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# Antibiotic Susceptibility Testing: Effects of Variability in Technical Factors on Minimum Inhibitory Concentration using Broth Microdilution

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## Abstract

### Background
Broth microdilution (BMD) is a gold-standard reference method to determine minimum inhibitory concentration (MIC) of antibiotics. For this, a standardized concentration of bacterial inoculum (2e5–8e5 colony-forming units, CFU/ml) is added to progressively higher concentrations of antibiotics. Bacteria stop growing at a particular antibiotic concentration termed MIC. Like other assays, various biological and/or technical factors can affect BMD results.

### Aims
To investigate the effects of inoculum concentration (5e4–5e6 CFU/ml), growth-medium concentration (cation-adjusted Mueller-Hinton Broth (CAMHB)), ranging 0.5x to 2x (1x as standard)) and age (<6-months or >1-year old) of fastidious medium on MIC results. And to compare BMD results using 5 different brands of CAMHBs and 1 cation-non-adjusted MH-broth (non-CAMHB).

### Methods
12 isolates of bacteria (gram-positive (n=3), gram-negative(n=5), fastidious isolates (n=7)) and custom-made antibiotics-containing plates for gram-positive (11 antibiotics) or gram-negative bacteria (10 antibiotics) were used. Overnight-grown colonies were used to prepare BMD solutions (MH-broth + inoculum +/- fastidious) which were plated on antibiotic-plates as well as diluted prior to plating on agar-plates. Antibiotic- and agar-plates were incubated (18–20hr, 35°C) and used to determine MICs (following European Committee on Antimicrobial Susceptibility Testing instructions) and actual number of viable bacteria in BMD solutions, respectively.

### Results
Increasing inoculum concentration increased MICs of all antibiotics except cefoxitin. Piperacillin–tazobactam, levofloxacin, benzylpenicillin and ampicillin were especially sensitive to increase in inoculum and showed a 4-fold increase in >50% isolates. MICs for tobramycin, tigecycline and gentamicin increased by 2-fold in >50% isolates every time MH-broth concentration increased. Age of fastidious medium had no decipherable pattern of effects on MIC. All MH-broths gave similar results except when testing daptomycin which gave higher MICs with non-CAMHB compared to CAMHB.

### Conclusion
This research reveals some technical factors affecting MIC results. These results could help define parameters for automated BMD-performing-systems. However, this research shows only trends as more replicates are needed to determine statistically significant results.

# New study might pave way for rapid diagnosis of sepsis

## Popular Science Summary

*Seemal Aziz*

Sepsis is a severe bacterial infection that is one of the leading causes of deaths all over the world. According to a report published by World Health Organization (The Global Report on the Epidemiology and Burden of Sepsis), around 49 million people were affected, and 11 million people died because of sepsis in 2017 alone. This makes sepsis responsible for 1 in every 5 deaths all over the world. The report further points out that sepsis affects low- and middle- income countries disproportionately. According to the data from 2017, almost 85% of the cases and deaths as a result of sepsis occurred in sub-Saharan Africa and South-East Asia.

The rise in the number of sepsis cases is closely linked with antimicrobial resistance. Antimicrobial resistance can be thought of as a phenomenon in which microbes develop a resistance against a certain antibiotic by gaining a mutation that helps them survive. Microbes divide and grow at a rapid rate e.g., a single bacterium will result in around 260,000 bacteria in just 6 hours. As they divide and grow, a single mutation in their DNA can make them resistant against a certain antibiotic which could have killed them previously and this mutation will then be transferred to the next generations. In low- and middle- income countries, bacterial infections are treated by broad-spectrum antibiotics which contributes to developing antimicrobial resistance.

This study focuses on a method called Broth Microdilution that is a standard method for finding out the minimum concentration of antibiotic which can stop the growth of bacteria, also called the Minimum Inhibitory Concentration (herein referred to as MIC). This method can help hinder the spreading of antimicrobial resistance by helping doctors prescribe only the minimum required concentration of antibiotics to the patients. However, the Broth Microdilution process is quite laborious and can take up to 2-3 days when done using conventional methods. For sepsis, there is an 8% increase in mortality with every passing hour that the antibiotics are not administered.

In this study, various technical factors involved in the Broth Microdilution process were studied in detail to determine a correlation between a slight variation in one of these factors and change of the MIC outcome. The results from the study could pave the way for rapid and automated sepsis diagnosis as it has outlined the effect of various technical factors on MIC results which can then be incorporated and compensated for in an automated diagnostic solution.

Although this study has shown some promising results and given some helpful insights towards the optimisation of Broth Microdilution process for rapid and automated diagnostic solutions, the preliminary results need to be further verified to evaluate the reproducibility of the results in different conditions. A rapid and automated diagnostic solution for sepsis is desperately needed to help curtail the increasing number of people being affected by this disease every year and the current study has taken a step towards that.

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## List of Abbreviations

| | |
|---|---|
| AMR | antimicrobial resistance |
| AST | antibiotic susceptibility testing |
| BMD | broth microdilution |
| CFU | colony forming unit |
| EUCAST | European committee on antimicrobial susceptibility testing |
| FDA | The Food and Drug Administration |
| MHB | Mueller-Hinton broth |
| MIC | minimum inhibitory concentration |
| CAMHB | cation-adjusted Mueller Hinton broth |
| NCCLS | National Committee for Clinical Laboratory Standards |
| PBS | Phosphate Buffered Saline |
| WHO | World Health Organization |

# 1. Introduction

## 1.1. Global burden of bacterial infections and antimicrobial resistance

Globally, severe bacterial infections (sepsis) kill many people every year. It is estimated that in 2017, around 49 million people suffered from sepsis out of which 11 million people died worldwide. This amounts to 19.7 percent of all deaths in the world [1]. The likelihood of dying of sepsis is significantly increased if the infection is caused by a resistant bacteria [2]. Resistance is of two types; one that is inherent and the other which is acquired. Inherent resistance is the type of resistance that is present in bacteria intrinsically. It occurs due to various factors such as efflux systems, low-permeability outer membrane [3] and genes that are intrinsically present in the bacteria[4]. Acquired resistance occurs when bacteria acquire mutations that resist the effect of antibiotics [5]. Antimicrobial resistance (AMR) results due to widespread, extensive, and unnecessary use of antimicrobials. Overuse of antibiotics puts a selective pressure on the bacteria. As a result, the bacteria which are resistant to the damaging effects of antibiotics survive and propagate. Eventually, whole population of bacteria becomes resistant [6]. Due to this reason, resistance to current antibiotics is increasing at an astronomical rate[7] (Figure 1).

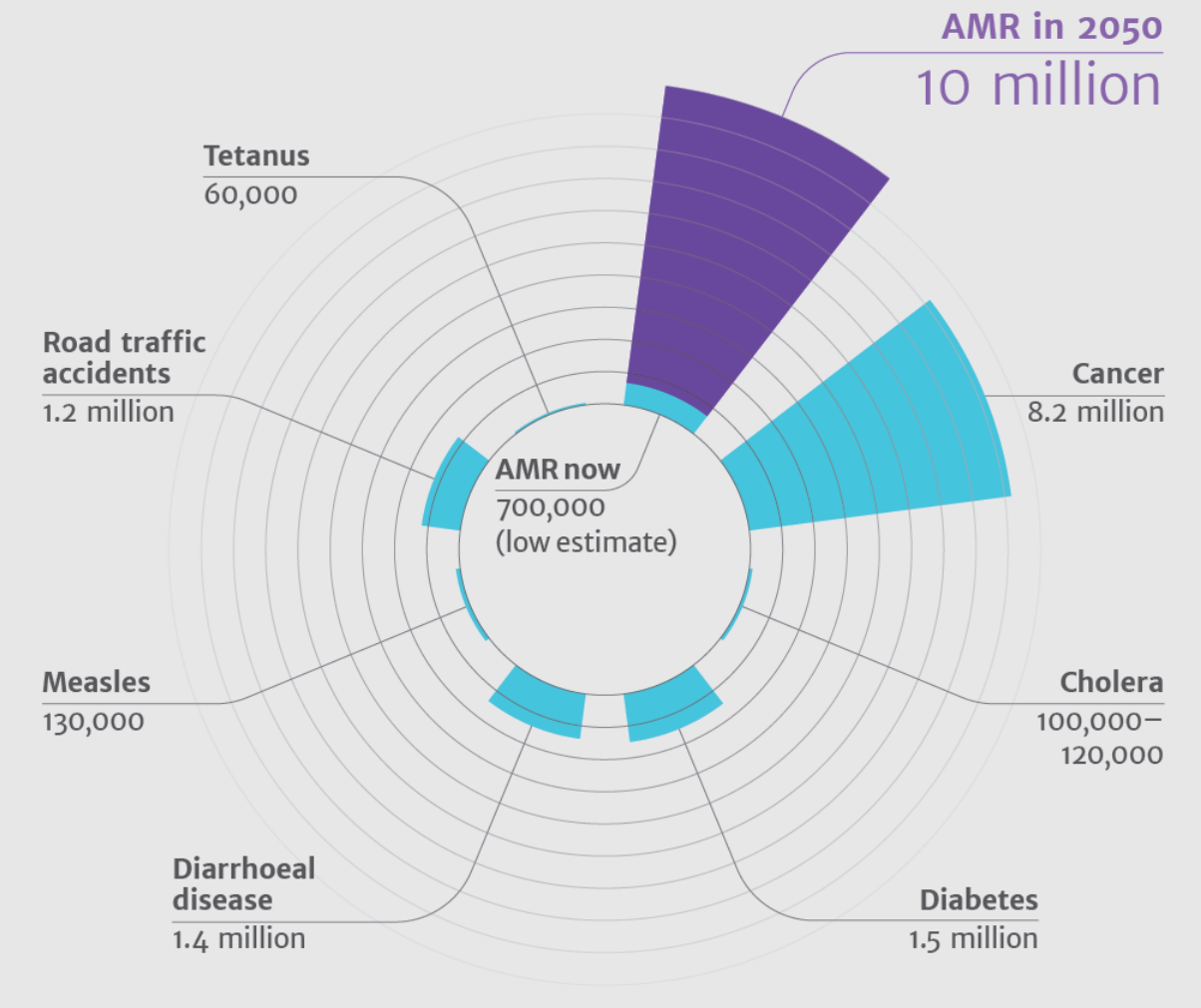


**Figure 1**: Deaths attributable to AMR every year compared to other major causes of deaths[7].

### 1.2. Consequences of AMR

As a result of wide-spread resistance, the antibiotic fails to eliminate the infection and eventually, becomes ineffective and obsolete. One of the solutions to this problem is the development of new antibiotics[8]. However, the rate of development of new antibiotics seems to be reducing. One of the reasons for this decline is that huge investment is required to bring a new drug into the market. Research into new drugs has relatively large costs but most of the new drugs fail to reach the market because of their various side-effects. This problem is further exacerbated by the fact that eventually most antibiotics would become obsolete due to growing antimicrobial resistance which further reduces the incentive for pharmaceutical companies to invest in development of new antibiotics [9].

