BIRZEIT UNIVERSITY

11/5/2018

DEPARTMENT OF BIOLOGY AND BIOCHEMISTRY

MICROBIOLOGY-BIOL243

Chemical Effects on Bacterial Growth

SUPERVISED BY: MS. ASEEL MHANA

INSTRUCTOR: MR. MUNTHER METANI

Date of Experiment: 26/11/2018

Muna Abed Rabbo
ID: 1171596
Partners names: Abdullah Lahham,
Yasmeen Ahmad

Contents

Objectives

The aim of this experiment is to examine the effects of certain chemicals and antibiotics on the growth of *Enterococcus faecalis* and *Staphylococcus aureus* bacterial species. As well as finding the Minimum Inhibitory Concentration (MIC), and Minimum Bactericidal Concentration (MBC) for certain antibiotics.

Introduction

The growth of microorganisms can be controlled by the usage of many chemical agents, that would kill microbes (bactericidal) or inhibit the growth of microbes (bacteriostatic). The effect of chemical agents may vary. Some chemicals decrease antimicrobial presence in an area or on a surface (decontaminant). Others are not used to remove all contaminants; instead, reduce the amount of contamination by killing some pathogenic bacteria and fungi (disinfectant). Others kill or inhibit microorganisms but are safe for human tissues (antiseptic). [1]

Antibiotics, however, are metabolic products of microorganisms that are used to kill or inhibit the growth of other microorganisms and can be used on or inside patient.

To determine the effectiveness of a certain antibiotic on a certain microbe **the Kirby-Bauer method** is used. It is done by measuring the diameter of the zone of inhibition (the zone where no microbial growth is observed) around a given antibiotic disc, after the disc is placed in a Mueller-Hinton agar petri dish cultured with a certain microbe. The measurement of the zone is then compared to standards, to determine if the bacteria is sensitive to the antibiotic (killed or inhibited by it), or resistant to it (unaffected).

As uncalculated large doses of antibiotics are not healthy for human use, medical microbiologists have defined the MIC and MBC as critical amounts of an antibiotic that can be depended on in drug production. Minimum Inhibitory Concentration (MIC) is the lowest concentration of an antimicrobial drug that will inhibit the visible growth of a microorganism after overnight incubation. It can be determined by broth dilution methods after a culture has been isolated, and identical doses of bacteria are cultured in wells of liquid media containing progressively lower concentrations of the drug. The MIC would be the last well containing clear (not turbid) medium.

However, Minimum Bactericidal Concentration (MBC) determines the lowest concentration at which an antimicrobial agent will kill a microorganism. It is complementary to the MIC test. That after determining the MIC, the liquid media well containing the microbe and antibiotic MIC and the previous two wells are plated onto agar dishes. And using the method of viable count as a proxy measure of bacterial viability, the number of colony-forming units (CFUs) can be determined; finally, the dish that has 0-10 CFUs is the one that its antibiotic concentration is considered as the MBC.

[1] A. Unfried (2013). *Difference Between Decontamination & Sterilization*. Retrieved October 29, 2018, from https://study.com/academy/lesson/difference-between-decontamination-sterilization.html

Material

- Effect of disinfectants and antiseptics on bacterial growth
  - ✓ *E. coli* and *S. aureus* stock cultures
  - ✓ McFarland standard*[2]
  - ✓ Nutrient-agar broth cultures
  - ✓ Sterile cotton swabs
  - ✓ 150 mm Muller-Hinton agar plates
  - ✓ Disposable sterile inoculating loops
  - ✓ Ethanol 95%
  - ✓ Stainless steel tweezers
  - ✓ Discs submerged in Disinfectants and antiseptics: Dettol, Listerine, 95% ethanol, 70% ethanol, H_2O (control).

