Evaluating Antiseptic and Disinfectant Susceptibilities of Microorganisms

Resource Type: Curriculum: Laboratory

Publication Date: 3/16/2001

Authors

Janet Cooper
Rockhurst University
Biology Department
Kansas City, Missouri
USA
Email: janet.cooper@rockhurst.edu

Abstract

In this lab exercise, students will work in cooperative teams to test the antimicrobial activity of an antiseptic or disinfectant using a modified use dilution assay as described by Benson (1). In the use dilution assay, paper clips are submerged in the antimicrobial agent, followed by a neutralization in sterile water and incubation in broth to determine the effectiveness of a particular agent. Vinyl paper clips are easier to manipulate than pins as recommended by Benson (1) and are preferable to metal substrates suggested by Johnson and Case (4) since metals can be inhibitory to the growth of most organisms (2). Students will use the scientific method to answer a question relating to the efficacy of a disinfectant or antiseptic. They will develop a hypothesis, design an experiment to test that hypothesis, and collect and analyze data. Efficacy of the disinfectant or antiseptic is determined by the inhibition of growth in broth tubes using optical density as a measure of growth. Following completion of the work, students will report their results to the class and a comparative analysis of the antimicrobic agents may be performed.

Activity

Invitation for User Feedback. If you have used the activity and would like to provide feedback, please send an e-mail to MicrobeLibrary@asmusa.org. Feedback can include ideas which complement the activity and new approaches for implementing the activity. Your comments will be added to the activity under a separate section labeled "Feedback." Comments may be edited.

INTRODUCTION

Time Required. Two 1-hour laboratory periods plus 1 discussion period are required for this exercise.

Pedagogical Function.
This study is designed to (i) teach a method for measuring antimicrobial susceptibility, (ii) allow students to become familiar with the scientific method, and (iii) allow students to gain experience in the manipulation of data and the use of spread sheet and graphing programs.

Background.
Various methods exist for the examination of the antimicrobial capabilities of antiseptics and disinfectants. These methods include disk diffusion, phenol coefficient, and use dilution assays as well as assays that determine minimal inhibitory and minimal bactericidal concentrations. While these assays are all simple to do and provide a means for examining antimicrobial effects, they are not without their limitations.

The disk diffusion assay utilizes a filter paper disk saturated with the chemical of choice and placed on a nutrient agar plate preswabbed with the organism. A zone of inhibition develops around the disk if the organism is unable to grow in the presence of the antimicrobial agent (1). The limitations of this assay, however, include the solubility and size of the molecule. A chemical insoluble in water will not diffuse into the agar and therefore will not exhibit a zone of inhibition. A large molecule will diffuse poorly while a smaller molecule may diffuse readily. While this assay is quick and easy to do, it is difficult to compare the effectiveness of the antimicrobial agents based on this experiment.

A phenol coefficient assay makes use of serially diluted samples of a chemical tested with various organisms. In standard phenol coefficient assays the traditional organisms used are *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and *Salmonella choleraesuis*. Following 5-, 10-, and 15-minutes exposure to the chemical and subsequent incubation in nutrient broth, the tubes are examined for the bactericidal properties of the chemical as compared to phenol. While this assay has been a traditional method for determining the bactericidal properties of a chemical, it is limited only to phenol-like chemicals (5).

Assays to determine minimum inhibitory concentrations (MIC) and minimum bactericidal concentrations (MBC) of antimicrobial chemicals are much more useful but involve much greater manipulation and a longer time period to perform (5). To determine MIC, a known amount of organism is incubated with increasing amounts of the antimicrobial agent. Following a 24-hour incubation period, the tube containing the lowest amount of the antimicrobial agent that exhibits no growth is defined as the MIC. Aliquots of each tube are then removed and placed in fresh broth containing no antimicrobial agent. Following an additional incubation period, the tubes can be examined for growth. The tube containing the least amount of the antimicrobial agent that still shows no growth is considered the MBC. While the MIC and MBC assays may provide more information on a particular chemical, unless the laboratory meets three times a week, these assays are impractical to perform.

