MICROBIOLOGY

Antimicrobic Sensitivity Testing:
The Kirby-Bauer Method

EXERCISE 34

Learning Outcomes

After completing this exercise, you should be able to

1. Define the terms *antimicrobial, antibiotic, semi-synthetic,* and *synthetic* as they relate to agents used to treat bacterial infections.
2. Demonstrate the effectiveness of antimicrobials using the Kirby-Bauer method.

Antimicrobials and antibiotics have been our first line of defense in the battle to conquer infectious diseases. Diseases caused by *Staphylococcus, Streptococcus, Pseudomonas,* and *Mycobacterium* that once were fatal to humans became manageable and curable with the discovery of antibiotics. Alexander Fleming's observation in 1929 that a species of *Penicillium* growing on an agar plate with *Staphylococcus aureus* inhibited the bacterium led to the subsequent purification of penicillin by Howard Florey, Norman Heatley, and Ernst Chain and the development of penicillin as the first antibiotic to be used in the clinical treatment of bacterial infections. Other antibiotics were subsequently found and were added to the list that could be used to treat bacterial diseases. Prior to the discovery of antibiotics, very few options were available to treat infectious agents except for some antimicrobial chemical compounds such as salvarsan, an arsenic compound, and the sulfa drugs, which were used with some success. Today we have a vast number of compounds that are effective against bacterial, fungal, and some viral agents. By definition, **antimicrobials** are compounds that kill or inhibit microorganisms. **Antibiotics** are antimicrobials, usually of low molecular weight, produced by microorganisms that inhibit or kill other microorganisms. Two common examples are penicillin and streptomycin. Many times antibiotics are chemically altered to make them more effective in their mode of action. These are referred to as **semi-synthetics.** Some antimicrobials are chemically synthesized in the laboratory and are not produced by microbial biosynthesis. The sulfa drugs are **synthetics** that were used to treat some bacterial diseases before the discovery of penicillin.

Our perceived defeat of the infectious microbe, however, has been short-lived. An increasing problem in medicine at the present time is the development of antibiotic-resistant strains of bacteria such as *Staphylococcus aureus, Pseudomonas aeruginosa, Mycobacterium tuberculosis, Neisseria gonorrhoeae,* and *Enterococcus* that do not respond to the antibiotics that have been normally used to treat these bacteria. *Staphylococcus aureus* has become especially problematic because both hospital and community strains of MRSA (MRSA: methicillin-resistant *S. aureus*) have been isolated that do not respond to penicillin, and hence other and more expensive drugs such as vancomycin must be used to manage infections caused by these organisms. As might be expected, strains of *Staphylococcus* and *Enterococcus* have also developed resistance to vancomycin. In hospitals MRSA is responsible for many **health-care acquired infections (HAIs),** formerly known as nosocomial infections because they were acquired in hospitals. In 2002 the Centers for Disease Control (CDC) estimated that there were 1.7 million HAIs in the United States. Penicillin has long been the drug of choice to treat gonorrhea. However, penicillin-resistant *N. gonorrhoeae* strains have been isolated that require more expensive drugs to treat this sexually transmitted disease. Strains of *Mycobacterium tuberculosis* have been isolated from street people and drug addicts that do not respond to any antimicrobials available. Needless to say, antibiotic resistance is a major public health problem affecting the treatment and spread of disease.

When a patient seeks medical assistance for a bacterial infection, the physician will normally take a conservative approach and prescribe an antimicrobial without isolating the pathogen. The physician bases the choice of antimicrobial on symptoms presented by the patient, probable causative agents associated with the symptoms, and drugs known to be effective against these pathogens. Usually this approach is sufficient to treat the infection, but occasionally a pathogen will not respond to the prescribed antimicrobial. It is then crucial that the causative agent be isolated in pure culture, specifically identified, and tested for its sensitivity and resistance to various antimicrobial

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agents. Antimicrobial agents can vary in their effectiveness against various pathogenic bacteria. Some antimicrobial agents are narrow in their spectrum and may be more effective against gram-positive bacteria, while others are more effective against gram-negative bacteria. Broad-spectrum antimicrobials are effective against both kinds of organisms. Based on test results, an antimicrobial that the pathogen is sensitive to would then be prescribed to treat the infection.

