Lab 10

Kirby-Bauer Assay (Disk-Diffusion Test)

Theory:

Antibiotics are natural antimicrobial agents produced by microorganisms. One type of penicillin, for example, is produced by the fungus *Penicillium notatum*. Today, because many agents that are used to treat bacterial infections are synthetic, the terms **antimicrobials** or **antibiotics** are used to describe all substances used for this purpose.

The **Kirby-Bauer Test**, also called the **Disk-Diffusion Test**, is a valuable standard tool for measuring the effectiveness of antimicrobial agents against pathogenic microorganisms. In the test, antimicrobial–impregnated paper disks are placed on a plate that is inoculated to form a bacterial lawn. The plates are incubated to allow growth of the bacteria and time for the agent to diffuse into the agar. As the drug moves through the agar, it establishes a concentration gradient. If the organism is susceptible to it, a clear zone will appear around the disk where the growth was inhibited by the drug.

The size of this **zone of inhibition** depends upon the sensitivity of the bacteria to the specific antimicrobial agent and the point at which the chemical's **minimum inhibitory concentration** (**MIC**) is reached. Some drugs kill the organism and are said to be **bactericidal**. Other drugs are **bacteriostatic**; they stop growth but don't kill the microbe.

All aspects of the Kirby-Bauer Test is standardized to ensure reliable results. A break in any of these parameters will lead to results that are less reliable. **Mueller-Hinton Agar** has a pH range of 7.2 — 7.4 and the agar is poured to a **depth of 4 mm** in either 150 mm or 100 mm diameter Petri dishes. The depth is important because of its effect upon diffusion. *Agar poured thicker than 4 mm will slow lateral diffusion and thus produces smaller zones while those poured too thin (<4 mm) will have zones of inhibition that are too large.*

The disks also have a set amount of antibiotics impregnated in them and incubation of the plates is done only for 16 to 18 hours at 35 ± 2°C (we will be using 37°C).

In this lab exercise, you will test the susceptibility of a Gram Negative and a Gram Positive bacteria to several standard antibiotics. The various drugs will have different **mechanisms of action** such as:

1. Disrupting a metabolic pathway.
2. Inhibiting cell wall synthesis.
3. Disrupting of the plasma membrane.
4. Inhibition of protein synthesis by targeting the bacterial ribosome or its subunits.
5. Inhibition of nucleic acid synthesis.

Materials:

Amount per Student	Material
2	Mueller-Hinton Agar Plates
1 vial	vial of _____.
1 vial (optional)	vial of _____.
2	sterile plastic pipettes
shared (on table)	Antibiotic Filter Disks*
1	Glass Hockey stick
1	Bottle of Ethanol (for sterilizing Glass Hockey sticks)
1	Bunsen Burner
1	Forceps

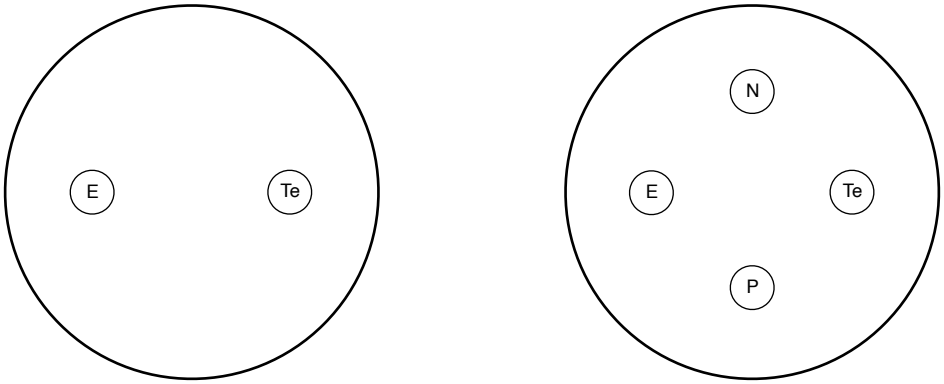
*Abbreviations on the Antibiotic Filter Disks:

E	**=**	**Erythromycin**	**15 µg**
N	**=**	**Neomycin**	**30 µg**
P	**=**	**Penicillin**	**10 units**
S	**=**	**Streptomycin**	**10 µg**
Te	**=**	**Tetracycline**	**30 µg**

