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Original Article

A novel method for early detection of MIC value – Broth dilution using indicator solution versus agar dilution: an original article

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Abstract

Introduction: The adequate protocol for treatment of an infection is often determined on the basis of the minimum inhibitory concentration (MIC) of the causative organism. Traditional methods (agar dilution, microbroth dilution, and gradient diffusion) are labour intensive and time consuming (they are usually take over 48 hours to report the results). On the other hand, automated systems (VITEK™, Phoenix™, MicroScan WalkAway™) and rapid methods of MIC detection (using dielectrophoresis (DEP), magnetic bead rotation sensors and microfluidic incubation) require expensive instruments. This study is aimed to develop a rapid MIC detection method with the ability to applied to a resource limited setting.

Methods: Agar dilution method and a novel broth dilution method (containing indicator solution) were simultaneously performed using amikacin, ceftriaxone, piperacillin-tazobactam, imipenem, cefoxitin and azithromycin.

Results: Isolates of *Escherichia coli*, *Enterobacter* spp, *Klebsiella* spp, *Staphylococcus aureus*, *Proteus mirabilis*, *Acinetobacter* spp and *Pseudomonas* spp were used. The MIC values for Enterobacteriaceae and *S. aureus* isolates for each antibiotic were obtained within 4 to 5 hours by a novel broth dilution method. The obtained MIC values were corresponded with the MIC shown on the following day by agar dilution method.

Conclusion: Broth dilution method with indicator solution is effective in rapid determination of the MIC for cephalosporins, penicillin, carbapenems, cephamycin, aminoglycosides and macrolides for most isolates of Enterobacteriaceae and *S. aureus*. Unfortunately this method did not work for the non-fermenter group of organisms like *Pseudomonas* spp and *Acinetobacter* spp, as their results could not be obtained before 24 hours. The method is time saving, relatively inexpensive and is applicable to resource limited settings.

Introduction

Minimum inhibitory concentration (MIC) is the lowest concentration of an antibiotic, inhibiting the growth of microorganisms after an overnight incubation. MIC value is not only used to confirm antibiotic resistance, but also it is an important tool to conduct determining researches for the *in vitro* activity of new antibiotics. Data obtained from these studies are utilised in determining MIC breakpoints. In clinical scenarios, achieving the MIC data of the infectious organism is important to regulate the suitable treatment protocol. MIC is a crucial part of the PK/PD (pharmacokinetic/pharmacodynamics) ratio that determines the amount of drug exposure which is necessary to ensure that a patient achieves a predefined PK/PD target that is associated with maximum efficacy of that drug.[1,2]

Various methods are available for MIC determination. The traditional methods include agar dilution method, macrobroth dilution method, microbroth dilution method and gradient diffusion method (Epsilometer test or Etest). While agar dilution uses solid media, broth dilution utilizes liquid media containing serial dilutions of the antibiotic. Growth of the bacteria on the agar plates or presence of turbidity in the test tubes/microtube wells (macrobroth/microbroth respectively) indicates resistance to the applied concentration of the drug. In Etest, MIC is a read off the linear scale printed on the upper side of the antibiotic impregnated strip where the zone of growth inhibition intersects with the edge of the strip.[3] These methods have some limitations e.g. being cumbersome as they are entirely manual, being time consuming because they need an overnight incubation, which means a reporting time of 24 hours, consuming the 24-48 hours required for primary processing and incubation of clinical samples, a total of three days is required for this test. During this time, to prevent

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the deterioration of the condition of the patient, the clinician often prescribes a high dose of broad spectrum antibiotic to ensure the efficacy on the target pathogen. This approach helps in the emergence of antimicrobial resistance.

Nowadays to bring down the labour costs and time, micro broth dilution method has been automated. These automated equipments, which perform antibiotic susceptibility, employ various methods to investigate bacterial growth against varying concentrations of the tested antibiotics. While, VITEK (Biomeriux) detects increased bacterial proliferation by means of increased culture solution turbidity, the Phoenix (BD Diagnostics) and the MicroScan WalkAway systems (Dade MicroScan) utilize colorimetric and fluorometric principles to detect the redox reactions of actively metabolising bacteria.[2] Although, these systems though are labour saving, susceptibility reports are still generated after a lot of time (in total ranging from 8-18 hours). Also these machines are not available in resource limited settings.[4]

