Integrating Statistics with a Microbiology Laboratory Activity

WILLIAM LOROWITZ,* ELIZABETH SAXTON,† MOHAMMAD SONDOSSI,
AND KAREN NAKAOKA

Department of Microbiology, Weber State University, Ogden, Utah 84408

Statistics is an important tool for microbiologists but is virtually absent from undergraduate laboratory activities. The variables in a stringent protocol, the antibiotic disk diffusion assay described by the National Committee for Clinical Laboratory Standards, were examined by the authors as a means for introducing hypothesis testing and the application of elementary statistical tools. After several experiments, a lab activity was developed where students examine the effect of cell concentration on antibiotic activity and analyze data with the *t* test. They also collect data independently from the same samples and compare their measurements using analysis of variance (ANOVA). The outcome of the activity, including an assessment tool, indicated that students learned the appropriate use of the *t* test and ANOVA, gained an appreciation for standardized protocols, and enjoyed the experience.

Statistics is an important tool for microbiology. Statistical tools are used to collect, organize, analyze, and interpret numerical data. Descriptive statistics allow investigators to summarize large amounts of data to more understandable levels using numerical descriptors (e.g., mean, mode, median, or standard deviation) or graphical methods. Inferential statistics allow investigators to make generalizations and predictions or to estimate the relationship between variables using incomplete information. A cursory review of the professional literature reveals the widespread application of descriptive and inferential statistics. Furthermore, there seems to be a consensus among microbiology educators regarding the importance of including statistics in a microbiology curriculum, determined through informal polling and recommendations made by professional organizations like the American Society for Microbiology or the Society for Industrial Microbiology (<http://www.asm.org/Education/index.asp?bid=10054>, http://www.simhq.org/pdf/career_req.pdf). In 2003, the National Research Council published recommendations for revising undergraduate biology education in *BIO2010: Undergraduate Education to Prepare Biomedical Research Scientists* that supported stronger backgrounds in physics and mathematics and suggested that biology faculty integrate these subjects into their courses.

Astonishingly, statistical analyses are virtually absent from undergraduate microbiology laboratory activities. When a formal requirement exists, it seems that statistics are left to being taught in a mathematics course, removed from practical application in microbiology. Teaching statistics from a mathematical rather than a practical approach is a recognized problem that produces students who learn **how** to apply statistical procedures but are challenged deciding **when** to use them (1). Recommendations by statistics educators include teaching statistics in more of a “hands on” manner and promoting statistics as an experimental

science and less as a mathematics course (1, 2). Another strategy is to establish a clear link between statistics and its application to the real world (7).

We thought that one approach to this problem could be modification of typical microbiology laboratory activities to include statistical analyses and enhance critical thinking and analytic skills. As a means of providing our undergraduate microbiology majors with a practical introduction to elementary statistics, an activity was developed that investigates variables within a stringent microbiological protocol. The National Committee for Clinical Laboratory Standards (NCCLS) guidelines for antibiotic testing using a disk diffusion assay are well established, highly detailed, and of clinical importance (5). In the current study, variables which might alter the outcome of this protocol were investigated by the authors with the result that one of these variables, cell concentration, was selected for use in the student lab activity. Using inocula with different cell concentrations, students were able to explore different statistical relationships, calculating means and standard deviations and testing hypotheses using the *t* test and ANOVA.

METHODS

Cultures and antibiotics. Bacteria examined for use in developing a lab activity were *Escherichia coli* ATCC 8739, *Staphylococcus aureus* ATCC 6538, and *S. aureus* ATCC 25923. Unless otherwise noted, plates of Mueller-Hinton agar were inoculated from suspensions of cells adjusted to McFarland turbidity standards (6) using sterile saline. The antibiotics used with *E. coli* and their concentrations were: ampicillin, 10 µg; chloramphenicol 30 µg; ciprofloxacin, 5 µg; nitrofurantoin, 300 µg; gentamicin, 10 µg; tetracycline, 30 µg; trimethoprim-sulfamethoxazole, 1.25 µg and 23.75 µg. The antibiotics used with *S. aureus* and their concentrations were: ampicillin, 10 µg; chloramphenicol 30 µg; ciprofloxacin, 5 µg; gentamicin, 10 µg; oxacillin, 1 µg; tetracycline, 30 µg; trimethoprim-sulfamethoxazole, 1.25 µg and 23.75 µg; vancomycin, 30 µg (BBL Sensi-Disk, Becton Dickinson and Company, Sparks, Md.).

