The End Zone: Measuring Antimicrobial Effectiveness with Zones of Inhibition

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Experimental Procedure

Working with Biological Agents

For health and safety reasons, science fairs regulate what kinds of biological materials can be used in science fair projects. You should check with your science fair's Scientific Review Committee before starting this experiment to make sure your science fair project complies with all local rules. Many science fairs follow Intel® International Science and Engineering Fair (ISEF) regulations. For more information, visit these Science Buddies pages: Project Involving Potentially Hazardous Biological Agents (http://www.sciencebuddies.org/science-fair-projects/competitions/biological-agents-regulations) and Scientific Review Committee (http://www.sciencebuddies.org/science-fair-projects/competitions/scientific-review-committee-src). You can also visit the webpage ISEF Rules & Guidelines (https://www.societyforscience.org/isef/international-rules/) directly.

This science fair project involves the use of the bacteria *E. coli*. While *E. coli* is not considered a biohazardous or dangerous bacteria, it is important to always properly clean and dispose of bacteria and supplies that come in contact with it. See the *Bacterial Safety* guidelines below for more details on how to handle bacterial cleanup and waste.

Preparing Plates for Disk Diffusion Test

For this experiment, it is important to inoculate the plate with a uniform distribution of bacterial colonies, and to use the exact same procedure for each plate. Here are the steps for inoculating the control and test plates.

1. Use a permanent marker to divide the bottom of 12 nutrient agar plates into four quadrants each. In one quadrant of each plate write "C" for control. Write the name of a different disinfectant in each remaining quadrant. In summary:
    a. Every plate should have one control quadrant and 3 different disinfectant quadrants.
    b. Each disinfectant should be represented on 6 different plates.
2. Follow the directions in the kit to reconstitute the dried *E. coli*. Let the reconstituted *E. coli* sit at room temperature throughout the next step.
3. Sterilize a cup of water by boiling it on the stove top for 5 minutes. Cover and wait for it to cool to room temperature. Allowing the water to cool is critical; if it is too hot then it will kill the bacteria in the next step.
4. Using proper sterile technique, inoculate each plate uniformly. While wearing gloves, gently shake the container of reconstituted *E. coli* so that it is uniformly mixed. Put two drops of the *E. coli* mixture on a plate. Dip a fresh (unused) sterile cotton swab into the sterile water. Use the cotton swab to wipe the drops of bacteria around the entire surface of the plate. Cover the plate and wait at least five minutes for the plate to dry. A few additional tips:
    a. The sterile water on the cotton swab makes it easier to spread the bacteria uniformly.
    b. Make sure to use a fresh swab for each plate. *Do not* dip a used swab back in to the sterile water.
5. Hold a single sterile disk by the edge with sterile forceps and dip it into the disinfectant solution to be tested. Touch the disk against the side of the container to drain off excess liquid.
6. Use sterile forceps to place a single disinfectant disk in the center of each of the quadrants on your test plates. Use the forceps to gently press each disk against the agar surface to insure good contact. Remember to use the exact same technique for each disk—consistency is very important for this experiment. Make sure to carefully match the label on the plate with the right disinfectant.
7. Place a plain (not dipped in any disinfectant) sterile disk in each control quadrant.
8. Incubate all of the plates, inverted (lid on the bottom and agar on top), overnight at 37°C. Use a longer incubation time if necessary (for example, for incubation at lower temperature).

Measuring Zones of Inhibition

1. After overnight incubation, examine your plates (keep them covered at all times).
    a. The control quadrants should show uniform colonies over the entire surface of the plate. If the distribution is highly uneven, you will need to improve your inoculation technique and repeat the experiment.
    b. If your disinfectants are effective at the concentrations you tested, you should see zones of inhibition around the disinfectant disks. The clear zones around each disk should have a uniform width, since diffusion of the compounds through the agar should be uniform in every direction. If this is not the case, suspect either your impregnation technique, or poor contact of the filter paper with the agar.
2. Measure the diameter of the zone of inhibition for each disk. Keeping the lid of the plate in place, use a ruler to measure the diameter of the disk plus the surrounding clear area in millimeters (mm).
    a. Include the diameter of the disk in your measurements. For example, if your disk has a diameter of 6 mm and the clear area has a width of 3 mm beyond the disk, the diameter of the zone of inhibition that you should measure and record would be 12 mm (6 mm + 3 mm + 3 mm). This is the standard way that zones of inhibition are measured.
    b. You will get six separate measurements for each disinfectant, one from each of the three test plates.
3. Are the diameters consistent across all three plates? Calculate the average and the standard deviation of the diameter of the zone of inhibition for each disinfectant.
4. Use the values from Table 1 (below) to evaluate the bacterial response to each compound (Johnson and Case, 1995).

	Diameter of Zone of Inhibition (mm)
Resistant	10 or less
Intermediate	11–15
Susceptible	16 or more

Table 1. Bacterial response.

Bacterial Safety

Bacteria are all around us in our daily lives and the vast majority of them are not harmful. However, for maximum safety, all bacterial cultures should always be treated as potential hazards. This means that proper handling, cleanup, and disposal are necessary. Below are a few important safety reminders.

- Keep your nose and mouth away from tubes, pipettes, or other tools that come in contact with bacterial cultures, in order to avoid ingesting or inhaling any bacteria.
- Make sure to wash your hands thoroughly after handling bacteria.
- **Proper Disposal of Bacterial Cultures**
    - Bacterial cultures, plates, and disposables that are used to manipulate the bacteria should be soaked in a 10% bleach solution (1 part bleach to 9 parts water) for 1–2 hours.
    - Use caution when handling the bleach, as it can ruin your clothes if spilled, and any disinfectant can be harmful if splashed in your eyes.
    - After bleach treatment is completed, these items can be placed in your normal household garbage.
- **Cleaning Your Work Area**
    - At the end of your experiment, use a disinfectant, such as 70% ethanol, a 10% bleach solution, or a commercial antibacterial kitchen/bath cleaning solution, to thoroughly clean any surfaces you have used.
    - Be aware of the possible hazards of disinfectants and use them carefully.