Whether an antimicrobial has a broad or narrow spectrum depends appreciably on its mode of action and its ability to be transported into the cell. Antimicrobials have different modes of action, and they affect different aspects of bacterial cell metabolism. They can target cell wall synthesis (penicillin), DNA and RNA synthesis (cipro, rifampin), protein synthesis (tetracyclines, streptomycin), and vitamin synthesis (sulfa drugs). For example, antibiotics that target 70S ribosomes tend to be broad spectrum because all bacteria have 70S ribosomes, which are necessary for protein synthesis. Sulfa drugs are more narrow in spectrum because not all bacterial cells can synthesize the vitamin folic acid, whose synthesis is inhibited by this drug. Another factor that affects the ability of an antibiotic to function is permeability. The outer membrane of gram-negative bacteria acts as a permeability barrier and can restrict the entry of antimicrobials into the cell.

Table 34.1 summarizes the modes of action of selected antimicrobials.

The **Kirby-Bauer method** (figure 34.1) is used to determine the sensitivity or resistance of a bacterium to an antimicrobial. This is a standardized test procedure that is reliable, relatively simple, and yields results in as short a time as possible. It is performed by uniformly streaking a standardized inoculum of the test organism on Mueller-Hinton medium, and then paper disks containing specific concentrations of an antimicrobial or antibiotic are deposited on the agar surface. The antimicrobial diffuses out from the disk into the agar, forming a concentration gradient. If the agent inhibits or kills the test organism, there will be a zone around the disk where no growth occurs called the **zone of inhibition** (figure 34.2). This zone can vary, however, with the diffusibility of the agent, the size of the inoculum, the type of medium, and other factors. All of these factors were taken into consideration in developing this test. The Kirby-Bauer method is sanctioned by the U.S. FDA and the Subcommittee on Antimicrobial Susceptibility Testing of the National Committee for Clinical Laboratory Standards. Although time is insufficient here to consider all facets of this test, its basic procedure will be followed to test both antibiotics and antimicrobials.

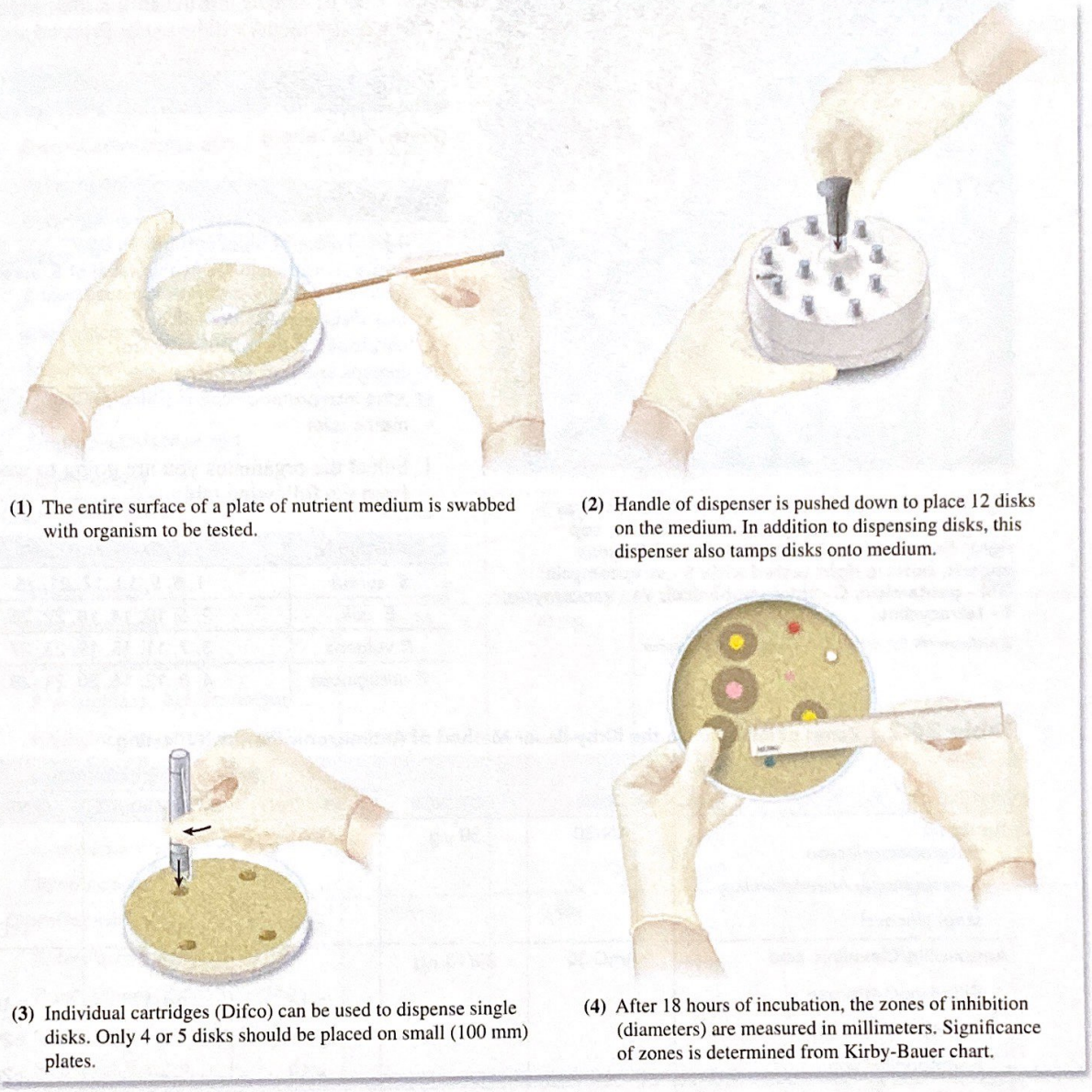
The recommended medium in this test is Mueller-Hinton II agar. Its pH should be between 7.2 and 7.4, and it should be poured to a uniform thickness of 4 mm in the petri plate. This requires 60 ml in a 150 mm plate and 25 ml in a 100 mm plate. For certain fastidious microorganisms, 5% defibrinated sheep's blood is added to the medium.

