



Protocol to Evaluate Antibacterial Activity MIC, FIC and Time Kill Method

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Introduction

In the evaluation of antibacterial activity using various antibiotics and ZnONPs nanoparticles alone and in combination, microdilution broth, checkerboard techniques and time kill curve assay are commonly used. Checkerboard data analysis depends on different mathematical and pharmacological models to detect deviations from theories of no-interaction and to evaluate antagonistic and synergistic interactions [1]. *In vitro* antimicrobial drugs formulations in combinations are generally assessed by fractional inhibitory concentration (FIC) index which denotes the sum of the FICs of each formulated drug tested, whereas, FIC is evaluated for each formulated drug by dividing the MIC of each formulated drug and used in combination by the MIC of each formulated drug used alone. FIC index is based on Loewe additivity zero interaction theory [2], based on the hypothesis that a drug cannot interact by itself and the effect of a self-drug combination will be additive, with an FIC index of 1, therefore, FIC index lower or higher than 1 indicates synergy or antagonism, as less or more drug would be required in order to have the same effect as the drugs alone [2].

Because of the twofold drug dilution scheme and the 1-dilution error of single-drug susceptibility testing methods used for FIC index determination, cutoffs of 0.5 and 4 were suggested for defining additivity/indifference. However, the actual variation of the FIC index in checkerboard microdilution methods was not well studied. In addition, most *in vitro* combination studies resulted in FIC indices within the range of 0.5 to 4 concluding no interaction (additivity/indifference), raising questions about the validity of this arbitrarily chosen FIC range given the absence of *in vitro-in vivo* correlation studies.

Bacterial suspension preparation

- The bacterial culture suspension must be prepared according to Clinical Laboratory Standards Institute (CLSI) guidelines [3], both positive and negative controls were used the positive control was used to test the growth conditions of bacteria and the negative control to check sterility of the media.
- Isolates that showed resistance or elevated MIC were kept for further investigation, stored at -80°C in skim milk media with glycerol.
- To test the MIC values against the antibiotics (e.g. Meropenem, Ciprofloxacin or Colistin etc) subcultures of isolates were revived by streaking on Blood/Nutrient agar plates, and incubated overnight at 37°C and 5% CO_2 incubator.
- To prepare the colony count to approximately 5×10^5 CFU/mL (CLSI, 2012), a direct saline suspension of isolated colonies selected from an 18 - 24 hour incubated blood/Nutrient agar plate was prepared. Then, the turbidity of this suspension was adjusted using a McFarland turbidity reader, equivalent to a 0.5 McFarland standard.
- This saline suspension was diluted 1:20 times in Muller Hinton Broth (MHB) ready-made liquid broth medium, by adding 100 μL of the saline suspension to a tube containing 1900 μL MHB, immediately before using it.
- 10 μL of the bacterial suspension was inoculated in wells containing 100 μL of the antibacterial agent, that is, the volume of the suspension was 10% of the final volume in each microtiter well [4].

Antibacterial agent and zinc oxide nanoparticles (ZnO NPs) preparation

Pure powdered antibiotics (e.g. Meropenem, Ciprofloxacin or Colistin etc) can be used as antibacterial agents.

First the antibiotic stock solutions would be prepared, by accurately weighing and dissolving in the appropriate diluents to yield the required concentrations.

The following formulas could be used to determine the amount of powder (1) or diluent (2) to make standard solution:

- $\text{Weight (mg)} = \text{Volume (mL)} \cdot \text{Concentration } (\mu\text{g/mL}) / \text{Potency } (\mu\text{g/mg})$
- $\text{Volume (mL)} = \text{Weight (mg)} \cdot \text{Potency } (\mu\text{g/mg}) / \text{Concentration } (\mu\text{g/mL})$

To determine the powder weight required, we used the formula:

$$W \text{ (mg)} = [V \text{ (mL)} \times C \text{ } (\mu\text{g/mL})] / P \text{ } (\mu\text{g/mg})$$

Where W= Weight of antibiotic powder, V= Volume of the diluent, C= Concentration of stock solution, and P= Potency of the antibiotic [4].

Antibiotic stock solution would be prepared as follows:

- Meropenem trihydrate by dissolving 1600 μg of the antibiotic dissolved in 1 mL Dimethyl sulfoxide (DMSO) and the concentration will be 1600 $\mu\text{g/mL}$ (1.6 mg/mL).
- Ciprofloxacin stock solution was prepared by dissolving 1600 μg of the antibiotic in 1 mL 0.1 N HCL, and the concentration will be 1600 $\mu\text{g/mL}$ (1.6 mg/mL).
- The Colistin is completely soluble in water, the Colistin stock solution was prepared by dissolving 3200 μg (3.2 mg) of the antibiotic in 1 mL sterile distilled water, and the concentration will be 3200 $\mu\text{g/mL}$.
- The ZnO/water suspension could be prepared by weighing 16 mg of ZnO nanoparticles in 1 mL of sterile distilled water and the suspension must be subjected to vigorous vortex.
- Suspending the ZnO NPs by sonication at different voltages could be attempted.
- However, after comparing the results it was found that better results were obtained by non-sonicated ZnO/water suspension, and that the ZnO NPs were well dispersed in the water.