- Effect of chemotherapeutic agents on bacterial growth
  - ✓ *E. coli* and *S. aureus* stock cultures
  - ✓ Nutrient-agar broth cultures
  - ✓ McFarland standard
  - ✓ Sterile cotton swabs
  - ✓ Disposable sterile inoculating loops
  - ✓ Ethanol 95%
  - ✓ Stainless steel tweezers
  - ✓ 150 mm Muller-Hinton agar plates
  - ✓ Antibiotic discs: ERYTHROMYCIN E15, AMIKACIN AK30, PENCILLIN G P10, CEFACLOR CEC30, VANCOMYCIN 30
- Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC)
  - ✓ Antibiotic stock solution 100 μg/ml (CIPROFLOXACIN)
  - ✓ Sterile 96-microdilution well plates
  - ✓ Stock culture of *E. coli*
  - ✓ Multichannel micropipette
  - ✓ Sterile pipette tips
  - ✓ A sterile diluent (Muller-Hinton broth)

    (After Incubation)
  - ✓ Nutrient agar plates
  - ✓ Micropipettes
  - ✓ Hook spreader
  - ✓ 95% ethanol

[2] "McFarland turbidity standards are prepared by mixing various volumes of 1% sulfuric acid and 1% barium chloride to obtain solutions with specific optical densities. 0.5 McFarland turbidity standard provides an optical density comparable to the density of a bacterial suspension 1.5x 10^8 colony forming units (CFU/ml)."

Acharya, T. (2017, May 13). *Preparation of McFarland Turbidity Standards* -. Retrieved November 1, 2018, from https://microbeonline.com/preparation-mcfarland-turbidity-standards/

Method

- Effect of disinfectants and antiseptics on bacterial growth
  - The Bunsen burner was lighted, and the flame was adjusted so that it had 2 blue cones (inner, and outer), to create aseptic conditions.
  - The nutrient-agar plates were labelled with all the disinfectants so that the control (water) was made in the middle.
  - Using the sterile inoculating loop a small amount of the stock culture of *E. coli* was taken off on the loop tip.
  - The inoculating loop was then submerged in nutrient-agar broth cultures and the solution was shaken several times to get a homogenous solution.
  - The turbidity of the culture was then compared with the McFarland standard (the turbidity of our solution ought to have been near the standard's turbidity).
  - A sterile cotton swab was dipped in the broth culture, then swabbed in several directions in a way that made the whole Muller-Hinton agar plate inoculated equally.
  - The tweezers were sterilized by submerging its tip with ethanol 95% and then it was gently passed above the flame and cooled under aseptic conditions.
  - Using the sterilized tweezers, a disc (from the previously submerged discs) of each disinfectant was drained briefly to eliminate excessive liquid and then placed on its labelled position on the plate.
  - The same method was done for the *S. aureus*.
  - Then the two plates were incubated at 37°C.

- Effect of chemotherapeutic agents on bacterial growth
  - The same method above was repeated for both *E. coli* and *S. aureus*, except that the antibiotic discs were dry rather than submerged in a liquid, and there is no control.
- Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC)
  - 100 µL of the sterile diluent was pipetted into the wells numbered 2-12.
  - 200 µL of the Ciprofloxacin antibiotic was pipetted into the well numbered 1.
  - Serial dilution was done by pipetting 100 µL of each well and transferring them to the next well (between 1-11). The last 100 µL from well #11 was discarded.
  - 100 µL of the bacterial inoculum was pipetted into 1-12.
  - Well #1 had a 500 µL concentration of the antibiotic, and well #12 had 0 µL (negative control), Positive control had only antibiotic in it.
  - The wells were put in the incubator at 37°C.
  - After incubation, the wells were examined, and the last clear well was considered the MIC.

- 100 µL of the cultures of the MIC well and the previous 2 wells, were pipetted on 3 nutrient-agar plates.
- The broth was spread using a sterile hook spreader (sterilized using the same method of sterilizing the tweezers in part 1).

Data and Results

Effect of disinfectants and antiseptics on bacterial growth

After 42 hours of incubation of both *E. coli* and *S. aureus* plates, containing the antimicrobial discs, the following results were obtained:

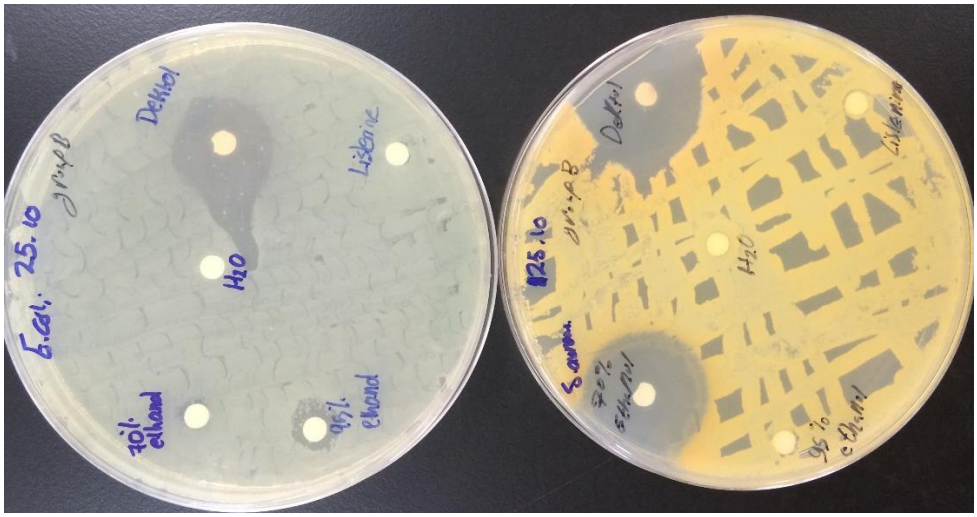

Fig 1: The results obtained after 42 hours of incubation of the E. coli plate (left), and S. aureus plate (right) containing the antimicrobial discs

From this picture we can obviously notice that the inhibition zone (the area where there is no bacterial growth) varies from one disc to another. And we can calculate the diameter of the inhibition zone of each antimicrobial agent in both plates, as shown in the table below:

Table 1: The diameter of the inhibition zone (I.Z.) around each antimicrobial disc in both E. coli and S. aureus plates:

Escherichia coli		***Staphylococcus aureus***
Chemicals	**Diameter of I.Z.**	**Diameter of I.Z.**
Water (control)	6 mm (diameter of the disk itself, no inhibition of growth)	6 mm (diameter of the disk itself, no inhibition of growth)
Dettol	26 mm	30 mm
95% Ethanol	10 mm	7 mm
70% Ethanol	7 mm	26 mm
Listerine	6 mm (diameter of the disk, no inhibition of growth)	6 mm (the diameter of the disk, no inhibition of growth)

For example: the inhibition zone of Dettol on *E. coli* plate was 26 mm, while on *S. aureus* plate was 30 mm, and so on for all the other agents.

❖ **Effect of chemotherapeutic agents on bacterial growth**

After 42 hours of incubation of both *E. coli* and *S. aureus* plates, containing the antibiotic discs, the following results were obtained:

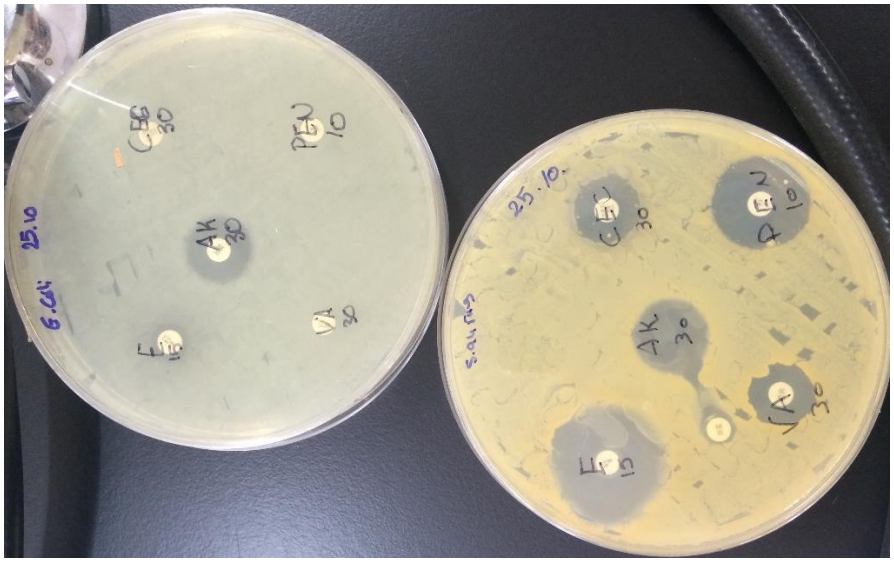

Fig 2: The results obtained after 42 hours of incubation of the E. coli plate (left), and S. aureus plate (right) containing the antibiotic discs