Inoculation of the surface of the medium is made with a cotton swab from a broth culture. In clinical applications, the broth turbidity has to match a defined standard. Care must also be taken to express excess broth from the swab prior to inoculation.

High-potency disks are used that may be placed on the agar with a mechanical dispenser or sterile forceps. To secure the disks to the medium, it is necessary to press them down onto the agar.

Table 34.1 Modes of Action of Antibiotics

ANTIBIOTIC	CELLS AFFECTED	CELL TARGET/SPECIFIC SITE
Penicillin	G+; G-	Cell wall/β-lactamase, peptidoglycan synthesis—amino acid side chain
Vancomycin	G+	Cell wall/Peptidoglycan synthesis
Bacitracin	G+	Cell wall/Transport of peptidoglycan monomer
Isoniazid	*Mycobacterium tuberculosis*	Cell wall/Mycolic acid synthesis in *Mycobacterium*
Fluroquinolones	G+; G-	DNA/Topoisomerase unwinding of DNA in DNA synthesis
Rifamycins	G+; some G-	RNA/RNA polymerase in RNA synthesis
Tetracyclines	G+; G-	Protein synthesis/30S subunit of 70S ribosome
Streptomycin	G+; G-	Protein synthesis/30S subunit of 70S ribosome
Chloramphenicol	G+; G-	Protein synthesis/50S subunit of 70S ribosome
Sulfa drugs	G+; G-	Structural analogue of para-amino benzoic acid (PABA)—inhibit enzyme linking pteridine to PABA in folic acid synthesis



(1) The entire surface of a plate of nutrient medium is swabbed with organism to be tested.

(2) Handle of dispenser is pushed down to place 12 disks on the medium. In addition to dispensing disks, this dispenser also tamps disks onto medium.

(3) Individual cartridges (Difco) can be used to dispense single disks. Only 4 or 5 disks should be placed on small (100 mm) plates.

(4) After 18 hours of incubation, the zones of inhibition (diameters) are measured in millimeters. Significance of zones is determined from Kirby-Bauer chart.

Figure 34.1 **Antimicrobic sensitivity testing.**

After 16 to 18 hours of incubation, the plates are examined and the diameters of the zones of inhibition are measured to the nearest millimeter. Diameters for tested bacteria are compared to those in a table that are based on values obtained for ATCC (American Type Culture Collection) reference cultures that are sensitive to the specific antibiotic. Cultures are designated as resistant, sensitive, or intermediate in their response to the antibiotic. These designations are derived by comparison to the response of the reference culture. (To determine the significance of the zone diameters, consult table 34.2.)