Another solution to AMR is to use antibiotics judiciously – it means using appropriate antibiotics only when necessary. Using targeted narrow-spectrum antimicrobials against an infection may help prevent the antimicrobial from becoming obsolete. Determining the right antibiotic for a specific infection requires the use of antibiotic susceptibility testing (AST) [10].

### 1.3. Importance of AST in AMR and sepsis

In case of severe infection, the need to prescribe the correct antibiotic in a short amount of time becomes of paramount importance as a number of these patients could be saved if timely and appropriate treatment is provided [11]. According to a study by Liu and colleagues, delaying antibiotic treatment in case of sepsis increases mortality rate by 9 percent for every hour of delay in treatment [12]. Concordantly, if antibiotic therapy is started immediately after the diagnosis of sepsis, the risk of mortality decreases by 33 percent [13]. Current internationally accepted guidelines for treatment of sepsis suggest that empirical broad-spectrum antibiotics that defeat all likely bacteria should be started within one hour of diagnosis of sepsis. Blood for culture (along with other routine microbiological cultures) should be sent for testing for both aerobic and anaerobic bacteria before starting antimicrobial therapy unless it causes delay in initiating therapy. Once the results of the blood culture and antibiotic susceptibility testing are attained, therapy should be narrowed down to target the causative agent [14]. Antibiotic susceptibility testing (AST) is performed to determine which antibiotic regimen is the most effective for treatment of a bacterial infection in an individual patient. Using traditional methods of antibiotic susceptibility testing, definitive results take up to 48 hours or more to attain [15]. Due to this reason, antibacterial therapy is initiated empirically meaning without knowing the causative bacterial species. But a disadvantage of empirical treatment is that the bacteria that caused infection might be discordant with or resistant to the antibiotics administered. A study performed at hospitals in the US suggests that one in five patients receive inappropriate antimicrobial therapy contributing to increased likelihood of mortality [16]. Another reason for performing antibiotic susceptibility testing is that it confirms whether the treatment being given should be altered and whether the treatment would be effective against the causative agent or not.

### 1.4. Gold-standard AST methods

There are two main reference methods to perform antibiotic susceptibility testing: disk diffusion method and broth dilution method.

**Disk diffusion method**

The disk diffusion method is simple and practical and performed by applying a standardised bacterial inoculum on a Mueller-Hinton agar plate. Bacterial inoculum refers to the small, standardised number of bacteria that is applied to the agar plate. Thereafter, paper disks containing standardised concentrations of antibiotics are placed on the agar surface. This assembly is then incubated for 16-24 hours at 35°C and subsequently results determined by measuring the zone of bacterial growth inhibition around the antibiotics disk. The measurement of size of the zone of inhibition is interpreted using guidelines given by EUCAST (European committee on antimicrobial susceptibility testing)[17].

Despite being simple, cost-effective, and easy to perform, this technique has some disadvantages as well. The biggest drawback of this technique is that the technique indicates only the degree of susceptibility of the bacteria and does not indicate the minimum inhibitory concentration of the antibiotics (MIC), which may lead to excessive dosage of antibiotics. Another drawback is that the technique does not differentiate between the different types of antibiotics i.e. bacteriostatic and bactericidal antibiotics [17]. Bacteriostatic antibiotics are the antibiotics that inhibit the growth of bacteria and bactericidal are the ones that kill bacteria [18].

**Broth microdilution method**

Broth microdilution (BMD) indicates if the bacteria that infected the patient are resistant or susceptible as well as the correct dose of antibiotics that needs to be supplemented. BMD is a reference gold-standard method to determine MIC using bacterial inoculum. Bacterial inoculum, in this case, is referred to the standardised amount (2e5 to 8e5 CFU/ml) of bacteria which is added to the growth medium (Mueller-Hinton broth). A hundred microlitres of the resulting mixture is added to each of the ninety-six wells (except for the negative control wells) on a microdilution plate. In the negative-control wells, only growth broth is added without adding inoculum. The wells contain progressively increasing concentrations of antibiotics, with twofold concentration increase between two adjacent wells containing the same antibiotic. These loaded plates are incubated overnight, and thereafter the antibiotic concentration at which bacteria stop growing (MIC) determined using standardised guidelines given by EUCAST [17].

### 1.5. Variability in AST methods

All assay-based procedures have some inherent variability in results. For example, a study was done to determine the contribution of strain and laboratory variability to variation in MIC measurements using E-test (a diffusion and dilution based AST technique) [19]. This study used linezolid as antibiotic and compared various strains of *Staphylococcus aureus* and suggested that the causes of close to 60 percent of the variation in MIC measurements can be explained: around 50 percent of this variation occurred as a result of strain-to-strain differences and around 10 percent happened due to differences between laboratories. The remainder of the variability was attributed to assay variance.

Similarly, broth microdilution also exhibits variability in MIC readings. This variability in results can be due to biological or technical factors. For example, research was conducted to study reproducibility of MIC results within the same laboratories and between different

laboratories [20]. This study used *Nocardia* isolates for different antibiotics susceptibility testing by broth microdilution method. The results suggested that the level of reproducibility, to some extent, was related to the antibiotic being used. For some antibiotics, the results were largely agreed upon among different laboratories. While for others, the MIC results varied largely among the different laboratories. However, no variation in results was found when assessing the lot-to-lot variation among the different lots of microdilution plates. This indicated that the microdilution plates were essentially functionally identical.

### 1.6. Purpose of this study

This thesis will focus on some technical factors that could affect results of broth microdilution. An effort is made to define various parameters for automated BMD-performing systems to increase precision in results. The experiments have explored how variation in various factors, such as inoculum concentration, growth broth (Mueller-Hinton broth) concentration, and age of a growth medium (fastidious medium) can affect observed MICs. Another part of this research was to compare the MIC reading observed using different Mueller-Hinton broths. Mueller-Hinton broth is recommended by FDA (Food and Drug Administration, USA), WHO (World Health Organisation) and NCCLS (National Committee for Clinical Laboratory Standards, USA) for testing commonly found aerobic and anaerobic bacteria.

The panel of chosen bacterial isolates contains a variety of different bacteria with distinct features. Some of the isolates are gram-positive, some gram-negative. Some are also fastidious and require fastidious medium for growth as explained above. While some bacterial isolates of this panel are non-fastidious and do not require this special medium for growth. Two of the isolates are resistant against many antibiotics while the majority of isolates are susceptible to commonly used antibiotics. The reason for choosing isolates with such a wide variety of features is to mimic the kinds of bacteria that most commonly cause infections in humans and are clinically significant.

## 2. Aim of the Thesis

The experiments done in this research attempt to study the effect of various technical factors on MIC readings. In addition, this study also compares the results from using different Mueller-Hinton broths.

In short, this study aims to:

1. determine the effect of variation in concentration of bacterial inoculum on MIC.
2. determine the effect of variation in concentration of cation-adjusted Mueller-Hinton broth on MIC.
3. determine the effect of age of fastidious medium on MIC.
4. compare MIC results using Mueller-Hinton broths from five different brands. Mueller-Hinton broths from four brands were cation-adjusted while Mueller-Hinton broth from one brand was cation-non-adjusted (i.e., deficient in cations).

## 3. Material and Methods

**Day 0: Selection, streaking and growth of isolates:**

On day zero, an isolate was chosen from a pre-selected panel of isolates that had been stored in a freezer (temperature range: -80°C +/- 5°C) long-term. The chosen isolate was streaked on a suitable agar plate as discussed in the following table.

The streaking was done as follows:

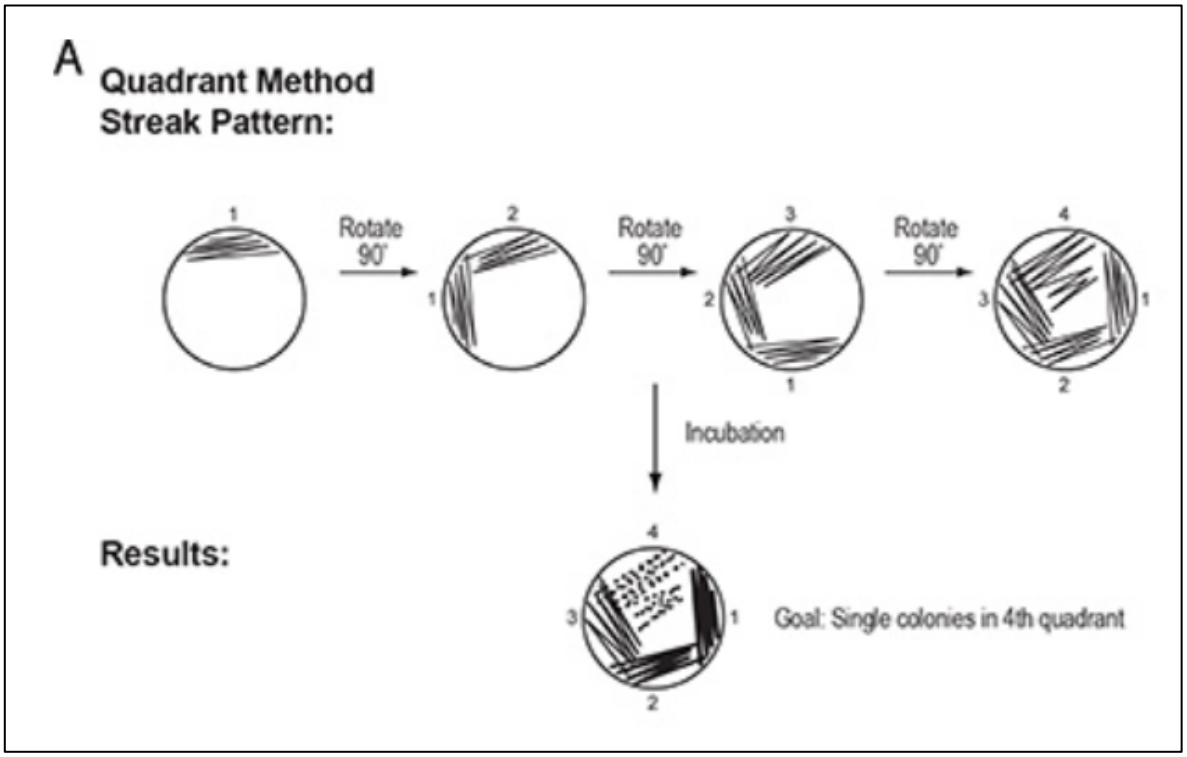


**Figure 2**: Method followed for streaking the agar plates [21].

The streaked plates were incubated for 18-20 hours at 35°C with or without $CO_2$ as required for the chosen bacterial species.
Agar plates were obtained from the following sources:

| Type of plate | Company | Article number |
|---|---|---|
| TSA-agar | B.D | 254087 |
| Chocolate Agar (Blood Agar No.2 Base) | B.D | 257456 |
| CLED | B.D | 254070 |

Table 1 lists the panel of bacterial isolates used for testing effect of inoculum concentration, effect of CAMHB (cation-adjusted Mueller-Hinton broth) concentration and comparison between different MHB (Mueller-Hinton broth) brands.

