‘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 ± 6.5 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. baumannii 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**

Growth deposits at the bottom of wells

**B**

(caption on next page)
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 Enterobacteriales, 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. coli 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 (%) |
|--------------------------------------------------|------------------------|
| Enterobacteriales                                | 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 to 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 Enterobacteriales, 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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