Minimum inhibitory concentration (MIC)

- The broth microdilution method is used to determine Minimum Inhibitory Concentration (MIC).
- Costar 96-well Polystyrene Cell Culture Cluster with flat bottom plate would be used for the experiment, antibiotic susceptibility testing results (if susceptible, intermediate, or resistant), were determined using MIC value breakpoints [3].
- To the microtiter plate 100 μL of Mueller Hinton Broth (MHB) would be added from column one to column twelve.
- The antibiotic stock solution prepared in section 3 stored at -20°C must be thawed and diluted to 1/100 in a tube containing MHB and add 20 μL of the stock solution to 1980 μL MHB and the concentration would be 16 $\mu\text{g/mL}$.
- 200 μL of the above dilution must be added to all the wells of the first column in the microtiter plate and then double dilute by taking 100 μL from the first to the second column using the multichannel pipette and mix properly.
- Lastly, 10 μL of the bacterial suspension must added to all columns except negative control.
- Negative control contains only 100 μL MHB and positive control contains MHB and bacterial suspension [4].
- MIC would be determined by comparing the growth rate of tested bacterial strains with the positive and negative controls.

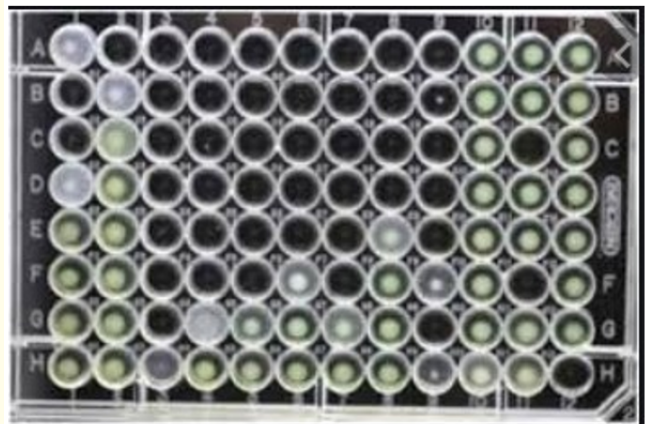


Figure 1: Microdilution broth plate 96 well.

Checkerboard method

The checker board method used to evaluate the interaction between the antibiotic or drug formulations (Meropenem, Ciprofloxacin and Colistin) and the ZnO NPs.

Procedure to prepare the checkerboard plate

- The antibiotic stock solution stored at -20°C should be taken out and thawed.
- The stock solution was diluted 1/100 in a tube containing MHB by adding $40\ \mu\text{L}$ of the stock solution to $3960\ \mu\text{L}$ of MHB and the concentration would be $16\ \mu\text{g}/\text{mL}$.
- Arrange eight tubes in a rack and add 2 mL MHB.
- The antibiotic concentration ($16\ \mu\text{g}/\text{mL}$) would be double diluted by adding 2 mL in a tubes containing 2 mL MHB and the concentration would be $8\ \mu\text{g}/\text{mL}$ and the process must be repeated to get the final concentration to $0.06\ \mu\text{g}/\text{mL}$.
- $100\ \mu\text{L}$ of the antibiotic concentration ($16\ \mu\text{g}/\text{mL}$) would be added to all the wells in column of microtiter plate.
- The process must be repeated for the rest of the dilutions, moving across the columns from the highest to the lowest concentration.

For the ZnO NP suspension the following steps were carried out

- Initially, the tubes filled with 1 mL sterile water must be arranged in a rack.
- Next, 1 mL of the stock ZnO/water suspension must be added to the first tube and double diluted with water till reach the last tube.
- And 1 mL of MHB must be added to each tube to support the growth of the bacteria.
- The concentration in first tube would be $4\ \text{mg}/\text{mL}$.
- The microtiter plate that already contained the antibiotic solutions were filled with $100\ \mu\text{L}$ solution from the above first tube prepared.
- Similar process must be followed for the second concentration also filling the next row till the final concentration, microtiter plate would be having a decreasing concentration of the antibiotic in the horizontal direction against a decreasing concentration of the ZnO NPs in the vertical direction.
- The bacterial suspension $20\ \mu\text{L}$ was added to the whole microtiter plate column except the negative control.

- Mixing must be done at each step using the multichannel pipette using sterile tips.
- All the plates must be was incubated 37°C in incubator, and optical density (OD) of themicroplate must be read before incubation using a microplate spectrophotometer (SpectraMax Plus 348) from Molecular Devices at a wavelength equal to 600 nm and after incubation for overnight the optical density (OD) must be taken at 600 nm.
- The microtiter plate could be incubated for 16 hours in the SpectraMax machine which automatically read the OD every five minutes and plotted the growth curve with the SoftMax Pro Software.
- MIC for the antibacterial agents/drugs could be determined by comparing the growth of tested bacteria strains with positive and negative controls, experiment must be performed three times for each bacterial strains.

