Determination of Minimum Inhibitory Concentration by Broth Dilution Method - A Review

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Abstract

Microbial resistance to antimicrobial agents is a growing problem, especially in the medical sector. Application of proper antimicrobial agent is important as resistance can develop quickly. For this reason, the concentration of antimicrobial agents has to be properly adjusted. Providing a higher than normal concentration can lead to organisms developing resistance quickly and transforming into 'superbugs'. Higher concentrations of antibiotics are biologically harmful. On the other hand, if the concentration of antimicrobial agents is too low, it may be ineffective. To tackle these issues, determination of the Minimum Inhibitory Concentration of antimicrobial agents is required. A review of the method to determine MIC is done. The tube (or broth) dilution method is used to determine the MIC of a known compound. Further, the same test is used in the case of the formulation of an unknown compound. A confirmatory test is done to support the results.

Key words

Minimum Inhibitory Concentration, Broth dilution method, Susceptibility, Antimicrobial agent, Diffusion.

Citation

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1.0 Introduction

To determine the susceptibility of any antimicrobial agent, diffusion tests are done. These tests only provide qualitative information. These tests cannot be used to quantify the concentration of antimicrobial agents that can inhibit the growth of a harmful agent but only if the agent possesses antimicrobial properties. Minimum Inhibitory Concentration (MIC) gives us the exact concentration of an antimicrobial agent that can inhibit the growth of a microorganism (1). Once the MIC of an antimicrobial agent is determined, it can be further implemented into drug dosage, etc.

There are two methods to determine MIC:

1.1 Broth dilution method

The broth dilution method is performed in tubes where the antimicrobial agent is serially diluted in a nutrient medium. To each tube, a known concentration of test culture is added and incubated. Post incubation, the lowest concentration which shows the absence of turbidity is considered the minimum inhibitory concentration. It has two advantages over the agar dilution method: the method can be used for determining Minimum Bactericidal Count and it minimizes the solidification risk that is possessed by the agar dilution method. (2)

1.2 Agar dilution method

The Agar dilution method is performed by preparing serial dilutions of the stock compound and pouring molten agar over it. The advantage of this method is that more than one organism can be tested simultaneously on one plate.

This review contains the broth dilution method. It was originally published by the University of Maryland. It is also known as BSCI 424 Method(4). It makes use of 2 fold dilutions. This method was adopted with certain changes wrt culture preparation changes. The review of the method was performed by maintaining proper environmental conditions.

2. Materials and equipment

Weighing balance, Laminar Airflow, and Biosafety cabinet was required for testing. The materials were incubated in an incubator capable of maintaining 37 ± 2˚C temperature. 10 test tubes with caps for each set, micropipettes that can measure 0.1-1.0 ml of liquid, test tube racks, and bottles were sterilized before use. Mueller Hinton Broth and Soyabean casein digest agar were chosen media which were also sterilized.

3. Method

The protocol was designed following the standard method (2).

3.1 Preliminary test:

Before identifying the MIC of the unknown compound, the compound was tested against Staphylococcus aureus to ensure its antimicrobial activity.

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Figure 1: Agar well diffusion method to determine Antimicrobial nature of the compound

3.2 Preparation of stock:

A known stock of commercial 100 µg/ml of penicillin G was used. For the unknown compound, PM, the stock of solution was calculated as below:

Calculation of stock solution:

$$W = \frac{1000}{P} \times V \times C$$

Where,

W = Weight of antimicrobial to be dissolved in V (in µg)

V = Volume required (in ml)

C = Final Concentration of solution (in µg/ml)

P = Potency of the compound (given by manufacturer)

$$W = \frac{1000}{990} \times 100 \times 100$$

W = 10101.01 µg

Thus, W = 0.01 mg

0.01mg of the unknown compound was weighed in dissolved in 100 ml of ethanol (solvent chosen by manufacturer).

3.3 Preparation of nutrient medium and sterilization:

The chosen nutrient medium for the test was Mueller Hinton Broth. Mueller Hinton Broth (Hi-Media brand) was prepared as per instructions on the bottle and dispensed into borosilicate bottles before sterilizing. The sterilization process was carried out at 121˚C for 15 minutes by autoclaving. For the confirmatory test, Mueller Hinton Agar (Hi-Media brand) plates were prepared by autoclaving the medium and pouring 15 ml each into sterilized plates. The plates were pre-incubated at 37 ˚C for 18-24 hours.

3.4 Preparation of stock culture:

4 bacterial cultures were used for the test. They were:

3.4.1 Staphylococcus aureus

3.4.2 Klebsiella pneumoniae

3.4.3 Escherichia coli

3.4.4 Pseudomonas aeruginosa

Klebsiella pneumoniae and *Escherichia coli* are well known for their resistance to Penicillin and Beta lactams and are used as negative controls. Many *Staphylococcus* species are resistant to Penicillin and a sensitive strain of *Staphylococcus aureus* was used. *Pseudomonas aeruginosa* is sensitive to penicillin and was used as a positive control.

