

Modeling Antimicrobial Activity of Clorox™ Using an Agar-Diffusion Test: A New Twist on an Old Experiment

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Abstract: The computer statistical software package Minitab™ is used to model sensitivity of several microbes to the disinfectant NaOCl (Clorox™) utilizing the Kirby-Bauer technique. Each group of students collects data for one microbe, conducts regression analyses (e.g. linear, quadratic and cubic), and then chooses the best-fit model based upon the highest r-value obtained. This experiment provides an opportunity to improve critical thinking skills of students by allowing them to determine which best-fit model is both mathematically and biologically acceptable.

Key Words: antibacterial, antifungal, best-fit model, disinfectant, sodium hypochlorite, Kirby-Bauer technique

A very simple way of determining the susceptibility of a microorganism to an antimicrobial agent is to use a microbe-seeded agar plate and to allow the agent to diffuse into the agar medium, which is known as the Kirby-Bauer technique. A filter disk impregnated with the agent is applied to the seeded agar surface. As the substance diffuses from the filter paper into the agar, the concentration decreases as a function of the square of the distance of diffusion. At some particular distance from each disk, the antimicrobial agent is diluted to a point that it no longer inhibits microbial growth. The effectiveness of a particular antimicrobial agent results in the production of growth-inhibition zones that appear as clear areas surrounding the disk from which the agent diffused. The diameter of the zones can be measured with a ruler and the results of such an experiment constitute an antibiogram (Atlas et al. 1995).

The agar diffusion test provides a rapid assessment of antimicrobial activity for any water soluble compound. It is often used commercially to supply basic antimicrobial data during the manufacture, as well as, quality control assurance of the finished product (Ascenzi 1996; Block 1991; Paulson 1999). Antiseptics and disinfectants used in sanitation for food processing and the medical/surgical arena, antibiotics and pesticides for clinical and agricultural use, and preservatives for food, metals, cosmetics, medicines,

and paints are all examples of compounds tested by this procedure. The purpose of this experiment is to measure antimicrobial activity of household bleach against several types of microbes in agar medium.

Materials & Methods

Seeded Agar Plates.

The bacteria *Staphylococcus aureus* (Gram-positive coccus) and *Escherichia coli* (Gram-negative rod) and the fungus *Saccharomyces cerevisiae* (Baker's yeast) can be used to test efficacy of an antimicrobial agent. Two methods of preparing microbe-seeded agar plates can be used. In the simplest method, microbes are streaked onto 15 X 100 mm Petri dishes (plates) containing sterilized plate count agar (PCA). Bacterial cultures are grown at 37°C for 16-24 hours while the yeast is cultured 24-48 hours at room temperature (23-27°C). A sterile cotton swab can be used to pick up and transfer the microbe to uninoculated PCA plates that will be used for the test. Mueller-Hinton is the standard medium used in testing antibiotics; however, one may use any non-selective (e.g. plate count or tryptic soy) agar medium that allows growth of test microbes. Best results are obtained with plates containing the same volume of medium (e.g. 10-15 ml). The plates should be cross-streaked/smeared in at least three different directions to cover most of the agar surface, which will result in confluent growth. These

seeded agar plates should be treated with antimicrobial agents immediately.

Microbe-seeded agar plates prepared by the second method may be used immediately, or stored up to one week within the refrigerator. One-hundred milliliters of PCA medium within a 250 ml Erlenmeyer flask fitted with aluminum foil lid is autoclaved for 15 minutes. Sterilized flasks are placed into a 50°C waterbath. Test microbes are cultured on a non-selective agar medium as before. The surface growth from one to several plates per microbe is aseptically removed with an inoculating loop and transferred to a test tube containing 5.0 ml of sterile 0.85% w/v NaCl in water (physiological saline). Sufficient inoculum should be added (with mixing) so that the tube is turbid or opaque in appearance or you may use McFarland standards 24 (Kerr & McHale 1994). Warn students that the following steps must be completed within 5 minutes. Label 10 empty 15 X 100 mm sterile Petri dishes with the test microbe. Remove one flask of acclimated medium from the water bath, aseptically add 0.5 ml of test tube suspension with a sterile pipette and gently swirl to mix. Acclimated (50°C) medium is used because agar solidifies at 45°C and most microbes will be killed at temperatures >50°C within a few minutes. After inoculating the flask, immediately dispense 10 ml medium per empty labeled Petri dish using a sterile pipette. You may need to tilt or swirl

each dish immediately after adding medium to disperse it evenly across the bottom. Set aside the seeded agar plates to solidify for 15-30 minutes without disturbing. If seeded plates are to be stored, seal within a plastic bag and place inverted into refrigerator immediately after they solidify.