The following equation was used to calculate FICI:

$$\text{FICI} = \text{FICAb} + \text{FICNP}$$

Where, FIC antibiotic = (MIC of Antibiotic in the presence of nanoparticle)/(MIC of antibiotic alone) and FIC nanoparticle= (MIC of nanoparticle in the presence of antibiotic)/(MIC of nanoparticle alone).

FICI value interpretation ranges as follows: FICI is less than or equal to 0.5 is consider as synergy and more than 4.0 it is considered as antagonism. If FICI in between > 0.5 and ≤ 1.0 consider as an additive and FICI between > 1 and ≤ 4.0 is consider as indifference.

Time-Kill method

The time-kill method is used to test the bactericidal activity of one or more antimicrobial agents against a particular bacterial strain. This is done by counting viability of bacterial strains at different time intervals. The experiment would be performed in a 96-well microtiter plate.

To obtain the time-kill curve the bacterial strains growth rate must be counted at different time intervals starting from 0 hours to 24 hours, and these time intervals are plotted as a semi-log plot or graph. The bacterial strains would be usually plotted on the ordinate (Y axis) in logarithmic scale while the corresponding time is on the abscissa (X axis) in arithmetic scale.

The Microtiter plate for the time-kill method contained the antibiotic alone in one row (row A), the ZnO NPs suspension alone in another row (row C), and the combination of these two agents in a separate row as well (row B). To prepare the microtiter plate, first, the antibiotic MIC for the tested strain was determined as described in section 4.

The stock solution of selected antibiotic was diluted to four times the MIC ($4\times\text{MIC}$) for tested bacterial strains.

The serial double dilutions of would be done by adding 0.5 mL MHB and 0.5 mL of the previous concentration which will be equal to double the MIC ($2\times\text{MIC}$).

In one row of each well 200 μL of the decreasing concentrations of the antibiotic solution will be added as ($4\times\text{MIC}$), ($2\times\text{MIC}$) and (MIC) concentration.

In another row of the well add 100 μL of antibiotic concentration ($4\times\text{MIC}$, $2\times\text{MIC}$, and MIC).

In some cases half the MIC and quarter the MIC ($0.5\times\text{MIC}$, $0.25\times\text{MIC}$) could also be added.

Time kill growth curve for ZnO NP suspension

- The stock ZnO/water solution prepared in the above previous section would be used.
- For serial dilution the tubes would be arranged containing 0.5ml sterile water.
- The Different concentrations would be prepared by double dilution technique, by adding 0.5 mL of ZnO stock solution to 0.5ml of sterile water and process would be repeated by transferring 0.5ml to the next tubes to get the desired concentration of (MIC).
- To support the bacterial growth of bacteria 0.5 mL MHB was added to each tube, the desired concentration would be in decreasing order as follows, equal to four times the MIC ($4\times\text{MIC}$), double the MIC ($2\times\text{MIC}$), MIC concentration (MIC), half the MIC ($0.5\times\text{MIC}$) and quarter the MIC ($0.25\times\text{MIC}$).
- For the microtiter plate assay the protocol must be followed.
- Add 100 μL of antibiotic solution to the wells filled with 100 μL of ($0.5\times\text{MIC}$) ZnO NP suspension.
- Then the next rows would be filled with 200 μL of ZnO NPs decreasing concentration suspensions prepared above.

- The positive control must contain 200 μL of the MHB and the bacterial suspension and the negative control with 200 μL of the MHB.
- To all the wells 20 μL of the prepared bacterial suspension were added, except for negative control and then incubate at 37°C in a 5% CO_2 incubator.

For colony counting assay the following steps must be performed:

- First 10 μL of the suspension were aspirated from each well at different time intervals starting from 0 hours to 24 hours during the incubation period and diluted to 1:10 in PBS and diluted samples were streaked on blood agar plate and the plates were incubated for 24 hours in a CO_2 incubator at 37°C .
- After incubation the colonies were counted and the data must be recorded in an Excel sheet and the graph of time-kill curve as semi-log plot would be plotted, to get the plot the colony counts as a Log_{10} of the CFU/mL would be on the Y axis in the logarithmic scale and the time on the X axis in arithmetic scale.
- Bacteriostatic activities are defined as $< 3 \log_{10}$ and bactericidal activities are defined as $\geq 3 \log_{10}$ reductions in CFU/mL during the incubation period of 24-hours (CLSI, 1999).
- Synergy values are $\geq 2 \log_{10}$ decrease in CFU.
- Indifference is defined as $\leq 1 \log_{10}$ CFU/mL.
- Additive is $< 2 \log_{10}$ CFU/mL.

Conclusion

As the combinations of drug therapy is being practiced in the laboratories to evaluate the efficacies of drugs and their combinations to check the sensitivity of the bacterial and fungal strains, the mentioned protocols of MIC and FICI could be valuable.

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