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'Breakpoint broth microdilution plate' for susceptibility testing of Gram negative bacilli against colistin sulfate

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ABSTRACT

The MIC method applicable to Gram negative bacilli including *Acinetobacter* spp. is broth microdilution (BMD). Cost and/or availability issues limit the use of commercial MIC panels in resource limited settings.

Objectives: To design and implement an in-house breakpoint BMD panel (BBMD) for colistin against Gram negative bacilli.

Design: BBMD panel was prepared in 96-well plate. MIC concentrations of 1, 2, & 4 μg/mL for test, and 0.25, 0.5, 1, 2 & 4 μg/mL for control strains were selected to accommodate 19 test and 3 quality control strains per plate. Plates were frozen at −80 °C until testing. Validation was performed using strains from a previously published study and compared with freshly prepared MIC panel of 16–0.03 μg/mL.

Results: Validation showed 100% agreement with the reference method and BBMD was introduced into routine laboratory practice for colistin susceptibility of carbapenem resistant *Enterobacterales* (CRE), *Acinetobacter baumannii* complex and *Pseudomonas aeruginosa*. From 2nd July-16th September 2018, a total of 1294 (mean 16.8 ± 5.5 isolates/day) clinical isolates were tested; 1157/1294 were reported (MIC ≤2 μg/mL) within 24-h, whereas 133 required resistance confirmation by full-range BMD. Resistance was confirmed for all but 24 isolates. These discrepancies were mostly due to contamination with bacterial genera inherently resistant to colistin.

Conclusion: This BBMD plate is a high through-put and practical method that could reliably be utilized in a routine microbiology laboratory for colistin susceptibility testing of CRE, *A. bauamanii* complex and *P. aeruginosa*.

1. Introduction

Antimicrobial resistance (AMR) has challenged the world since the introduction of the first antimicrobial agent into clinical use, it continues to this day and will continue to do so in future. Extended spectrum β-lactamases (ESBLs) rendered third generation cephalosporins ineffective against Gram negative bacilli. Emergence of resistance to carbapenem group of β-lactam antimicrobials among Gram negative bacilli led to the revival of old antimicrobials for clinical use especially polymyxins (polymyxin B and polymyxin E or colistin). Susceptibility testing for these agents is problematic. Owing to their large molecular size, diffusion in agar is unreliable, thus

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	1	2	3	4	5	6	7	8	9	10	11	12
A	4	2	1	O1 GC	4	2	1	O7 GC	4	2	1	O13 GC
B	4	2	1	O2 GC	4	2	1	O8 GC	4	2	1	O14 GC
C	4	2	1	O3 GC	4	2	1	O9 GC	4	2	1	O15 GC
D	4	2	1	O4 GC	4	2	1	O10 GC	4	2	1	O16 GC
E	4	2	1	O5 GC	4	2	1	O11 GC	4	2	1	O17 GC
F	4	2	1	O6 GC	4	2	1	O12 GC	4	2	1	O18 GC
G	4	2	1	O19 GC	4	2	1	0.5	0.25	*P. aeruginosa* ATCC27853 GC	CAMHB Control	Antibiotic Control
H	4	2	1	0.5	0.25	*E. coli* NCTC 13846 GC	4	2	1	0.5	0.25	*E. coli* ATCC 25922 GC

A

(caption on next page)

Fig. 1. 1A. Map of 96-well microtiter plate used for determining breakpoint broth microdilution (BBMD) of colistin sulfate. Numbers in the top row represent columns (1-12) and first column on the left represents rows (A-H). Numbers 4, 2, 1, 0.5 and 0.25 in wells represent concentration (μg/mL) of colistin sulfate in the respective wells. Three concentrations (4, 2 & 1 μg/mL) were chosen for test organisms and five concentrations (4, 2, 1, 0.5, 0.25 μg/mL) were chosen for quality control strains. Designations O1 to O19 represent test organisms from 1 to 19. Columns 4, 8 and 12 serve as growth control wells (GC) for test organisms. Wells G10, H6 and H12 serve as growth control wells for *P. aeruginosa* ATCC 27853, *E. coli* NCTC 13846 and *E. coli* ATCC 25922 respectively. Wells G11 and G12 serve as sterility controls for cat-ion adjusted Muller Hinton broth (CAMHB) and antibiotic (colistin sulfate) respectively.
1B. BBMD plate 24 hours after inoculation showing MIC values of three quality control strains and 15 test isolates. Sterility control. Antibiotic and CAMHB control wells (G11 & G12 respectively) are clear. MIC values for three QC strains are: *P. aeruginosa* ATCC 27853, 1 μg/mL (range: 0.25-2 μg/mL)), *E. coli* NCTC 13846, 4 μg/mL (4 μg/mL)); and *E. coli* ATCC 25922, 0.5 μg/mL (range: 0.5-4 μg/mL). Refer to Fig. 1A for an explanation of BBMD plate wells and orientation of QC and test organisms. This figure also shows four colistin resistant organisms i.e. O1, O2, O5 and O7 (all have colistin MIC of >4 μg/mL). Rest of the organisms are susceptible to colistin. Wells for O16-O19 do not contain any test isolate and hence do not show any growth in corresponding wells.

