicrobial susceptibility testing (AST):

As for the antibiotic susceptibility testing, we noticed that although not all isolates included in our study were tested for all antibiotics, yet, all of those tested recorded 100% resistance rate against almost all β-lactams with exception of ceftazidime (97.62%) and imipenem (96.43%) by disk diffusion method. The results of the rest of the antibiotics are shown in Table (3).

Table 3: Antimicrobial susceptibility testing for 62 studied isolates in the current study disk diffusion method.

Antibiotic sensitivity	Total N	Susceptible	Resistant	Intermediate
		N (%)	N (%)	N (%)
Ampicillin	9	0 (0%)	9 (100%)	0 (0%)
Ampicillin-Sulbactam	8	0 (0%)	8 (100%)	0 (0%)
Amoxicillin-clavulanic	52	0 (0%)	52 (100%)	0 (0%)
Piperacillin-Tazobactam	9	0 (0%)	9 (100%)	0 (0%)
Cefoxitin	8	0 (0%)	8 (100%)	0 (0%)
Cefotaxime	53	0 (0%)	53 (100%)	0 (0%)
Ceftazidime	42	1 (2.38%)	41 (97.62%)	0 (0%)
Cefoperazone	24	0 (0%)	24 (100%)	0 (0%)
Ceftriaxone	51	0 (0%)	51 (100%)	0 (0%)
Cefpodoxime	5	0 (0%)	5 (100%)	0 (0%)
Cefepime	13	0 (0%)	13 (100%)	0 (0%)
Imipenem	28	1 (3.57%)	27 (96.43%)	0 (0%)
Meropenem	23	0 (0%)	23 (100%)	0 (0%)
Amikacin	35	10 (28.57%)	21 (60%)	4 (11.43%)
Gentamicin	17	3 (17.65%)	13 (76.47%)	1 (5.88%)
Tobramycin	26	0 (0%)	26 (100%)	0 (0%)
Ciprofloxacin	36	0 (0%)	35 (97.22%)	1 (2.78%)
Levofloxacin	34	4 (11.76%)	28 (82.35%)	2 (5.88%)
Doxycycline	39	9 (23.08%)	30 (76.92%)	0 (0%)
Trimethoprim-sulfamethoxazole	47	6 (12.77%)	41 (87.23%)	0 (0%)
Nitrofurantoin	9	1 (11.11%)	8 (88.89%)	0 (0%)

Results of conducted tests:

a. ***Colistin MIC by colistin broth microdilution Test:*** For colistin MIC results, we found that MIC results ranged between 0.5 to 32 ug/ml. 37 isolates out of 62 were resistant to colistin (59.68%) and 25 isolates were susceptible (40.32%) as shown in Table (4).

Table 4: Colistin MIC results among the 62 studied isolates in the current study.

		N = 62
Colistin MIC (ug/ml)	Median (IQR)	4 (1 - 8)
	Range	0.5 – 32
	0.5	5 (8.06%)
	1	14 (22.58%)
	2	6 (9.68%)
	4	7 (11.29%)
	8	17 (27.42%)
	16	10 (16.13%)
	32	3 (4.84%)
Colistin MIC interpretation	Susceptible	25 (40.32%)
	Resistant	37 (59.68%)

MIC: Minimal inhibitory concentration

b. ***Colistin broth disk elution (CBDE):***

For CBDE test, we found that among the tested 62 isolates 26 (41.94%) isolates were susceptible and 36 (58.06%) were resistant as shown in Table (5).

c. ***Colistin agar dilution test (CAT):***

Among the 62 tested isolates by CAT 26 (41.94%) were susceptible and 36 (58.06%) were resistant as shown in Table (5).

Table 5: Colistin broth disk elution test and Colistin Agar Test results among the 62 studied isolates in the current study.

		N (%)
CBDE (ug/ml)	1	22 (35.48%)
	2	4 (6.45%)
	4	10 (16.13%)
	>4	26 (41.94%)
CBDE interpretation	Susceptible	26 (41.94%)
	Resistant	36 (58.06%)
Agar dilution (ug/ml)	1	15 (24.19%)
	2	10 (16.13%)
	4	9 (14.52%)
	>4	28 (45.16%)
Agar dilution interpretation	Susceptible	25 (41.94%)
	Resistant	37(58.06%)

Association studies with MIC:

a. Association between Phenotypic methods and MIC: The association between the MIC method for colistin versus CBDE and CAT was highly statistically significant as shown in Table (6).

Table 6: Association between colistin MIC versus CBDE and CAT.

