ANTIBIOTIC SENSITIVITY TESTING

I. OBJECTIVES

- To utilize specific monitoring techniques to evaluate the susceptibility of a microbe to different antibiotics.
- To distinguish the range of activity of an antibiotic.
- To recognize and define advantages and limitations of two different susceptibility testing procedures.

II. INTRODUCTION

There is a large number of antimicrobial agents available for treating diseases caused by microorganisms. Such drugs are now an essential part of modern medical practice. The antimicrobial agents used in medical practice are aimed at eliminating the infecting microorganisms or at preventing the establishment of an infection. To be of therapeutic use, an antimicrobial agent must exhibit selective toxicity; it must exhibit greater toxicity to the infecting pathogens than to the host organism. A drug that kills the patient is of no use in treating infectious diseases, whether or not it also kills the pathogens. As a rule, antimicrobial agents are of most use in medicine when the mode of action of the antimicrobial chemicals involves biochemical features of the invading pathogens not possessed by normal host cells.

Antibiotics represent a major class of antimicrobial agents. By definition, antibiotics are biochemicals produced by microorganisms that inhibit the growth of, or kill, other microorganisms. By their very nature, antibiotics must exhibit selective toxicity because they are produced by one microorganism and exert varying degrees of toxicity against others. The discovery and use of antibiotics have revolutionized medical practice in the twentieth century. The formal definition of an antibiotic distinguishes biochemicals that are produced by microorganisms from organic chemicals that are synthesized in the laboratory. This distinction is no longer meaningful because organic chemists can synthesize the biochemical structures of many naturally occurring antibiotics. Additionally, many antibiotics in current medical use are chemically modified forms of microbial biosynthetic products.

An antibiotic should have the following characteristics:

- It should be toxic to the infecting organism while harmless to the host cells and the microbiota of the host.
- It should stay in toxic form for a sufficient amount of time to affect the infecting microorganism. If it changes to another form or is broken down in the body, it may not be useful.

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- Sufficient amounts of it should reach the site of infection to kill the infecting agent.
- The infecting agent should be sensitive to it.

The determination of antibiotic susceptibility of a pathogen is important in selecting the most appropriate one for treating a disease. There are several different procedures used by clinical microbiologists to determine the sensitivity of microorganisms to antibiotics. Two such procedures are described below. The first one (the Kirby-Bauer Disc Method) is used to determine which antibiotic is the most effective against a certain pathogen. The second (MIC) is used to determine the lowest concentration that is needed to kill the pathogen at the site of infection.

The Kirby-Bauer Disc Method

This method is also called the agar diffusion method or the disk diffusion method. The procedure followed is simply that a filter disk impregnated with an antibiotic is applied to the surface of an agar plate containing the organism to be tested and the plate is incubated at 37°C for 24-48 hours. As the substance diffuses from the filter paper into the agar, the concentration decreases as a function of the square of the distance of diffusion. At some particular distance from each disk, the antibiotic is diluted to the point that it no longer inhibits microbial growth. The effectiveness of a particular antibiotic is shown by the presence of growth-inhibition zones. These zones of inhibition (ZOIs) appear as clear areas surrounding the disk from which the substances with antimicrobial activity diffused. The diameter of the ZOI can be measured with a ruler and the results of such an experiment constitute an antibiogram.

The agar diffusion method uses commercially available filter paper disks, each containing a defined concentration of a specific antibiotic. The relative effectiveness of different antibiotics provides the basis for a sensitivity spectrum of the organism. This information, together with various pharmacological considerations, is used in the selection of an antibiotic for treatment.

It should be emphasized that chemotherapeutic agents are not chosen simply on the basis of the drug producing the widest ZOI. This is because of the nature of the growth-inhibition substances. The size of the zone may be affected by the density or viscosity of the culture medium, the rate of diffusion of the antibiotic, the concentration of the antibiotic on the filter disc, the sensitivity of the organism to the antibiotic, and the interaction between the antibiotic and the medium. In addition, an agent that has been found to have a significant antibiotic effect may not be therapeutically useful because it may also have significant adverse effects in the system for which it is intended. The disk diffusion method represents a simple procedure for screening substances to determine if they have significant antibiotic activity.

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Interpretation of zones of inhibition (in mm) for Kirby-Bauer antibiotic susceptibility test.