**Table 1**: List of bacterial isolates used in experiments testing the effect of inoculum concentration, effect of CAMHB concentration and comparison of MHB brands.

| Species | Isolate ID number(s) | Gram type | Fastidious or non-fastidious | Incubation | Type of agar |
|---|---|---|---|---|---|
| *Staphylococcus epidermidis* (SE) | QM358 | positive | non-fastidious | Standard | TSA-agar |
| *Staphylococcus aureus* (SA) | QM079 | positive, QC isolate | non-fastidious | Standard | TSA-agar |
| *Escherichia coli* (EC) | QM309 | negative, resistant | non-fastidious, | Standard | TSA-agar |
| *Klebsiella pneumoniae* (KP) | QM385 | negative, resistant, QC isolate | non-fastidious | Standard | TSA-agar |
| *Pseudomonas aeruginosa* (PA) | QM276 | negative QC isolate | non-fastidious | Standard | CLED |
| *Streptococcus pneumoniae* (SP) | HV431 | positive, $CO_2$ required | fastidious | 5% $C0_2$ | TSA-agar |
| *Haemophilus influenzae* (HI) | QM021 QM346 | negative, $CO_2$ required | fastidious | 5% $CO_2$ | Chocolate agar |

Table 2 lists the bacterial isolates used for testing the effect of age of fastidious medium.

**Table 2**: List of bacterial isolates used for testing effect of age of fastidious medium.

| Species | Isolate ID number | Gram Type | Incubation | Agar type |
|---|---|---|---|---|
| Streptococcus pneumoniae (SP) | HV431 QM145 QM328 | positive, QM145 is QC isolate | $CO_2$ | TSA-agar |
| Haemophilus influenzae (HI) | QM021 QM346 QM664 QM333 | negative, QM664 is QC isolate | $CO_2$ | Chocolate agar |

**Day 1: Mixing and plating Broth microdilution (BMD) solutions:**

Next step is to make "BMD solution" by mixing inoculum and growth medium as explained later. By the next day (day 1), the streaked agar plate had grown colonies and was taken out of the incubator. 3-5 (or more as in case of *Streptococcus pneumoniae*) individual colonies were picked using an inoculation loop and mixed with PBS (phosphate buffered saline, Gibco™, Thermofischer Scientific) in a McFarland tube (VWR, article number 216-1045) to form McFarland solution. The density of the solution was adjusted to 0.5 McFarland using a McFarland densitometer (Grant Bio™, Thermofischer Scientific). A pre-calculated amount of McFarland solution was mixed into Mueller-Hinton broth (Table 3) +/- fastidious medium (depending on the type of bacteria being tested) to make 11 ml of BMD solution so that the target range of inoculum was achieved. For testing the effect of change in inoculum on MIC, 3 different inoculum concentrations were: 5e4 (range 2e4-8e4), 5e5 (range 2e5-8e5) and 5e6

(range 2e6-8e6) CFU/ml. For the rest of the experiments, target inoculum concentration used was 5e5 (range 2e5-8e5) CFU/ml.

To test the effect of concentration of CAMHB from different manufacturers on MIC, 3 concentrations were selected: 0.5x, 1x and 2x. As 1x is the standard concentration, the results from 0.5x and 2x concentrations were compared with results from 1x concentration. For experiments testing the rest of the technical factors, the CAMHB concentration used was 1x. The name and specifics of the standard MHB used for all experiments cannot be stated here due to confidentiality, however, the MHBs from different brands that were compared to the standard are listed in Table 3. Furthermore, 2 different batches from Thermofischer were used.

**Table 3**: List of MHBs used to compare results.

| **Manufacturer** | **Reference number** | **Used for isolates** |
|---|---|---|
| BioMerieux CAMHB | AEB110699 | SP HV431<br>HI QM021 & 346<br>KP QM385<br>PA QM276<br>SE QM358<br>SA QM079<br>EC QM309 |
| Merlin CAMHB | E2-331-100 | SP HV431<br>HI QM021 & 346<br>KP QM385<br>PA QM276<br>SE QM358<br>SA QM079<br>EC QM309 |
| ThermoFischer CAMHB (Batch A) | T3462 | SP HV431<br>HI QM021 & 346<br>KP QM385<br>PA QM276<br>SE QM358<br>SA QM079<br>EC QM309 |
| ThermoFischer CAMHB (Batch B) | T3462 | KP QM385<br>EC QM309<br>SP HV431<br>SA QM079 |
| BD BBL CAMHB | 298268 | SP HV431<br>HI QM021 & 346<br>KP QM385<br>PA QM276<br>SE QM358<br>SA QM079<br>EC QM309 |
| Difco (cation-non-adjusted) | 275730 | SP HV431 |

| | | |
|---|---|---|
| | | HI QM021 & 346<br>KP QM385<br>PA QM276<br>SE QM358<br>SA QM079<br>EC QM309 |

The fastidious medium used for all experiments (other than those testing the effect of age of fastidious medium on MIC) came from a batch that was stored for less than 1 year in the freezer (temperature: -20°C +/-5°C). For testing the effect of age of fastidious medium, 2 different batches were used: 1 that had been stored in the freezer for approximately 20 months (termed as "old fastidious") and the other that had been in the freezer for less than 6 months (termed as "new fastidious"). All fastidious media were manufactured in-house at Q-Linea AB as described in EUCAST guidelines[22].

This mixture of inoculum and MHB +/-fastidious medium is termed as "BMD solution". BMD solution was loaded on the microdilution plates containing dried antibiotics, as well as plated on suitable agar plates. 100µl of BMD solution was added to each well of the microdilution plate. For plating on agar plates, the BMD solution having ~5e4 CFU/ml was diluted 100 times, ~5e5 CFU/ml is diluted 1000 times and ~5e6 CFU/ml is diluted 10,000 times and plated on agar plates using an automatic plater (Easyspiral™, Interscience). The plates were incubated at 35°C for 18-20 hours. The antibiotic plates used in these experiments were expired, however tested for QC isolates and determined to give correct results. QC isolates are isolates from specific bacterial species and are well characterised bacteria that have defined resistance or susceptibility to antimicrobial agents. Hence, it was concluded that these plates were still usable, and the results considered as valid. This has been explained further in the "Discussion" section.

The antibiotics plates used for all experiments are listed in Table 4.

**Table 4**: List of antibiotics used in all experiments.

| **Manufacturer** | **Plate type** | **Expiry date** | **Antibiotics** |
|---|---|---|---|
| Merlin | Gram positive | 03-04-2019 | Erythromycin<br>Daptomycin<br>Levofloxacin<br>Trimethoprim:sulfamethoxazole<br>Benzylpenicillin<br>Tetracycline<br>Vancomycin<br>Clindamycin<br>Ampicillin<br>Tigecycline<br>Cefoxitin |
| Merlin | Gram negative | 30-03-2019 | Gentamicin |

| | | | |
|---|---|---|---|
| | | | Cefotaxime<br>Ciprofloxacin<br>Piperacillin - tazobactam<br>Ceftazidime<br>Meropenem<br>Tobramycin<br>Ceftolozane-tazobactam<br>Amoxicillin-clavulanic acid<br>Colistin |

**Day 2: MIC and inoculum determination:**

The next day (day 2), BMD plates were read according to EUCAST guidelines [23] and results noted. Agar plates were also analysed using automatic colony counter (Scan 300™, Interscience) to count the number of colonies on each plate. This count was then used to calculate the actual inoculum concentration of each BMD plate.

# 4. Results

The results from all experiments are summarised below. For some isolates change in MIC could not be observed because the MIC was outside the antibiotic concentration range on the plate. In some cases, skips were observed while reading the BMD plates. Skips refer to the phenomenon of alternating presence and absence of growth in multiple adjacent wells and as a result MIC cannot be determined.

## 4.1. Effect of variability in inoculum concentration

The inoculum range used for these experiments was between 1.7e4 to 4.5e6. It was observed that increasing inoculum concentration increased MICs in all isolates for majority of antibiotics. For the resistant isolate EC QM309, more than 50% of its MIC readings fell outside the antibiotic concentration range of the plate. So, the trend of increasing MICs as a result of increase in inoculum could not be observed for this isolate in many instances. Analysing all antibiotics separately, they could be classified into three categories:

1. Antibiotics that were more sensitive to inoculum change
2. Antibiotics that were less sensitive to inoculum change
3. Antibiotics that were insensitive to inoculum change

**Antibiotics that were more sensitive to inoculum change**

These are the antibiotics, for which, increasing inoculum concentration 10 times resulted in at least one 4-fold or more increase in MIC reading for more than half of the isolates. The antibiotics and the number of isolates that fall into this category are given in Table 5.