Over the years a few rapid methods have been developed for MIC detection, including those using dielectrophoresis (DEP), magnetic bead rotation sensors, and microfluidic incubation. The DEP-based method monitors elongation of the bacterial cells under the effect of antibiotics, which causes changes in the DEP properties. The AST method which is based on magnetic-bead rotation sensing, employs an organism specific antibody conjugated to the magnetic beads. The desired bacteria adhere to the rotating magnetic beads, which are then identified by sensing platforms. Therefore, both these tests are highly specific, and cannot be commonly generalized to every bacteria-antibiotic combination. As these methods are applicable only after the pathogen has been identified, thus the saved time comes to be clinically invaluable. The microfluidic incubation method utilizes optical density (OD) to monitor the presence or absence of bacterial growth in 10 µL volume microreactors. Hence, it is a microfluidic counterpart of the classical broth dilution method, with the superadded advantage of a shorter test time. It is noteworthy that the mentioned emerging techniques, require expensive instruments that are not readily available.[5-7]

The current study was undertaken in light of the fact that in a scenario where the traditional phenotypic methods for MIC detection are cumbersome, rapid methods which can be applied to resource limited settings do not exist. Therefore, this study aimed at developing a rapid MIC detection method which can be applied to a resource limited setting.

Methods

Broth dilution method using indicator solution

This method involves preparation of an aqueous indicator mix containing 0.05% phenol red solution, 0.1 mmol per litre zinc sulphate and 1% D-glucose. pH was adjusted to 7.8 ± 0.1 using sodium hydroxide solution with a concentration of 0.1 N. The bacterial isolates used in this study were obtained from patients during the processing of various clinical specimens. 1 µL loopfull of a 24-hour old pure bacterial culture (that had been prepared from bacterial colonies isolated from Mueller-Hinton agar) was taken and suspended in 1.5 mL Eppendorf tubes containing 100 µL aqueous indicator mix that had been supplemented with serial dilutions of the antibiotics (standard powder form/injectable form) just before use. Two-fold serial dilutions were prepared as per the Clinical and Laboratory Standards Institute (CLSI) recommendation for MIC breakpoints of each of the antimicrobial agent used.[8] The inoculum was matched to the turbidity of the 0.5 McFarland. Before incubation, the tubes were vigorously mixed for 5 to 10 seconds using a vortex. Finally, the capped Eppendorf tubes were incubated aerobically at 35°C and were monitored over the next 4 to 5 hours for colour change from original red to orange or yellow in the antibiotic-containing tube. Uninoculated reagent controls were used for each sample, which contained aqueous indicator mix and serial dilutions of antimicrobials. A colour change was interpreted as the organism being resistant to the applied concentration of antibiotic (indicating the growth of organism and utilization of glucose). As well, observation of samples with no colour change was interpreted as susceptible (meaning that the organism did not grow and did not utilize any glucose in the culture medium). MIC value was determined by the indicator tube containing the lowest dilution of antibiotic that showed no colour change (Figure 1).

Agar dilution method

Mueller Hinton agar plates were prepared using two-fold serial dilutions of the studied antibiotics as per CLSI guidelines. A standardized inoculum was set for the agar dilution method by growing microorganisms matched to 0.5 McFarland standard turbidity, containing approximately 1- 2×10^8 CFU/mL, so that the resultant inoculum is 10^4 CFU/spot of about 5- 8 mm diameter. The plates were inoculated starting from the lowest dilution of the antibiotic and then incubated at a temperature of 35°C for 16 to 20 hours.[8] Microorganisms resistant to the applied concentration of the drug, produced a circle of growth at the inoculum site, while the susceptible organisms did not. MIC was determined by the lowest concentration of the antibiotic inhibiting the growth on MHA plate (Figure 2).

The MIC results obtained after 4-5 hours by the rapid method were compared with the results obtained from the standard agar dilution method after overnight incubation.

The antimicrobial agents used were amikacin, ceftriaxone, piperacillin-tazobactam, imipenem, cefoxitin (only for isolates of *Staphylococcus aureus*), and azithromycin.