Antibiotic	Disk Conc.	Diameter of zone of inhibition (ZOI)		
		Resistant	Intermediate	Susceptible
Amikacin	10 μg	≤11	12-13	≥14
Ampicillin	10 μg	≤11	12-13	≥14
Bacitracin	10 units	≤8	9-11	≥13
Cephalothin	30 μg	≤14	15-17	≥18
Chloramphenicol	30 μg	≤12	13-17	≥18
Clindamycin	2 μg	≤14	15-16	≥17
Erythromycin	15 μg	≤13	14-17	≥18
Gentamicin	10 μg	≤12	13-14	≥15
Kanamycin	30 μg	≤13	14-17	≥18
Lincomycin	2 μg	≤9	10-14	≥15
Methicillin	5 μg	≤9	10-13	≥14
Nalidixic acid	30 μg	≤13	14-18	≥19
Neomycin	30 μg	≤12	13-16	≥17
Nitrofurantoin	0.3 mg	≤14	15-16	≥17
Penicillin				
vs. staphylococci	10 units	≤20	21-28	≥29
vs. other organisms	10 units	≤11	12-21	≥22
Polymyxin	300 units	≤8	9-11	≥12
Streptomycin	10 μg	≤11	12-14	≥15
Sulfonamides	0.3 mg	≤12	13-16	≥17
Tetracycline	30 μg	≤14	15-18	≥19
Vancomycin	30 μg	≤9	10-11	≥12

The Minimum Inhibitory Concentration (MIC) Method

The minimum inhibitory concentration (MIC), which is the lowest concentration that still inhibits the growth of a particular organism, can be determined using serial dilution methods. This procedure establishes the concentration of an antibiotic that is effective in preventing the growth of the pathogen and gives an indication of the dosage of that antibiotic that should be effective in controlling the infection in the patient. A standardized microbial inoculum is added to the tubes containing serial dilutions of an antibiotic, and the growth of the microorganism is monitored as a change in turbidity. In this way, the break point, titer, or minimum inhibitory concentration (MIC) of the antibiotic that prevents growth of the microorganism at the site of infection can be determined.

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By knowing the MIC and the theoretical levels of the antibiotics that may be achieved in body fluids, such as blood and urine, the physician can select an appropriate antibiotic, the dosage schedule, and the route of administration. Generally, a margin of safety of ten times the MIC is desirable to ensure successful treatment of the disease.

The use of microtiter plates and automated inoculation and reading systems makes the determination of MIC feasible for use in the clinical laboratory. MIC can even be performed on normally sterile body fluids without isolating and identifying the pathogenic microorganisms. For example, blood or cerebrospinal fluid containing an infecting microorganism can be added to tubes containing various dilutions of an antibiotic and a suitable growth medium. An increase in turbidity would indicate that the microorganism is growing and that the antibiotic at that concentration was ineffective in inhibiting microbial growth. Conversely, a lack of growth would indicate that the pathogenic microorganisms were susceptible to the antibiotic at the given concentration.

III. LABORATORY SUPPLIES

Kirby-Bauer:

Cultures, 4 ml/tt	
Staph. aureus	1/table
E. coli	1/table
Jar of 95% Ethanol + forceps	2/table
BHI plates	2/group
Dispenser with antibiotic discs	1 of each kind/table
Sterile Swabs	2/group

MIC:

Cultures, diluted 100 times, 15 ml	
Staph. aureus	1/group
E. coli	1/group
TSB (Tryptic Soy Broth), 30 ml/bottle	1/group
Gentamicin, 100 μg/ml, 3 ml	1/group
Tetracycline HCl, 100 μg/ml, 3 ml	1/group
Microwell plates (24 well)	2/group

V. PROCEDURE (Students at each side of a table will form a group. Strict aseptic technique should be followed!)

The Kirby-Bauer Disc Method

1. Obtain 2 plates and the cultures of *E. coli* and *Staph. aureus*.

2. Obtain a swab and dip it into the *E. coli* broth culture. Roll the swab against the inside of the tube to remove excess liquid.

3. Streak one of the plates with the swab in even strokes to obtain a uniform growth pattern across the entire surface of the plate. Rotate the plate 90 degrees and using the same swab, streak the plate again. Rotate the plate 45 degrees and reswab. Replace the lid. Discard the swab. Label the plate.

4. Repeat the above procedure for *Staph. aureus* with a new plate.

5. Allow the plates to dry for 2-5 minutes.

6. Remove the forceps from the alcohol beaker and pass through the flame of a bunsen burner. When all the alcohol has burned off, use the sterile forceps to aseptically remove one of each antibiotic disc from the dispenser and place it on each plate. You can draw pie lines on the back to divide each plate into 6 sections. The antibiotic discs used are: gentamicin, tetracycline, penicillin G, chloramphenicol, ampicillin and erythromycin.

7. Repeat the alcohol-flame sterilization of the forceps and tap each disc gently onto the plate.

8. Replace the lid, and invert the plate. Complete the label at the bottom of plates and incubate at 37°C for 2 days.

9. Record the results by measuring the diameters of the zone of inhibition (ZOI). The data is recorded and interpreted using tables supplied at the introduction section of this lab exercise.