Preparing Dilutions. Serial 1:2 dilutions (2^0 to 2^6) of the disinfectant bleach Clorox™ (Clorox Company, Oakland, CA) will provide a sufficient range of concentrations (0.01-0.71 molarity NaOCl) which may be tested for antimicrobial activity. Each group of students is given a rack containing 7 small (e.g. 15 x 100 mm) test tubes. One group of students should be given an extra test tube to prepare a $2^{0.5}$ (1/1.41) dilution. The purpose of this dilution will be explained in the results section. An example dilution series is shown in Table 1. Using aseptic techniques (e.g. sterile test tubes, water, and pipettes) when preparing dilutions is recommended but not required. Many antimicrobial agents (e.g. Clorox™ or Providone/Iodine™) sanitize the dilution blanks (especially at higher concentrations or lower dilutions). Furthermore, the high titer of test microbes on/within seeded plates will hinder growth of any microbe contaminants introduced when non-aseptic techniques are used to prepare dilutions.

Table 1: Two-fold dilutions and corresponding concentrations tested of Clorox™ (active ingredient NaOCl).

Dilution (Scientific Notation)	Fraction	Decimal	Molarity	mg NaOCl/disk (Moles/L)
2^0 (stock)	1	1	0.7095	525.0
$2^{0.5}$	1/1.41	0.71	0.5037	371.2
2^{-1}	1/2	0.50	0.3548	262.5
2^{-2}	1/4	0.25	0.1774	131.3
2^{-3}	1/8	0.125	0.0887	65.7
2^{-4}	1/16	0.0625	0.0444	32.9
2^{-5}	1/32	0.03125	0.0222	16.5
2^{-6}	1/64	0.015625	0.0111	8.3

The active ingredient in Clorox™ is sodium hypochlorite (NaOCl; formula weight = 74 g/mole) at a concentration of 5.25% w/v. Molarity (moles/liter) of the stock solution is calculated as:

$$\frac{5.25 \text{ g}}{100 \text{ ml}} \times \frac{1 \text{ mole}}{74 \text{ g}} \times \frac{1000 \text{ ml}}{1 \text{ L}} = 0.7095 \text{ moles/L}$$

Agar-Diffusion Test. Using forceps aseptically select a sterile 6 mm diameter paper (susceptibility) disk by its outer edge and dip it into one of the prepared dilutions. [Disks may be purchased from VWR Scientific Products (Chicago, IL; Cat.# 28446-002) or you can use a hole punch to cut your own disks

from any type of absorbent filter paper. Disks are autoclaved for 15 minutes in glass Petri dishes.] Remove excess solution by touching disk to inside lip of test tube and place the saturated disk onto surface of seeded agar plate. A different disk is used for each dilution, including the stock solution, and 3 disks per concentration tested are plated. Disks are arranged approximately 2-3 cm within the outer perimeter of microbe-seeded agar plates (Figure 1) as follows: plate #1 (2^0 and control disk without antimicrobial); plate #2 (2^{-1} and 2^{-6} dilutions); and plate #3 (2^{-2} through 2^{-5} dilutions). Make sure not to reposition or allow the disk to slide across medium surface once contact is made. Treated plates are inverted and immediately placed into

a 37°C incubator. After 24 hours incubation, the diameter of inhibition zone surrounding each disk is measured with a metric ruler. When recording zone of inhibition, subtract 6 mm diameter of the control disk (or inhibition zone if observed) from each measurement. For example, if the inhibition zone diameter is 13 mm, the actual inhibition is 7 mm (13 mm - 6 mm).

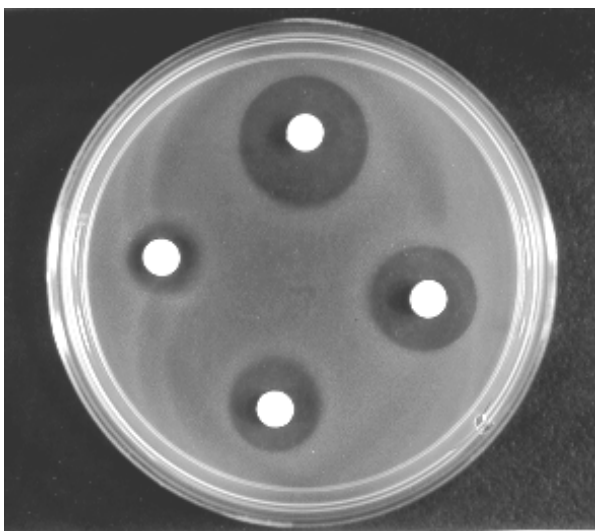


Figure 1: Agar-diffusion test on the effect of sodium hypochlorite against *Staphylococcus aureus* grown on PCA medium. Two-fold dilutions tested include (clockwise from top): 2^2 through 2^5 ($1/4$ through $1/32$).

