Experiment 8

Determination of the minimal inhibitor concentration (MIC) of a bacteriostatic substance by agar diffusion and broth dilution method

Introduction:

Each bacterium has a level of antibiotic which will inhibit growth but not kill the organism. This is called the minimum inhibitory concentration **(MIC)**. Related to this, a higher antibiotic concentration will kill the organism. This is called the minimum bactericidal concentration **(MBC)**.

Bacteriocides are expected to have equal or very close MIC and MBC values, but bacteriostatics have a difference in these values mainly MBC concentrations are higher than MIC concentrations.

MIC is defined as "the lowest concentration of antimicrobial agent required to inhibit or stop visually the organism from growing after 18-24 hrs incubation". It is usually expressed in (mg/L or µg/ml).

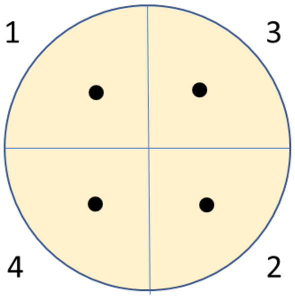
MBC is the lowest concentration of a given antimicrobial that will kill 99.9 % of the organism after 18-24 hrs of incubation. It is the lowest concentration of antimicrobial agent that will prevent the growth of an organism after subculture on to antibiotic- free media.

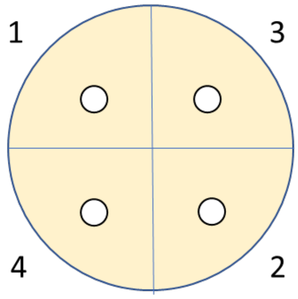
Now that we know the MIC and MBC for certain organism and antibiotic, we can put the patient on oral antibiotics and see what antibiotic levels can be achieved in the patient's bloodstream. The peak plasma level should be several times higher (e.g. 8 times higher) than the MBC, depending on the type of infection. If such levels cannot be obtained by oral antibiotics, then I.V antibiotics must be maintained for the duration of therapy.

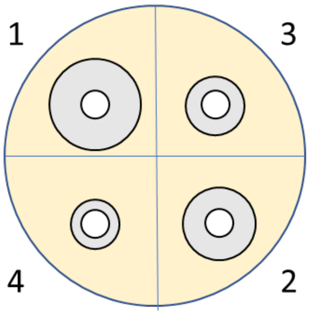
For highly resistant microorganism with continuously changing profile of resistance, there are no constant parameters (MIC & MBC) identified, and thus their MIC & MBC should be checked all the time.

The sensitivity of a desired culture can be measured by using a tube dilution technique or by an agar diffusion method, which determines the minimum inhibitory concentration (MIC) of an anti-microbial agent.

- The MIC is specific to each combination of antibiotic and microbe
- The MIC of an antibiotic against a microbe is used as a measure of the sensitivity of the microbe to the antibiotic
- The MIC is an inverse measure of sensitivity: the lower the MIC value of the antibiotic the greater sensitivity of the bacterium



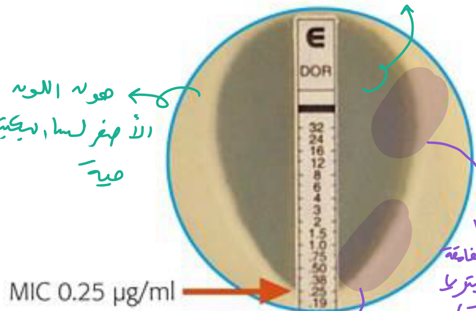


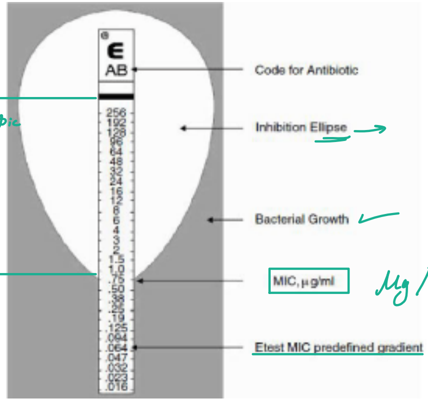


ii. The Epsilometer test (E- test)

The E- test is a quantitative method used to determine MIC which applies both the dilution and diffusion of antibiotic into the medium. E-test is a 'ready-to-use' reagent strip with a predefined gradient of antibiotic for the determination of precise MIC values of a wide range of antimicrobial agents against different organism groups, when E-test is applied to the surface of an agar plate inoculated with the test strain, there is an instantaneous release of the antimicrobial gradient from the plastic carrier to the agar to form a stable and continuous gradient beneath and in the immediate vicinity of the strip.

When the E-test strip is placed on an agar surface, the antibiotic gradient on the strip is simply transferred to the agar matrix creating an imprint of the gradient on the strip in the agar. The bacterial growth becomes visible after incubation and a symmetrical inhibition ellipse centered along the strip is seen. The MIC value is read from the scale in terms of µg/mL where the ellipse edge intersects the strip.





iii. Determination of the minimal inhibitor concentration (MIC) of a bacteriostatic substance by broth dilution method

The MIC procedure for testing antibiotics involves an antibiotic dilution assay, using either the wells of a micrometer plate or culture tubes. Each tube or well contains a different concentration of the agent and is inoculated with a fixed amount of the organism being tested. After incubation, the visible growth in the presence of the antibiotic is observed. The tube or well completely inhibiting growth of the organism in the presence of the smallest concentration of the antibiotic is expressed as the MIC.