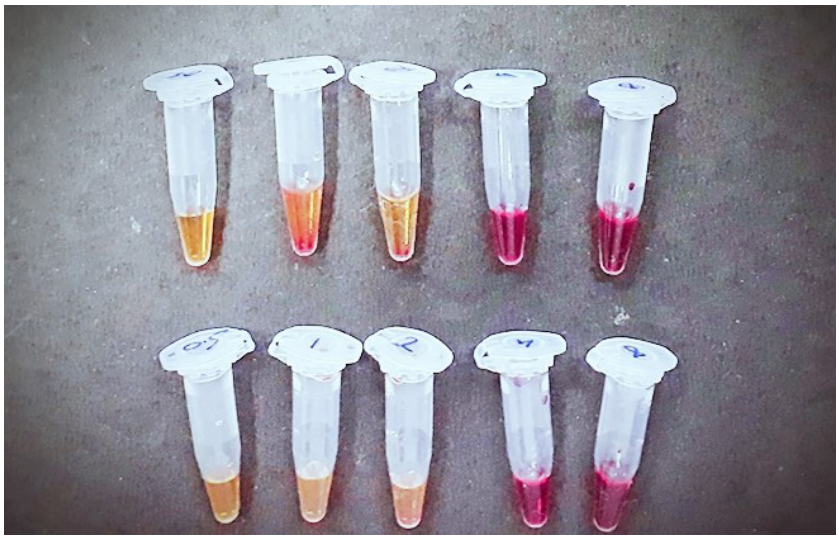

Figure 1. Broth dilution method

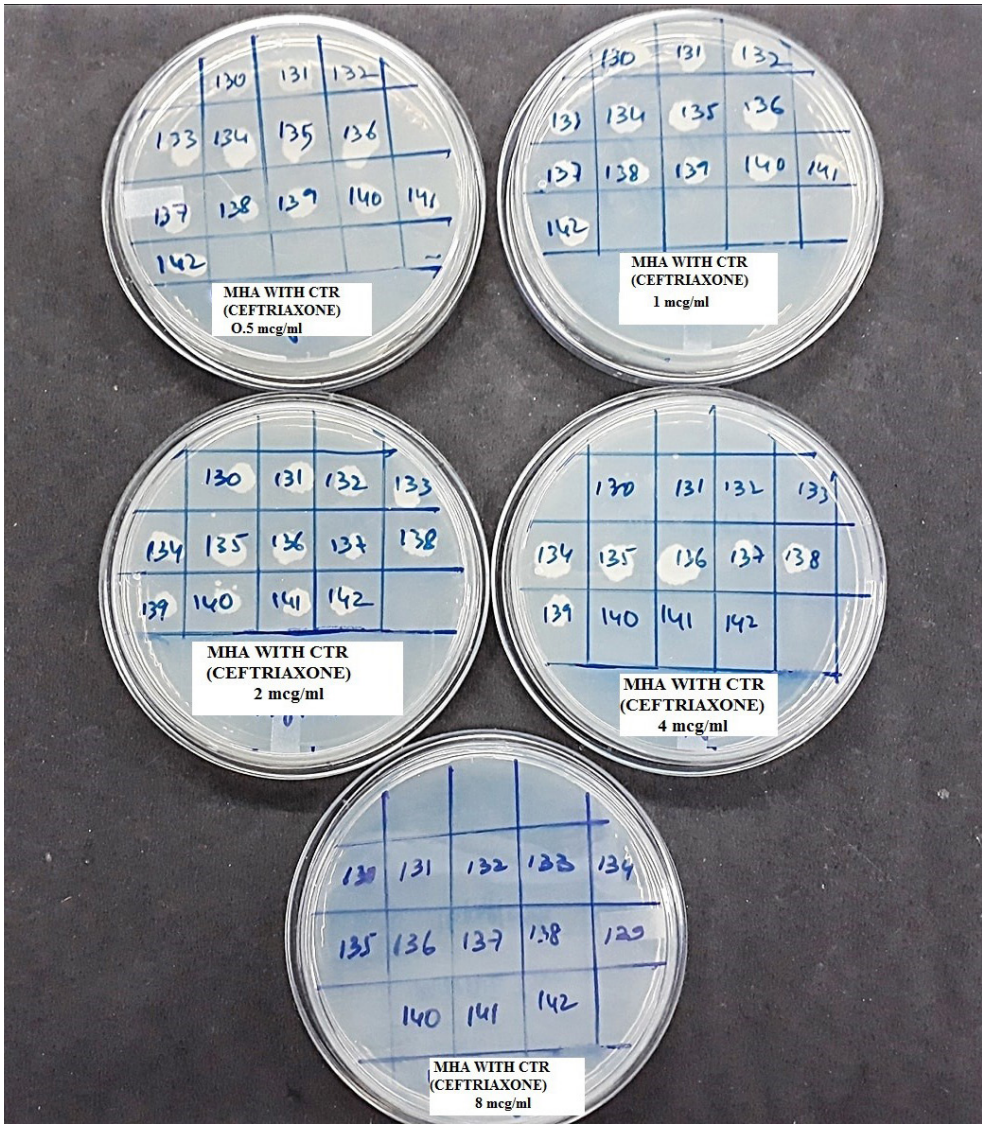

Figure 2. Agar dilution method

Results

One hundred isolates of *Escherichia coli,* 100 isolates of *Klebsiella* spp, 20 isolates of *Enterobacter* spp, 30 isolates of *Proteus mirabilis,* 70 isolates of *S. aureus,* 30 isolates of *Pseudomonas* spp, and 50 isolates of *Acinetobacter* spp were used for the study. The gram-negative bacilli were tested for susceptibility to amikacin, imipenem, ceftriaxone, piperacillin-tazobactam and azithromycin. *S. aureus* was tested for susceptibility to cefoxitin and azithromycin.

All Enterobacteriaceae and *S. aureus* isolates showed MIC values using broth dilution method for all of the antibiotics which were tested within 4-5 hours. the obtained MIC values were corresponded with the MIC values shown by agar dilution method on the next day (Table 1).

On the other hand, the isolates of *Pseudomonas* spp and *Acinetobacter* spp were failed to show any kind of colour change. In the case the non-fermenter organisms, the indicating method was modified using bromothymol blue instead of phenol Red, where the results were corresponded with agar dilution values but only after overnight incubation of the inoculated indicator solution. Hence, their MIC could not be detected within 4-5 hours.

Discussion

MIC values have many applications in clinical settings. Usually, to ensure that the antibiotic is effective *in vivo*, the administered drug concentration is about 4-5 times higher than its MIC value. It has also been observed that in regards to certain antibiotics like beta-lactams, macrolides, linezolid and clindamycin, the clinical efficacy is strongly dependent on the duration for which the serum antibiotic concentrations were above their respective MICs. On the other hand, in the case of other antibiotics like aminoglycosides and fluoroquinolones, the ratio of the serum peak concentration to MIC turns out to be the major factor determining the efficacy of drug. Base on the MIC value, laboratories classify the organisms as susceptible, intermediate, susceptible dose dependent and resistant to antibiotics.