The use dilution assay described in this experiment usually employs stainless steel cylinders as carriers for microorganisms to examine the effectiveness of the antimicrobial agents. Following an exposure period to the antimicrobial agent, the cylinders are placed in fresh broth tubes and examined for growth following an incubation period. This assay was adopted in 1950 by the Association of Official Agricultural Chemists (1) and provides a measure of the bactericidal properties of antimicrobial agents.

This laboratory is designed to evaluate the antimicrobial activity of disinfectants and antiseptics using a modified use dilution assay. Most assays determine growth qualitatively, by visually inspecting tubes for growth, while this activity provides a quantitative measure of the antimicrobial activity. This assay also substitutes paper clips for the steel cylinders or brass pins as suggested by Benson (1). Paper clips are easier to manipulate than either steel cylinders or brass pins. The assay, however, is limited to bactericidal compounds. This activity is also unique from similar activities in that it introduces students to the scientific method by asking them to formulate a hypothesis based on their knowledge of the antimicrobial agents and the organisms they have studied. Students should have a familiarity with terms such as bactericidal and bacteriostatic and should be aware of the mechanisms by which antiseptics and disinfectants act. This laboratory should be conducted after a discussion of the various classes of antimicrobial agents and their actions. The students then test their hypotheses by designing an experiment and collecting the data for analysis. The growth of the microorganisms is quantitatively measured by turbidity using a spectrophotometer.

PROCEDURE

Materials.
Sterile vinyl paper clips, coated with microorganisms prior to the laboratory (10 per group)
Tubes of Mueller-Hinton broth, 10 ml each (10 per group)
Selection of disinfectants and antiseptics
Tubes of sterile water, 10 ml each (5 per group)
Spectrophotometer (wavelength = 550 nm)
Inoculating loop
Bunsen burner

The microorganisms used in this activity depend on the hypothesis being examined by the group. For example, teams may choose to examine the effects of a specific antimicrobic agent against both gram-negative and gram-positive bacteria or they can examine the effects of several agents on one particular species of bacteria. Microorganisms suggested for this study include: *Escherichia coli*, *Micrococcus luteus*, *Micrococcus roseus*, *Staphylococcus epidermidis*, and *Bacillus megaterium*.

Mueller-Hinton broth is the suggested medium since it grows most aerobic and facultatively anaerobic bacteria. It has also been recommended by the World Health Organization Committee on Standardization and Susceptibility Testing, since it reduces the antagonism to sulfonamide activity (3). While this is more important for the testing of antibiotics, it does provide an opportunity to discuss media interactions to consider in an experiment. If additional supplementation is necessary for the microbes used, Mueller-Hinton broth can be supplemented as recommended by the Difco Manual (3). However, the recommended organisms grow well in this medium.

Instructor Version.
Prelab. The instructor should precoat the paper clips with the organism by incubating them overnight in nutrient broth cultures (125-ml flasks) of microbes chosen for the study. Paper clips should be air-dried before the laboratory period starts (a 1 to 2 hour drying time is suggested for best results).

Day one. Duplicate, microbe-coated, paper clips are incubated in the chosen disinfectant or antiseptic for various periods of time. Suggested incubation periods for antimicrobial treatment are 0, 1, 5, and 10 minutes. It is important for students to understand the value of the time 0 point as a control for comparing their subsequent treatment times. Since time 0 points can vary from one species to another, it is important that each team collect their own time 0 point data. Following the incubation periods, duplicate paper clips should be removed from the disinfectant or antiseptic and placed in the tubes of sterile water for one minute to neutralize the disinfectant or antiseptic. The duplicate paper clips are then placed in separate Mueller-Hinton broth tubes for overnight incubation. See Fig. 1 for a diagram of this procedure. Paper clips can be manipulated from one tube to another with inoculating loops. Overnight incubation is preferable for this activity to insure that differences will be observed at the various exposure times. With longer incubation periods, tubes from highly bactericidal chemicals may have a few remaining microbes, which are capable of growth reaching the stationary phase of the growth curve after 48 hours. In this instance, the quantitative differences of the assay will be lost. If a lab does not meet within 24 hours, the instructor may remove the tubes from the incubator and store them at 4°C until the analysis can be performed.