		MIC		Fisher's Exact test		
		Resistant (positive)	Susceptible (negative)			
		N (%)	N (%)	Value	p-Value	Sig.
CBDE	Resistant	36 (97.3%)	0 (0%)	62.5	<0.001	S
	Susceptible	1 (2.7%)	25 (100%)			
CAT	Resistant	35 (94.59%)	1 (4%)	55.04	<0.001	S
	Susceptible	2 (5.41%)	24 (96%)			

b. The diagnostic performance and evaluation of colistin broth disk elution and colistin agar test: The diagnostic performance of CBDE and CAT compared to the reference method MIC for colistin are summarized in Table (7).

Table 7: Diagnostic performance of CBDE and agar dilution tests compared to the reference method MIC for colistin among 62 tested isolates in the current study.

	TP	**TN**	**FP**	**FN**	**Sensitivity**	**Specificity**	**PPV**	**NPV**	**Accuracy**
CBDE	36	25	0	1	97.3%	100%	100	96.15	98.39%
Agar dilution	35	24	1	2	94.59%	96%	97.2	92.3	95.16%

For CBDE, the sensitivity of the test was 97.3% and the specificity was 100%. For CAT, the sensitivity of the test was 94.59% and the specificity was 96%.

c. Comparison of colistin broth disk elution and colistin agar test with the reference colistin broth microdilution test: In our study we found that CBDE categorical agreement (CA) 98.4%, Essential agreement (EA)

95.1%, one Very major error was observed (VME) 2.7% and no Major error (ME) of 0%.

For CAT we found Categorical agreement of 95.2%, Essential agreement of 88.7%, two Very major errors were observed of 5.4% and one Major error of 4%.

DISCUSSION:

Polymyxin resistance is growing because they are used as a remediation for carbapenem-resistant Gram-negative bacterial infections. BMD is recommended by CLSI and EUCAST for polymyxin susceptibility testing [13]. However, it is very tiresome and time-consuming [14].

Most laboratories depend on disk diffusion or gradient diffusion susceptibility testing methods. Regrettably, both tests cannot precisely diagnose colistin resistance. Tan and Ng demonstrated that all disk diffusion methods yielded false susceptibility in most colistin-resistant isolates [15].

So, alternative methods with satisfactory performance are required for routine laboratory work. Both CBDE and CAT have been studied on a wide scale after CLSI approved them for testing colistin, but each has its advantages and disadvantages which must be studied in Egyptian hospitals so that one of these tests can be used routinely [16].

We aimed to evaluate the accuracy of the CBDE test and CAT compared to the reference broth microdilution (BMD) to determine colistin MICs.

As regards CBDE test, we performed the test as per the CLSI using 10 μg colistin disks to obtain a final concentration of 0 (growth control), 1, 2, and 4 μg/mL. Then we made a minor modification by using 1 mL per tube instead of the 10 ml recommended by CLSI and that yielded the same results.

In our study we found that MIC results ranged between 0.5 to 32 ug/ml. 37 isolates out of 62 were resistant to colistin (59.68%) and 25 isolates were susceptible (40.32%).

Studies done by various other authors also showed a high prevalence of colistin resistance as our study [17,18,19]. Monaco and colleagues from Italy found that among their 191 CRE isolates, carbapenemase-producing *K. pneumoniae* represented 178 (93%) isolates, with 76 (43%) resistant to colistin[17].

Similarly, *Bardet et al.* (2019) from France reported 63.4% of colistin resistance among gram-negative bacilli out of 235 bacterial strains[18]. Additionally, in a prospective study in Italy from a total of 97 isolates, Capone and his colleagues (2013) reported that 36.1% of carbapenem-resistant *K. pneumoniae* were colistin-resistant [19].

Other researchers found lower rates of colistin resistance in their work. In two multicenter analyses of *carbapenem-resistant K. pneumoniae*, 13% to 16% of isolates were colistin-resistant [20&21]. In India, a study demonstrated 11% resistance by the reference BMD among their 100 CRE isolates [16].

In the light of results stated worldwide, we found that they agreed to a great extent with those reported by our study and this affirms the fact of increasing resistance against colistin which are considered the last resort for treatment of MDR organisms, this foreshadows an upcoming worldwide disaster.

As regards CBDE test, we found that among the tested 62 isolates, 26 (41.94%) isolates were susceptible and 36 (58.06%) were resistant. The sensitivity of the test was 97.3% and specificity 100%, categorical agreement (CA) 98.4%, Essential agreement (EA) 95.1%. One Very major error was observed (VME) 2.7% with no Major error (ME) obtained (0%).