making disk diffusion and minimum inhibitory concentration (MIC) by gradient diffusion strips unsuitable for susceptibility testing of colistin [1].

Commercial automated systems for colistin susceptibility testing, especially Vitek-2 (Biomerieux, France), currently do not allow testing colistin on their testing instrument [2]. Both the European Committee on Antimicrobial Susceptibility Testing (EUCAST) and the Clinical and Laboratory Standards Institute (CLSI) recommend broth microdilution (BMD) for determination of colistin MIC for Gram negative bacilli [3,4]. However, in the year 2020, in 30th edition of M100 document of CLSI [5], colistin agar test (CAT) and colistin broth disk elution (CBDE) tests were included and approved for Enterobacterales and *Pseudomonas aeruginosa*. For *Acinetobacter* spp. BMD is still the preferred method for colistin susceptibility testing.

The emergence and spread of carbapenem resistance among Gram negative bacilli [6–9], especially carbapenem resistant Enterobacteriales, *Acinetobacter* spp. and *Pseudomonas aeruginosa*, in low middle income settings, has led to widespread clinical use of colistin for treatment of these infections, which requires a cost-effective method for determining colistin susceptibility in clinical laboratories.

Commercially available colistin BMD panels offer satisfactory determination of colistin susceptibility. Commercial freeze-dried panels for colistin BMD, such as Sensititre plate (Sensititre, Thermofisher) offer testing one isolate per 96-well plate for colistin along with other antimicrobials. UMIC (Biocentric, France) and Micronaut MIC-Strip colistin (MERLIN Diagnostika, Germany) are freeze-dried 12-wells panels dedicated for single isolate per panel. Similarly ComASP™ colistin (Liofilchem, Italy) is another BMD panel for testing 4 isolates per panel. Rapid Polymyxin™ NP (ElitechGroup, France) is another commercial test offering rapid detection of polymyxin resistance but not the MIC values. Microscan walkaway (Beckman Coulter, USA) also offers colistin MIC testing on its system. However, higher cost and issues of availability especially shipment delays hinder their applicability in resource-limited settings, which paradoxically, are also areas with higher incidence and impact of AMR, making laboratory detection even more imperative. We present an in-house, frozen BMD panel for breakpoint susceptibility testing against colistin for use in high-volume laboratories with an intention to maximize the number of strains that could be tested in one 96-well microtiter plate. We present data on validation against full-range MIC and implementation in routine clinical practice in our laboratory.

2. Materials and methods

2.1. Designing of breakpoint BMD (BBMD) plate

A 96-well microtiter plate was used to design a template for breakpoint susceptibility testing. Keeping in mind the susceptibility breakpoint of colistin for *Enterobacterales*, *Acinetobacter* spp. and *Pseudomonas aeruginosa* of ≤2 μg/mL [3,4], 3 dilutions i.e. 4, 2 and 1 μg/mL were chosen for test strains [10]. The susceptibility breakpoints were revised by CLSI in the year 2020 as follows: a susceptible category no longer exists, intermediate at ≤2 μg/mL and resistant at ≥4 μg/mL. For quality control strains, the range of dilutions was expanded to 5 dilutions i.e. 4, 2, 1, 0.5 and 0.25 μg/mL to cover their MIC ranges. Designing the plate in this way, 19 test isolates and 3 quality control strains could be adjusted in one plate (Fig. 1A) (for details see supplementary material). For conventional BMD, colistin dilutions ranged from 16 to 0.03 μg/mL.

2.2. Preparation of BBMD plates

This is described in supplementary material [3,11].