Instructions:

1. Label each of your Petri dishes (on the bottom with your name, the date and write *Pseudomonas aeruginosa* on one dish and *Staphylococcus epidermidis* on the other.
2. Inoculate one petri dish with **0.1 mL** of fluid from the _____ vial.
3. Sterilize the Glass Hockey Stick with the Ethanol and Flame method previously used and then spread the inoculum evenly over the plate. Be careful not to damage the agar.
4. Inoculate one petri dish with **0.1 mL** of fluid from the _____ vial (optional).
5. Sterilize the Glass Hockey Stick with the Ethanol and Flame method previously used and then spread the inoculum evenly over the plate. Be careful not to damage the agar.
6. *Gently* place one set of antibiotic disks on each plate with as much separation from each other as possible and DO NOT force them into the agar. DO NOT move the disk once it has made contact with the agar, just "tap it" down flat if needed. Only handle the disks with the sterilized ethanol–flamed forceps.
7. Place both your petri dishes in the incubator (37°C) with the **lid side up**. *If you put them in upside down as in previous labs, you run the risk of your disk falling off the agar and ruining your results for next week.*
8. In 16 to 18 hours, I will remove your plate and put them in the refrigerator to preserve your results as much as is possible till you interpret them next week. If I don't the zones of inhibition can get too big and be inaccurate.

Figure 11–1: Examples of antibiotic disk spacing on a Petri dish using two or more disks.



Lab 10 Questions (Due at the end of lab)

Name:_____ **Grade: _____ of 10 points**

1. (2 points) Copy the chart of information obtained from the class on the board. All products at every concentration listed were tested against _____(name of bacteria tested from previous lab).

Name of Product:	1%	50%	100%

2. (1 point) Which disinfectant(s) is(are) effective (bactericidal) at 1%?

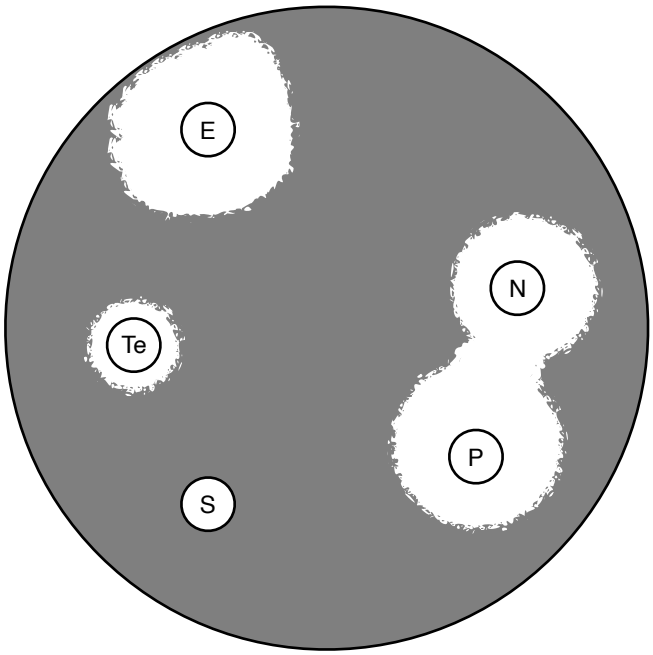
3. (1 point) From the information obtained above list the name of the product and additional percentages which would benefit from additional testing to determine with more precision where the minimum inhibitory concentration is at.

3. (2 points) What would happen to the <u>diameter</u> of the *zone of inhibition* if the depth of the Muller–Hinton agar was accidentally poured only 1 mm deep? What would happen if it was poured to 6 mm?

— 1 mm: too small, no change, too big (circle one choice)

— 6 mm: too small, no change, too big (circle one choice)

4. (4 points) Measure in millimeters, the Zones of Inhibition for all 5 antibiotics below. Show your work by drawing lines where each of your measurements were made and write next to the line what those measurements are. Some are easy and a simple diameter in mm will do, but others will require using a radius to calculate the final diameter. Fill in the chart with your final Zone of Inhibition measurements for each antibiotic. The areas shaded gray is where the bacteria is still alive and growing. The white areas in the sketch are locations where the antibiotic killed the bacteria.



Antibiotic	Zone of Inhibition (in millimeters)
E	
N	
P	
S	
Te	