

Lab Exercise #4**Control of Microorganisms: Physical, Chemical and Chemotherapeutic****I. OBJECTIVES:**

- ✓ Investigate the effectiveness various agents of control.
- ✓ Assess the effectiveness of heat in killing vegetative cells and endospores.
- ✓ Evaluate ultraviolet radiation as a mechanism of control.
- ✓ Examine the fundamentals of antibiotic sensitivity testing.

II. TERMINOLOGY:

Students should define and use the following terms:

agents of control	principles for testing
Chemical agents of control	sensitive
Chemotherapeutic agents of control	resistant
Disinfectant	Selective toxicity
Physical agents of control	Sterilize
Microbial Growth	Spectrum of activity for various antibiotics
Mode of action for tetracycline, erythromycin, sulfadiazine, penicillin, ciprofloxacin, bacitracin	Turbidity
	zone of inhibition

III. INTRODUCTION:

Control of microbial growth (inhibiting or killing microbes) is accomplished through physical, chemical and chemotherapeutic agents.

Physical Agents of Microbial Control

Physical agents of control include heat, freeze-drying, ultraviolet radiation and filtration. Heat denatures cellular proteins. Sterilization, the complete destruction of all viable cells, viruses and endospores, is accomplished by using the autoclave that combines the effects of heat and pressure. Autoclaved items are exposed to 121° C at 15 pounds per square inch (psi) of pressure for 15-20 minutes. Except for photosynthetic bacteria, most bacteria are harmed by ultraviolet radiation. Although sunlight contains the complete spectrum of short to long wavelengths of light, it is only the short invisible ultraviolet wavelengths that are injurious to these bacteria. Ultraviolet radiation is strongly absorbed by proteins and nucleic acids in cells; hence, the indications are that cellular damage involves changes in DNA. Depending on the dosage, ultraviolet radiation may cause enzyme inactivation, genetic mutation or death of the cell.

Chemical Agents of Microbial Control

Chemical agents of control like the disinfectants Lysol or Clorox destroy most vegetative cells and viruses. Chlorine is the active ingredient in Clorox. This is probably one of the most widely used disinfectants. It is used to disinfect water and for cleaning surfaces (e.g. floors, counters) and has proven effective in destroying HIV. The "killing" of microorganisms by chlorine and its compounds is due to the direct combination of chlorine with some cellular substances, thus 'poisoning' the cell. The mode of action of chlorine is generally considered to be the inhibition of enzyme activity and oxidization of cellular constituents to such an extent that they no longer perform normal metabolic functions. When chlorine reacts with organic materials, it is used up. Therefore, in order to be effective, chlorine concentrations must be high enough to allow the chlorine to attach to all the organic material present and still have some residual. Phenol is the active ingredient in Lysol. This disinfectant was first used by Lister in the mid 1800's to sterilize surgical instruments (aka: carbolic acid) There are many brand names and chemical

cousins: cresols, hexylresorcinol, hexachlorophene to name a few. Phenols exert their germicidal effect (i.e. mode of action) by denaturing proteins and destroying the selective permeability of the cell membrane (which permits "leakage" of cellular contents).

Limits of Physical and Chemical Microbial Control

Microbes respond differently to the affects of chemical and physical control agents. Endospores are very difficult to destroy. The autoclave is the most reliable way to destroy them. Some vegetative cells are more difficult to destroy than others. Cells with mycolic acid, for instance, are more resistant to destruction than others. Viruses are generally easier to destroy than vegetative cells. Naked viruses are more difficult to destroy than enveloped viruses.

Both chemical and physical agents of control are not particularly stealth agents. The deleterious effects they exert on the microbe are a bit like bombing the target---everything (microbe, host cells, environment) is prone to the toxic effects of the agent that comes in contact with it. In contrast, chemotherapeutic agents are selectively toxic. They target some aspect of microbial metabolism (e.g. protein synthesis, cell wall production). Different antibiotics target different aspects of bacterial metabolism. The chemotherapeutic agent is an antimicrobial or antibiotic that should not harm the host cell.