MIC values are of special importance to the critically ill patients admitted in Intensive care units (ICUs). ICU acquired infections are usually caused by organisms with higher reported MICs as compared to other clinical settings. The Package Insert (Product Information) of the antibiotic is often the basis for choosing the dose of antibiotic for critically ill patients. However, this is based on dose determining studies that are performed on non-critically ill patients and then extrapolated to critically ill patients. Critically ill patients have significant PK variability, so the antibiotics may be at the risk of not achieving the PK/PD targets that are required for positive clinical outcomes, in spite of being reported as "susceptible". Thus, knowledge of MIC data of the pathogen in individual patient is essential to accurately calculate the PK exposure that the patient needs.[2]

The available classical methods for MIC detection (agar dilution, broth dilution, and gradient diffusion methods) are inexpensive but time consuming and cumbersome. The automated systems (VITEK, Phoenix and the MicroScan WalkAway) on the other hand, are less cumbersome but expensive and time consuming. The newly developed rapid methods in use nowadays (based on DEP, magnetic bead rotation sensors and microfluidic incubation) show drawbacks of being expensive, requiring special equipment and expertise to perform.

Broth dilution method using an indicator solution is a rapid method (results within 4-5 hours) determining the MIC for fluoroquinolone, penicillin, aminoglycoside, cephalosporin, carbapenem, cephamycin, and macrolide group of antibiotics for most Enterobacteriaceae and *S. aureus.* It suffers from the drawback of not working for the non fermenting bacteria like *Acinetobacter* spp *and Pseudomonas* spp, as their results could not be obtained before 24 hours.