*Corresponding author. Mailing address: Department of Microbiology, Weber State University, 2506 University Circle, Ogden, UT 84408-2506. Phone: (801) 626-7782. Fax: (801) 626-7445. E-mail: wlrowitz@weber.edu.

†Present address. Pro Pac Labs, Ogden, UT 84405.

Media. Cultures were routinely grown in tryptic soy broth (Difco Laboratories, Becton Dickinson and Company). Mueller-Hinton agar (Difco Laboratories, Becton Dickinson and Company) was used for disk diffusion assays.

Disk diffusion assay. Except where noted, the disk diffusion assay was performed according to the protocol recommended by NCCLS (5). General details of the protocol include using a culture diluted to a 0.5 McFarland turbidity standard, plates of Mueller-Hinton agar poured to a depth of 4 mm, inoculation using a sterile cotton swab (three passes over the agar with 60° rotation each time), and incubation immediately following introduction of the antibiotic disks at 37°C for 18 hours. The McFarland turbidity standards are solutions of BaSO₄ made by mixing BaCl₂ with H₂SO₄ (6). McFarland turbidity standards are visually compared to bacterial cultures as a means of estimating bacterial concentration. For example, a bacterial culture with a turbidity equivalent to the 0.5 McFarland turbidity standard contains about 10⁸ CFU/ml. A digital caliper was used to measure the diameters of the zones of inhibition.

Statistics. The concept of hypothesis testing was reinforced by explaining the null hypothesis, or the hypothesis of no difference, and alternative hypotheses. In the activity that was developed, one null hypothesis was that the diameters of zones from antibiotic inhibition would not change due to cell concentration, and the other null hypothesis was that those diameters would not be different regardless of the person making the measurement. The importance of using statistics to determine significant differences between treatments was emphasized. The appropriate statistical test, either the *t* test or ANOVA, was applied to test each null hypothesis, as noted. The *t* test is a powerful and robust test for determining if two populations have different means when samples are independent and random and the measured variable is continuous and normally distributed. ANOVA is more appropriate (and less error prone) than running multiple *t* tests when there are more than two samples to compare. In ANOVA there are two types of variance to consider: error variance (within-groups variance) and treatment variance (between-groups variance). Since the zones of inhibition being measured by each group member are the same, the within-groups variance should be the same. Therefore, any difference between the variances of the measurements would be due to the treatment (in this activity, the individual making the measurement). ANOVA can indicate a difference between the treatments but additional tests are necessary to find which treatments are significantly different. An alpha value of 0.05 was used for all analyses. This is the level usually selected as it offers a good compromise to avoid Type I (rejecting a null hypothesis that is true) or Type II (not rejecting a null hypothesis that is false) errors.

Assessment. The effectiveness of the activity on reinforcing basic laboratory skills (e.g., aseptic technique, dilutions, using pipettes), understanding the importance of a standardized protocol, and using elementary statistics was assessed using a pre- and posttest. The pretest contained

15 multiple-choice questions; the posttest added six additional questions, including open-ended questions asking students to identify the best part of the activity and how the activity could be improved (Table 1). This assessment was approved by the Institutional Review Board of Weber State University, and anonymity of the participants was maintained throughout the assessment process. The pre- and posttests did not impact student grades.