Individual colonies from each culture were added to separate sterilized flasks with sterilized Mueller Hinton Broth and incubated at 35 ± 2˚C for 18 hours. After the incubation period is over, the cultures were adjusted with McFarland's standard to obtain a density of 1.8×10^8 CFU/ml. This density was further diluted to 10^5 CFU/ml for better visibility since the cultures were no longer turbid at this density(3).

3.5 Preparation of stock dilution:

12 sterilized and capped tubes numbered from 1 to 12. To the first tube, 2 ml of 100µg/ml of penicillin stock was added. 1 ml of sterilized Mueller Hinton Broth was added to tubes 2 to 10. 1ml of stock penicillin was transferred from tubes 1 to 2 with a micropipette and mixed well. Once mixed, 1 ml of the mixed stock was transferred from tube 2 to 3 and so on to tube 10. The first tube containing equal amounts of 100 µg/ml of Penicillin and culture was labeled as 50 µg/ml. Tube no. 2 was considered as 25 µg/ml and so on till tube no 10 was labeled as 0.391 µg/ml.

Micropipette tips were discarded after every tube to avoid

The following table shows tube numbers corresponding to the concentration of penicillin used.

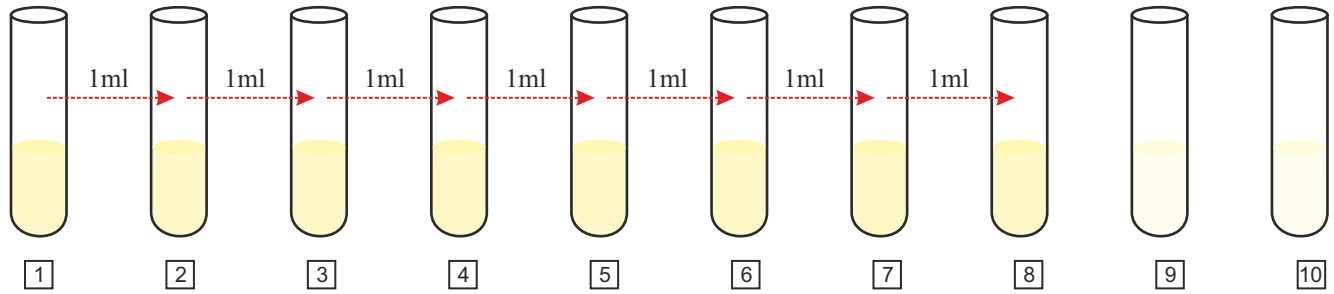
Tube No.	1	2	3	4	5	6	7	8	9	10
Concentration of antimicrobial agent (µg/ml)	50	25	12.5	6.25	3.125	1.563	0.781	0.391	0.196	0.098

the transfer of penicillin from the outer surface of the tip. 1 ml from tube 10 was discarded. Tube 11 and 12 consisted only of Mueller Hinton Broth. Tube 11 was a positive control for the culture. Tube 12 was medium control. The procedure was repeated for all cultures.

For an unknown compound, PM, the procedure was slightly altered as per the manufacturer's instructions that the compound's MIC was not low. Hence, concentrations of 0.196 µg/ml and 0.098 µg/ml concentration tubes were skipped.

The following table shows tube numbers corresponding to the concentration of unknown compound, PM, used.

Tube No.	1	2	3	4	5	6	7	8
Concentration of antimicrobial agent (µg/ml)	50	25	12.5	6.25	3.125	1.563	0.781	0.391



Diagrammatic representation of tube dilutions of an unknown compound, PM.

3.6 Culture addition:
1 ml each of prepared culture of 10^5 CFU/ml was added to all tubes except for medium control. The procedure was repeated for all cultures and unknown compounds, PM.

3.7 Incubation:
All tubes were incubated in racks at 35 ± 2˚C for 18-24 hours. After the incubation period was over, the tubes were observed for turbidity.

3.8 Confirmatory tests

Based on the results of the broth dilution test, tubes were selected and streaked on sterile Mueller Hinton Agar. The tubes were incubated at 35 ± 2˚C for 18-24 hours.

4. Results and discussion

4.1 Tube dilution results:

4.1.1 Known compound (Penicillin) results:

For standard penicillin, all tubes showed turbidity when tested against *Klebsiella pneumoniae* and *Escherichia coli*. Both cultures are popularly known to be resistant to Penicillin G and hence were used specifically as negative controls. Tubes inoculated with *Pseudomonas aeruginosa* showed no turbidity, apart from the positive control tube which contained no Penicillin. The tube containing 0.098 µg/ml penicillin showed turbidity. Other tubes showed growth.