**Table 5**: MICs of antibiotics more sensitive to inoculum change.

*Yellow-shading indicates MIC increase of 4-fold or more.*

| Isolate name | Antibiotic name | Concentration range on the plate (mg/L) | 5e4 | 5e5 | 5e6 |
|---|---|---|---|---|---|
| SE QM358 | Ampicillin | 0.06-32 | 0.125 | M.S.* | 32 |
| SA QM079 | Ampicillin | 0.06-32 | 0.5 | 1 | 16 |
| SP HV431 | Ampicillin | 0.06-32 | ≤0.0625 | ≤0.0625 | ≤0.0625 |
| PA QM276 | Piperacillin - tazobactam | 0.5-64 | 2 | 8 | 8 |
| HI QM021 | Piperacillin - tazobactam | 0.5-64 | ≤0.5 | ≤0.5 | ≤0.5 |
| EC QM309 | Piperacillin - tazobactam | 0.5-64 | 8 | >64 | >64 |
| HI QM346 | Piperacillin - tazobactam | 0.5-64 | ≤0.5 | ≤0.5 | ≤0.5 |
| KP QM385 | Piperacillin - tazobactam | 0.5-64 | 8 | 8 | 32 |
| SE QM358 | Benzylpenicillin | 0.015-8 | 0.125 | M.S.* | >8 |
| SA QM079 | Benzylpenicillin | 0.015-8 | 0.25 | 2 | >8 |
| SP HV431 | Benzylpenicillin | 0.015-8 | ≤0.015625 | ≤0.015625 | 0.03125 |
| SE QM358 | Levofloxacin | 0.06-8 | 0.25 | 0.25 | 1 |

| | | | | | |
|---|---|---|---|---|---|
| SA QM079 | Levofloxacin | 0.06-8 | 0.25 | 0.25 | 0.25 |
| SP HV431 | Levofloxacin | 0.06-8 | 1 | 1 | >8 |

* Multiple skips (M.S.) observed so exact MIC could not be determined. However, it can be safely assumed that the increase was 4-fold or higher.

**Antibiotics that were less sensitive to inoculum change**

These are the antibiotics, for which, increasing inoculum concentration 10 times resulted in at least one 2-fold or more jump in observable MIC readings (yellow-shaded boxes in Tables 6 (for gram-positive bacteria) and Table 7 (for gram-negative bacteria)) for half or more than half of the observable isolates. Observable MICs refer to those readings that were within the antibiotic concentration range of the BMD plates. For the remaining isolates, change was not or could not be observed because the MICs were outside the antibiotic concentration range on the plates.

**Table 6**: MICs of antibiotics less sensitive to inoculum change in gram-positive bacteria.

| Isolate name (Gram Positive) | Antibiotic name | Concentration range on the plate (mg/L) | 5e4 | 5e5 | 5e6 |
|---|---|---|---|---|---|
| SE QM358 | Erythromycin | 0.015-8 | >8 | >8 | >8 |
| SA QM079 | Erythromycin | 0.015-8 | 0.5 | 1 | 1 |
| SP HV431 | Erythromycin | 0.015-8 | 0.03125 | 0.0625 | 0.0625 |
| SE QM358 | Daptomycin | 0.06-4 | 1 | M.S.* | >4 |
| SA QM079 | Daptomycin | 0.06-4 | 0.5 | 0.5 | 1 |
| SP HV431 | Daptomycin | 0.06-4 | 0.25 | 0.25 | 0.5 |
| SE QM358 | Tetracycline | 0.06-8 | 0.25 | 0.25 | 1 |
| SA QM079 | Tetracycline | 0.06-8 | 0.25 | 0.5 | 0.5 |
| SP HV431 | Tetracycline | 0.06-8 | 0.125 | 0.125 | 0.25 |
| SE QM358 | Clindamycin | 0.008-2 | 0.0625 | 0.25 | 0.5 |
| SA QM079 | Clindamycin | 0.008-2 | 0.0625 | 0.125 | 0.125 |
| SP HV431 | Clindamycin | 0.008-2 | 0.0625 | 0.0625 | 0.0625 |
| SE QM358 | Trimethoprim:sulfamethoxazole | 0.125-16 | ≤0.125 | ≤0.125 | 0.25 |
| SA QM079 | Trimethoprim:sulfamethoxazole | 0.125-16 | ≤0.125 | ≤0.125 | ≤0.125 |
| SP HV431 | Trimethoprim:sulfamethoxazole | 0.125-16 | ≤0.125 | 0.25 | 2 |
| SE QM358 | Vancomycin | 0.25-16 | 1 | 1 | >16 |
| SA QM079 | Vancomycin | 0.25-16 | 1 | 1 | 1 |
| SP HV431 | Vancomycin | 0.25-16 | ≤0.25 | ≤0.25 | 0.5 |
| SE QM358 | Tigecycline | 0.015-4 | 0.125 | 0.125 | 0.25 |
| SA QM079 | Tigecycline | 0.015-4 | 0.125 | M.S.* | 0.5 or 1** |

| | | | | | |
|---|---|---|---|---|---|
| SP HV431 | Tigecycline | 0.015-4 | ≤0.015625 | ≤0.015625 | 0.125 |

*Multiple skips (M.S.) observed so MICs could not be determined. However, it can be safely assumed that the increase was 2-fold or higher.
** 0.5 concentration well was left empty and 1 concentration well was negative so MIC could not be determined exactly but it can be safely assumed that the increase was 2-fold or higher.

**Table 7:** MICs of antibiotics less sensitive to inoculum change in gram-negative bacteria.

| **Isolate name (Gram-negative)** | **Antibiotic name** | **Concentration range on the plate (mg/L)** | **5e4** | **5e5** | **5e6** |
|---|---|---|---|---|---|
| PA QM276 | Gentamicin | 0.125-32 | 0.5 | 1 | 1 |
| HI QM021 | Gentamicin | 0.125-32 | 0.5 | 0.5 | 0.5 |
| EC QM309 | Gentamicin | 0.125-32 | >32 | >32 | >32 |
| HI QM346 | Gentamicin | 0.125-32 | 0.5 | 0.5 | 0.5 |
| KP QM385 | Gentamicin | 0.125-32 | 4 | 4 | 8 |
| PA QM276 | Cefotaxime | 0.015625-16 | 16 | 16 | >16 |
| HI QM021 | Cefotaxime | 0.015625-16 | 0.0625 | 0.0625 | 0.125 |
| EC QM309 | Cefotaxime | 0.015625-16 | 8 | 8 | >16 |
| HI QM346 | Cefotaxime | 0.015625-16 | 1 | 1 | 1 |
| KP QM385 | Cefotaxime | 0.015625-16 | 2 | 16 | >16 |
| PA QM276 | Ciprofloxacin | 0.0078125-8 | 0.25 | 0.5 | 0.5 |
| HI QM021 | Ciprofloxacin | 0.0078125-8 | ≤0.0078125 | ≤0.0078125 | 0.015625 |
| EC QM309 | Ciprofloxacin | 0.0078125-8 | >8 | >8 | >8 |
| HI QM346 | Ciprofloxacin | 0.0078125-8 | ≤ 0.0078125 | ≤0.0078125 | ≤0.0078125 |
| KP QM385 | Ciprofloxacin | 0.0078125-8 | 1 | 1 | 1 |
| PA QM276 | Ceftazidime | 0.125-32 | 2 | 2 | 8 |
| HI QM021 | Ceftazidime | 0.125-32 | ≤0.5 | ≤0.5 | 0.5 |
| EC QM309 | Ceftazidime | 0.125-32 | >32 | >32 | >32 |
| HI QM346 | Ceftazidime | 0.125-32 | ≤0.5 | ≤0.5 | ≤0.5 |
| KP QM385 | Ceftazidime | 0.125-32 | 16 | 32 | >32 |
| PA QM276 | Meropenem | 0.03125-32 | 0.25 | 0.25 | 1 |
| HI QM021 | Meropenem | 0.03125-32 | 0.0625 | 0.0625 | 0.5 |
| EC QM309 | Meropenem | 0.03125-32 | ≤0.03125 | ≤0.03125 | ≤0.03125 |
| HI QM346 | Meropenem | 0.03125-32 | 0.25 | 0.25 | 0.5 |
| KP QM385 | Meropenem | 0.03125-32 | ≤0.03125 | 0.0625 | 0.0625 |
| PA QM276 | Tobramycin | 0.125-16 | ≤0.125 | 0.25 | 0.25 |
| HI QM021 | Tobramycin | 0.125-16 | 0.25 | 0.25 | 0.5 |
| EC QM309 | Tobramycin | 0.125-16 | 16 | 16 | >16 |
| HI QM346 | Tobramycin | 0.125-16 | 0.25 | 0.25 | 0.5 |
| KP QM385 | Tobramycin | 0.125-16 | 2 | 2 | 4 |

| | | | | | |
|---|---|---|---|---|---|
| PA QM276 | Amoxicillin-clavulanic acid | 0.25-32 | >32 | >32 | >32 |
| HI QM021 | Amoxicillin-clavulanic acid | 0.25-32 | 0.5 | 0.5 | 4 |
| EC QM309 | Amoxicillin-clavulanic acid | 0.25-32 | >32 | >32 | >32 |
| HI QM346 | Amoxicillin-clavulanic acid | 0.25-32 | 4 | 8 | 16 |
| KP QM385 | Amoxicillin-clavulanic acid | 0.25-32 | 8 | 8 | 16 |
| PA QM276 | Colistin | 0.0625-8 | 1 | 1 | 2 |
| HI QM021 | Colistin | 0.0625-8 | 0.25 | 0.25 | 0.5 |
| EC QM309 | Colistin | 0.0625-8 | 0.25 | 0.25 | 1 |
| HI QM346 | Colistin | 0.0625-8 | 0.25 | 0.25 | 0.25 |
| KP QM385 | Colistin | 0.0625-8 | 0.5 | 1 | 1 |
| PA QM276 | Ceftolozane-tazobactam | 0.125-16 | 0.5 | 1 | 1 |
| HI QM021 | Ceftolozane-tazobactam | 0.125-16 | ≤0.125 | 0.25 | 0.5 |
| EC QM309 | Ceftolozane-tazobactam | 0.125-16 | 0.5 | >16 | >16 |
| HI QM346 | Ceftolozane-tazobactam | 0.125-16 | 2 | 2 | 4 |
| KP QM385 | Ceftolozane-tazobactam | 0.125-16 | 1 | 1 | 4 |

**Antibiotics that were insensitive to inoculum change**

Only one antibiotic fell in this category: cefoxitin. Increasing the inoculum concentration had no effect on the MIC of cefoxitin for any isolate.