From this picture we can obviously notice that the inhibition zone varies from one disc to another. And we can calculate the diameter of the inhibition zone of each antibiotic in both plates, as shown in the table below:

Table 2: The diameter of the inhibition zone (I.Z.) around each antibiotic disc in both E. coli and S. aureus

Staphylococcus aureus		***Escherichia coli***
Antibiotic	**Diameter of I.Z.**	**Diameter of I.Z.**
AMIKACIN AK30	18 mm	17 mm
ERYTHROMYCIN E15	29 mm	9 mm
VANCOMYCIN 30	15 mm	6 mm (no inhibition of growth)
CEFACLOR CEC30	17 mm	7 mm
PENCILLIN G P10	27 mm	6 mm (no inhibition of growth)

❖ **Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC)**

After examining the wells that had been incubated for 42 hours, the well #7 was the last clear well, as shown in (fig 3) - we performed duplicate tests to decrease the error -, thus the MIC is the concentration of the antibiotic in well #7 which is 7.812 µg/ml as shown in table 3.

Fig 3: The results of the MIC and MBC test for Ciprofloxacin antibiotic against E. coli

Table 3: The serial concentration of the Ciprofloxacin in the well plates of MIC test

Well #	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
Antibiotic concentration (µg/ml)	500.0	250.0	125.0	62.50	**31.25**	15.63	**7.812**	3.906	1.9531	0.9767	0.4883	0.0000

However, after culturing the broths of wells #7, #6, and #5 in nutrient-agar dishes and incubating them for 42 hours the following results were obtained as shown in (fig 4):

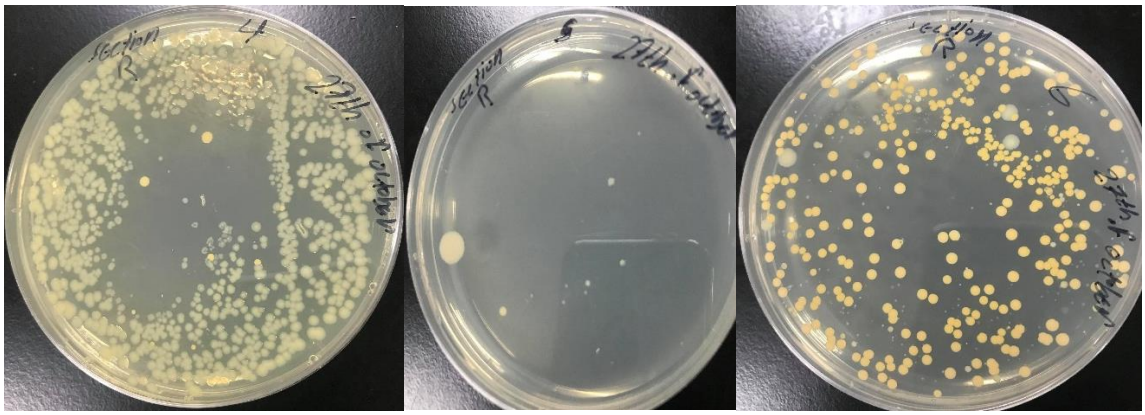

Fig 4: Results obtained after 42 hours of incubation, of cultured broths in wells #4, #5, and #6 of the MIC and MBC test.

From these pictures, we can comprehend that plate #4 obviously contained more than 10 colonies, plate #5 contained 9 colonies, which means that we can consider that the MBC is 31.25 µg/ml, which is equal to the antibiotic concentrtaion inside well #5 of the microdilution well plates used in the test.

Discussion

The results show that different types of microorganisms vary in their response to antimicrobial agents, antiseptics, and antibiotics.