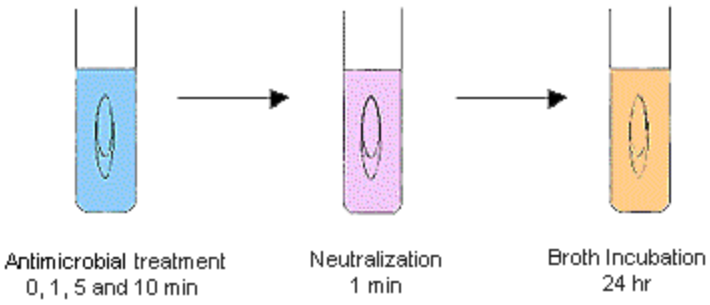


FIG. 1. Diagram showing the steps for Day one of the use dilution assay.

Day two. Following overnight incubation, the turbidity of the sample is assessed using a spectrophotometer (wavelength = 550 nm) to determine optical density. For analysis, students may average the duplicate samples and then evaluate their results. Their data may then be graphed using a spread sheet and graphing program (e.g., Microsoft Excel).

Day three. Following analysis, one day should be used for students to present their results. Students should report their hypothesis and experimental design and discuss their conclusion with the class for evaluation. For comparison of the data with other lab groups using different organisms, the students may normalize their data as percent of controls.

Safety Issues.
Students should show proficiency in aseptic laboratory techniques and demonstrate common laboratory safety skills.

ASSESSMENT and OUTCOMES

Suggestions for Assessment.
Students report their results both orally to the class and in written form as a research paper. The concepts of antimicrobial evaluation are incorporated on subsequent exams and quizzes.

Examples of Student Data.
See Fig. 2 for an example of student data collected during this lab which shows observed differences of 20 and 50 percent following overnight incubation (2).

FIG. 2.

Problems and Caveats.
Benson (1) suggests using brass-plated straight pins for this experiment, however, pins are very hard to manipulate. Paper clips are much easier to manipulate with an inoculating loop, but nickel-coated paper clips are actually inhibitory to the growth of microbes (2). Vinyl paper clips provide an excellent substitute because they will not inhibit the growth of microbes and can withstand the sterilization process.

