

Antibiotics in Action

Background

Antibiotics and Man

The term **antibiotic** was originally coined to describe chemicals produced by microorganisms with the capacity to kill or to inhibit the growth of other microorganisms. Today the definition also includes **antimicrobial compounds**, which are produced not in nature but in the laboratory. There are three kinds of antibiotics in use in the medical field today:

1. Natural antibiotics such as penicillin, which are produced by natural agents such as fungi.
2. Semi-synthetic antibiotics such as ampicillin, which are chemically-modified variants of natural antibiotics, altered to enhance their efficacy, lower their side effects, or alter the range of microbes that are susceptible to them.
3. Synthetic antibiotics such as the sulfonamides, which are designed and produced in the laboratory.

Antibiotics work in different ways. They can be **bacteriostatic**, in that they inhibit microbial cell replication and thus give the host a chance to activate its immune response and clear the infection. Antibiotics can also be **bactericidal**, in that they cause the microbes to die. The spectrum of activity of each antibiotic reflects the range of microorganisms that are susceptible to the antibiotic. Antibiotics can be specific to one or a select few species, or they can be general, **broad range antibiotics**, meaning they affect a wide range of microbes. The susceptibility of microbes to antibiotics depends upon their genetic characteristics and (in a patient) the site of infection. A successful antibiotic should have as much **selective toxicity** as possible, i.e., it should destroy the **pathogen** (the disease-causing microbe) yet not harm the host. There are no completely selective antibiotics; all of them have some effect on the host. However, the widely used penicillin G comes close; it is selective for inhibiting bacterial peptidoglycan cell wall synthesis, a process absent in mammalian cells. However, some people are allergic to penicillin and cannot use penicillin G.

It is important to note that antibiotics, which prevent the growth or reproduction of bacteria within or on an organism, are distinct from antiseptics, which are only used externally to discourage the growth of microorganisms, and from disinfectants, which are substances used to prevent the growth or reproduction of microbes on inanimate objects.

The human body contains numerically more microbial cells than human cells, and if a microbe ends up in the wrong location, there is potential for disease. Our first line of defense is our skin, which prevents many microbes from entering our bodies. Similarly, our mucous membranes act as traps for microbes and provide our immune system a chance to destroy them. If microbes get past these first lines of defense, additional immune defenses come into play to eliminate the microbes from our body. In fact, most of the time we have been infected, we don't even realize it! However, if our defenses alone aren't effective, we make our way to a physician and look for a quick cure...

A physician considers several factors before deciding on a course of action. These include

- the patient's condition. What is ailing the patient, and where? Also, what condition is the patient in (e.g., general health, age, genetic risk factors, previous drug interactions, allergies, etc.)?
- the causative agent for the ailment. Gram staining, culturing, and other testing can identify the causative agent.

- the microbe's sensitivity, if any. Knowing the microbe's drug sensitivity enables the physician to use the best drug as well as the proper dosage. Giving the proper dosage of the drug means using the correct amount of drug over a long enough period of time such that the pathogen is cleared from the patient's body. Determining the proper dose is aided by knowing the **minimum inhibitory concentration** (MIC) and/or the **minimum bactericidal concentration** (MBC) of an antibiotic with respect to the pathogen. The minimum inhibitory concentration of an antimicrobial agent is the lowest concentration at which visible microbial growth is inhibited. The minimum bactericidal concentration is the lowest concentration of antibiotic needed for it to be lethal within a defined period for 99% of the isolated pathogen tested.

Chemotherapy, the use of chemical compounds (including herbal remedies) to treat diseases has been in use since ancient times. However, antibiotics were first discovered in the 1930s through the famous accident of Sir Alexander Fleming, in which the area around a contaminating mold (later named *Penicillium notatum*) on an old petri plate was found to be free of the staphylococcus bacteria Fleming was growing. Further investigation into the phenomenon led to the discovery of penicillin and, later, other antimicrobial agents. Initially, crude extracts were used, but in time, the pure antimicrobial compounds were isolated and identified. These "miracle drugs" were used effectively to treat many ailments. With the aid of antibiotics, patients who otherwise would have succumbed to sepsis (a toxic condition that can result when bacteria or bacterial toxins spread beyond the focus of the initial infection) were often able to make a full recovery.

Antibiotics have gained widespread use, as well as, some would argue, overuse. In agriculture, livestock animals, some used for human consumption, are often fed low doses of antibiotics prophylactically. Initially, it was believed that the general health and growth of the animals would increase, because the antibiotics would inhibit harmful microbes that would otherwise stunt the growth of the animal. The question of risk versus benefit for these practices has since been hotly debated.

