EXPERIMENT 10

ANTIMICROBIAL AGENTS

Agents that kill or inhibit microorganisms may be classified as disinfectants, antiseptics or antibiotics. Antibiotics are molecules that are produced by one microorganism that kill (bactericidal) or inhibit (bacteriostatic) other microorganisms. Antiseptics and disinfectants are commercially prepared chemicals and the distinction between them is that antiseptics can be exposed to mucosal surfaces for at least a short time and disinfectants should not as they could impart harm.

Whether an agent acts by killing or inhibiting growth is dependent upon its concentration and/or length of contact with the target microorganism. Additionally, the metabolic state of the target microbe can affect its response. Dormant cells, and of course the spore, are usually less affected than growing cells.

Among some of the factors which influence the action of a disinfectant are: concentration, type of chemical, time of contact, type of microbe, number of microbes/ml, presence of protective substances, temperature, and other physical and chemical parameters. In this experiment, we will do a kinetic assay (time of contact) with two concentrations of several disinfectants. The effectiveness of the disinfectant will be measured by its concentration and how much time of contact is required to kill a bacterial culture.

Antiseptic and antibiotic assays can be performed in much the same way: finding the **minimal inhibitory concentration** (the lowest concentration that stops growth of the test organism is a **MIC**) or by measuring the **zone of inhibition** produced by inhibiting growth of a lawn of test organism (Figure 1.). To do a MIC, the antiseptic or antibiotic is first serially diluted and then each dilution in mixed with a standard inoculum of the test organism. After growth in the controls (usually 18-24 hours at 37^{o}C), the tubes are examined for the presence or absence of growth.

Figure 1. Zones of inhibition around the discs containing the test substances

The classical antibiotic assay is done by first spreading the test organism over the surface of an appropriate nutrient agar medium; we will use BHI agar. Spreading is accomplished by smearing a broth culture with a sterile cotton swab. After spreading the organism on the plate, paper discs containing the test substances are placed on the surface of the plate. The plate is then incubated at a temperature appropriate for growth of the microbe. After incubation, the plates are examined for the zone of inhibition (area of no growth) around the discs containing the test substances. The size of the zone of inhibition is dependent upon the concentration of the test substance, its potency, and rate of diffusion in the medium. Most antiseptics and antibiotics are small molecules and have identical diffusion rates. The zone of inhibition is therefore a good measure of the effectiveness of the test substance against the test organism especially in pair wise comparisons where the concentrations of the test substances are nearly identical.

Antibiotic susceptibility of the test organisms is also measured by this method. Some test organisms are susceptible to just about all substances tested while others can be amazingly resistant. Gram negative bacteria often respond differently to some antibiotics than do Gram positive bacteria. It is common practice in most hospital microbiology labs to test organisms isolated from infections against a number of different antibiotics so that the most effective antibiotics against various disease causing agents are used. These labs can employ either MIC testing (often automated) or measuring zones of inhibition.

MATERIALS (per pair)

1. Antiseptics: 5 different commercial mouthwashes.
2. Antibiotics: Difco or BBL antibiotic discs and dispenser.
3. 3 BHI Agar plates.
4. 1 Nurtient Agar plate.
5. 1 sterile 13x100 mm capped tube.
6. 1 1X strength BHI broth - 1 ml/13x100 mm tube.
7. Sterile cotton swabs, parafilm.
8. Forceps, beaker with 95% ethanol.
9. Sterile paper discs (from Whatman No. 1 paper).
10. Onion, garlic, red pepper, razor blades.

11. Test Cultures (BHI broth cultures):

Escherichia coli	*Bacillus megaterium*
Staphylococcus aureus	*Pseudomonas aeruginosa*
Serratia marcescens	*Proteus vulgaris*

Pure Culture isolated in Experiment 8

PROCEDURE

Antiseptics - Zone of Inhibition

1. With a sterile swab, swab your teeth. Place the swab into the BHI tube containing 0.5 ml of broth: leave the swab in the broth (close with some parafilm) and incubate at 37^{o}C for two hours. This represents a mixed culture of bacteria from your mouth.

2. Swab the surface of a BHI plate with the mixed culture of mouth bacteria. When swabbing the plate make sure to thoroughly cover the surface of the plate by completely as possible swabbing with back and forth motions over the plate, then turning the plate 90^{o} and repeating the swabbing.

3. With alcohol sterilized forceps, take a sterile paper disc and inset it into one of the commercial mouthwashes, express excess mouthwash on the inside of the mouthwash bottle and then place in on the swab-inoculated plate. Mark the disc on the back of the plate. Repeat this with the remaining four mouthwashes. Try to evenly space the discs around the plate.

4. Repeat steps 1-3 with a known test culture in place of the mouth bacterial culture.

5. Incubate both plates at 37^{o}C for 48 hours and observe the size of the zone of inhibition by measuring the distance from the side of each disc to the beginning of growth

Antibiotics - Zone of Inhibition

1. Swab a plate with the test organism used in the antiseptic assay above. Swab another plate with the culture you isolated in Experiment 8 from either your throat or skin.

2. After swabbing both plates, dispense eight antibiotic discs onto their surfaces. Make sure each is in firm contact with the agar by touching the disc with a sterile (alcohol) forceps.

3. Incubate at 37^{o}C for 48 hours and measure the sizes of the zones of inhibition for each antibiotic.

Action of Garlic, Onion and Pepper

1. Swab a Nutrient Agar plate with your known test organism.

2. Cut small, equal sections of garlic and onion and place on the swab inoculated plate. Place a few pepper seeds on another section of the plate. Incubate at 37^{o}C for 48 hours and observe for zones of inhibition.

RESULTS

ANTISEPTICS

Known Test Bacterium:_____

Size of the Zone of Inhibition (mm)

Antiseptic	**Mouth Bacteria**	**Known Test Bacterium**
_____	_____	_____
_____	_____	_____
_____	_____	_____
_____	_____	_____
_____	_____	_____
_____	_____	_____
_____	_____	_____

ANTIBIOTICS

Size of the Zone of Inhibition (mm)

Antibiotic	**Expt. 8 Bacterium**	**Known Test Bacterium**
_____	_____	_____
_____	_____	_____
_____	_____	_____
_____	_____	_____
_____	_____	_____
_____	_____	_____
_____	_____	_____

ACTION of ONION, GARLIC AND PEPPER:

Describe the growth on the plate in relationship to these three natural products (onion, garlic and pepper).

QUESTIONS

1. How would you determine if a zone of inhibition was due to the bacteriocidal (killing) or bacteriostatic (growth inhibiting) action of an antimicrobial?

2. Sometimes, one observes a few bacterial colonies growing within a zone of inhibition. How would you explain this? How would you conclusively prove your hypothesis?

3. How do antibiotics differ from other natural products of microbes such as lactic acid, acetic acid, ethanol and others?

4. How are new antibiotics discovered? What properties must they possess in order to be more desirable than previously discovered antibiotics?