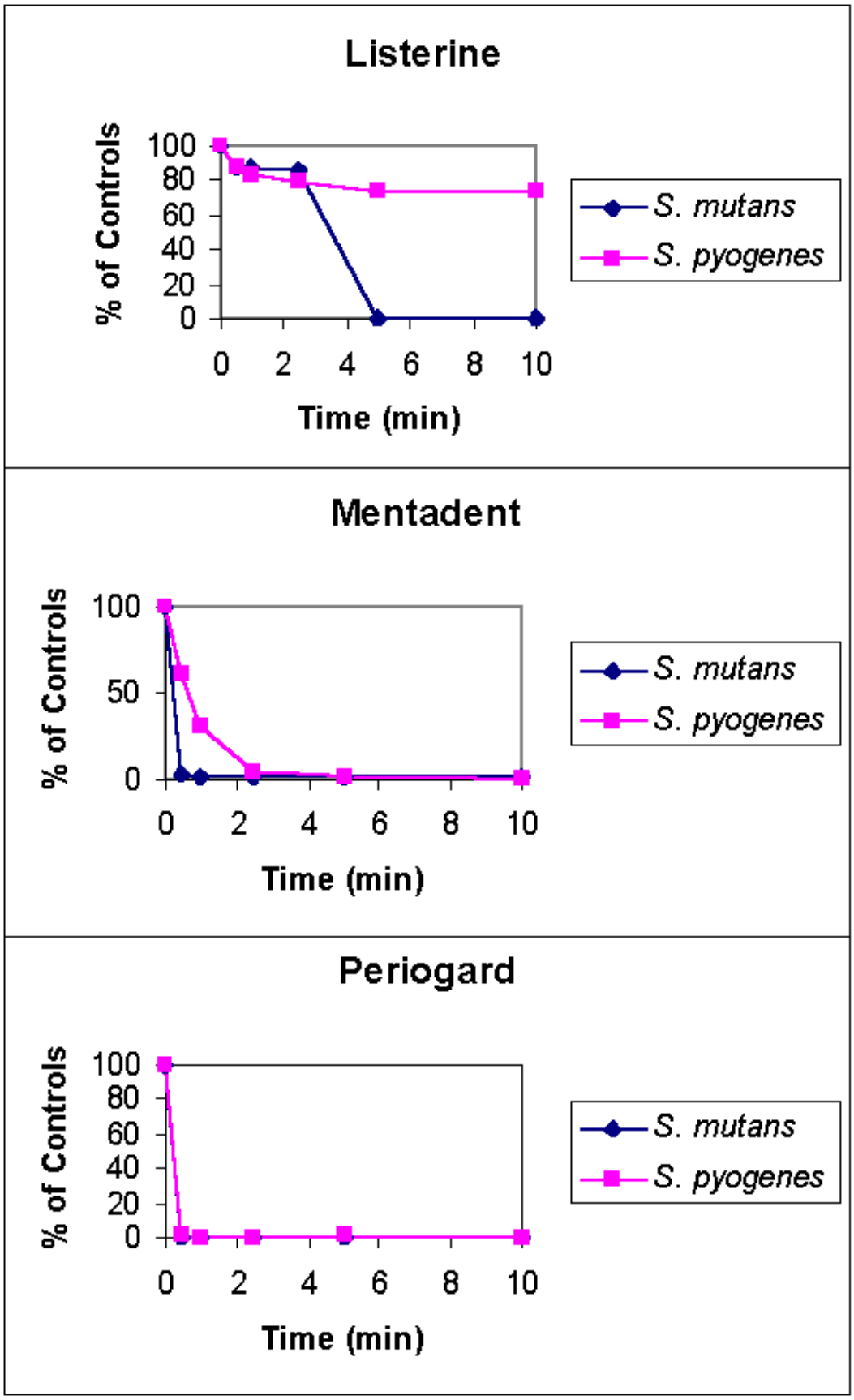
Finally, a common mistake made by students is to omit the control (time 0) sample from the experiment. Without knowing the number of organisms that grew from the original paper clip prior to treatment, it is difficult to compare the results.

SUPPLEMENTARY MATERIALS

Possible Modifications.
Students may use a gram-negative and a gram-positive organism to assess the differences of a particular disinfectant or antiseptic on various cell wall types, or they may compare a potential spore former with a nonspore former. Students may also assess the activity of various antimicrobial agents on a given species. For example, Lysol claims it is effective against *Staphylococcus*. Students may want to compare the abilities of Lysol and bleach to kill *S. epidermidis*. In laboratories with facilities capable of growing pathogenic organisms, *Staphylococcus aureus* may be used. Some undergraduate research students have utilized *Streptococcus mutans* and *S. pyogenes* to evaluate the antimicrobial activities of mouthwashes but only using approved facilities.

Related Activities.
Disk diffusion, phenol coefficient, and Kirby Bauer assays can all be used in conjunction with this activity to show other mechanisms for evaluating antimicrobic agents.

References.
1. **Benson, H. J.** 1998. Microbiological applications, short version, 7th ed. WCB-McGraw-Hill Publishers, Boston, Mass.
2. **Cooper, J. L.** 1999. Anti-microbic testing in microbiology laboratory using vinyl paper clips. Association of College and University Biology Educators, 43rd annual meeting.
3. **Difco Laboratories**. 1998. Difco manual, 11th ed. Detroit, Mich.
4. **Johnson, T. R., and C. Case.** 1995. Laboratory experiments in microbiology, brief edition, 4th ed. Benjamin/Cummings Publishing Company, Inc., Redwood, CA.
5. **Talaro, K. P., and A. Talaro**. 1999. Foundations in microbiology, 3rd ed. WCB-McGraw-Hill Publishers, Boston, Mass.


Listerine
% of Controls
100
80
60
40
20
0
0
2
4
6
8
10
Time (min)
S. mutans
S. pyogenes
Mentadent
% of Controls
100
50
0
0
2
4
6
8
10
Time (min)
S. mutans
S. pyogenes
Periogard
% of Controls
100
80
60
40
20
0
0
2
4
6
8
10
Time (min)
S. mutans
S. pyogenes