**Table 8**: MICs of antibiotic insensitive to inoculum change.

| **Isolate name** | **Antibiotic name** | **Concentration range on the plate (mg/L)** | **5e4** | **5e5** | **5e6** |
|---|---|---|---|---|---|
| SE QM358 | Cefoxitin | 1-16 | 2 | 2 | 2 |
| SA QM079 | Cefoxitin | 1-16 | 4 | 4 | 4 |
| SP HV431 | Cefoxitin | 1-16 | 2 | 2 | 2 |

### 4.2. Effect of variability in CAMHB concentration

The inoculum range used for these experiments was between 2.6e5 to 6.2e5. The MIC results for experiments done with 0.5x and 2x CAMHB were compared with results obtained from experiments done with 1x CAMHB. The antibiotics analysed in this this experiment were classified into following categories:

1. Antibiotics that were more sensitive to change in CAMHB concentration
2. Antibiotics that were less sensitive to change in CAMHB concentration
3. Antibiotics with unclear pattern
4. Antibiotics insensitive to change in CAMHB concentration
5. Antibiotics showing decrease in MICs with increase in CAMHB concentration

**Antibiotics that were more sensitive to change in CAMHB concentration**

These antibiotics had 2 increases (2-fold or more) in observable MICs on the 0.5x to 2x range for >50% isolates. The antibiotics in this category are Tobramycin, Tigecycline and Gentamicin and the results are shown in Table 9.

**Table 9:** MICs of antibiotics more sensitive to change in CAMBH concentration.

*Readings showing an increase of 2-fold or more are highlighted in yellow.*

| Isolate name | Antibiotic name | Concentration range on the plate (mg/L) | 0.5x | 1x | 2x |
|---|---|---|---|---|---|
| PA QM276 | Tobramycin | 0.125-16 | ≤0.125 | 0.5 | 0.5 |
| EC QM309 | Tobramycin | 0.125-16 | 4 | 8 | >16 |
| HI QM021 | Tobramycin | 0.125-16 | ≤0.125 | 0.125 or 0.25* | 0.5 |
| HI QM346 | Tobramycin | 0.125-16 | ≤0.125 | 0.25 | 0.5 |
| KP QM385 | Tobramycin | 0.125-16 | ≤0.125 | 2 | 8 |
| PA QM276 | Gentamicin | 0.125-32 | 0.25 | 1 | 4 |
| EC QM309 | Gentamicin | 0.125-32 | 16 | >32 | >32 |
| HI QM021 | Gentamicin | 0.125-32 | ≤0.125 | 0.5 | 1 |
| HI QM346 | Gentamicin | 0.125-32 | ≤0.125 | 0.5 | 1 |
| KP QM385 | Gentamicin | 0.125-32 | 0.5 | 4 | 16 |
| SP HV431 | Tigecycline | 0.015-4 | <0.015625 | <0.015625 | 0.03125 |
| SE QM358 | Tigecycline | 0.015-4 | 0.03125 | 0.125 | 0.25 |
| SA QM079 | Tigecycline | 0.015-4 | 0.0625 | 0.125 | 0.25 |

*0.125 well was empty and 0.25 was negative. MIC could be either of the two. In any case, a 2-fold or higher increase was detected in the 2x concentration.

**Antibiotics that were less sensitive to change in CAMHB concentration**

These are the antibiotics which either showed an increase in MIC or the MIC remained the same on the 0.5x to 2x range of CAMHB concentration. The antibiotics in this category are: Erythromycin, Trimethoprim:sulfamethoxazole, Tetracyclin, Clindamycin, Ampicillin, Amoxicillin-clavulanic acid, Levofloxacin and Vancomycin and the results are shown in Table 10.

**Table 10:** MICs of antibiotics less sensitive to change in CAMBH concentration.

*Yellow-highlighted boxes indicate increase.*

| Isolate name | Antibiotic name | Concentration range on the plate (mg/L) | 0.5x | 1x | 2x |
|---|---|---|---|---|---|
| SP HV431 | Erythromycin | 0.015-8 | 0.03125 | 0.0625 | 0.0625 |
| SE QM358 | Erythromycin | 0.015-8 | >8 | >8 | >8 |
| SA QM079 | Erythromycin | 0.015-8 | 0.5 | 0.5 | 0.5 |
| SP HV431 | Trimethoprim:sulfamethoxazole | 0.125-16 | ≤0.125 | 0.25 | 0.25 |
| SE QM358 | Trimethoprim:sulfamethoxazole | 0.125-16 | ≤0.125 | ≤0.125 | ≤0.125 |
| SA QM079 | Trimethoprim:sulfamethoxazole | 0.125-16 | ≤0.125 | ≤0.125 | ≤0.125 |
| SP HV431 | Tetracycline | 0.06-8 | 0.125 | 0.125 | 0.25 |
| SE QM358 | Tetracycline | 0.06-8 | 0.5 | 0.5 | 0.5 |
| SA QM079 | Tetracycline | 0.06-8 | 0.5 | 0.5 | 1 |
| SP HV431 | Clindamycin | 0.008-2 | 0.0625 | 0.0625 | 0.0625 |
| SE QM358 | Clindamycin | 0.008-2 | 0.25 | 0.25 | 0.25 |
| SA QM079 | Clindamycin | 0.008-2 | 0.125 | 0.125 | 0.25 |
| SP HV431 | Ampicillin | 0.06-32 | ≤0.0625 | ≤0.0625 | ≤0.0625 |
| SE QM358 | Ampicillin | 0.06-32 | 0.5 | 0.5 | 1 |
| SA QM079 | Ampicillin | 0.06-32 | 1 | 1 | 1 |
| SP HV431 | Vancomycin | 0.25-16 | ≤0.25 | ≤0.25 | 0.5 |
| SE QM358 | Vancomycin | 0.25-16 | 1 | 1 | 2 |
| SA QM079 | Vancomycin | 0.25-16 | 0.5 | 1 | 2 |
| SP HV431 | Levofloxacin | 0.06-8 | 0.5 | 1 | 1 |
| SE QM358 | Levofloxacin | 0.06-8 | 0.25 | 0.25 | 0.5 |
| SA QM079 | Levofloxacin | 0.06-8 | 0.125 | 0.25 | 0.25 |

**Antibiotics with Unclear patterns**

These antibiotics showed MICs that increased, decreased, or remained the same with increase in CAMHB concentration. No specific pattern was observed for the results of these antibiotics. The antibiotics in this category are Cefotaxime, Ciprofloxacin, Piperacillin-tazobactam,

Ceftazidime, Meropenem, Ceftolozane-tazobactam, Amoxicillin-clavulanic acid and Colistin, and the results are shown in Table 11.

**Table 11**: MICs of antibiotics with unclear patterns.

| Isolate name | Antibiotic name | Concentration range on the plate (mg/L) | 0.5x | 1x | 2x |
|---|---|---|---|---|---|
| PA QM276 | Cefotaxime | 0.015625-16 | 16 | 16 | 16 |
| EC QM309 | Cefotaxime | 0.015625-16 | 8 | >16 | 8 |
| HI QM021 | Cefotaxime | 0.015625-16 | 0.0625 | 0.0625 | 0.03125 |
| HI QM346 | Cefotaxime | 0.015625-16 | 0.5 | 1 | 0.5 |
| KP QM385 | Cefotaxime | 0.015625-16 | 4 | 2 | 2 |
| PA QM276 | Ciprofloxacin | 0.0078125-8 | 0.25 | 0.5 | 0.5 |
| EC QM309 | Ciprofloxacin | 0.0078125-8 | >8 | >8 | >8 |
| HI QM021 | Ciprofloxacin | 0.0078125-8 | 0.007813 | ≤0.0078125 | 0.015625 |
| HI QM346 | Ciprofloxacin | 0.0078125-8 | ≤0.0078125 | ≤0.0078125 | 0.0156 |
| KP QM385 | Ciprofloxacin | 0.0078125-8 | 0.5 | 0.5 | 0.5 |
| PA QM276 | Piperacillin - tazobactam | 0.5-64 | 4 | 2 | 2 |
| EC QM309 | Piperacillin - tazobactam | 0.5-64 | 16 | >64 | 16 |
| HI QM021 | Piperacillin - tazobactam | 0.5-64 | ≤0.125 | ≤0.125 | ≤0.125 |
| HI QM346 | Piperacillin - tazobactam | 0.5-64 | ≤0.5 | ≤0.5 | ≤0.5 |
| KP QM385 | Piperacillin - tazobactam | 0.5-64 | 16 | 8 | 8 |
| PA QM276 | Ceftazidime | 0.125-32 | 2 | 2 | 2 |
| EC QM309 | Ceftazidime | 0.125-32 | >32 | >32 | >32 |
| HI QM021 | Ceftazidime | 0.125-32 | ≤0.125 | ≤0.125 | ≤0.125 |
| HI QM346 | Ceftazidime | 0.125-32 | 0.5 | 0.25 | 0.25 |
| KP QM385 | Ceftazidime | 0.125-32 | 32 | >32 | 16 |
| PA QM276 | Meropenem | 0.03125-32 | 0.25 | 0.25 | 0.5 |
| EC QM309 | Meropenem | 0.03125-32 | ≤0.03125 | ≤0.03125 | ≤0.03125 |
| HI QM021 | Meropenem | 0.03125-32 | ≤0.03125 | 0.0625 | 0.0625 |
| HI QM346 | Meropenem | 0.03125-32 | ≤0.03125 | 0.25 | 0.125 |
| KP QM385 | Meropenem | 0.03125-32 | ≤0.03125 | ≤0.03125 | 0.0625 |
| PA QM276 | Ceftolozane-tazobactam | 0.125-16 | 0.5 | 0.5 | 1 |
| EC QM309 | Ceftolozane-tazobactam | 0.125-16 | 0.25 | >16 | 8 |
| HI QM021 | Ceftolozane-tazobactam | 0.125-16 | ≤0.125 | 0.25 | ≤0.125 |
| HI QM346 | Ceftolozane-tazobactam | 0.125-16 | 2 | 2 | 2 |

| | | | | | |
|---|---|---|---|---|---|
| KP QM385 | Ceftolozane-tazobactam | 0.125-16 | 1 | 1 | 4 |
| PA QM276 | Colistin | 0.0625-8 | 0.5 | 1 | 2 |
| EC QM309 | Colistin | 0.0625-8 | 1 | 0.25 | 2 |
| HI QM021 | Colistin | 0.0625-8 | ≤0.0625 | 0.5 | 0.5 |
| HI QM346 | Colistin | 0.0625-8 | 0.5 | 0.5 | 0.5 |
| KP QM385 | Colistin | 0.0625-8 | 0.5 | 1 | 1 |
| PA QM276 | Amoxicillin-clavulanic acid | 0.25-32 | >32 | >32 | >32 |
| EC QM309 | Amoxicillin-clavulanic acid | 0.25-32 | 32 | >32 | >32 |
| HI QM021 | Amoxicillin-clavulanic acid | 0.25-32 | ≤0.25 | 0.5 | 0.5 |
| HI QM346 | Amoxicillin-clavulanic acid | 0.25-32 | 0.5 | 4 | 1 |
| KP QM385 | Amoxicillin-clavulanic acid | 0.25-32 | 4 | 8 | 16 |

**Antibiotics insensitive to change in CAMHB concentration**

Cefoxitin is the only antibiotic that falls in this category. No change in MIC was observed with the change in CAMHB concentration as can be seen in Table 12.