To optimize accuracy, 10 μ l of each disinfectant concentration tested may be added per disk (before placing disk onto agar) using an automatic pipette. This allows for the exact concentration of test agent (e.g. μ g) to be determined. However, using saturated disks as described above will result in acceptable data.

Results

Antimicrobial concentration (X) and average inhibition zone diameter (Y) values can be analyzed by either linear, binomial (e.g. quadratic) or polynomial (e.g. cubic) regression. The best-fit line for each microbe tested against Clorox™, including standard deviations (error bars) for each set of data, is presented in Figure 2. All three microbes were sensitive to this disinfectant since inhibition zones developed on PCA medium. *Staphylococcus aureus* appears more sensitive than *E. coli* or *S. cerevisiae* at sodium hypochlorite concentrations ranging from 0.02-0.35 molarity. However, undiluted Clorox™ (0.71 molar NaOCl) produces a larger inhibition zone against *S. cerevisiae* compared to either of the two bacteria.

Validation of the data is presented in Table 2. Sensitivity of the two bacteria followed a quadratic

function or model while the yeast followed a linear model. The correlation coefficient (r) values were very high for all sodium hypochlorite concentrations tested. A perfect correlation between two variables (e.g. concentration vs. inhibition zone diameter) is an r value equal to 1.0 (-1.0 is a perfect negative correlation). In other words, by knowing the value of one variable, the value of the other variable can be predicted with 100% accuracy. The further the r-value is from 1.0, the less reliable any prediction will be.

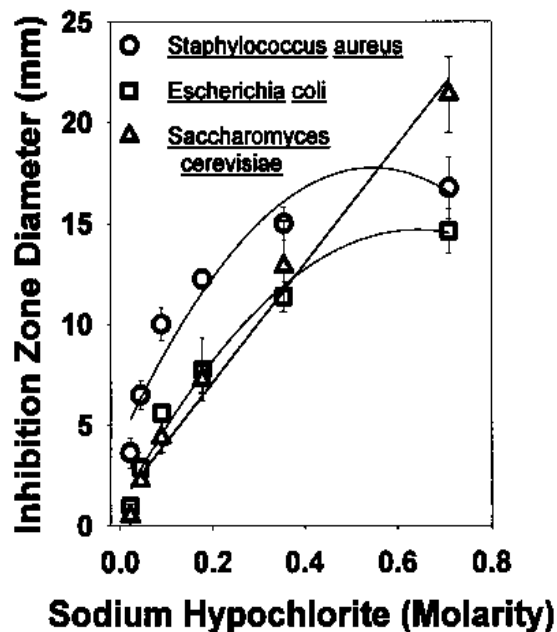


Figure 2: Effect of sodium hypochlorite on inhibition of selected test microbes.

An important aspect of this experiment is to teach students how to choose which mathematical model best fits the data collected. In the senior author's classroom students use the computer statistical software Minitab™ (Minitab Inc, State College, PA) because it is available in computer classrooms located on campus. Any computer software package that provides best-fit regressions for linear, binomial and polynomial models may be utilized. Using a 1:2 serial dilution series of Clorox™ provides an interesting challenge. Once data are collected, the only information students are given to help them determine which model to choose is that the higher the r-value, the better the model describes the data. Students enter data and run best-fit lines for all three mathematical models as shown in Figure 3A-3C. Validation of data is provided on the computer screen with each graph (model) and is shown in Table 3. Based upon the 'mathematical' analysis (r-value calculation), students are inclined to accept the cubic

Table 2: Agar-diffusion test results on the effect of sodium hypochlorite.

Microbe	Mathematical Model	Equation	R-value
<i>Staphylococcus aureus</i>	Quadratic	$Y = 4.2 + 49.5X - 45.4X^2$	0.96
<i>Escherichia coli</i>	Quadratic	$Y = 1 + 42.6X - 33.1X^2$	0.98
<i>Saccharomyces cerevisiae</i>	Linear	$Y = 1.24 + 29.5X$	0.98

Table 3: Comparison of different mathematical models used to analyze the effect of sodium hypochlorite against *Staphylococcus aureus*.

