# CONTROL OF MICROBIAL GROWTH - DISINFECTANTS AND ANTISEPTICS

Specific control measures can be used to kill or inhibit the growth of microorganisms. A procedure which leads to the death of cells is broadly termed **cidal**, whereas a procedure which inhibits growth is termed **static**. If the organism being killed is a bacterium, the term would be **bactericidal**; killing of a fungus would be referred to as **fungicidal**, and so forth. Cidal measures are used when living microorganisms must be completely eliminated; for example, when canning food or preparing media for laboratory use. Static measures are used when the organisms need not be killed, but when their growth must be slowed or stopped. An example of static control is the use of refrigeration to slow microbial growth in foods, thus increasing the storage or shelf life of the food item.

Many chemicals are used to control microbial growth. The modes of action of these chemicals vary, but the most common actions are disruption of cell membranes or interference with nucleic acid or protein synthesis. Some of the most commonly used chemicals are classified as antiseptics, disinfectants, or antibiotics. Literally, the word antiseptic means "opposition to sepsis or decay." **Antiseptics** are chemicals which kill or inhibit the growth of microorganisms and are safe to use on animal tissue. Antiseptics may be used to treat wounds or may be used as mouth washes. **Disinfectants** are bactericidal or bacteristatic chemicals which are generally not safe to use on animal tissue. Disinfectants find wide use in the home, laboratory, hospital, and food industries and are used to kill or remove microorganisms on surfaces. The term disinfectant is also used when describing chemicals such as iodine solutions (common trademark: Betadine) used on the skin in preparation for surgery. **Antibiotics** are chemicals produced by one microorganism which kill or inhibit the growth of another microorganism. Antibiotics are used both internally and externally in the treatment of infectious disease. Antibiotics will not be studied here but will be studied in the next exercise.

Keep in mind that chemical treatment is only one of many methods available to control microbial growth. Other methods include: (a) "cidal" control measures such as sterilization by heat (e.g. autoclaving) or by ultraviolet radiation, and (b) "static" control measures such as freezing and drying. The method of choice depends on the material to be treated, the requirement for cell death vs. inhibition of growth, the organism to be controlled, and characteristics of the method such as ease of

use, danger to the user, and cost.

In this exercise, you will use a filter paper test to determine the effectiveness of a variety of antiseptics and disinfectants on bacterial growth. The test consists of spreading a bacterial inoculum on a nutrient agar plate, and placing paper discs that have been soaked with various concentrations of antiseptics or disinfectants on the plate. If the substance is inhibitory, a clear zone of inhibition will surround the paper disc. The size of the zone is an expression of the agent's effectiveness and can be compared quantitatively against other substances. In this exercise you will test the relative effectiveness of the "standard" concentration of various chemicals against both Gram-positive and Gram-negative organisms.

**FIRST PERIOD**

**Material:**

1. Broth cultures of *Staphylococcus aureus*, *Escherichia coli*, and *Pseudomonas aeruginosa, Salmonella enteritidis*
2. Four nutrient agar plates
3. Sterile cotton-tipped swabs
4. 95% ethyl alcohol in glass beaker
5. Sterile filter paper discs
6. Various antiseptics and disinfectants
7. Forceps

**Procedure: (work in groups of four)**

1. Dip a sterile swab into one of the bacterial cultures.

2. Inoculate the surface of a nutrient agar plate with the swab (after removing excess fluid from the swab by pressing against the inside of the tube). Cover the surface of the agar evenly by swabbing in three directions. (Note: Mark the plate with the name of the organism). Allow the plate to dry at room temperature for 3 to 5 minutes.

3. Repeat steps 1 and 2 for each culture (one culture per plate).

**4.** Divide each of the four plates into six divisions.

**5.** Flame-sterilize the forceps as you would sterilize a glass spreader. Pick up a sterile filter paper disc with the forceps and saturate the disc with a particular antiseptic or disinfectant. Allow excess liquid to drain by holding the disc against the side of the dish.

**6.** Place the saturated disc on one of the inoculated plates. Resterilize the forceps and repeat this procedure to apply discs to the other three plates.