Chemotherapeutic Agents of Microbial Control

Antimicrobics are drugs used to treat patients diagnosed with an infectious disease. Once the causative organism of a specific disease has been isolated, the physician needs to know, as soon as possible, which antibiotic is most effective in treating the disease. The laboratory uses the antibiotic sensitivity test to provide information regarding the effectiveness of various antibiotics to the physician. Disks are impregnated with the antibiotic. A nutrient agar plate is uniformly inoculated with bacteria and the disks are placed on the media. Over the incubation period, the antimicrobial diffuses in all directions out from the disk. If the microbe is sensitive to the specific antimicrobial in question, a zone of inhibited growth will occur around the antibiotic.

How to Test The Effectiveness of Microbial Control Agents

The basic principle for testing the effectiveness of an agent of control is as follows:

1. Expose the organism to the agent.
2. After a set amount of time, remove the agent.
3. Put the organisms in favorable growth media.
4. Look for reproduction/growth of organisms.
Organisms sensitive to the agent will not grow; resistant organisms will continue to reproduce.

IV. MATERIALS (In addition to supplies found in your supply drawer):

18 sterile nutrient broth tubes
Broth culture of *E. coli*, *Staph epi* and *Bacillus subitius*
Microincinerator
4 water baths set at 40° C, 60° C, 80° C and 100°C.
Test Tube racks
TSY agar in the warming oven
4 sterile Petri plates
UV box
Antibiotic discs
Metric ruler
Dilution series of Clorox or Lysol
10 sterile blank tubes

V. PROCEDURE:

- A. **HEAT:** Objective - To assess the effects of heat on vegetative cells and endospores:
1. Label each tube with the name of the organism (*E. coli* or *Bacillus*), your initials, and the temperature (40° C, 60° C, 80° C, 100° C).
 2. Each group of 2 students should inoculate 4 tubes of nutrient broth with *E. coli* and 4 with *Bacillus*.
 - a. Light the Bunsen Burner.
 - b. Sterilize the loop (in microincinerator or flame of Bunsen burner).
 - c. Remove plastic top to the nutrient broth tube containing *E. coli*. (Take care not to spill the culture. Do not set the cap down and take care not to contaminate it.)
 - d. Flame the top of the test tube that contains the *E. coli*. Insert the cooled loop into the test tube careful not to touch the side of the tube as you insert the loop.
 - e. Retrieve a loopful of *E. coli*.
 - f. Flame the top of the tube after removing the loop and replace the cap.
 - g. Remove the plastic cap from the sterile nutrient broth tube and flame the top of the tube.
 - h. Insert the loop containing *E. coli* into the nutrient broth.
 - i. Remove the loop, flame the top of the test tube and replace the cap, sterilize your loop.
 3. Repeat A1 as you transfer *E. coli* to 3 more sterile nutrient broth tubes, for a total of 4 tubes containing a loopful of *E. coli*.
 3. Inoculate an additional 4 tubes with a loopful of broth containing *Bacillus subtilis* as per directions A1.
 5. Incubate the tubes at the temperature indicated on the tube for 10 minutes.
 6. Remove the tubes after 10 minutes at temperature. Incubate the tubes in the green 'save' bin until the next lab meeting.
- B. **UV RADIATION:** Objective - To evaluate the effects of ultraviolet radiation as a mechanism of microbial control.
1. Pour 2 TSY plates and allow them to come to room temperature.
 2. Inoculate 2 plates with *E. coli*.
 - a. Obtain a sterile "Q-tip" .
 - b. Aseptically dip the cotton swab in a broth culture of *E. coli*.
 - c. Swab the entire surface of a TSY plate to inoculate the entire surface of the plate to allow for a "carpet" of bacterial growth.
 3. Place the plates inside the UV box; one without a lid, the other covered with the lid. Expose both plates to UV light for 5 minutes.
 4. Store the plates upside down in the green 'save' bin.
- C. **ANTIMICROBIALS** - To practice the basic technique of Antibiotic Sensitivity Testing:
1. Pour two TSY media plates.
 2. Obtain a broth culture of *E. coli* and *S. epidermis*.
 3. Using a sterile cotton swab, inoculate a plate of *E. coli* with the cultures using the directions for swabbing the plates above. Label the plate with your initials and '*E. coli*'.
 4. Repeat the steps for inoculating the surface of a plate using *Staphylococcus epidermis*. Label the plate with your initials and '*S. epi*'
 5. Secure the antibiotic disks from either list I or II as directed.
 6. Gently drop a disk on the plate. Use a flamed loop to position the disc if needed. Gently tap the disc to adhere it to the media plate.
 7. Drop a second and third disk each equidistant from the one another.
 8. Place the plates upside down in the green 'save' bin until the next lab meeting.