Table 1: Observations of MIC test for Penicillin G

Culture	Dilution of Penicillin G (µg/ml)											
	50	25	12.5	6.25	3.125	1.563	0.781	0.391	0.196	0.098	Culture control	Media control
K. pneumoniae	+	+	+	+	+	+	+	+	+	+	+	-
E. coli	+	+	+	+	+	+	+	+	+	+	+	-
S. aureus	-	-	-	-	-	-	-	-	+	+	+	-
P. aeruginosa	-	-	-	-	-	-	-	-	-	+	+	-

Key: "–" = No growth; " + " = Growth (turbidity)

Based on the observation, Penicillin G has a Minimum Inhibitory Concentration of 0.391 µg/ml for *Staphylococcus aureus* and 0.196 µg/ml for *Pseudomonas aeruginosa*. Both, *Klebsiella pneumoniae* and *Escherichia coli* are resistant to Penicillin G. This result is from studies of Penicillin G which shows that the method can be used for other compounds.

4.1.1 Unknown compound (PM) results:

For an unknown compound, PM, tubes 5 to 8 showed turbidity in case of *Klebsiella pneumoniae* and tubes 4 to 8 showed turbidity in case of *Escherichia coli, Staphylococcus aures*, and *Pseudomonas aeruginosa*.

Table 2: Observations of MIC test for unknown compound, PM

Culture	Dilution of PM (µg/ml)									
	50	25	12.5	6.25	3.125	1.563	0.781	0.391	Culture control	Media control
K. pneumoniae	-	-	-	-	+	+	+	+	+	-
E. coli	-	-	-	+	+	+	+	+	+	-
S. aureus	-	-	-	+	+	+	+	+	+	-
P. aeruginosa	-	-	-	+	+	+	+	+	+	-

Key: "–" = No growth; " + " = Growth (turbidity)

Based on the observation, Sample PM has a Minimum Inhibitory Concentration of 6.25 µg/ml for *Klebsiella pneumoniae*, 12.50 µg/ml for *Eschericha coli*, 12.50 µg/ml for *Staphylococcus aureus*, and 12.50 µg/ml for *Pseudomonas aeruginosa*.

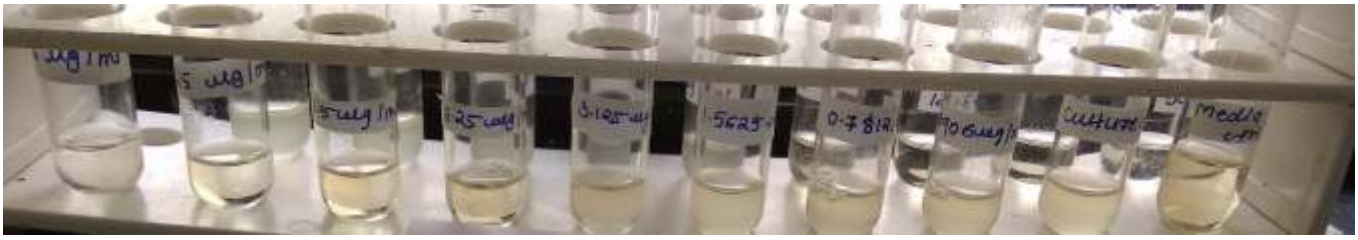


Figure 2: Tubes showing dilutions of PM tested against Klebsiella pneumoniae

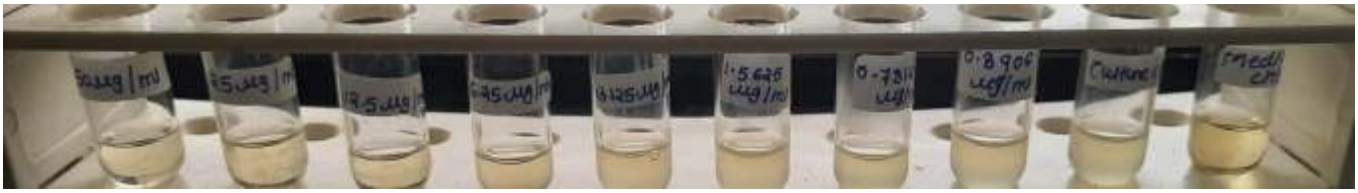


Figure 3: Tubes showing dilutions of PM tested against Escherichia coli

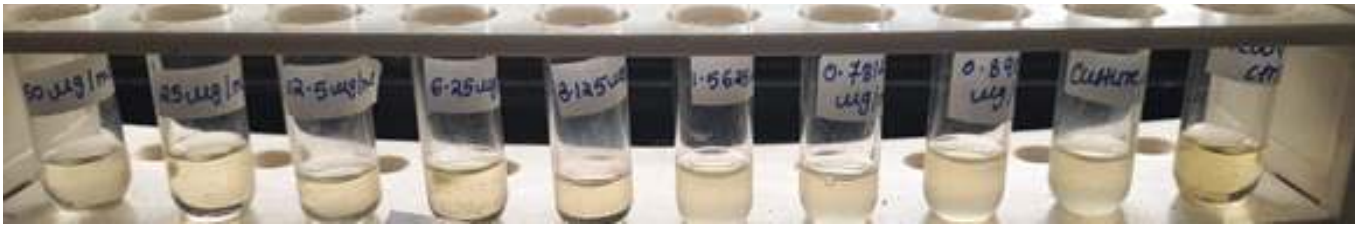


Figure 4: Tubes showing dilutions of PM tested against Staphylococcus aureus.