**7.** Repeat this procedure for each of the various chemicals.

**8.** Incubate all plates at 37°C for 2-5 days.

## SECOND PERIOD

### Procedure:

**1.** Examine each plate and measure the zone of inhibition (clear area) around each disc.

**2.** Record results.

**Table: Example of Results**

| | Zone of inhibition (in mm) | | | | |
|---|---|---|---|---|---|
| | Dilute Soap soln | 70% EtOH | Iodine soln | Dilute Lysol | Listerine |
| *E. coli* | | | | | |
| *S. aureus* | | | | | |
| *P. aeruginosa* | | | | | |
| *S. enteritidis* | | | | | |

# CONTROL OF MICROBIAL GROWTH - EVALUATION OF ANTIBIOTICS

The principal drugs used in the treatment of infectious disease fall into three categories: antibiotics, sulfonamides and chemotherapeutics. In this exercise, the susceptibility of four microbial cultures to different antibiotics will be determined by the Kirby-Bauer, or disc agar diffusion method. Recall that **antibiotics** are chemicals produced by one microorganism which kill or inhibit the growth of another microorganism. Before a particular antibiotic is prescribed, a physician should know that the antibiotic is effective against the organism causing the disease. It is often necessary to isolate and establish the identity of the causative organism. If the physician is still in doubt about which antibiotic to prescribe, the clinical laboratory can conduct an antibiotic sensitivity test, in which the isolated organism is challenged by various antibiotics of different concentrations. The antibiotic that inhibits the growth of the organism to the greatest extent is usually the antibiotic of choice.

In this exercise, the standardized disc susceptibility test will be used to test cultures for antibiotic sensitivity. The recommended procedure is called the **Kirby-Bauer method**, after the workers who developed it. An agar plate of suitable culture medium is inoculated by spreading an aliquot of a bacterial culture evenly across the agar surface. Filter-paper discs containing known concentrations of different antimicrobial agents are then placed on the plate. After incubation, the presence of a zone of inhibition around the filter-paper disc is noted. The susceptibility of the organism to the antimicrobial agent is determined by reference to a standard chart of typical zone sizes.

The size of the zone of inhibition is influenced by a number of factors such as the size of the inoculum, the rate of growth of the organism, the rate of diffusion of the antibiotic through agar and, finally, the susceptibility of the organism to the antibiotic. In order for this test to be valid in a clinical setting, there are certain precautions that must be observed. Some of the precautions are as follows:

1. The culture medium should be **Mueller-Hinton agar**, which gives reproducible zones of inhibition and does not inhibit sulfonamides. The initial depth of medium in the plate should be 4 mm. If deeper medium is used,

smaller zones of inhibition will result from greater diffusion of the antibiotic downward than outward. If the medium is too thin, larger zones of inhibition will result.

2. Normally in clinical labs, the inoculum size must be standardized against a barium sulfate turbidity standard (prepared by adding 0.5 ml of 48 mM $BaCl_2$ (11.7 g of $BaCl_2 \cdot 2H_2O$ per liter) to 99.5 ml of 1% (vol/vol) $H_2SO_4$). About 10 ml of the standard is dispensed into each of several test tubes, and is usually reliable for 30 days. If the inoculum is too small, the antibiotics will have a distinct advantage. Conversely, if the inoculum is too large, the zones of inhibition will be smaller than under standardized conditions, and hence difficult to interpret. We will omit this step for our purposes.

3. Incubation of the Mueller-Hinton plates must be at 37°C for 18-24 hrs. Lower incubation temperatures will impede the growth of the organisms, and the potency of some of the antibiotics at 37°C may vary with time. Also, after approximately 20-24 hrs, the organisms may show overgrowth, which would result in smaller zones of inhibition than under standard conditions.