Because of the prolonged use of most antibiotics, some microbial strains are no longer susceptible to these miracle drugs. Why is this? One mechanism is through natural selection of spontaneously occurring **resistant** strains that can develop from an originally antibiotic-sensitive strain. These spontaneously occurring resistant strains arise through a natural mutation process. This mechanism of developing drug resistance may take a while, because resistant strains may rarely arise, or a patient's immune system may eliminate the mutated sensitive strain before it has a chance to multiply. Another mechanism of acquiring antibiotic resistance is through transfer from another microbe of a **plasmid** containing an antibiotic-resistance gene. Plasmids are circular, extrachromosomal pieces of DNA that can be transferred from microorganism to microorganism. Plasmids containing antibiotic resistance genes may also be referred to as **resistance factors**, or R factors. In an environment in which antibiotics are present, a microorganism acquiring resistance to the antibiotic via either of these mechanisms has a selective advantage over those that do not, and becomes the predominant strain. Because of the prevalence of antibiotic use in hospitals, many "hospital strains" of microbes have become resistant to one or more antibiotics. This creates a major problem for health care providers and their patients.

Because of the appearance of new infectious agents and dangerous, antibiotic resistant strains, studying the basis of antibiotic resistance and development of new antibiotics are extremely important. Academic researchers and pharmaceutical companies are seeking more and novel antibiotics by using modern tools, including synthetic chemistry processes, computational drug design, and **metagenomics**. Metagenomics is the study of genomes from mixed populations of organisms gleaned directly from the environment, and is being used to discover novel genes and proteins.

Measuring Antibiotic Susceptibility Using Antibigrams

In the course of the search for new antibiotics, scientists use multiple tools and techniques. One of these tools is the **antibiogram**, an assay used to determine a microbe's sensitivity to antibiotics. Antibigrams are also used in the health care and agricultural fields. In the agricultural field, they are used to examine strains of infectious agents found on and in animal patients as well as in food products. In medicine, they are often used to determine the best drug to treat a patient who has an infection.

In the clinical setting, the **disk diffusion test** (also called the Kirby-Bauer test) is used to create the antibigrams. The test requires that certain conditions be held constant [e.g., young cultures (2–5 hours old) must be used at particular concentrations or at a particular optical density, and must be incubated for a specific length of time, etc.]. To set up the test, cultures are plated onto agar plates that will support the growth of the culture. Antibiotic-impregnated disks are then placed on the agar surface. As the microbes grow to create a lawn, their growth is inhibited to varying degrees by the different antibiotics diffusing from each of the various disks. As a result, in areas around some of the antibiotic disks, **zones of clearing** can be observed. The size of these zones depends on several conditions, including

1. the microbe's sensitivity to the antibiotic.
2. the rate of antibiotic diffusion through the medium.
3. the rate of microbial growth.
4. the type of culture medium.
5. the growth conditions (e.g., temperature).
6. the concentration of microbes in the inoculation medium.
7. the age of the culture.

Which antibiotic disks the zones of clearing are around, and the size of these zones (which can indicate the MIC), are critical in determining which antibiotic and what concentration should be used for treatment. However, as discussed above, in order to obtain accurate and clinically useful information from this test, specific and multiple conditions are standardized and carefully controlled.

In this laboratory exercise, we will create an antibiogram using the halophilic microbe, *Halobacterium* sp. NRC-1. This organism requires a high-salt environment to thrive and is different from most of the microbes encountered in a doctor's office or hospital. Its genome has been fully sequenced and studied, and it is not known to cause any human diseases. Not only that, it will lyse at salinities lower than 2.5 M NaCl, even in seawater.

Halobacterium sp. NRC-1 in the Classroom

Halobacterium sp. NRC-1 is an ideal microbe with which to study antibiotic action. There is no risk of passing on to a human pathogen any antibiotic resistance that may arise during the experiment, as could occur if working with a strain (however nonpathogenic) that grows under the same conditions as many pathogens. This is because *Halobacterium* sp. NRC-1 will not grow in the same medium as most of the more commonly encountered microbes that are pathogenic to humans, and because most human pathogens cannot grow on the hypersaline (high salt) medium required by *Halobacterium* sp. NRC-1.

You will study the effects of the antibiotics ampicillin, bacitracin, novobiocin, and tetracycline on *Halobacterium* sp. NRC-1.

Sterile Technique

1. Wipe your work area with a disinfectant such as alcohol or bleach, and then wash your hands with soap and water.
2. Put on gloves (if provided by your instructor).
3. Light the Bunsen burner. The openings or "lips" of test tubes and flasks must be flamed during the transfer of media or cultures. Heating the container directs air convection currents upward and away from the opening, momentarily preventing airborne contaminants from entering. It is essential to perform the transfer quickly, before the opening cools.
4. Hold the test tube at a 45° angle in your left hand if you are right-handed, or in your right hand if you are left-handed. Use your other hand to hold the transferring instrument (in this case, a pipet). Use the little finger of the hand not holding the test tube to remove and hold the cap of the culture container. Flame the top of the test tube. Take out the inoculum (the culture to be transferred), re flame the opening of the culture container, and replace the cap.
5. When working with petri plates, always hold the lid over the plate to prevent contaminants from landing on the surface of the agar. Always replace the lid as soon as possible. Realize that the lid is designed to fit loosely to allow diffusion of air around the edges, while minimizing the possibility of contamination. When you lift the lid, hold it directly over the plate. Never place the lid on the bench top.
6. Turn off the burner.
7. Once again, wipe your work area with a disinfectant, and then wash your hands thoroughly.