In the part of antimicrobial agents against E. coli and S. aureus, Listerine had no effect on both bacteria, as its inhibition zone was the same as the inhibition zone of the control (water). For the first glance this may be surprising, however, the sample of Listerine we had been using had been expired for three years now. This will explain that it lost its antimicrobial activity, so it hadn't had any effect on bacterial growth. On the other hand, Dettol was found to be very effective on both bacterial types, where the zones of inhibition were so close 26 mm for E. coli (inhibition zone on E. coli plate was not a uniform circle, as the disc may have moved during incubation) , and 30 mm for S. aureus. This is mainly due to the active ingredient in Dettol, which is Chloroxylenol, a chemical that is an antiseptic and disinfectant agent, which is shown to be most effective against Gram positive bacteria where it disrupts the cell wall due to its phenolic nature, however gram negative bacteria may be more resistant because of its more complex cell wall.*3Ethanol had also a cidal effect on bacteria, as it dissolves its plasma membrane, and denatures cell's proteins. It is clear in the S. aureus plate, that 70% ethanol had more antimicrobial activity than 95% ethanol. This correlates with the fact that as the concentration of water increases in the ethanol content (with an adequate concentration of ethanol itself) its cidal effect increases, as water decreases its evaporation rate and increases its penetration towards the inside of the cell. However, E. coli plate had a different result, where the disc of 95% ethanol had a larger inhibition zone than 70% ethanol disc. This may be because the disc of 95% ethanol had had excess of ethanol and wasn't drained well before placing it on the plate, thus created a larger inhibition zone. However, ethanol generally has more effect on gram positive bacteria than gram negative as the later has an outer membrane that needs to be disrupted before disrupting the inner plasma membrane.

In the part of chemotherapeutic agents against *E. coli* and *S. aureus*, AMIKACIN 30 had similar effects on both *S. aureus* and *E. coli,* as its mechanism of action targets the 16S rRNA, and the RNA-binding S12 protein of the 30S subunit of prokaryotic ribosome and inhibits protein synthesis by changing the ribosome's shape so that it cannot read the mRNA codons correctly, thus it is bactericidal with a broad spectrum of activity.*[4, 5]However, ERYTHROMYCIN 15 had different effects on *S. aureus* and *E. coli*, in which the inhibition zone on the *S. aureus* plate was 29 mm, while it was 9 mm on the *E. coli* plate. This can be explained by the fact that E15 is from the family of Macrolides which are inhibitors of protein synthesis. They impair the elongation cycle of the peptidyl chain by specifically binding to the 50 S subunit of the ribosome, thus this is the reason behind the bactericidal effect it had on *S. aureus*. However, *E. coli* has macrolide-resistance mutations in the ribosomal protein L22 genes, which alters the ribosomal target sites and prevents binding. This explains its reduced antimicrobial activity against *E. coli*. *[6] Similarly, VANCOMYCIN, PENCILLIN, CEFACLOR greatly affected *S. aureus* growth, while *E. coli* was mildly affected or wasn't

[3] Chloroxylenol. (n.d.). Retrieved November 3, 2018, from https://www.drugbank.ca/drugs/DB11121

[4] Amikacin. (2018, October 27). Retrieved from https://en.wikipedia.org/wiki/Amikacin.

[5] Papich, M. G. (2016). *Saunders Handbook of Veterinary Drugs* (Fourth Edition) (Fourth ed.). Retrieved November 3, 2018, from https://www.sciencedirect.com/book/9780323244855/saunders-handbook-of-veterinary-drugs#book-info

[6] Moore, S. D., & Sauer, R. T. (2008, November 25). Revisiting the *mechanism of macrolide-antibiotic resistance mediated by ribosomal protein L22*. Retrieved November 3, 2018, from http://www.pnas.org/content/105/47/18261

affected at all. VA 30 has a unique mode of action inhibiting the second stage of cell wall synthesis of susceptible gram-positive bacteria (*S. aureus* in this case). Similarly, Penicillin and Cefaclor exert bactericidal activity by inhibiting the enzyme that catalyzes the final step in cell wall synthesis, the cross-linking of peptidoglycan. However, *E. coli* and other gram-negative bacteria are mildly affected by some or resistant to others, since the outer membrane of Gram-negative bacteria acts as a barrier to its entry into the cell. *[7,8,9,10]

In the last part, we found that the MIC for CIPROFLOXACIN against E. coli was approximately 8 µg/ml, while the MBC was approximately 31 µg/ml. From this result, we can tell that the (MBC/MIC) ratio is equal to 4. This means that ciprofloxacin has a static effect on E. coli. This can be explained by the understanding its mechanism of action. It functions by inhibiting DNA gyrase, and topoisomerase types II and IV, necessary for DNA separation, thus inhibiting cell division. *[11]

Conclusion

To conclude, microbial growth may be adversely affected by many chemicals, that may either kill the microorganisms, or inhibit its growth. However, our experiment showed that the antimicrobial activity of each chemical is influenced by a variety of factors, including the concentration of the chemical agent, the type and nature of the microorganism itself, and its resistance ability to the mechanism used by the agent against the microbe.