**Table 12**: MICs of antibiotic insensitive to change in CAMHB concentration.

| Isolate name | Antibiotic name | Concentration range on the plate (mg/L) | 0.5x | 1x | 2x |
|---|---|---|---|---|---|
| SP HV431 | Cefoxitin | 1-16 | 2 | 2 | 2 |
| SE QM358 | Cefoxitin | 1-16 | 2 | 2 | 2 |
| SA QM079 | Cefoxitin | 1-16 | 4 | 4 | 4 |

**Antibiotics showing decrease in MICs with increase in CAMHB concentration**

These antibiotics showed decrease in MICs with increase in CAMHB concentration for >50% isolates. The antibiotics in this category are daptomycin and benzylpenicillin, and the results shown in Table 13.

**Table 13:** Antibiotics showing decrease in MICs with increase in CAMHB concentration.

*Yellow-highlighted values indicate decrease in MIC.*

| Isolate name | Antibiotic name | Concentration range on the plate (mg/L) | 0.5x | 1x | 2x |
|---|---|---|---|---|---|
| SP HV431 | Benzylpenicillin | 0.015-8 | 0.03125 | 0.03125 | ≤0.015625 |
| SE QM358 | Benzylpenicillin | 0.015-8 | 2 | 1 | 1 |
| SA QM079 | Benzylpenicillin | 0.015-8 | 0.5 | 0.5 | 0.5 |
| SP HV431 | Daptomycin | 0.06-4 | 0.5 | 0.25 | 0.25 |
| SE QM358 | Daptomycin | 0.06-4 | 1 | 0.5 | 0.5 |
| SA QM079 | Daptomycin | 0.06-4 | 1 | 0.5 | 2 |

### 4.3. Effect of age of fastidious medium

The inoculum range used for these experiments was from 1.1e5 to 5.6e5. There was 1 microdilution plate which had inoculum lower than the optimal range. No pattern of effects in MIC was observed with the change in the age of fastidious medium. Gentamicin, cefotaxime, piperacillin-tazobactam, ceftazidime, levofloxacin, benzylpenicillin, tetracycline, vancomycin, clindamycin, tigecycline showed no difference in MICs when using old and new fastidious medium (Table 14). The rest of the antibiotics showed some differences, but no clear pattern could be identified (Table 15).

**Table 14**: Antibiotics that showed no difference to change in age of fastidious medium.

| Isolate name | Antibiotic name | Concentration range on the plate (mg/L) | New Fastidious Medium | Old Fastidious Medium |
|---|---|---|---|---|
| HI QM021 | Gentamicin | 0.125-32 | 0.5 | 0.5 |
| HI QM346 | Gentamicin | 0.125-32 | 0.5 | 0.5 |
| HI QM333 | Gentamicin | 0.125-32 | 0.25 | 0.25 |
| HI QM664 | Gentamicin | 0.125-32 | 0.5 | 0.5 |
| HI QM021 | Cefotaxime | 0.015625-16 | 0.0625 | 0.0625 |
| HI QM346 | Cefotaxime | 0.015625-16 | 1 | 1 |
| HI QM333 | Cefotaxime | 0.015625-16 | ≤0.015625 | ≤0.015625 |
| HI QM664 | Cefotaxime | 0.015625-16 | 0.25 | 0.25 |
| HI QM021 | Piperacillin - tazobactam | 0.5-64 | ≤0.5 | ≤0.5 |
| HI QM346 | Piperacillin - tazobactam | 0.5-64 | ≤0.5 | ≤0.5 |
| HI QM333 | Piperacillin - tazobactam | 0.5-64 | ≤0.5 | ≤0.5 |
| HI QM664 | Piperacillin - tazobactam | 0.5-64 | ≤0.5 | ≤0.5 |
| HI QM021 | Ceftazidime | 0.125-32 | ≤0.125 | ≤0.125 |
| HI QM346 | Ceftazidime | 0.125-32 | 0.5 | 0.5 |
| HI QM333 | Ceftazidime | 0.125-32 | ≤0.125 | ≤0.125 |

| | | | | |
|---|---|---|---|---|
| HI QM664 | Ceftazidime | 0.125-32 | 0.5 | 0.5 |
| SP HV431 | Levofloxacin | 0.06-8 | 1 | 1 |
| SP QM145 | Levofloxacin | 0.06-8 | 0.5 | 0.5 |
| SP QM328 | Levofloxacin | 0.06-8 | 1 | 1 |
| SP HV431 | Benzylpenicillin | 0.015-8 | ≤0.015625 | ≤0.015625 |
| SP QM145 | Benzylpenicillin | 0.015-8 | 0.25 | 0.25 |
| SP QM328 | Benzylpenicillin | 0.015-8 | 2 | 2 |
| SP HV431 | Tetracycline | 0.06-8 | 0.125 | 0.125 |
| SP QM145 | Tetracycline | 0.06-8 | 0.125 | 0.125 |
| SP QM328 | Tetracycline | 0.06-8 | 0.5 | 0.5 |
| SP HV431 | Vancomycin | 0.25-16 | ≤0.25 | ≤0.25 |
| SP QM145 | Vancomycin | 0.25-16 | ≤0.25 | ≤0.25 |
| SP QM328 | Vancomycin | 0.25-16 | 0.5 | 0.5 |
| SP HV431 | Clindamycin | 0.008-2 | 0.0625 | 0.0625 |
| SP QM145 | Clindamycin | 0.008-2 | 0.0625 | 0.0625 |
| SP QM328 | Clindamycin | 0.008-2 | >2 | >2 |
| SP HV431 | Tigecycline | 0.015-4 | ≤0.015625 | ≤0.015625 |
| SP QM145 | Tigecycline | 0.015-4 | ≤0.015625 | ≤0.015625 |
| SP QM328 | Tigecycline | 0.015-4 | 0.03125 | 0.03125 |

**Table 15:** Antibiotics that showed difference in some isolates to change in age of fastidious medium.

*Yellow-highlighted boxes show increase in MIC.*

| **Isolate name** | **Antibiotic name** | **Concentration range on the plate (mg/L)** | **New Fastidious Medium** | **Old Fastidious Medium** |
|---|---|---|---|---|
| HI QM021 | Meropenem | 0.03125-32 | 0.0625 | ≤0.03125 |
| HI QM346 | Meropenem | 0.03125-32 | 0.25 | 0.25 |
| HI QM333 | Meropenem | 0.03125-32 | ≤0.03125 | ≤0.03125 |
| HI QM664 | Meropenem | 0.03125-32 | 0.0625 | 0.125 |
| HI QM021 | Tobramycin | 0.125-16 | 0.25 | 0.25 |
| HI QM346 | Tobramycin | 0.125-16 | 0.5 | 0.5 |
| HI QM333 | Tobramycin | 0.125-16 | ≤0.125 | 0.25 |
| HI QM664 | Tobramycin | 0.125-16 | 0.25 | 0.5 |
| HI QM021 | Ceftolozane-tazobactam | 0.125-16 | ≤0.125 | 0.25 |
| HI QM346 | Ceftolozane-tazobactam | 0.125-16 | 2 | 4 |
| HI QM333 | Ceftolozane-tazobactam | 0.125-16 | ≤0.125 | ≤0.125 |
| HI QM664 | Ceftolozane-tazobactam | 0.125-16 | 1 | 1 |
| HI QM021 | Amoxicillin-clavulanic acid | 0.25-32 | 0.5 | 0.5 |
| HI QM346 | Amoxicillin-clavulanic acid | 0.25-32 | 4 | 8 |

| | | | | |
|---|---|---|---|---|
| HI QM333 | Amoxicillin-clavulanic acid | 0.25-32 | ≤0.25 | ≤0.25 |
| HI QM664 | Amoxicillin-clavulanic acid | 0.25-32 | 2 | 4 |
| HI QM021 | Colistin | 0.0625-8 | 0.25 | 0.25 |
| HI QM346 | Colistin | 0.0625-8 | 0.25 | 0.25 |
| HI QM333 | Colistin | 0.0625-8 | 0.125 | 0.25 |
| HI QM664 | Colistin | 0.0625-8 | 0.125 | 0.125 |
| SP HV431 | Erythromycin | 0.015-8 | 0.0625 | 0.03125 |
| SP QM145 | Erythromycin | 0.015-8 | 0.0625 | 0.0625 |
| SP QM328 | Erythromycin | 0.015-8 | >8 | >8 |
| SP HV431 | Daptomycin | 0.06-4 | 0.5 | 0.25 |
| SP QM145 | Daptomycin | 0.06-4 | 0.5 | 0.25 |
| SP QM328 | Daptomycin | 0.06-4 | 0.25 | 0.5 |
| SP HV431 | Trimethoprim:sulfamethoxazole | 0.125-16 | ≤0.125 | ≤0.125 |
| SP QM145 | Trimethoprim:sulfamethoxazole | 0.125-16 | 0.25 | ≤0.125 |
| SP QM328 | Trimethoprim:sulfamethoxazole | 0.125-16 | ≤0.125 | ≤0.125 |
| SP HV431 | Cefoxitin | 1-16 | 2 | 2 |
| SP QM145 | Cefoxitin | 1-16 | 4 | 8 |
| SP QM328 | Cefoxitin | 1-16 | >16 | >16 |
| SP HV431 | Ampicillin | 0.06-32 | ≤0.0625 | ≤0.0625 |
| SP QM145 | Ampicillin | 0.06-32 | 0.125 | ≤0.0625 |
| SP QM328 | Ampicillin | 0.06-32 | 4 | 4 |
| HI QM021 | Ciprofloxacin | 0.0078125-8 | ≤0.0078125 | ≤0.0078125 |
| HI QM346 | Ciprofloxacin | 0.0078125-8 | ≤0.0078125 | ≤0.0078125 |
| HI QM333 | Ciprofloxacin | 0.0078125-8 | ≤0.0078125 | ≤0.0078125 |
| HI QM664 | Ciprofloxacin | 0.0078125-8 | ≤0.0076125 | 0.015625 |

### 4.4. Comparison among different MHB brands

The inoculum range used for these experiments was between 1e5 to 6.8e5. Bacterial inoculum in 3 antibiotic plates was found to be below the optimal inoculum range. No significant difference was found between the MICs obtained in experiments with different MHBs (cation adjusted or not) except for daptomycin. Significant difference refers to a difference in MIC of 4-fold or higher for all isolates tested for the antibiotic.