2.3. Bacterial strains

Sixty clinical isolates were selected from an archived repository of a previously published study [12]. This included equal numbers of colistin-susceptible and -resistant clinical isolates from *Enterobacterales* family. Three quality control strains were used for this study and included *Pseudomonas aeruginosa* ATCC 27853 (colistin MIC: 0.25–2 μg/mL), *Escherichia coli* NCTC 13846 (colistin MIC: 4 μg/mL, positive for *mcr-1* gene by PCR) and *Escherichia coli* ATCC 25922 (colistin MIC: 0.5–4 μg/mL).

2.4. Inoculation of plates

A template of the microtiter plate was designed to help identify wells of different types (Fig. 1A). Inoculated BBMD plates were covered with lids and incubated along with inoculum purity and inoculum control plates at 35–37 °C in air for 18–24 h at which time MIC reading was performed visually (Fig. 1A & B. See supplementary material for details).

Two laboratory technologists who were experienced in performing broth microdilution, were initially assigned for validation of this BBMD procedure. The BBMD plate was validated against full range colistin BMD which always included QC strains as listed above [13].

Once the validation results were approved, a group of competent laboratory technologists for this breakpoint susceptibility testing were required. For this purpose, dedicated training sessions, that included weighing the drug, making stock solution, preparation of plate, inoculation of plate, reading and recording of results, were conducted for each step of this process and competency assessment was performed at the end of the training period. It was mandatory for each technologist to achieve a competency level of 4 and above [14].

Categorical agreement, very major and major errors, accuracy and precision values were calculated. For accuracy and precision calculations of the BBMD following formulae were used:

Accuracy (%) = number of true positives/total number of results x 100

Precision (%) = number of replicated results in agreement/total number of results x 100

A categorical agreement rate of >90% between full range BMD and BBMD was used as an acceptable criterion for initial validation. All colistin resistant clinical isolates encountered during ongoing validation were subjected to full range of colistin BMD. Trouble shooting for discrepancies (major errors = resistant on BBMD but susceptible on full range BMD) included a careful inspection of inoculum purity plate for contamination, subculture of all the BBMD wells for the discrepant isolate, looking for other colistin resistant isolates tested on the same BBMD plate that did not show discrepancy, re-testing of isolate by full range colistin MIC from inoculum purity plates of: a) BBMD & b) full range BMD and, from primary inoculation plate of the specimen where the strain was first isolated.

3. Results

3.1. Initial validation using reference strains

A total of 120 MIC readings for 60 clinical isolates were obtained. This included equal number of readings for colistin-susceptible and -resistant isolates. Twenty-four QC readings were also obtained and all MIC values for QC strains were found to be within range. Breakpoint susceptibility readings thus obtained were compared with full range colistin MICs for colistin resistant isolates published previously [12]. There was 100% categorical agreement between BBMD and the reference method (table A in supplementary material). No very major or major errors were observed and BBMD showed 100% accuracy and precision when compared to full range BMD.

3.2. Ongoing validation in routine clinical use

Once the results of validation study were analyzed and approved by the laboratory director, this breakpoint susceptibility method was put into routine use for testing of clinical isolates. We have been using this method to test clinical isolates of *Enterobacterales*, *P. aeruginosa*, and *Acinetobacter baumannii* complex since July 2nd, 2018. In addition to daily quality control testing and inoculum controls, other cumulative indicators recorded to reflect the robustness of the process include number and types of bacteria tested, results reported or discarded, and source (specimen type). Personnel competency assessments are performed annually.

A total of 1294 clinical isolates were tested at an average of 16.8 ± 5.5 isolates/day by BBMD along with 297 QC readings from 2nd July to September 16, 2018. Distribution of these clinical isolates is shown in Table 1. It was noted during ongoing validation that colistin MIC values for *E. co*li NCTC13846 tend to go down to 2 μg/mL on 5th or 6th passage from the original QC culture.

Out of 1294 clinical isolates, 1157 (mean 15.0 ± 4.8 isolates/day) were communicated to clinicians at 24 h as susceptible to colistin, whereas, 133 isolates (mean 1.7 ± 1.5 isolates/day) had an MIC of ≥4 μg/mL and were subjected to full range colistin BMD for confirmation of resistance. Among 133 colistin resistant isolates on BBMD, 109 (81.9%) were confirmed by full range colistin BMD as resistant (mean 1.7 ± 1.2 isolates/day). The remaining 24 isolates out of 133 (18%) were found to be susceptible by full range of colistin BMD. A discrepancy investigation was undertaken (see table B for details of individual isolates in supplementary material). BBMD inoculum purity plates of all these discrepant isolates were satisfactory at 24-h reading. However, upon subculture of BBMD wells for these isolates, 10/24 were found to have test isolate and another colistin resistant Gram negative rod, 11/24 grew Gram positive cocci

Table 1
Distribution of clinical isolates tested for colistin MIC by breakpoint broth microdilution (BBMD).