Reading and Interpretation

After incubation, the plates are examined and the diameter of the zones of inhibition is measured to the nearest whole millimeter by use of sliding calipers, a ruler, or a template prepared for this purpose. When supplemented medium is used, the measuring device is held on the back of the petri plate, which is illuminated with reflected light. Zones on blood-containing media are measured at the agar surface. The end point by all reading systems is complete inhibition of growth as determined visually, ignoring faint growth or tiny colonies which can be detected by very close scrutiny. Large colonies growing within the clear zone of inhibition may represent resistant variants or a mixed inoculum and may require re-identification and retesting. The zone diameters for individual antibiotics are translated into prefixed susceptible, intermediate, or resistant categories by referring to an interpretative table, such as the table seen in the Introduction.

The Minimum Inhibitory Concentration (MIC) Method

1. Label two titer plates, one for *Staph. aureus* and one for *E. coli*. In both plates, wells A1 to A6 and B1 to B6 are for Tetracycline (T), while wells C1 to C6 and D1 to D6 are for Gentamicin (G). Wells B6 and D6 are controls with no antibiotic.

2. Aseptically add 0.5 ml TSB to all wells except A1 and C1.

3. Aseptically add 0.5 ml tetracycline to wells A1 and A2 and 0.5 ml gentamicin to wells C1 and C2. Serial 2 fold dilutions are prepared in wells 3 through 6 in A and C and wells 1 through 5 in B and D (see the following table). A new pipette should be used for each mixing and transfer but for the sake of economy, you may do all your mixing and transfer with the same pipette.

4. Add 0.5 ml of 100 times diluted culture to each well. Your setup should be as shown in the following table.

5. Incubate 37°C for 2 days.

6. Calculate concentration of antibiotic per ml. The endpoint of the assay is the highest dilution of the antibiotic that is inhibitory for the microbe.

Tetracycline Well#	**Broth ml**	**Antibiotic 100 µg/ml**	**Diluted *E. coli* or *Staph. aureus***	**Antibiotic Conc. (µg/ml)**
A1	0	0.5	0.5	50
A2	0.5	0.5	0.5	
A3	0.5	0.5 from A2	0.5	
A4	0.5	0.5 from A3	0.5	
A5	0.5	0.5 from A4	0.5	
A6	0.5	0.5 from A5	0.5	
B1	0.5	0.5 from A6	0.5	
B2	0.5	0.5 from B1	0.5	
B3	0.5	0.5 from B2	0.5	
B4	0.5	0.5 from B3	0.5	
B5	0.5	0.5 from B4 (Discard 0.5 ml)	0.5	
B6	0.5	0	0.5	
Gentamycin Well#	**Broth ml**	**Antibiotic 100 µg/ml**	**Diluted *E. coli* or *Staph. aureus***	**Antibiotic Conc. (µg/ml)**
C1	0	0.5	0.5	50
C2	0.5	0.5	0.5	
C3	0.5	0.5 from C2	0.5	
C4	0.5	0.5 from C3	0.5	
C5	0.5	0.5 from C4	0.5	
C6	0.5	0.5 from C5	0.5	
D1	0.5	0.5 from C6	0.5	
D2	0.5	0.5 from D1	0.5	
D3	0.5	0.5 from D2	0.5	
D4	0.5	0.5 from D3	0.5	
D5	0.5	0.5 from D4 (Discard 0.5 ml)	0.5	
D6	0.5	0	0.5	

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Results of the Antibiotic Sensitivity Lab Exercises

NAME _____DATE _____ GROUP NAME _____

PARTNER(S) _____

1. Kirby-Bauer Test: Fill in the following table:

Microorganism	Antibiotic Name	Antibiotic Amount on disc	Diameter of ZOI	Interpretation* (R-I-S)
Staph. aureus	Tetracycline			
	Gentamicin			
	Penicillin G			
	Chloramphenicol			
	Ampicillin			
	Erythromycin			
E. coli	Tetracycline			
	Gentamicin			
	Penicillin G			
	Chloramphenicol			
	Ampicillin			
	Erythromycin			

*Refer to the table in the Introduction of this chapter.

Therefore, of the 6 antibiotics tested:
Staph. aureus is most sensitive to:
While *E. coli* is most sensitive to:

2. Based on Kirby-Bauer test, is *E. coli* more sensitive to gentamicin or tetracycline? What about *Staph. aureus*?

3. Based on MIC method, is *E. coli* more sensitive to gentamicin or tetracycline? What about *Staph. aureus*?

4. Look at the values of ZOI for tetracycline and gentamicin for each species and compare them with the titers of the antibiotics obtained in the MIC test. Are there any correlations? Discuss your findings.