- D. CHEMICAL: Objective - To investigate of the effectiveness of Clorox and Lysol in destroying vegetative cells and endospores. Your group will be assigned one agent, either Clorox or Lysol, and one bacterial culture, either *E.coli* or *Bacillus subtilis*.
1. Obtain the following materials:
 - a. The chemical agent assigned to your group.
 - b. 10 tubes of sterile nutrient broth
 - c. 10 blank (empty) tubes

LABELING AND PREPARING TEST TUBES

BLANK TUBES (The test tubes with nothing in them)

2. You will have one set of 5 blank tubes that will be used for *E. coli* and one set of 5 blank tubes that will be for *Bacillus subtilis*. For each set of 5 blank tubes:
 - a. Label one set of blank tubes "EC" for *E. coli* and one BS" for *Bacillus subtilis*.
 - b. For each set, label the first tube LS for 'label strength'.
 - c. Label the second tube 10^{-1} for the 1/10 dilution of the agent.
 - d. Label the third tube 10^{-2} for the 1/100 dilution of the agent.
 - e. Label the fourth tube 10^{-3} for the 1/1000 dilution of the agent.
 - f. Label the fifth tube 10^{-4} for the 1/10000 dilution of the agent.
3. Pipet the various concentrations of the agent of control to the blank tubes.
 - a. Pipet 10 mL of each dilution of your agent into the appropriately labeled tube (LS, 10^{-1} , 10^{-2} , 10^{-3} , 10^{-4})

BROTH TUBES

4. You have 2 sets of 5 broth tubes, one set of 5 for *E. coli* and one set of 5 for *Bacillus subtilis*. Label the broth tubes.
 - a. Label each with the dilutions listed above (LS, 10^{-1} , 10^{-2} , 10^{-3} , 10^{-4})
 - b. Label one set of broth tubes "EC" for *E. coli* and one BS" for *Bacillus subtilis*.

INOCULATING TUBES

BLANK TUBES

5. Note the time, then inoculate each of the blank tubes that now contains the various dilutions of your agent with one loopful of the bacteria. One set of blank tubes you will inoculate with *E. coli*, the other set with *B. subtilis*.

BROTH TUBES

6. After 10 minutes transfer bacterium exposed to agent to nutrient broth.
 - a. Transfer one loopful from the agent dilution tube labeled "label strength" to a culture broth tube that is appropriately labeled with the corresponding label.
 - b. Repeat this step for both sets of each of the dilutions.

STORING & DISPOSING

7. Place the serial dilution tubes (the ones that were originally the BLANKS) of the agents that were inoculated with *E. coli* or *Bacillus subtilis* in the DISCARD rack on the side bench.
8. Place the culture broth tubes in the labeled green 'Save' bin for incubation and storage until next week when we will determine the patterns of growth.

Think about what you are doing for this chemical control experiment and why you are doing it. You have placed different concentrations of a chemical control agent into 5 tubes, then you

added bacteria. After 10 minutes you took a sample out of each of your BLANK tubes that had the chemical and bacteria and put these samples into tubes of broth.

You then exposed the bacteria to the chemical agent for 10 minutes to see if the agent would kill the bacteria. In order to find out if the bacteria in each tube is dead or alive, you need to put a sample from each tube into another tube that contains nutrients and incubate it so that, next week, we can see if there was growth or not.

NOTE: All dilutions were made in the following manner: A 10^{-1} dilution contains 1 mL of agent in 9 mLs of water. So the 1 mL becomes part of a total volume of 10 mLs (1ml + 9ml) OR 1 ml in 10 ml which equals $1/10$ or 10^{-1} .