Isolate type	Number of isolates (%)
Enterobacterales	667 (51.5)
Acinetobacter spp.	317 (24.5)
Pseudomonas aeruginosa	273 (21.1)
Inherent colistin resistant organisms (*Proteus*, *Providencia*, *Morganella* and *Serratia marcescens*)	37 (2.9)
Total	1294

(*Staphylococcus* spp. and/or *Streptococcus* spp.) in addition to test isolate. For the remaining three isolates (3/26), two had only test isolate on wells subculture (repeat MICs from primary plate of these test isolates were in sensitive range) and one had BBMD & purity plates discarded (well subculture could not be performed).

4. Discussion

To address the scourge of AMR, detection of antimicrobial resistance is an essential first step. With the rising cost of healthcare, it is paramount that this be done cost-effectively, while also maintaining the quality of standards of susceptibility testing. Last-resort antimicrobial agents for Gram negative bacilli, such as colistin, require BMD for accurate determination of resistance [3,4]. In settings with high rates of AMR and frequent encounters with carbapenem resistant Gram-negative bacilli, it is prudent to devise a susceptibility test method for colistin that is both cost-effective and high throughput. Here we have described breakpoint susceptibility testing for colistin by BMD in laboratory prepared frozen 96-well plates and validated this method against the reference (full range of colistin MIC by BMD). This improvisation could accommodate 19 test isolates and 3 QC strains in a single 96-well plate. In our laboratory, BBMD is preferred over recently approved CAT & CBDE CAT & CBDE so that a single method could be utilized across Enterobacterales, *P. aeruginosa* and *Acinetobacter* species. An initial validation study proved its utility and BBMD was then implemented for routine testing of clinical isolates. The validation study did not find any discrepancies between test and reference methods. However, when this method was placed into routine clinical use, some issues were encountered.

We faced a big challenge in resolving discrepancies (24/133 202 or 18% isolates) between breakpoint susceptibility results and full range colistin MICs. Some of the isolates that were resistant on BBMD showed MIC values that were in susceptible range when retested with full range BMD. The probable reasons for these discrepancies might have been contamination from primary plate while preparing inoculum, when making final inoculum dilution before inoculating BBMD plate, from adjacent wells, inoculation of incorrect bacterial strain (very unlikely in our case as well subculture grew both the test isolate and contaminant), or double inoculation of same wells, etc. (see table B in supplementary material).

Another plausible reason for the contamination may have been related to workload. Initial validation was performed in a controlled setting whereas ongoing validation is observed in routine laboratory practice. The microbiology laboratory at our hospital is a very busy service, performing approximately 65 000–70,000 tests per month including bacteriology cultures, mycology cultures, mycobacteriology cultures, serology, urinalysis, parasitology, and food and water cultures, all under one roof.

In our experience, *E. coli* NCTC 13846 is a difficult strain to maintain. We observed that with even 5th or 6th passage from the original QC culture, its colistin MIC value tends to drop to 2 μg/mL. To resolve this, more frequent revival of this strain was performed from −80 °C and not more than 2–3 passages were allowed from the parent vial [15].

We recommend retesting all colistin resistant isolates before reporting the results. Resolution of discrepancies (major errors) need careful evaluation of isolate identification, inoculum purity plate, BBMD plate for presence of other colistin resistant isolates and any procedural breaches. Strengths of this BBMD plate include ease of use, the capacity to test 19 isolates and 3 QC strains in one 96-well plate and use of recommended BMD method. A considerable amount of time is required for training of laboratory technologists for preparing BBMD plates which could be considered a limitation, however, once prepared, plates may be kept at −80 °C and used later. However, this time investment was paid back as time saving at inoculation of plates since BBMD plates were already prepared and only bacterial inocula remained to be added to the plates. Another limitation of this study was lack of detection of genetic markers of colistin resistance in the clinical isolates. Studies from Pakistan have shown that *mcr* gene may not be responsible for colistin resistance in some gram negatives, and other genetic markers representing efflux pumps are common [16]. Whole genome sequencing of isolates to identify genetic markers, or for routine clinical reporting is costly, time consuming and difficult to implement in high volume laboratories.

5. Conclusions

This study demonstrated validation and implementation of a high throughput method of performing colistin breakpoint susceptibility by the recommended broth microdilution in a routine clinical microbiology laboratory. Our results support utilization of this BBMD plate.