**FIRST PERIOD**

**Material:**

**1.** Broth cultures of *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, and *Salmonella enteritidis*.
**2.** Four Mueller-Hinton plates
**3.** Sterile cotton-tipped swabs
**4.** Antibiotic discs
**5.** 95% ethyl alcohol in glass beaker
**6.** Forceps

## Procedure: (work in groups of four)

1. Dip a sterile cotton swab into one of the bacterial cultures.

2. Inoculate the surface of a Mueller-Hinton plate with the swab (**after** removing excess fluid from the swab by pressing it against the inside of the tube). Cover the surface of the agar evenly by swabbing in three directions. (NOTE: Mark the plate with the name of the organism). Allow the plate to dry at room temperature for 3 to 5 minutes.

3. Repeat steps 1 and 2 for each culture (one culture per plate).

4. Flame-sterilize the forceps as you would sterilize a glass spreader. Remove one of the discs containing a known antibiotic from the dispenser and place the disc on one of the inoculated plates. Keep the discs evenly spaced and at least 10-15 mm from the edge of the plate!

5. Resterilize the forceps and repeat this procedure to apply discs to the other three plates. In the eighth sector, add a single streak of your *Streptomyces* culture or another antibiotic disk (if not available).

6. Invert all plates and incubate them at 37°C for 18-24 hours.

## SECOND PERIOD

### Procedure:

1. Measure the diameter of the zone of inhibition in millimeters, including the disc. Preferably the zone should be read from the bottom of the plate without removing the lid of the petri dish.

2. Use the “zone-size interpretative chart” at the end of this protocol to determine whether the organism is resistant, intermediate, or susceptible to the antibiotics tested.

**Results:**

| | **Zone of inhibition** (in mm) | | | |
|---|---|---|---|---|
| Antibiotic; amount | *S. aureus* | *Sal. enter.* | *E.coli* | *P. aerug.* |
| Ampicillin; 10 µg | | | | |
| Bacitracin; 10 Units | | | | |
| Erythromycin; 15 µg | | | | |
| Oxycillin; 5 µg | | | | |
| Streptomycin; 10 µg | | | | |
| Tetracycline; 30 µg | | | | |
| Penicillin G; 10 Units | | | | |
| *Streptomyces* culture or another antibiotic | | | | |

## ZONE-SIZE INTERPRETATIVE CHART

| Antibiotic | Disc Potency | Inhibition Zone Diameter in mm | | |
|---|---|---|---|---|
| | | Resistant (mm or less) | Intermediate | Susceptible (mm or larger) |
| Amikacin | 10 mcg | 11 | 12-13 | 14 |
| Ampicillin[1] | | | | |
| Gram-negative micro-organisms & enterococci | 10 mcg | 11 | 12-13 | 14 |
| Staphylococci & highly penicillin-susceptible organisms | 10 mcg | 20 | 21-28 | 29 |
| *Hemophilus* | 10 mcg | 19 | - | 20 |
| Bacitracin | 10 U | 8 | 9-12 | 13 |
| Carbenicillin | | | | |
| *Pseudomonas aeruginosa* | 50 mcg | 12 | 13-14 | 15 |
| | 100 mcg | 13 | 14-16 | 17 |
| *Escherichia coli* & *Proteus* species | 50 mcg | 17 | 18-22 | 23 |
| | 100 mcg | 17 | 18-22 | 23 |
| Cephalothin[2,3] | 30 mcg | 14 | - | 15 |
| Chloramphenicol | 30 mcg | 12 | 13-17 | 18 |
| Clindamycin | 2 mcg | 14 | 15-16 | 17 |
| Colistin[4] | 10 mcg | 8 | 9-10 | 11 |
| Doxycycline | 30 mcg | 12 | 13-15 | 16 |
| Erythromycin | 15 mcg | 13 | 14-17 | 18 |
| Gentamicin | 10 mcg | 12 | - | 13 |
| Kanamycin | 30 mcg | 13 | 14-17 | 18 |
| Lincomycin | 2 mcg | 9 | 10-14 | 15 |
| Methicillin[5] | 5 mcg | 9 | 10-13 | 14 |
| Nafcillin & Oxacillin | 1 mcg | 10 | 11-12 | 13 |
| Nalidixic acid[2] | 30 mcg | 13 | 14-18 | 19 |
| Neomycin | 30 mcg | 12 | 13-16 | 17 |
| Novobiocin[6] | 30 mcg | 17 | 18-21 | 22 |
| Oleandomycin | 15 mcg | 11 | 12-16 | 17 |
| Oxolinic Acid[2] | 2 mcg | 10 | - | 11 |
| Penicillin G[7] | | | | |
| Staphylococci | 10 U | 20 | 21-28 | 29 |
| Other organisms | 10 U | 11 | 12-21 | 22 |
| Polymyxin B[4] | 300 U | 8 | 9-11 | 12 |
| Streptomycin | 10 mcg | 11 | 12-14 | 15 |
| Tetracycline | 30 mcg | 14 | 15-18 | 19 |
| Tobramycin | 10 mcg | 11 | 12-13 | 14 |
| Vancomycin | 30 mcg | 9 | 10-11 | 12 |