Lab 1: Establishing a Lawn of *Halobacterium* sp. NRC-1

Procedure

1. Turn over the petri plate in order to label the bottom of the plate as follows:
 - a. Divide the plate into quarters using an indelible marker and a ruler.
 - b. On the outer edge of one segment, write "CT." In this quarter of the plate, you will place your control disk. On the outer edge of the other three segments, write the name or abbreviation for the three antibiotics you will be testing.
 - c. In small letters along the edge of the plate, write your initials, station number, or team name (as directed by your teacher), and the current date.
2. Turn the plate over. Follow these steps to plate out a lawn of *Halobacterium* sp. NRC-1:
 - a. Remove the cap of the starter culture. Flame the lip of the container. (If no Bunsen burner is available, you may, for the purposes of this lab, omit the flaming step.)
 - b. Aspirate several drops of culture up into a sterile dropping pipet and replace the lid on the starter culture.
 - c. Lift the lid of the petri plate. Hold the lid over the plate while you work. Pipet 5 or 6 drops (~250 μ L) onto the center of the plate.
 - d. With a sterile swab, spread the starter culture evenly across the surface of the agar. It is critical that the culture be spread evenly, and that no areas of the plate are left uncovered.
 - e. Replace the cover of the petri plate. Dispose of the applicator stick as directed by your teacher.
3. Use forceps to gently press the appropriate disk onto the agar surface in the center of each quarter of the plate. According to the markings on the edge of the plate (see Step 1b), place one control disk and three different antibiotic-infused disks. Make sure each disk is securely contacting the agar and will not fall off. Do not invert the plate.
4. Allow the plate to sit out overnight. (On the following day, you or your teacher must place the plate into an airtight container and then into an incubator.)

Data Collection and Analysis

1. Describe the appearance of the starter culture of *Halobacterium* sp. NRC-1.

2. What is the purpose of the control disk in this experiment?

3. Draw a schematic of your plate (as seen from above). Label each section clearly.

Lab 2: Lab Analysis

Procedure :

1. After the appropriate length of time has passed, closely observe your plate. Look for the antibiotic susceptibility pattern of *Halobacterium* sp. NRC-1:
 - If there is growth right up to the antibiotic disk, *Halobacterium* sp. NRC-1 is **resistant** to the antibiotic.
 - If there is a zone of clearing around the antibiotic disk, *Halobacterium* sp. NRC-1 is **sensitive** to the antibiotic.
2. The zone of clearing may be devoid of colonies. On the other hand, small, "pinpoint" colonies may have arisen. These are colonies of resistant spontaneous mutants. Count the number of pinpoint colonies around each antibiotic disk, if any.
3. Measure the diameter of the zone of clearing. Remember to subtract the diameter of the antibiotic disk.

Data Collection and Analysis

1. Draw a schematic of your plate as seen from above. Label each section clearly.

2. Complete the table below based upon your observations of your plate.

Disk Used	Appearance on Plate Is there growth right up to the antibiotic disk, or is there a zone of clearing around the disk?	Diameter of Cleared Area (Remember to subtract the disk diameter.)	Number of Pinpoint Colonies	Is <i>Halobacterium</i> sp. NRC-1 resistant or susceptible to the antibiotic?

3. Using data gathered by your classmates, complete the tables on the following pages.

Worksheet

Name _____

Date _____

Ampicillin

[illegible]

Bacitracin

Initials, Station Number, or Team Name	Appearance on Plate Is there growth right up to the antibiotic disk, or is there a zone of clearing around the disk?	Diameter of Cleared Area (Remember to subtract the disk diameter.)	Number of Pinpoint Colonies	Is <i>Halobacterium</i> sp. NRC-1 resistant or susceptible to the antibiotic?

Worksheet

Name _____

Date _____

[illegible]

Worksheet

Name _____

Date _____

[illegible]

Discussion Questions

1. Using what you know about the response of *Halobacterium* sp. NRC-1 to several antibiotics, explain the order in which you would use the four antibiotics tested, if you suspected *Halobacterium* sp. NRC-1 to be a pathogen. Support your answer with data from your classroom results.

2. a. Were the numbers of spontaneous resistant colonies the same for all the antibiotic disks?

- b. Why?

3. a. For each antibiotic disk type, were the numbers of spontaneous resistant colonies the same for all the plates in your class?

b. Why do you think this is so?

4. How do you think this type of information regarding the occurrence of spontaneous antibiotic resistant mutants should figure into the use of antibiotics in a medical setting? (This is in part a bioethics question and should not be seen as having an absolute answer.)

5. Livestock farmers often give their animals prophylactic doses of antibiotics in order to ward off or prevent infection. What do you think of this practice, and what impact do you think this has on the microbes found in the food supply? You may wish to research this issue further before answering, in order to give a more complete answer.