Conclusion

Broth dilution method with an indicator solution gives numerous advantages over conventional methods for antimicrobial susceptibility testing. Firstly, it gives antimicrobial susceptibility results within 4-5 hours. Secondly, it rapidly gives results for MIC determination, the results of which are comparable to that of the standard methods. Thirdly, unlike the traditional broth dilution methods, which needed to check for turbidity (the perception of which might change from observer to observer), the results here are based on a colour change (which is easily perceivable), eliminating the subjective variation. Fourthly, this method is not too labour intensive and no special expertise is required to perform it, making the method easy to be employed in any laboratory.

Table 1. Number of isolates with MIC detected by broth dilution method using indicator solution corresponding with the results of agar dilution method

	Ceftriaxone No. (%)	Amikacin No. (%)	Azithromycin No. (%)	Piperacillin-Tazobactam No. (%)	Imipenem No. (%)	Cefoxitin No. (%)
E. coli (n = 100)	100 (100%)	100 (100%)	100 (100%)	100 (100%)	100 (100%)	N.A.
Klebsiella spp (n = 100)	100 (100%)	100 (100%)	100 (100%)	100 (100%)	100 (100%)	N.A.
Enterobacter spp (n = 20)	20 (100%)	20 (100%)	20 (100%)	20 (100%)	20 (100%)	N.A.
Proteus mirabilis (n = 30)	30 (100%)	30 (100%)	30 (100%)	30 (100%)	30 (100%)	N.A.
Staphylococcus aureus (n = 70)	N.A.	N.A.	70 (100%)	N.A.	N.A.	70 (100%)

Study Highlights

What is current knowledge?

- Conventional methods of MIC determination are time consuming whereas rapid methods require an expensive setup.

What is new here?

- A novel method for MIC determination has been discovered which is both time saving and can be performed in resource limited settings

Fifthly, it is inexpensive and does not require any setup, instruments or machines which are not available in resource limited settings. As the test is performed in Eppendorf tubes, the media required is also less. Finally, this method has an additional advantage of the capability of predicting methicillin resistance for *S. aureus* faster than any other existing conventional method.

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Author Contributions
Both authors were equally involved in designing of the study, processing of samples, analysis of results, preparation, checking and final approval of this manuscript.

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Ethical Approval
None.

Conflict of Interests
None.

References

1. Andrews JM. Determination of minimum inhibitory concentrations. J Antimicrob Chemother. 2001;48 Suppl 1:5-16. doi: 10.1093/jac/48.suppl_1.5.
2. Roberts JA, Abdul-Aziz MH, Lipman J, Mouton JW, Vinks AA, Felton TW, et al. Individualised antibiotic dosing for patients who are critically ill: challenges and potential solutions. Lancet Infect Dis. 2014;14(6):498-509. doi: 10.1016/s1473-3099(14)70036-2.
3. Koneman EW, Procop GW, Church DL, Hall GS, Schrekenberger PC, Janda WM, et al. Antimicrobial susceptibility testing. In: Koneman's Color Atlas and Textbook of Diagnostic Microbiology.7th ed. Jones & Bartlett Learning; 2017. p. 1075-171.
4. Puttaswamy S, Lee BD, Amighi B, Chakraborty S, Sengupta S. Novel electrical method for the rapid determination of minimum inhibitory concentration (MIC) and assay of bactericidal/bacteriostatic activity. J Biosens Bioelectron. 2011;S2:1-6. doi: 10.4172/2155-6210.s2-003.
5. Jorgensen JH, Ferraro MJ. Antimicrobial susceptibility testing: general principles and contemporary practices. Clin Infect Dis. 1998;26(4):973-80. doi: 10.1086/513938.
6. Horstkotte MA, Knobloch JK, Rohde H, Dobinsky S, Mack D. Evaluation of the BD PHOENIX automated microbiology system for detection of methicillin resistance in coagulase-negative staphylococci. J Clin Microbiol. 2004;42(11):5041-6. doi: 10.1128/jcm.42.11.5041-5046.2004.
7. Smith JM, Serebrennikova YM, Huffman DE, Leparc GF, García-Rubio LH. A new method for the detection of microorganisms in blood cultures: part I. Theoretical analysis and simulation of blood culture processes. Can J Chem Eng. 2008;86(5):947-59. doi: 10.1002/cjce.20095.
8. Clinical and Laboratory Standards Institute (CLSI). Performance Standards for Antimicrobial Susceptibility Testing (CLSI M100). 32nd ed. Wayne, PA: CLSI; 2022.