Daptomycin showed a more than 4-fold increase in MIC for all isolates tested for cation non-adjusted MHB as compared with CAMHBs, the results are shown in Table 16 for gram-positive and Table 17 for gram-negative isolates.

**Table 16**: Comparison among MICs of BMDs performed using different CAMHB brands for gram-positive isolates.

| Isolate name (Gram-positive) | Antibiotic name | BioMerieux | Merlin | Thermo Fischer (A) | Thermo Fischer (B) | BD BBL | Difco |
|---|---|---|---|---|---|---|---|
| SP HV431 | Erythromycin | 0.0625 | 0.0625 | 0.0625 | 0.0625 | 0.0625 | 0.0625 |
| SA QM079 | Erythromycin | 0.5 | 0.5 | 1 | 0.5 | 0.5 | 2 |
| SE QM358 | Erythromycin | >8 | >8 | >8 | | >8 | >8 |
| SP HV431 | Daptomycin | 0.25 | 0.25 | 0.25 | 0.5 | 0.25 | 2 |
| SA QM079 | Daptomycin | 0.5 | 0.5 | 0.5 | 0.5 | 0.5 | >4 |
| SE QM358 | Daptomycin | 1 | 0.5 | 1 | | 0.5 | >4 |
| SP HV431 | Levofloxacin | 1 | 1 | 1 | 1 | 1 | 1 |
| SA QM079 | Levofloxacin | 0.25 | 0.25 | 0.125 | 0.25 | 0.25 | 0.125 |
| SE QM358 | Levofloxacin | 0.25 | 0.25 | 0.25 | | 0.25 | 0.25 |
| SP HV431 | Trimethoprim:sulfamethoxazole | ≤0.125 | ≤0.125 | ≤0.125 | ≤0.125 | ≤0.125 | ≤0.125 |
| SA QM079 | Trimethoprim:sulfamethoxazole | ≤0.125 | ≤0.125 | ≤0.125 | ≤0.125 | ≤0.125 | ≤0.125 |
| SE QM358 | Trimethoprim:sulfamethoxazole | ≤0.125 | ≤0.125 | ≤0.125 | | ≤0.125 | ≤0.125 |
| SP HV431 | Benzylpenicillin | 0.03125 | 0.03125 | 0.03125 | 0.03125 | 0.03125 | 0.03125 |
| SA QM079 | Benzylpenicillin | 0.5 | 0.5 | 0.5 | 0.5 | 0.5 | 0.5 |
| SE QM358 | Benzylpenicillin | 2 | 1 | 1 | | 1 | 1 |
| SP HV431 | Tetracycline | 0.125 | 0.125 | 0.125 | 0.125 | 0.125 | 0.125 |
| SA QM079 | Tetracycline | 0.5 | 0.25 | 0.5 | 0.5 | 0.5 | 0.25 |
| SE QM358 | Tetracycline | 0.5 | 1 | 0.5 | | 0.5 | 0.25 |
| SP HV431 | Cefoxitin | ≤1 | 2 | 2 | 2 | 2 | 2 |
| SA QM079 | Cefoxitin | 4 | 4 | 4 | 4 | 4 | 4 |
| SE QM358 | Cefoxitin | 2 | 2 | 2 | | 2 | 4 |
| SP HV431 | Vancomycin | ≤0.25 | 0.5 | ≤0.25 | ≤0.25 | ≤0.25 | ≤0.25 |
| SA QM079 | Vancomycin | 1 | 0.5 | 0.5 | 1 | 0.5 | 0.5 |
| SE QM358 | Vancomycin | 2 | 2 | 2 | | 2 | 2 |
| SP HV431 | Clindamycin | 0.0625 | 0.0625 | 0.0625 | 0.0625 | 0.0625 | 0.0625 |
| SA QM079 | Clindamycin | 0.125 | 0.125 | 0.125 | 0.125 | 0.125 | 0.25? |
| SE QM358 | Clindamycin | 0.25 | 0.25 | 0.125 | | 0.25 | 0.25 |
| SP HV431 | Ampicillin | ≤0.0625 | ≤0.0625 | ≤0.0625 | ≤0.0625 | ≤0.0625 | ≤0.0625 |
| SA QM079 | Ampicillin | 1 | 1 | 1 | 2 | 1 | M.S.* |
| SE QM358 | Ampicillin | 0.5 | 0.5 | 0.5 | | 0.5 | 1 |
| SP HV431 | Tigecycline | ≤0.015625 | ≤0.015625 | ≤0.015625 | ≤0.015625 | ≤0.015625 | ≤0.015625 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| SA QM079 | Tigecycline | 0.125 | 0.125 | 0.125 | 0.0625 | 0.125 | 0.0625 |
| SE QM358 | Tigecycline | 0.125 | 0.125 | 0.125 | | 0.125 | 0.0625 |

*Multiple skips (M.S.) observed so MIC could not be determined.

**Table 17**: Comparison among MICs of BMDs performed using different CAMHB brands for gram-negative isolates.

| **Isolate name (Gram-negative)** | **Antibiotic name** | **BioMerieux** | **Merlin** | **ThermoFischer (A)** | **ThermoFischer (B)** | **BD BBL** | **Difco** |
|---|---|---|---|---|---|---|---|
| KP QM385 | Gentamicin | 4 | 4 | 4 | 2 | 4 | 2 |
| EC QM309 | Gentamicin | >32 | >32 | >32 | 32 | 32 | 32 |
| PA QM276 | Gentamicin | 1 | 2 | 1 | | 1 | 0.25 |
| HI QM346 | Gentamicin | 0.5 | 0.5 | 0.5 | | 0.5 | 0.5 |
| HI QM021 | Gentamicin | 0.5 | 0.5 | 0.5 | | 0.5 | 0.5 |
| KP QM385 | Cefotaxime | 2 | 4 | 4 | 2 | 4 | 2 |
| EC QM309 | Cefotaxime | 4 | 8 | 4 | 4 | 8 | 8 |
| PA QM276 | Cefotaxime | 16 | >16 | 16 | | 16 | 8 |
| HI QM346 | Cefotaxime | 1 | 1 | 1 | | 1 | 1 |
| HI QM021 | Cefotaxime | 0.0625 | 0.0625 | 0.0625 | | 0.0625 | 0.0625 |
| KP QM385 | Ciprofloxacin | 0.5 | 0.5 | 0.25 | 0.5 | 0.5 | 0.5 |
| EC QM309 | Ciprofloxacin | >8 | >8 | >8 | >8 | >8 | >8 |
| PA QM276 | Ciprofloxacin | 0.25 | 0.5 | 0.25 | | 0.5 | 0.5 |
| HI QM346 | Ciprofloxacin | ≤0.00078125 | ≤0.007812 5 | ≤0.0078125 | | ≤0.007812 5 | ≤0.007812 5 |
| HI QM021 | Ciprofloxacin | ≤0.00078125 | ≤0.007812 5 | ≤0.0078125 | | ≤0.007812 5 | ≤0.007812 5 |
| KP QM385 | Piperacillin - tazobactam | 8 | 8 | 8 | 8 | 8 | 8 |
| EC QM309 | Piperacillin – tazobactam | 16 | 16 | 8 | 8 | | 8 |
| PA QM276 | Piperacillin – tazobactam | 2 | M.S* | 2 | | 4 | 2 |
| HI QM346 | Piperacillin – tazobactam | ≤0.5 | ≤0.5 | ≤0.5 | | ≤0.5 | ≤0.5 |
| HI QM021 | Piperacillin - tazobactam | ≤0.5 | ≤0.5 | ≤0.5 | | ≤0.5 | ≤0.5 |
| KP QM385 | Ceftazidime | 32 | >32 | 32 | 32 | 32 | 32 |
| EC QM309 | Ceftazidime | >16 | >32 | >32 | >32 | >32 | >32 |
| PA QM276 | Ceftazidime | 2 | 2 | 2 | | 2 | 1 |
| HI QM346 | Ceftazidime | 0.5 | 0.5 | 0.5 | | 0.5 | 0.5 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| HI QM021 | Ceftazidime | ≤0.125 | ≤0.125 | ≤0.125 | | ≤0.125 | ≤0.125 |
| KP QM385 | Meropenem | ≤0.03125 | ≤0.03125 | ≤0.03125 | 0.0625 | ≤0.03125 | ≤0.03125 |
| EC QM309 | Meropenem | ≤0.03125 | ≤0.03125 | ≤0.03125 | ≤0.03125 | ≤0.03125 | ≤0.03125 |
| PA QM276 | Meropenem | 0.25 | 1 | 2 | | 0.5 | 0.25 |
| HI QM346 | Meropenem | 0.25 | 0.25 | 0.125 | | 0.25 | 0.125 |
| HI QM021 | Meropenem | ≤0.03125 | 0.0625 | 0.0625 | | ≤0.03125 | 0.0625 |
| KP QM385 | Tobramycin | 4 | 2 | 2 | 2 | 2 | 2 |
| EC QM309 | Tobramycin | 8 | >16 | >16 | 16 | 16 | 8 |
| PA QM276 | Tobramycin | ≤0.125 | 0.25 | 0.25 | | 0.25 | ≤0.125 |
| HI QM346 | Tobramycin | 0.5 | 0.5 | 0.5 | | 0.5 | 0.25 |
| HI QM021 | Tobramycin | 0.25 | 0.25 | 0.25 | | 0.25 | 0.25 |
| KP QM385 | Ceftolozane-tazobactam | 1 | 2 | 1 | 1 | 1 | 1 |
| EC QM309 | Ceftolozane-tazobactam | 1 | 0.5 | 0.25 | M.S* | 0.5 | 0.25 |
| PA QM276 | Ceftolozane-tazobactam | 0.5 | 0.5 | 0.5 | | 2 | 0.25 |
| HI QM346 | Ceftolozane-tazobactam | 2 | 2 | 2 | | 2 | 2 |
| HI QM021 | Ceftolozane-tazobactam | 0.25 | 0.25 | 0.25 | | 0.25 | 0.25 |
| KP QM385 | Amoxicillin-clavulanic acid | M.S* | 4 | 8 | 8 | 8 | 8 |
| EC QM309 | Amoxicillin-clavulanic acid | >32 | >32 | >32 | >32 | >32 | 32 |
| PA QM276 | Amoxicillin-clavulanic acid | >32 | >32 | >32 | | >32 | >32 |
| HI QM346 | Amoxicillin-clavulanic acid | 4 | 4 | 2 | | 4 | 4 |
| HI QM021 | Amoxicillin-clavulanic acid | 0.5 | 0.5 | 1 | | 0.5 | 0.5 |
| KP QM385 | Colistin | 0.5 | 0.5 | 2 | 0.5 | 1 | 1 |
| EC QM309 | Colistin | 0.5 | 1 | 2 | 0.5 | 0.5 | 0.5 |
| PA QM276 | Colistin | 1 | 1 | 2 | | M.S* | 0.5 |
| HI QM346 | Colistin | 0.25 | 0.25 | 0.25 | | 0.5 | 0.25 |
| HI QM021 | Colistin | 0.25 | 0.25 | 0.5 | | 0.25 | 0.25 |