Authors' contributions

SS conceived the idea and provided overall supervision. IA helped supervise the project, analyzed data and wrote manuscript. SL & NS performed bench work. SL performed initial data analysis. KW & AS provided logistic support, helped in running the project and made available overall technical support. JF helped supervise the project and provided help in data analysis and manuscript writing. RH provided critical support in designing the BBMD plate, supported project by giving critique to data and manuscript. All authors helped in manuscript writing and approved the final draft.

Declaration of competing interest

None.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.plabm.2020.e00192.

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References

[1] R.M. Humphries, Susceptibility testing of the polymyxins: where are we now? Pharmacotherapy 35 (1) (2015) 22–27.
[2] FDA, Class 2 Device Recall VITEK 2 Gram Negative test kit containing colistin (cs01n). https://www.accessdata.fda.gov/scripts/cdrh/cfdocs/cfRes/res.cfm?id=155857, 2017.
[3] CLSI, Performance standards for antimicrobial susceptibility testing, in: CLSI Supplement M100, 28th ed., Clinical and Laboratory Standards Institute, Wayne, PA, 2018.
[4] The European Committee on Antimicrobial Susceptibility Testing, Breakpoint Tables for Interpretation of MICs and Zone Diameters, 2018. http://www.eucast.org.
[5] CLSI, Performance standards for antimicrobial susceptibility testing, in: CLSI Supplement M100, 30th ed., Clinical and Laboratory Standards Institute, Wayne, PA, 2020.
[6] Y.Y. Liu, Y. Wang, T.R. Walsh, L.X. Yi, R. Zhang, J. Spencer, et al., Emergence of plasmid-mediated colistin resistance mechanism MCR-1 in animals and human beings in China: a microbiological and molecular biological study, Lancet Infect. Dis. 16 (2) (2016) 161–168.
[7] M. Mohsin, S. Raza, N. Roschanski, S. Guenther, A. Ali, P. Schierack, Description of the first Escherichia coli clinical isolate harboring the colistin resistance gene mcr-1 from the Indian subcontinent, Antimicrob. Agents Chemother. 61 (1) (2017).
[8] L. Poirel, N. Kieffer, A. Brink, J. Coetze, A. Jayol, P. Nordmann, Genetic features of MCR-1-producing colistin-resistant Escherichia coli isolates in South Africa, Antimicrob. Agents Chemother. 60 (7) (2016) 4394–4397.
[9] S.K. Chiu, T.L. Wu, Y.C. Chuang, J.C. Lin, C.P. Fung, P.L. Lu, et al., National surveillance study on carbapenem non-susceptible Klebsiella pneumoniae in Taiwan: the emergence and rapid dissemination of KPC-2 carbapenemase, PLoS One 8 (7) (2013), e69428.
[10] European Committee for Antimicrobial Susceptibility Testing (EUCAST) of the European Society of Clinical Microbiology and Infectious Diseases (ESCMID), Determination of minimum inhibitory concentrations (MICs) of antibacterial agents by broth dilution, Clin. Microbiol. Infect. 9 (8) (2003) 1–7.
[11] CLSI, Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically, in: CLSI Standard M07, eleventh ed., Clinical and Laboratory Standards Institute, Wayne, PA, 2018.
[12] S. Qamar, N. Shaheen, S. Shakoor, J. Farooqi, K. Jabeen, R. Hasan, Frequency of colistin and fosfomycin resistance in carbapenem-resistant Enterobacteriaceae from a tertiary care hospital in Karachi, Infect. Drug Resist. 10 (2017) 231–236.
[13] CLSI, Verification of commercial microbial identification and antimicrobial susceptibility testing systems, in: CLSI Guideline M52, first ed., Clinical and Laboratory Standards Institute, Wayne, PA, 2015.
[14] Elder, B. L., and S. E. Sharp. 2003. Cumitech 39, competency assessment in the clinical microbiology laboratory. Coordinating ed., S. E. Sharp. ASM Press, Washington, D.C.
[15] CLSI, Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically, in: CLSI Standard M07, eleventh ed., Clinical and Laboratory Standards Institute, Wayne, PA, 2018.
[16] S. Lomonaco, M.A. Crawford, C. Lascols, R.E. Timme, K. Anderson, D.R. Hodge, et al., Resistome of carbapenem- and colistin-resistant Klebsiella pneumoniae clinical isolates, PLoS One 13 (6) (2018), e0198526.