In this exercise, we will work with four microorganisms: *Staphylococcus aureus, Escherichia coli, Proteus vulgaris,* and *Pseudomonas aeruginosa.* Each student will inoculate one plate with one of the four organisms and place the disks on the medium by whichever method is available. Since each student

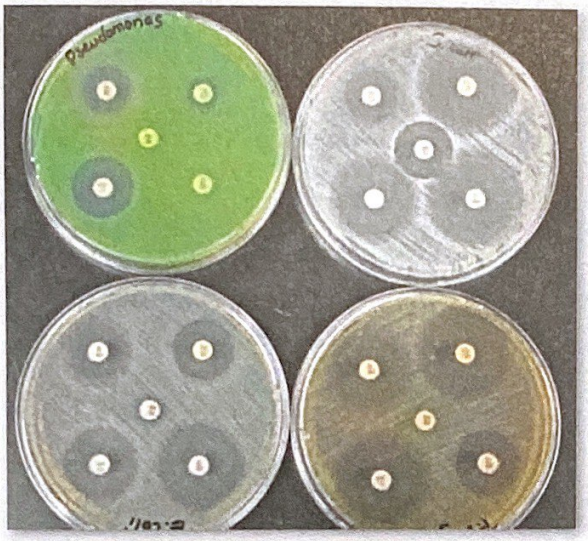


Figure 34.2 **Kirby-Bauer plates for *Pseudomonas aeruginosa*, top left; *Staphylococcus aureus*, top right; *Escherichia coli*, bottom left; and *Proteus vugaris*, bottom right tested with: S - streptomycin; GM - gentamicin; C - chloramphenicol; Va - vancomycin; T - tetracycline.**

will be doing only a portion of the total experiment, student assignments will be made. Proceed as follows:

First Period

(Plate Preparation)

Materials

- 1 petri plate of Mueller-Hinton II agar
- nutrient broth cultures (with swabs) of *S. aureus*, *E. coli*, *P. vulgaris*, and *P. aeruginosa*
- disk dispenser (BBL or Difco)
- cartridges of disks (BBL or Difco)
- forceps and Bunsen burner
- zone interpretation charts (Difco or BBL)
- metric ruler

1. Select the organisms you are going to work with from the following table.

ORGANISM	STUDENT NUMBER
S. aureus	1, 5, 9, 13, 17, 21, 25
E. coli	2, 6, 10, 14, 18, 22, 26
P. vulgaris	3, 7, 11, 15, 19, 23, 27
P. aeruginosa	4, 8, 12, 16, 20, 24, 28

Table 34.2 Zones of Inhibition in the Kirby-Bauer Method of Antimicrobic Sensitivity Testing

ANTIBIOTIC	CODE	POTENCY	Zone of Inhibition (mm)		
			RESISTANT	INTERMEDIATE	SENSITIVE
Amikacin	AN-30	30 μg			
Enterobacteriaceae					
P. aeruginosa, Acinetobactor					
staphylococci			≤14	15–16	≥17
Amoxicillin/Clavulinic acid	AmC-30	20/10 μg			
Enterobacteriaceae			≤13	14–17	≥18
Staphylococcus spp.			≤19	—	≥20
Haemophilus spp.			≤19	—	≥20
Ampicillin	AM-10	10 μg			
Enterobacteriaceae			≤13	14–16	≥17
Staphylococcus spp.			≤28	—	≥29
Enterococcus spp.			≤16	—	≥17
Listeria monocytogenes			≤19	—	≥20
Haemophilus spp.			≤18	19–21	≥22
β-hemolytic streptococci			—	—	≥24
Aziocillin	AZ-75	75 μg			
P. aeruginosa			≤17	—	≥18
Bacitracin	B-10	10 units	≤8	9–12	≥13

Table 34.2 Zones of Inhibition in the Kirby-Bauer Method of Antimicrobic Sensitivity Testing (continued)