To determine MBC: all visually negative tubes or wells (no visible growth after 18-24 hrs of incubation) are sub-cultured into agar plates and incubated for another 18-24 hrs at 37 ºC. The lowest dilution which shows a minimum of 99.9% killing on subculture is the MBC. Sub-culturing on an agar plate will dilute the antibiotic and if there were still surviving bacteria, growth on the plate will determine it.

The figure below is an example on serial dilution susceptibility testing for MIC determination.

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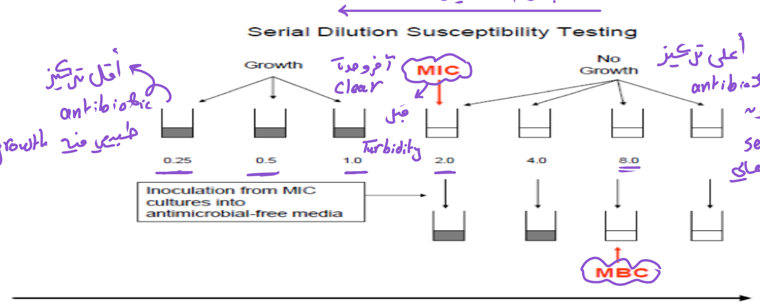


Example:

An antimicrobial susceptibility testing – the well (broth) dilution method was performed as following:

- 100 µL broth solution were first added to 8 wells.
- 100 µL of Gentamicin were added from a stock solution 512 µg/mL to the first well followed by 2-fold serial dilution in wells 2-8.
- 100 µL of bacterial inoculum was added after that to each well to give a final bacterial concentration of 5×10^{5} CFU/mL.

1. Calculate the concentration of gentamycin in each well in µg/mL

	1st well	**2nd**	**3th**	**4th**	**5th**	**6th**	**7th**	**8th**
Concentration after 2-fold serial dilution	256	128	64	32	16	8	4	2
Concentration after the addition of bacterial inoculum	128	64	32	16	8	4	2	1

2. These wells were incubated over night at 37°C, after result inspection, the turbidity started at the 5th diluted well. Determine the MIC.

	1st well	2nd	3th	4th	5th	6th	7th	8th
Results	C	C	C	C	T	T	T	T

C: Clear, T: Turbid

MIC = 16 µg/mL (4th test tube)

3. 10 µl of each of the first four wells were sub-cultured on an agar plate using surface inoculation technique. The 3rd and 4th test tubes showed bacterial growth while the 2nd and 1st test tubes showed no growth at all. Determine the MBC.

	1st well	2nd	3th	4th
Results	Zero colonies	Zero colonies	4 colonies	10 colonies

0.1% viable cells = 5×10^5 CFU/mL× 10 µl (0.01 ml) × 0.1%= 5 CFU

This means that you can see up to 5 colonies on the agar plates to state that this nominated concentration killed at least 99.9% of the CFUs

MBC= 32 µg/mL (2nd test tube)

Practical part

Materials Required:

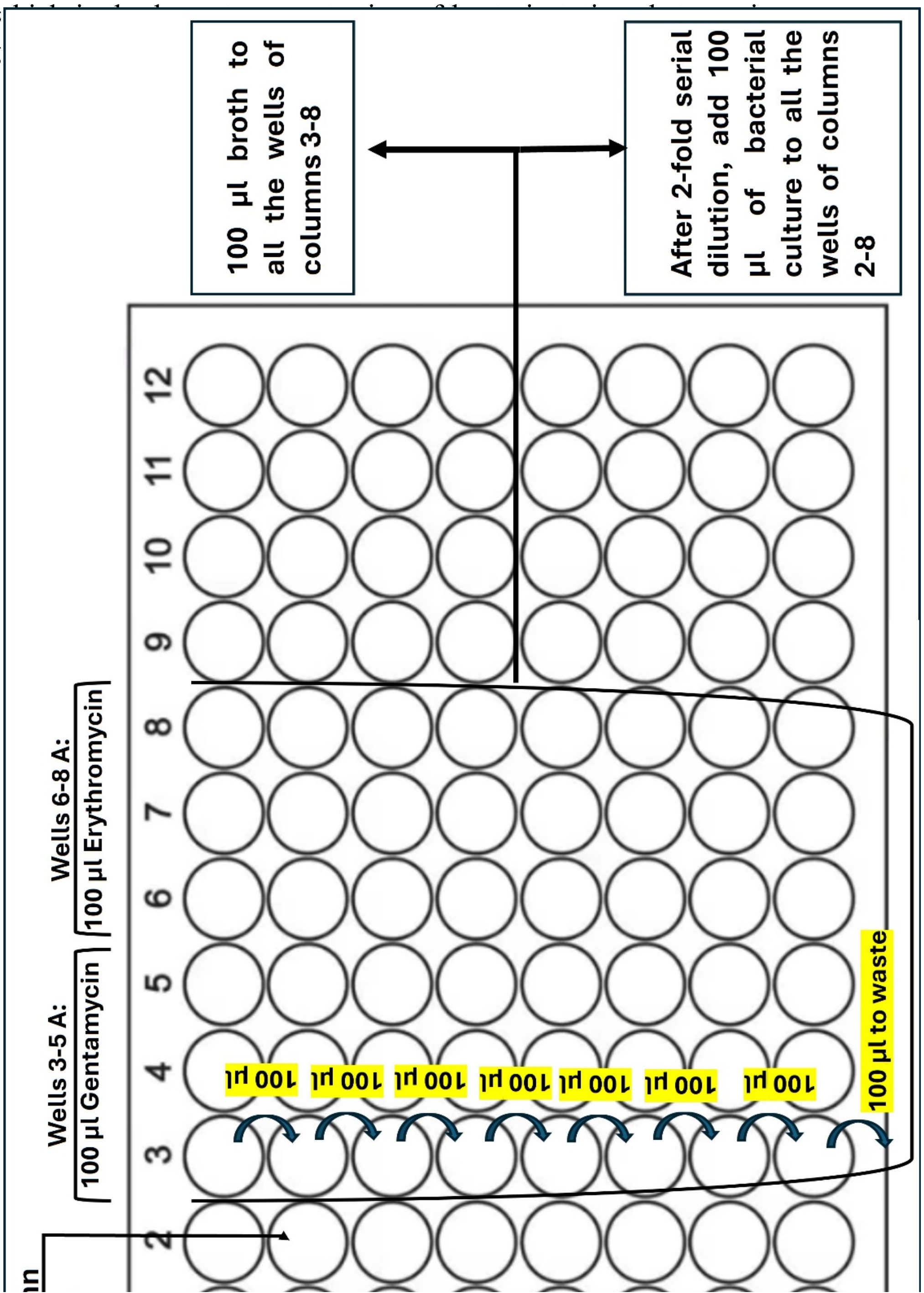
- 96-well microtiter plate with lid (sterile, flat-bottomed)
- Sterile micropipette tips
- Micropipettes (100 µL)
- Sterile broth media (nutrient broth or Mueller-Hinton Broth)
- Bacterial culture (adjusted to 0.5 McFarland standard, ~1×10^8 CFU/mL)
- Antimicrobial agent (Gentamycin solution 32µg/mL or Erythromycin solution 128 µg/mL)
- **Incubator set at 37°C**

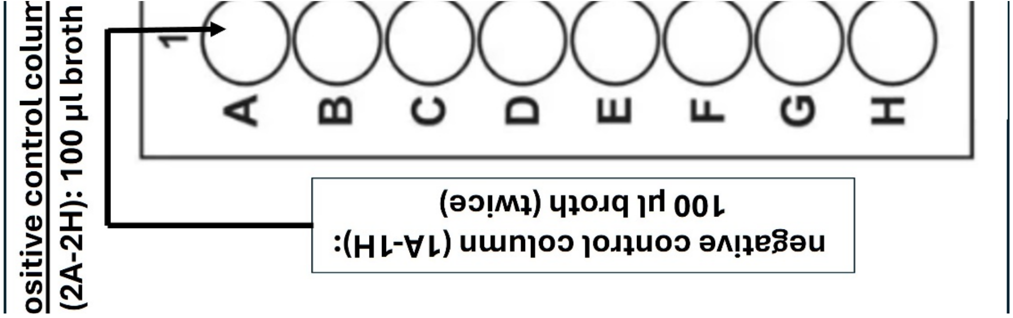
Procedure:

1. Add 100 µL of sterile broth to all wells in columns 1 to 8.
2. Add 100 µL of sterile broth to all wells of the first column. This will be your negative control.
3. Do not add any solution to column 2 at this stage. This will be considered your positive control.

4. Add 100 µL of gentamycin solution to well 3-5 of the first row.
5. Add 100 µL of gentamycin solution to well 6-8 of the first row.
6. Mix well by pipetting up and down several times.
7. Transfer 100 µL from well 1A to well 1B, mix thoroughly.
8. Continue this two-fold serial dilution across the row (wells B to H), transferring 100 µL each time and mixing.
9. After well H, discard 100 µL to maintain equal volumes.
10. Dilute the standardized bacterial culture 1:100 in broth to get ~1×10^{6} CFU/mL.
11. Add 100 µL of the diluted bacterial inoculum to each well from columns 2 to 8.
12. Incubate the 96 well plate at 37°C overnight
13. Read your results and record them in a table using signs (+) and (-) for presence and absence of growth, respectively
14. Calculate the MIC, w
the well showing no g

well number	1	2	3	4	5	6	7	8
Concentration								
Results								





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- اللهم وحّد صفوف المسلمين وانصرهم واهدِ اللهم شباب وشابات الإسلام.
- اللهم صلِّ وسلم وبارك على حبيبنا محمد ♡