Simner and his colleagues evaluated the performance of the CBDE test for assessing the susceptibility to colistin compared to the gold standard BMD. They used 172 *Enterobacteriaceae, A. baumannii, and Ps.*

aeruginosa isolates, including 38 colistin-resistant isolates. They found a CA of 98%, an EA of 99%, and no ME. They did find an 8% very major error rate, and this was because three of the six *E. coli* isolates with *mcr-1* that had MICs of 4 µg/ml by BMD (resistant) had MICs of 2 µg/ml by CBDE (susceptible) [5].

In June 2019, based on results obtained by Humphries and his team, the CLSI Antimicrobial Susceptibility Testing (AST) Subcommittee provisionally approved the CBDE for testing *Enterobacterales and P. aeruginosa.* In their study, a total of 348 *Enterobacteriaceae* isolates were tested. The CBDE showed EA of 94.3% and CA of 98.6% with reference broth microdilution MICs. Five VME (2.5%) and no ME (0%) were observed [9].

Another study conducted in India by Sujatha and his team reported that among the 100 *CRE* isolates, two isolates had MIC of 2 µg/ml in the CBDE method and MIC of 4 µg/ml in the reference method. The CA of CBDE versus the reference method was 98%[16]. Also, a study conducted in two different research centres in Brazil reported a CA of 91.18% and VME of 4.95% compared to the reference method [22]. The CBDE test requires only colistin disks and Mueller-Hinton broth. They can be easily obtained by any clinical microbiology laboratory, including those in resource-constrained settings, where colistin may be needed the most and colistin resistance may be the most prevalent. The elution method, such as the CBDE test, was proposed using glass tubes to avoid polymyxin binding, which was one of the limitations of the BMD test. Also, it showed high CA and EA with low VME, making it a reliable method for colistin susceptibility testing [13].

One of the limitations of this test is that it requires large volumes of MHB (40 ml per isolate). Preparing such large volumes of MHB and requiring many test tubes per sample while performing CBDE on a routine basis is very laborious and might not be economically feasible. We avoided this limitation in our work by using 1 ml of broth per tube (4 ml per isolate). Another remarkable issue noticed while doing CBDE is that we should use colistin disks of high standards with appropriate potency to guarantee proper disk elution.

In the study conducted by Simner and his team (2019), they found some *mcr-1*-producing isolates yielded MICs of 2 µg/ml by CBDE, while 4 µg/ml by BMD. As such, the results for isolates with colistin MICs of 2 µg/ml by CBDE should be confirmed by the reference BMD method and isolates with MICs of ≥2 µg/ml should be evaluated for the presence of *mcr* genes [5].

As for the CAT, we evaluated colistin susceptibility using homemade colistin agar plates with final colistin concentrations of 4, 2, 1 and 0 µg/ml. Up to 10 isolates could be tested per colistin plate.

Among the 62 tested isolates in the present work by CAT, 26 isolates (41.94%) were susceptible, and 36 (58.06%) were resistant. The test exhibited a sensitivity and specificity of 94.59% and 96%, respectively. A CA of 95.2%, EA of 88.7%, two VME of 5.4% and one ME of 4% were observed.

Like our study, Humphries and his team conducted research using 348 *Entero-bacteriaceae* isolates and reported CA of 99.7% and EA of 99.7% with 0.5% VME for CAT [9]. A study conducted in India by Sujatha and his team on 100 *CRE* isolates showed one isolate with MIC of 2 µg/ml in the CAT method and MIC of 8 µg/ml in the reference method (very major error), and the CA of CAT was 99% with reference BMD[16]. Similarly, Lellouche and his coworkers conducted a study on 364 isolates and reported a CA of 97.3%, EA of 91.5%, VME of 10.2% and ME of 1.6% for CAT[23].

In a study conducted by Ali and his team in Pakistan, they recommended that Colistin agar can be employed routinely as a credible

method for the identification of Colistin resistance for early medication and infection control in small laboratories where BMD and genetic sequencing are not available, as well as those with a high workload [24].

Additionally, CAT is relatively easier to execute than CBDE because up to ten isolates can be inoculated per dilution plate. Also, this test is economical with precise results and can be used in laboratories lacking enough lab technicians and instruments for BMD or genetic sequencing.

A limitation of our study is the relatively small sample size. This may cause the relatively high VME and ME values for CAT. This VME rate of 5.4% would likely have been much lower if a more representative sample of organisms were used. Another limitation was not detecting the different *mcr* genes in the tested isolates and correlating them with test results.