ANTIBIOTIC	CODE	POTENCY	Zone of Inhibition (mm)		
			RESISTANT	INTERMEDIATE	SENSITIVE
Carbenicillin	CB-100	100 μg			
Enterobacteriaceae and *Acinetobacter*			≤19	20–22	≥22
P. aeruginosa			≤13	14–16	≥17
Cefaclor	CEC-30	30 μg			
Enterobacteriaceae and staphylococci			≤14	15–17	≥18
Haemophilus spp.			≤16	17–19	≥20
Cefazolin	CZ-30	30 μg			
Enterobacteriaceae and staphylococci			≤14	15–17	≥18
Cephalothin	CF-30	30 μg			
Enterobacteriaceae, and staphylococci			≤14	15–17	≥18
Chloramphenicol	C-30	30 μg			
Enterobacteriaceae, *P. aeruginosa*, *Acinetobactor*, staphylococci, enterococci, and *V. cholerae*			≤12	13–17	≥18
Haemophilus spp.			≤25	26–28	≥29
S. pneumoniae			≤20	—	≥21
Streptococci			≤17	18–20	≥21
Ciprofloxacin	CIP-5	5 μg			
Enterobacteriaceae, *P. aeruginosa*, *Acinetobacter*, staphylococci and enterococci			≤15	16–20	≥21
Haemophilus spp.			—	—	≥21
N. gonorrhoeae			≤27	28–40	≥41
Clarithromycin	CLR-15	15 μg			
Staphylococcus spp.			≤13	14–17	≥18
Haemophilus spp.			≤10	11–12	≥13
S. pneumoniae and other streptococci			≤16	17–20	≥21
Clindamycin	CC-2	2 μg			
Staphylococcus spp.			≤14	15–20	≥21
S. pneumoniae and other streptococci			≤15	16–18	≥19

(continued)

Table 34.2 Zones of Inhibition in the Kirby-Bauer Method of Antimicrobic Sensitivity Testing (continued)

ANTIBIOTIC	CODE	POTENCY	Zone of Inhibition (mm)		
			RESISTANT	INTERMEDIATE	SENSITIVE
Doxycycline	D-30	30 μg			
Enterobacteriaceae, *P. aeruginosa*, *Acinetobactor*, staphylococci and enterococci			≤12	13–15	≥16
Erythromycin	E-15	15 μg			
Staphylococcus spp. and enterococci			≤13	14–22	≥23
S. pneumoniae and other streptococci			≤15	16–20	≥21
Gentamicin	GM-120	120 μg			
Enterobacteriaceae					
P. aeruginosa, *Acinetobacter*, and staphylococci			≤12	13–14	≥15
Imipenem	IPM-10	10 μg			
Enterobacteriaceae, *P. aeruginosa*, *Acinetobacter*, and staphylococci			≤13	14–15	≥16
Haemophilus spp.			—	—	≥16
Kanamycin	K-30	30 μm			
Enterobacteriaceae and staphylococci			≤13	13–17	≥18
Lomefloxacin	LOM-10	10 μg			
Enterobacteriaceae, *P. aeruginosa*, *Acinetobacter*, and staphylococci			≤18	19–21	≥22
Haemophilus spp.			—	—	≥22
N. gonorrhoeae			≤26	27–37	≥38
Loracarbef	LOR-30	30 μg			
Enterobacteriaceae and staphylococci			≤14	15–17	≥18
Haemophilus spp.			≤15	16–18	≥19
Meziocillin	MZ-75	75 μg			
Enterobacteriaceae and *Acinetobacter*			≤17	18–20	≥21
P. aeruginosa			≤15	—	≥16

Table 34.2 Zones of Inhibition in the Kirby-Bauer Method of Antimicrobic Sensitivity Testing (continued)