Model	Equation	R-value
Linear	$Y = 6.8 + 16.5X$	0.85
Quadratic	$Y = 4.2 + 49.5X - 45.4X^2$	0.96
Cubic	$Y = 2.2 + 98.1X - 243.7X^2 + 189.2X^3$	0.98
Quadratic ^a	$Y = 4.5 + 46.2X - 41.6X^2$	0.96

^a Includes 0.5 molarity ($2^{-0.5}$ dilution) data point in the analysis. This concentration is prepared by adding 1 ml stock to 0.41 ml water.

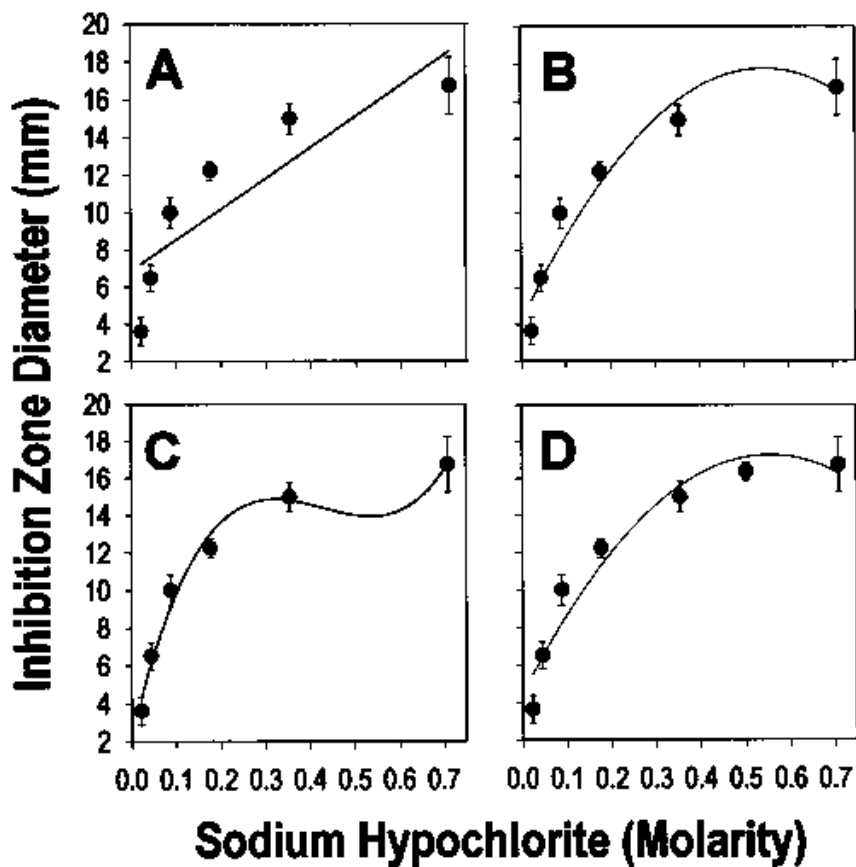


Figure 3: Different mathematical models tested to describe the effect of sodium hypochlorite on inhibition of *Staphylococcus aureus*. Models include (A) linear, (B) quadratic, (C) cubic, and (D) quadratic that includes 0.5 molarity data point in the analysis.

model since this function results in the highest r-value (0.98). The question arises as to whether it is 'biologically' feasible for a dip to occur in microbe susceptibility between 0.35 and 0.71 NaOCl molarities as this model predicts (Figure 3C). Some students believe purely in the mathematical analysis while others challenge the biological accuracy of the cubic model. Testing a sodium hypochlorite concentration that lies between 0.35-0.71 molarity (e.g. $2^{0.5}$ dilution or 0.5 molarity) provides a means to answer this question. Results indicate that the quadratic model best fits the data (Figure 2D), and the dip is of mathematical not biological origin. It should be noted that the mathematical model that describes the data most accurately is a rectangular hyperbola (binomial function). This latter model is not available with the software students use; however, the quadratic model (also a binomial function) sufficiently describes the data to enable students in formulating accurate predictions.

Discussion

The antiseptic Providone/Iodine Scrub Solution (1.17-75 μg) and antibiotic streptomycin sulfate (0.16-20 μg) also produce similar response models when tested against microbes using the Kirby-Bauer technique. One could use this technique to compare efficacy of different disinfectants, antiseptics, and/or antibiotics or investigate differences in antimicrobial activity between several brands of one specific antimicrobial agent (e.g. mouthwash).