Additionally, our study did not evaluate polymyxin B tests. Polymyxin B usage is preferred to colistin, and polymyxin B is the only accessible drug to multiple countries. However, CLSI recently asserted that it is possible to predict the polymyxin B results using the colistin tests instead, whether CBDE or CAT. Although we did not perform any polymyxin B tests either by broth disk elution or agar test, it is worth pointing out that a disk containing 300 U of polymyxin B is equivalent to 30 μg of the antimicrobial. Accordingly, to adapt these techniques to polymyxin B, laboratories should be aware that to reach the same concentrations of polymyxin B as for colistin, they ought to utilize three times the amount of CA-MHB. Humphries and colleagues expected that a polymyxin B agar test would be effective for *Enterobacterales* and *P. aeruginosa* [9].

Conclusion and Recommendations:

The CBDE is a precise, uncomplicated, and practical test for identifying colistin MICs that subdue various restraints of colistin AST. The CBDE test demands only colistin disks and Mueller-Hinton broth. They can be easily obtained by any microbiology laboratory, including those in resource-constrained settings. Also, it showed high CA and EA with low VME, rendering it a reliable method for colistin susceptibility testing.

The CAT is practical and can be implemented as a part of the routine AST of colistin. It is relatively easier to execute than CBDE because up to ten isolates can be inoculated per dilution plate. Also, this test is economical and can be implemented in organizations lacking enough lab workers and sophisticated instruments required for BMD or genomic sequencing. Our study showed a VME of 5.4% for CAT, which needs further evaluation and studies for this test.

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We declare that all listed authors have made substantial contributions to all of the following three parts of the manuscript:

- Research design, or acquisition, analysis or interpretation of data.
- drafting the paper or revising it critically;
- approving the submitted version.

We also declare that no-one who qualifies for authorship has been excluded from the list of authors.

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None.

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تقييم اختبار الفصل بالتصفية واختبار أجار لتحديد نشاط الكوليستين في المختبر في البكتيريا المعوية

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الخلفية وهدف الدراسة: يمثل اختبار الحساسية للكوليستين تحديًا للمختبرات السريرية، وقد أدى استخدامه دون اختبار مسبق إلى مقاومة الأدوية. أوصى معهد المعايير السريرية والمخبرية باختبار الفصل بالتصفية لاقراص الكوليستين واختبار كوليستين أجار لاختبار حساسية الكوليستين. تهدف الدراسة الحالية إلى تقييم دقة اختبار الفصل بالتصفية لاقراص الكوليستين واختبار كوليستين أجار مقارنة مع اختبار تخفيف المرق الدقيق لتحديد التخفيف المثبط الادني للكوليستين .

المواد والطرق: تم إجراء هذه الدراسة على 62 عزلة من البكتيريا المعوية المقاومة للأدوية والتي تم جمعها من عينات سريرية مختلفة تم تقديمها للزرع الروتيني والحساسية في مختبر الأحياء الدقيقة الرئيسي بمستشفيات جامعة عين شمس، في الفترة من يوليو 2022 إلى يناير 2023.

النتائج: بالنسبة لـ اختبار الفصل بالتصفية لاقراص الكوليستين ، كان الاتفاق القاطع 98.4%، و الاتفاق الأساسي 95.1%، ولوحظ خطأ كبير جدًا 2.7%، ولم يكن هناك خطأ كبير 0%. بالنسبة لـ اختبار كوليستين أجار ، كان الاتفاق القاطع بنسبة 95.2%، و الاتفاق الأساسي بنسبة 88.7%، وخطأين كبيرين للغاية بنسبة 5.4% وخطأ كبير بنسبة 4%.

الخلاصة: إن اختبار الفصل بالتصفية لاقراص الكوليستين هو اختبار دقيق وغير معقد وعملي لتحديد التخفيف المثبط الادني للكوليستين. لقد أظهر توافقًا قاطعًا عاليًا واتفاقًا أساسيًا مع انخفاض نسبة الأخطاء الجسيمة، مما يجعلها طريقة موثوقة لاختبار حساسية الكوليستين. يعد اختبار كوليستين أجار مفيدًا ويمكن تنفيذه كجزء من الحساسية الروتينية للميكروبات المضادة للكوليستين. يعد تنفيذ اختبار كوليستين أجار أسهل نسبيًا من اختبار الفصل بالتصفية لاقراص الكوليستين لأنه يمكن زرع ما يصل إلى عشر عزلات في كل طبق تخفيف. أظهرت دراستنا خطأً كبيرًا جدًا بنسبة 5.4% وخطأً كبيرًا بنسبة 4% في اختبار كوليستين أجار ، مما يتطلب مزيدًا من التقييم والدراسة لهذا الاختبار.