Figure 5: Tubes showing dilutions of PM tested against Pseudomonas aeruginosa.

4.2 Confirmatory test results:

4.2.1 Confirmatory result of Penicillin G

In the case of Penicillin G, the tubes containing 0.391 µg/ml, 0.196 µg/ml, and 0.098 µg/ml concentration of Penicillin G were streaked on Mueller Hinton Agar medium plates. The results matched with the broth dilution test.

Table: confirmatory results of broth dilution test.

Culture	Dilution of Penicillin G (µg/ml)		
	0.391	0.196	0.098
K. pneumoniae	+	+	+
E. coli	+	+	+
S. aureus	-	+	+
P. aeruginosa	-	-	+

Key: "–" = No growth; " + " = Growth (turbidity)

Based on the results, the resistance of *Escherichia coli* and *Klebsiella pneumoniae* to *Penicillin G* was confirmed. The MIC of Penicillin G of 0.391 µg/ml for *Staphylococcus aureus* and 0.196 µg/ml for *Pseudomonas aeruginosa* was confirmed.

4.2.2 Confirmatory result of PM

In the case of PM, the tubes containing 3.125 µg/ml, 6.25 µg/ml, 12.5 µg/ml, and 25 µg/ml were streaked on Mueller Hinton Agar medium plates. The results matched with the broth dilution test.

Table: confirmatory results of broth dilution test.

Culture	Dilution of PM (µg/ml)			
	25	12.5	6.25	3.125
K. pneumoniae	-	-	-	+
E. coli	-	-	+	+
S. aureus	-	-	+	+
P. aeruginosa	-	-	+	+

Figure 6: Confirmatory test broth dilution test of PM against Klebsiella pneumoniae

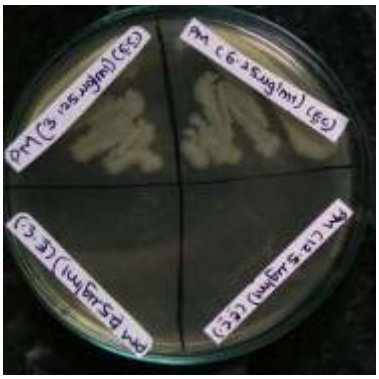


Figure 7: Confirmatory test broth dilution test of PM against Escherichia coli.

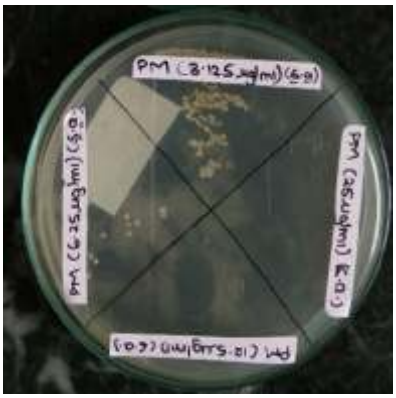


Figure 8: Confirmatory test broth dilution test of PM against Staphylococcus aureus

Figure 9: Confirmatory test broth dilution test of P M against Pseudomonas aeruginosa

5. Conclusion

Minimum Inhibitory Concentration was successfully determined using the BSCI 424 Method(4). MIC of the previously known antibiotic (Penicillin G) was confirmed. MIC of an unknown compound (PM) was found. Four bacteria were used for testing and all four showed varying reactions to the products tested. The method was reviewed and it works by the behavior of the compounds.

References

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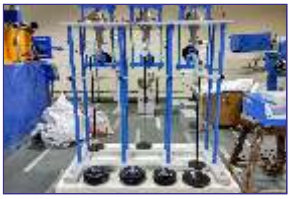
Installation Damage of Geosynthetics

The geosynthetics are prone to some amount of damage during their installation. To assess the quantity of the installation damage, a standard method was initially developed by Watts and Brady of the Transport Research Laboratory in the United Kingdom. The procedure has also discussed in the ASTM D 5818 with similar requirements. We are at BTRA doing the test following same ASTM D 5818 method followed by respective tensile strength. For the time being we are using the construction site for the sample preparation. If customer will agree, BTRA will collect the sample from site after standard procedure and provide ethe report.









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