A discussion with students of possible test microbes may be required before conducting this experiment. Choice of microbe may be based upon culture availability, student interest, or type of antimicrobial agent investigated. For example, one favorite experiment of students is to compare antimicrobial activity of different mouthwash brands. *Staphylococcus aureus* (a human skin inhabitant) and *E. coli* (an enteric of warm-blooded animals) may be logical choices for testing iodine or bleach, but improper choices for a mouthwash (Prescott et al. 1999). A solution for testing mouthwash is to prepare seeded agar plates from mouth swabbings. Another aspect one might consider when choosing a test microbe is whether the antimicrobial acts specifically

against a prokaryote (e.g. bacterium), eukaryote (e.g. fungus), or both.

This exercise provides an excellent tool to demonstrate the importance of utilizing an equation (compared to exclusively visualizing from a graph) to make an accurate prediction. Assume that a particular antimicrobial agent must be used at a rate that will result in an inhibition zone of at least 13 mm. Ask your students to answer the following question: "What minimum concentration of sodium hypochlorite will result in an inhibition zone = 13 mm for each microbe?" This is a simple calculation for the yeast because the linear equation (Table 2) is easy to use and understand. To calculate this for the two bacteria, students must first enter 13 mm (Y), rearrange the equation (Table 2) and use the quadratic formula to solve for X (molarity). A concentration of 0.22 molarity is predicted for *S. aureus*; whereas, higher concentrations of 0.42 and 0.40 molarity are required to produce the same effect with *E. coli* and *S. cerevisiae*, respectively. Utilizing the equation in the simulated exercise will allow the student to more precisely determine the exact amount of active ingredient which must be added to a formulation; therefore, not wasting chemical.

This exercise also teaches students to critically evaluate mathematical modeling in biology. Students must decide which model is both mathematically and biologically acceptable. Creative discussions result in the students formulating a rule to choose the polynomial of the smallest degree (simplest mathematical model) that has an r-value of at least 0.9 (90% accuracy) or greater. Analyzing first a linear, next quadratic and finally cubic model (Table 3) results in choosing quadratic since it is the simplest mathematical model with an acceptable r-value (0.96).

Additionally, students learn the importance of utilizing standardized methods when conducting experiments. Medium type, volume and age; disk size and composition; inoculum history and type; and incubation time and temperature are all important parameters which may affect inhibition zone diameter and ultimately interpretation and recommendations. The agar-diffusion test is a fun, problem-solving and knowledge-gathering exercise with an applied aspect. Hopefully your students will have as much fun as ours do in testing antimicrobial agents!

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Describes using a computer statistical software package called Minitab to model the sensitivity of several microbes to the disinfectant NaOCl (Clorox') using the Kirby-Bauer technique. Each group of students collects data from one microbe, conducts regression analyses, then chooses the best-fit model based on the highest r-values obtained. (Author/SAH). Mitchell, James K.; Carter, William E. Bioscene , v26 n3 p9-13 Aug 2000. Describes using a computer statistical software package called Minitab to model the sensitivity of several microbes to the disinfectant NaOCl (Clorox') using the Kirby-Bauer technique. Each group of students collects data from one microbe, conducts regression analyses, then chooses the best-fit model based on the highest r-values obtained. Antimicrobial activity assessment Antimicrobial activity was evaluated by both qualitative and quantitative test methods. The following are the descriptions of test methods employed for this study. Antimicrobial testing. Antimicrobial testing was carried out by using the shake flask method. The finished samples were washed using a standard detergent (2% on weight of fabric) and sodium carbonate (1% on weight of fabric) at 60°C. The antimicrobial activity was assessed after 50 washes by Challenge test. RESULTS AND DISCUSSION Antimicrobial activity of Aloe gel treated sample (Agar Diffusion Test) Figures 1, 2 and 3 shows the result of Agar Diffusion Test for antimicrobial effectiveness against standard test cul-. 230 Afr. J. Microbiol. Modeling antimicrobial activity of Clorox[®] using an agar-diffusion test: A new twist on an old experiment Mitchell et al. Bioscene 2000. Effect of gonococcal lipooligosaccharide variation on human monocytic cytokine profile Patrone et al. BMC Microbiol 2007. Member Articles. Paper: In vitro antimicrobial and antioxidant activities of anthrone and chromone from the latex of Aloe harlana Reynolds. To: Gelila Asamenew, Daniel Bisrat, Avijit Mazumder, Kaleab Asres. From (Name): E-mail: Only shared with authors of paper. Please enter a personalized message to the authors. More detailed explanations for your need are more likely to get a response. Send Request. Load Form Load Form. Request PDF from Authors.