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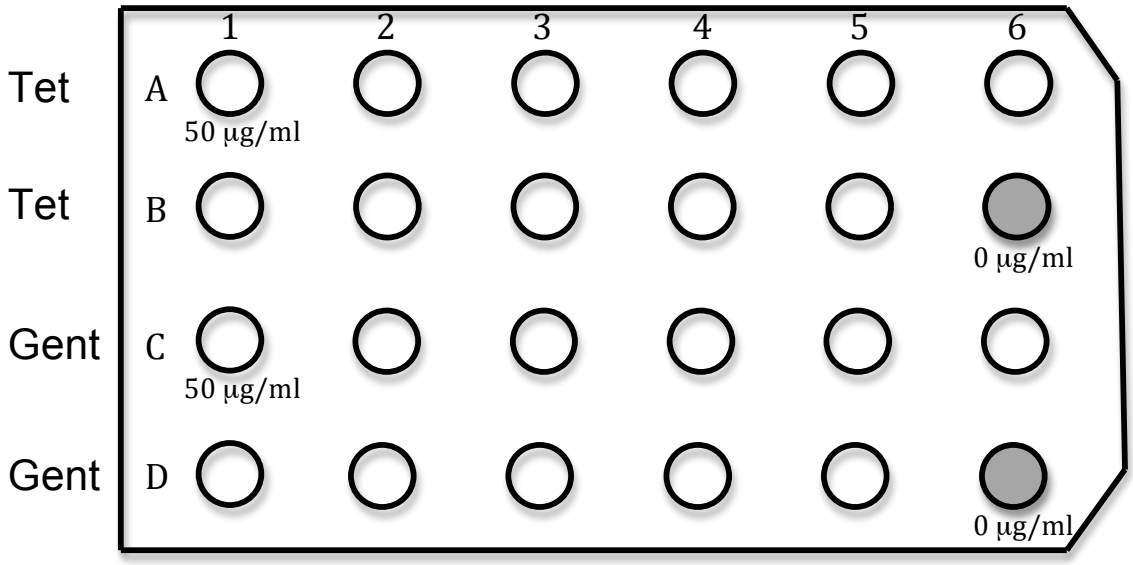
5. MIC Test: Write the concentration of antibiotic under each well in *μ*g/ml (Those for the first and last wells of each antibiotic are given). Shade the wells in which growth occurred (The control wells are already shaded).

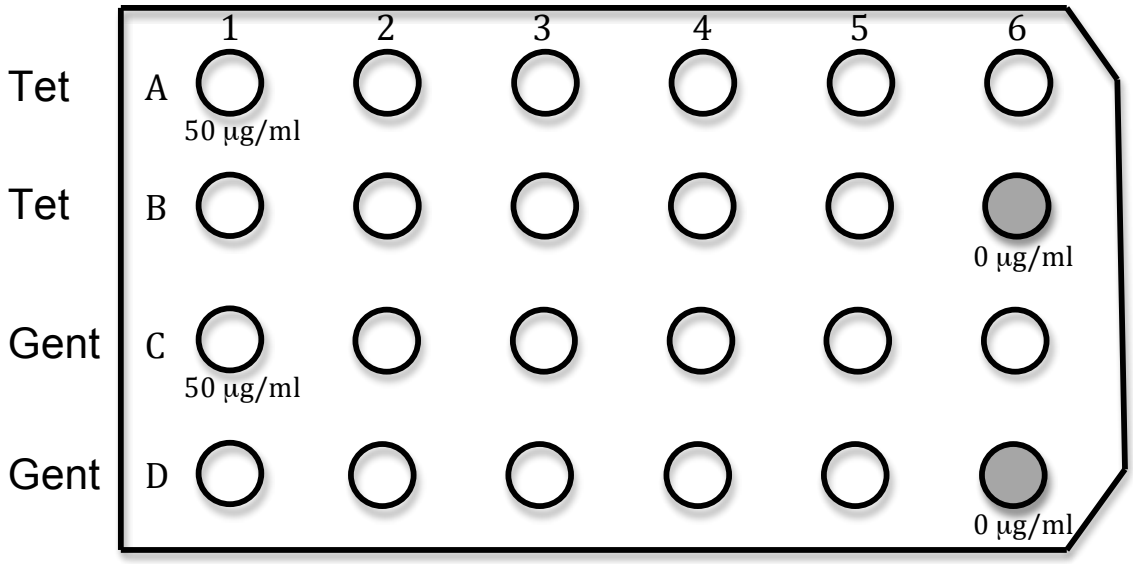
For *Staph. aureus*:



MIC of tetracycline for *Staph. aureus* = Well # _____ = _____ *μ*g/ml.

MIC of gentamicin for *Staph. aureus* = Well # _____ = _____ *μ*g/ml.

For *E. coli*:



MIC of tetracycline for *E. coli* = Well # _____ = _____ *μ*g/ml.

MIC of gentamicin for *E. coli* = Well # _____ = _____ *μ*g/ml.