ANTIBIOTIC	CODE	POTENCY	Zone of Inhibition (mm)		
			RESISTANT	INTERMEDIATE	SENSITIVE
Minocycline	MI-30	30 μg			
Enterobacteriaceae, *P. aeruginosa*, *Acinetobacter*, staphylococci, and enterococci			≤14	15–18	≥19
Moxalactam	MOX-30	30 μg			
Enterobacteriaceae, *P. aeruginosa*, *Acinetobacter*, and staphylococci			≤14	15–22	≥23
Nafcillin	NF-1	1 μg			
Staphylococcus aureus			≤10	11–12	≥13
Nalidixic Acid	NA-30	30 μg			
Enterobacteriaceae			≤13	14–18	≥19
Neomycin	N-30	30 μg	≤12	13–16	≥17
Netilmicin	NET-30	30 μg			
Enterobacteriaceae, *P. aeruginosa*, *Acinetobacter* and staphylococci			≤12	13–14	≥15
Norfloxacin	NCR-10	10 μg			
Enterobacteriaceae, *P. aeruginosa*, *Acinetobacter*, staphylococci and entercocci			≤12	13–16	≥17
Novobiocin	NB-30	30 μg	≤17	18–21	≥22
Oxacillin	OX-1	1 μg			
Staphylococcus aureus			≤10	11–12	≥13
staphylococcus (coagulase negative)			≤17	—	≥18
Penicillin	P-10	10 units			
Staphylococcus spp.			≤28	—	≥29
Enterococcus spp.			≤14	—	≥15
L. monocytogenes			≤19	20–27	≥28
N. gonorrhoeae			≤26	27–46	≥47
β-hemolytic streptococci			—	—	≥24
Piperacillin	PIP-100	100 μg			
Enterobacteriaceae, and *Acinetobacter*			≤17	18–20	≥21
P. aeruginosa			≤17	—	≥18

Table 34.2 Zones of Inhibition in the Kirby-Bauer Method of Antimicrobic Sensitivity Testing (continued)

ANTIBIOTIC	CODE	POTENCY	Zone of Inhibition (mm)		
			RESISTANT	INTERMEDIATE	SENSITIVE
Polymyxin B	PB-300	300 U	≤8	9–11	≥12
Rifampin	RA-5	5 μg			
Staphylococcus spp.			≤16	17–19	≥20
Enterococcus spp. and *Haemophilis* spp.			≤16	17–18	≥19
S. pneumoniae					
Spectinomycin	SPT-100	100 μg			
N. gonorrhoeae			≤14	15–17	≥18
Streptomycin	S-300	300 μg			
Enterobacteriaceae			≤11	12–14	≥15
Sulfisoxazole	G-25	250 μg			
Enterobacteriaceae, *P. aeruginosa*, *Acinetobacter*, *V. cholerae*, and staphylococci			≤12	13–16	≥17
Tetracycline	Te-30	30 μm			
Enterobacteriaceae, *P. aeruginosa*, *Acinetobacter*, *V. cholerae*, staphylococci and enterococci			≤14	15–18	≥19
Haemophilus spp.			≤25	26–28	≥29
N. gonorrhoeae			≤30	31–37	≥38
S. pneumoniae and other streptococci			≤18	19–22	≥23
Tobramycin	NN-10	10 μg			
Enterobacteriaceae, *P. aeruginosa*, *Acinetobacter*, and staphylococci			≤12	13–14	≥15
Trimethoprim	TMP-5	5 μg			
Enterobacteriaceae and staphylococci			≤10	11–15	≥16
Vancomycin	Va-30	30 μg			
Staphylococcus spp.			—	—	≥15
Enterococcus spp.			≤14	15–16	≥17
S. pneumoniae and other streptococci			—	—	≥17

2. Label your plate with the name of your organism.
3. Inoculate the surface of the medium with the swab after expressing excess fluid from the swab by pressing and rotating the swab against the inside walls of the tube above the fluid level. Cover the surface of the agar evenly by swabbing in three directions. A final sweep should be made of the agar rim with the swab.
4. Allow **3 to 5 minutes** for the agar surface to dry before applying disks.
5. Dispense disks as follows:
   a. If an automatic dispenser is used, remove the lid from the plate, place the dispenser over the plate, and push down firmly on the plunger. With the sterile tip of forceps, tap each disk lightly to secure it to medium.
   b. If forceps are used, sterilize them first by flaming before picking up the disks. Keep each disk at least 15 mm from the edge of the plate. Place no more than 13 on a 150 mm plate, no more than 5 on a 100 mm plate. Apply light pressure to each disk on the agar with the tip of a sterile forceps or inoculating loop to secure it to the medium (see figure 34.1).
6. Invert and incubate the plate for 16 to 18 hours at 37°C.