*M.S = Multiple skips.

# 5. Discussion

## 5.1. General

The panel of chosen bacterial isolates includes a variety of bacteria as mentioned earlier. All these bacteria are clinically significant and are known to cause sepsis. Some of the selected bacterial isolates were already known to show inoculum effect. It is reasonable to assume that MIC can vary depending on inoculum but how much and at what concentrations is difficult to predict. It can also vary for the different antibiotics and pathogens - some combinations are more sensitive to inoculum than others. Experiments were performed to identify different bug/drug combinations where the inoculum had an effect. From what was seen in the results of this study, in some cases there was a large difference in MIC i.e., >1 or <1 showing how important it is to use a correct inoculum. If the correct inoculum is not obtained, an incorrect MIC value would result in consequences during treatment of bacterial infections. Another reason to test isolates that have earlier shown an inoculum effect, was to test the reproducibility of the data by conducting the experiments that showed the effect of change in inoculum on MIC. That way when other factors were changed, a baseline that indicated the extent of effects of change in inoculum already existed, indicating if the change in results was due to change in different factors or due to the change in inoculum.

The antibiotic plates used in these experiments were old and expired. The plates used to test gram positive bacteria expired on 03-04-2019 and the plates used to test gram negative bacteria expired on 03-03-2019 (i.e. both expired for approximately 1.5-2 years prior performing the experiments). To ensure that the results from these plates were valid, experiments were first performed with QC isolates and the results compared with previous data. Since no discrepancy was found between the results from these experiments and the results from previous experiments, the plates were deemed to be “usable”, and the results obtained using these plates were considered valid.

One of the shortcomings of this study is that as no experiment was performed more than once, there are no replicates in this study because of time constraints. This means that the data generated through this study can, at best, indicate a general trend and is of no statistical significance. However, this study does give a general idea about how different factors might affect MICs and sheds some light on the areas that might need closer inspection in the future.

Other than in the experiments that tested the effect of change in inoculum, there were four plates that contained inoculum which was lower than the intended and standard range (2e5-8e5 CFU/ml). It should be noted that other than the technical factor being tested and manipulated, this low inoculum could also influence the results. However, it was decided to keep the results from these plates as there was not enough time to repeat the experiments. For future studies, it might be worthwhile to keep account of the fact that these particular plates had low inoculum and therefore, the results from these specific plates need to be analysed in that context.

## 5.2. Effect of change in inoculum on MIC

That inoculum affects MIC is well-known from scientific literature [24,25]. It is called inoculum effect and is defined as the significant increase in minimum inhibitory concentration of an antibiotic when the number of inoculated bacteria increases [24]. This effect was also

observed in our study with all isolates and antibiotics except for cefoxitin. Specific examples from previous research have been explained in the following paragraphs that relate further with our study and corroborate our results.

According to one research study [26], benzylpenicillin was found to be the most sensitive to inoculum effect as compared to 13 other antibiotics when tested with *Staphylococcus aureus*. The MIC results of tests performed with 10,000 times diluted inoculum and undiluted inoculum differed by a factor of up to 16,384. The reason for this is described to be production of β-lactamases by the bacteria which degrades the antibiotic. Another study by Udekwu and colleagues [27], suggests that vancomycin and daptomycin are affected by various enzymes produced by bacteria and show inoculum effect. The concentration of free antibiotics (which is also the active and effective form) in the medium is reduced due to degradation by various enzymes or due to binding with dead or alive bacterial structures. This bound form of antibiotics is ineffective against bacteria. As the bacterial concentration increases, so does the level of enzymes that degrade antibiotics and the number of structures that bind antibiotics. As more and more of the antibiotic becomes ineffective, inoculum effect manifests. The same study also describes that for gentamicin and ciprofloxacin, the inoculum effect results because fewer molecules of antibiotic are available for each cell of bacteria and thus the efficacy of antibiotic is affected. For example, using same concentration of antibiotic in each case, the number of antibiotic molecules available per cell decreases 1000 times when inoculum concentration increases from 10e5 CFU/ml to 10e8 CFU/ml. The inoculum effect has been demonstrated with levofloxacin also. One study has found that bactericidal effect of levofloxacin is significantly affected by the size of inoculum [28]. On the other hand, the MIC of cefoxitin was found to be unaffected by the increase in inoculum concentration and by the change in CAMHB concentration in our study. This conclusion is in line with other research conducted previously. According to a study, cefoxitin was found to be particularly stable against variations in inoculum concentration, pH and growth medium [29]. Another study suggests that cefoxitin is very resistant to β-lactamases which is a degradative enzyme produced by various bacteria making it a very stable antibiotic. The reason for this stability was found to be the presence of a methoxy group at 7a position of the β-lactam nucleus of cefoxitin. This makes cefoxitin more stable than other β-lactam compounds [30].

### 5.3. Effect of change in CAMHB concentration on MIC

The results of our study suggested that broadly speaking, increase in concentration of CAMHB caused an increase in MIC readings. It could be postulated that this occurs due to increase in concentration of cations in the growth medium. For example, a study [31] suggested that increasing the concentrations of magnesium and calcium ions in Mueller-Hinton broth increased MICs for gentamicin when tested for 18 isolates of *Pseudomonas Aeruginosa*. The reason for this increase in MIC is thought to be due to change in bacterial cell-wall - cations are thought to stabilize the lipopolysaccharide units of the bacterial cell wall thus decreasing the permeability of the cell-wall to antibiotics. This evidence for the stabilization of the cell-wall by cations is further strengthened by the fact that exposure of *Pseudomonas Aeruginosa* to EDTA removes cations from the cell-wall. And as a result, the cell-wall becomes more permeable to the antibiotic and the MIC decreases. Another study [32] conducted using isolates of *Pseudomonas Aeruginosa* and antibiotics including gentamicin and tobramycin confirms the same finding that increase in cations like calcium and magnesium increases MIC readings.

Increase in CAMHB concentration had opposite effect on the antibacterial activity of daptomycin as demonstrated in our study as a decrease in MIC was observed when concentration of CAMHB was increased. An educated guess can be made that this occurred due to an increase in the concentration of cations. This result is also mirrored by another study explained as follows. As stated by the study, the standard concentration of the cations in CAMHB is set as follows: 10-12.5 mg of magnesium per liter and 20-25 mg of calcium per liter. But for better determination of MIC, calcium ion concentration should ideally be increased to 50mg/l. Doing so mimics the concentration of calcium ions in human blood. This study also showed that increasing calcium concentration in the growth broth decreased MIC by 2- to 4-fold [33].

### 5.4. Comparison between results from different MHBs

As expected, when daptomycin was tested with different batches from different MHB brands, the MIC increased significantly only when cation deficient MHB (Difco) was used. This result from our study is also in line with other studies that suggest that calcium ions are necessary for the antibacterial activity of daptomycin and without calcium, the MIC of daptomycin increases significantly [34]. This study also suggests that when calcium interacts with daptomycin, it induces a structural change in daptomycin that in turn decreases its charge and allows it to interact with bacterial membrane. Furthermore, calcium also helps daptomycin to insert deeper into the membrane thus allowing daptomycin to target multiple sites inside the cell and exerting its antibacterial effect.

For the rest of the antibiotics, the difference between MICs was not very large when different batches of CAMHB were used in our study. However, some studies contradict this finding. It has been suggested that significant differences exist between MICs obtained by experiments using different brands of MHB. These differences occur due to differences in cation concentrations. For example, one study found that the concentration of zinc ions differed significantly among different broth brands. This resulted in a difference of up to 8-fold in MIC of meropenem when using *Enterobacteriaceae* [35].

### 5.5. Conclusion

To conclude, various technical factors have been shown to affect MICs of antibiotics. Some factors, like the concentration of inoculum and the concentration of CAMHB have clear effects on MICs except for that of cefoxitin. The age of fastidious medium has no observable effect on MIC. A comparison between the BMD results obtained from using multiple MHBs shows that MICs remained the same despite any MHB being used except for daptomycin which showed higher MICs with a MHB that lacked cations. These results are not statistically significant as no replicates were included. Further research is needed to confirm the results from this study. This study could help define parameters for automated AST systems that use BMD as their method of determining susceptibility and MICs. Automated AST systems are a useful technology as they can determine susceptibility, resistance, and MIC of an antimicrobial in lesser time than is required by the conventional methods. Such systems require lesser manpower and expertise to operate. This technology can be useful in determining the correct treatment in a timely manner which can save the lives of patients suffering from sepsis.

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