1. The ampicillin disk is used for testing susceptibility to ampicillin, hetacilin and amoxacilin.

2. Urinary tract infections only.

3. Staphylococci exhibiting resistance to the penicillinase-resistant penicillin class disks should be reported as resistant to cephalosporin class antibiotics. The 30 mcg cephalothin disk cannot be relied upon to detect resistance of methicillin-resistant staphylococci to cephalosporin class antibiotics.

4. Colistin and polymyxin B diffuse poorly in agar and the accuracy of the diffusion method is thus less than with other antibiotics. Resistance is always significant, but when treatment of systemic infections due to susceptible strains is considered, it is wise to confirm the results of a diffusion test with a dilution method.

5. The methicillin disk may be used for testing susceptibility to all penicillinase-resistant penicillins, that is, methicilin, cloxacillin, dicloxacillin, oxacilin and nafcilin.

6. Not applicable to blood-containing media.

7. The penicillin G disk is used for testing susceptibility to all penicillinase-susceptible penicillins except ampicillin and carbenicillin; that is, penicillin G, phenoxymethyl penicillin, and phenethicillin.

# LACTOBACILLI IN SALIVA: THE SNYDER TEST

The Snyder test is used to detect the presence of *Lactobacillus* in saliva. Only lactobacilli seem to be capable of lowering the pH enough to dissolve tooth enamel. Decalcification of the enamel plays an important role in the formation of dental caries (tooth decay). The Snyder test measures dental caries susceptibility by detecting the presence of lactobacilli in saliva.

The Snyder test medium is designed to favor growth of lactobacilli and discourage growth of most other species. This is accomplished by adjusting the pH of the medium to 4.8 and by adding glucose, a carbohydrate easily fermented by *Lactobacillus*. Lactobacilli thrive in the low pH environments and ferment the glucose, producing more acid which reduces the pH even more. The medium includes the pH indicator, bromcresol green, which is green at pH 4.8 and above, and yellow below pH 4.8.

The medium is autoclaved, cooled to just over 40°C and maintained in a water bath until needed. The molten agar is then inoculated with 0.2 ml of saliva, mixed well, and incubated at 37°C for up to 72 hours. The agar tubes are checked at 24-hour intervals for any change in color. Yellow color indicates that fermentation has taken place and is a positive result. High susceptibility to dental caries is indicated if the medium turns yellow within 24 hours. Moderate and slight susceptibility are indicated by a change within 48 and 72 hours, respectively. No change within 72 hours is considered a negative result.

## FIRST PERIOD

### Material:

**1.** Four small dixie cups
**2.** Five molten Snyder agar tubes (50-55°C)
**3.** 1.0 ml pipets

## Procedure: (work in groups of four)

1. Collect a small sample of saliva (about 0.5 ml) in a dixie cup for each person in the group.

2. Transfer 0.2 ml of saliva to a Snyder agar tube and roll the tube between your hands until the saliva is uniformly distributed throughout the agar.

3. Allow the agar tube to cool to room temperature. Do not slant the tube.

4. Incubate each of the four Snyder tubes with an uninoculated control tube at 37°C for up to 72 hours.

5. Check the tubes at **24-hour intervals** for yellow color formation (glucose fermentation).

6. Record your results and determine your susceptibility to tooth decay. Compare your results with those from other students in your group.