Second Period

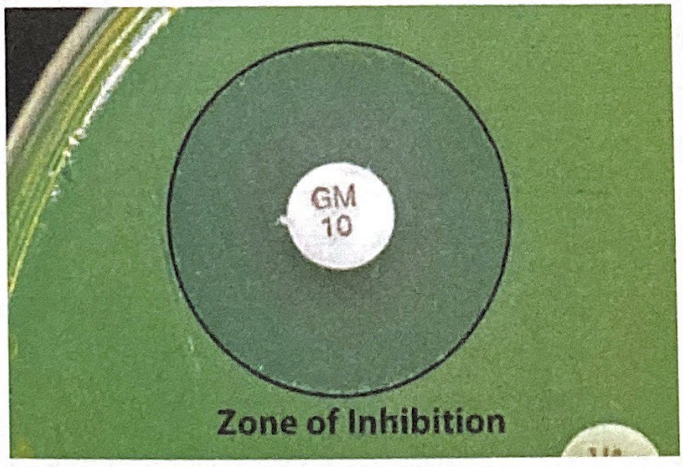
(Interpretation)

After incubation, measure the zone diameters with a metric ruler to the nearest whole millimeter (figure 34.3). The disk is included in the measurement. The zone of complete inhibition is determined without magnification. Ignore faint growth or tiny colonies that can be detected by very close scrutiny. Large colonies growing within the clear zone might represent resistant variants or a mixed inoculum and may require reidentification and retesting in clinical situations. Ignore the "swarming" characteristics of *Proteus*, measuring only to the margin of heavy growth.

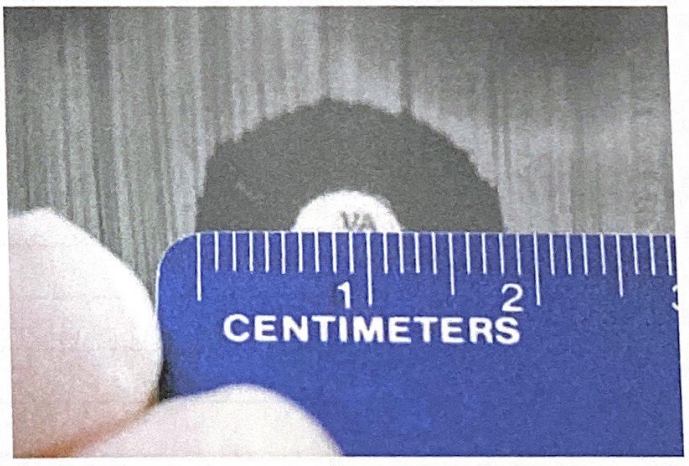
Record the zone measurements on the table of Laboratory Report 34 and on the chart on the demonstration table, which has been provided by the instructor.

Use table 34.2 for identifying the various disks.

To determine which antibiotics your organism is sensitive to (S), or resistant to (R), or intermediate (I), consult table 34.2. It is important to note that the significance of a zone of inhibition varies with the type of organism. If you cannot find your antibiotic on the chart, consult a chart that is supplied on the demonstration table or bulletin board. Table 34.2 is incomplete.



(a)



(b)

Figure 34.3 **(a) Zone of inhibition for gentamicin on *Pseudomonas aeruginosa*. (b) Measuring the zone of inhibition.**

2. Which antimicrobics would be suitable for the control of the following organisms?

   S. aureus: _____

   E. coli: _____

   P. vulgaris: _____

   P. aeruginosa: _____

B. Short-Answer Questions

1. Differentiate between the following and provide one example of each:

   a. antibiotics and antimicrobial drugs

   _____

   b. broad- and narrow-spectrum antibiotics

   _____

2. What factors influence the size of the zone of inhibition for an antibiotic?

   _____

   _____

3. Why are certain gram-negative bacteria more resistant than gram-positive bacteria to antibiotics that attack cytoplasmic targets?

   _____

   _____

4. Why are gram-positive bacteria typically more resistant than gram-negative bacteria to antibiotics that disrupt plasma membranes, such as polymyxin B?

   _____

   _____

5. If a bacterial isolate shows intermediate to moderate resistance to an antibiotic, how might this antibiotic still be successfully used in the treatment of this microbe?

   _____

   _____

6. What specific medium must be used in testing the effectiveness of antibiotics?

   _____

   _____