[7] Watanakunakorn, C. (1984, December). *Mode of action and in-vitro activity of vancomycin*. Retrieved from https://www.ncbi.nlm.nih.gov/pubmed/6440886

[8] Zhou, A., Kang, T. M., Yuan, J., Beppler, C., Nguyen, C., Mao, Z., Minh Quan Nguyen. (2015, January 01). *Synergistic Interactions of Vancomycin with Different Antibiotics against Escherichia coli: Trimethoprim and Nitrofurantoin Display Strong Synergies with Vancomycin against Wild-Type E. coli*. Retrieved November 3, 2018, from https://aac.asm.org/content/59/1/276

[9] Kaplan, M. (2000*). The problem with gram-negative bacteri*a. Retrieved November 3, 2018, from http://www.anapsid.org/gramnegative.html

[10] Yocum, R. R., Rasmussen, J. R., & Strominger, J. L. (1980, May 10). The mechanism of action of penicillin. Penicillin acylates the active site of Bacillus stearothermophilus D-alanine carboxypeptidase. Retrieved November 3, 2018, from https://www.ncbi.nlm.nih.gov/pubmed/7372662

[11] Ciprofloxacin. (2018, October 27). Retrieved November 3, 2018, from https://en.wikipedia.org/wiki/Ciprofloxacin

References

[1] A. Unfried (2013). *Difference Between Decontamination & Sterilization*. Retrieved October 29, 2018, from https://study.com/academy/lesson/difference-between-decontamination-sterilization.html

[2] Acharya, T. (2017, May 13). *Preparation of McFarland Turbidity Standards* -. Retrieved November 1, 2018, from https://microbeonline.com/preparation-mcfarland-turbidity-standards/

[3] *Chloroxylenol*. (n.d.). Retrieved November 3, 2018, from https://www.drugbank.ca/drugs/DB11121

[4] *Amikacin*. (2018, October 27). Retrieved from https://en.wikipedia.org/wiki/Amikacin.

[5] Papich, M. G. (2016). *Saunders Handbook of Veterinary Drugs* (Fourth Edition) (Fourth ed.). Retrieved November 3, 2018, from https://www.sciencedirect.com/book/9780323244855/saunders-handbook-of-veterinary-drugs#book-info

[6] Moore, S. D., & Sauer, R. T. (2008, November 25). Revisiting the *mechanism of macrolide-antibiotic resistance mediated by ribosomal protein L22*. Retrieved November 3, 2018, from http://www.pnas.org/content/105/47/18261

[7] Watanakunakorn, C. (1984, December). *Mode of action and in-vitro activity of vancomycin*. Retrieved from https://www.ncbi.nlm.nih.gov/pubmed/6440886

[8] Zhou, A., Kang, T. M., Yuan, J., Beppler, C., Nguyen, C., Mao, Z., Minh Quan Nguyen. (2015, January 01). *Synergistic Interactions of Vancomycin with Different Antibiotics against Escherichia coli: Trimethoprim and Nitrofurantoin Display Strong Synergies with Vancomycin against Wild-Type E. coli*. Retrieved November 3, 2018, from https://aac.asm.org/content/59/1/276

[9] Kaplan, M. (2000*). The problem with gram-negative bacteri*a. Retrieved November 3, 2018, from http://www.anapsid.org/gramnegative.html

[10] Yocum, R. R., Rasmussen, J. R., & Strominger, J. L. (1980, May 10). *The mechanism of action of penicillin. Penicillin acylates the active site of Bacillus stearothermophilus D-alanine carboxypeptidase*. Retrieved November 3, 2018, from https://www.ncbi.nlm.nih.gov/pubmed/7372662

[11] *Ciprofloxacin*. (2018, October 27). Retrieved November 3, 2018, from https://en.wikipedia.org/wiki/Ciprofloxacin