RESULTS

Screening organisms and antibiotics. Cultures and antibiotics were screened to select an appropriate combination that would produce distinct zones of inhibition that would be easy to measure. Duplicate spread plates of Mueller-Hinton agar inoculated from overnight cultures of *S. aureus* ATCC 6538, *S. aureus* ATCC 25923, and *E. coli* ATCC 8739 were tested against several antibiotics, and diameters of the zones of inhibition were recorded after overnight incubation at 37°C (Table 2). *E. coli*, rather than *S. aureus*, was selected for further experiments because the circumferences of the zones of inhibition were more distinct. Gentamicin, ciprofloxacin, and trimethoprim-sulfamethoxazole were selected as the antibiotics to use, because they produced the zones of inhibition with the largest diameters. During development of the class activity, the effects of several variables (e.g., medium volume, preincubation of plates, and culture age) were investigated and antibiotic selection was narrowed to gentamicin and trimethoprim-sulfamethoxazole because the effect of cell concentration was distinctly different (diameters of zones of inhibition displayed a much larger change with trimethoprim-sulfamethoxazole than with gentamicin) and using two antibiotics instead of three antibiotics simplified and lowered the cost of the exercise without compromising the outcome.

Agar volume. The NCCLS protocol calls for plates of Mueller-Hinton agar that are 4 mm deep (5). With the petri plates used, that was equivalent to just under 25 ml. Thirty plates of Mueller-Hinton agar were prepared for each volume of 20, 25, or 30 ml. Average weights of the plates were 34.86 ± 0.23 g, 39.51 ± 0.19 g, and 44.55 ± 0.20 g, respectively. Plates were inoculated with an *E. coli* suspension adjusted to a 0.5 McFarland turbidity standard and gentamicin, ciprofloxacin, and trimethoprim-sulfamethoxazole disks were placed on each plate between 5 and 15 minutes after inoculation. After 18 hours of incubation at 37°C, the diameters of the zones of inhibition were recorded (Table 3). ANOVA revealed no significant differences between the diameters of the zones of inhibition between plates with different volumes of Mueller-Hinton agar.

Cell concentration. It was observed that the diameters of the zones of inhibition were larger with cultures adjusted to the 0.5 McFarland turbidity standard (agar volume experiment) than those that had occurred previously with the approximately 10-fold denser overnight cultures (screening experiment). Thus, cell concentration in the inoculum seemed a good variable to investigate. Plates inoculated with cultures adjusted to 0.5, 2, and 5 McFarland

TABLE 1. Selected pre- and posttest questions with scores

Question	% Correct	
	Pretest	Posttest
If you determined the average height of students in two classes and wanted to know if they were the same, the appropriate statistical test to use would be	31	88
A. ANOVA		
B. linear regression		
C. the <i>t</i> test		
D. chi-square		
Ten students each poured plates with one liter of molten tryptic soy agar. Which statistical test would be most appropriate to determine if they all poured the same number of plates?	8	85
A. ANOVA		
B. standard deviation		
C. the <i>t</i> test		
D. chi-square		
What is the advantage of using a McFarland standard instead of a spectrophotometer as a means of standardizing the concentration of bacteria?	80	96
A. The McFarland standard is easier to use in the field.		
B. The McFarland standard is less expensive.		
C. Many common tests have a standardized protocol that uses the McFarland standard.		
D. All of the above.		
E. None of the above.		
What is the measurement used in the disk diffusion assay to assess antibiotic resistance or sensitivity of a bacterial culture?	65	100
A. radius of the zone of inhibition from the edge of the antibiotic disc		
B. area of the zone of inhibition		
C. diameter of the zone of inhibition		
D. circumference of the zone of inhibition		
Using <i>Staphylococcus aureus</i> as the test organism, you measure the zone showing no growth around a disk of vancomycin and determine that it indicates resistance to vancomycin. The zone around a disk with a different antibiotic is twice as large. This indicates that	19	73
A. <i>Staphylococcus</i> is resistant to the other antibiotic.		
B. <i>Staphylococcus</i> is not resistant to the other antibiotic.		
C. The effect of the other antibiotic on <i>Staphylococcus</i> cannot be determined from this information.		
Why is it important that the NCCLS disk diffusion procedure be followed precisely?	23	73
Posttest only:	Yes	No
Did this exercise help you understand the use of statistical tools such as the chi-square test, student's <i>t</i> test, and ANOVA?	96	4
Did this exercise help you understand the importance of following instructions when doing standardized methods?	96	4
What do you think was the best part(s) of this experiment?	67% of the students stated that the best part of the activity was learning how to analyze data with statistics.	

turbidity standards had zones of inhibition with diameters of 23.53 ± 0.36 mm, 23.03 ± 0.41 mm, and 21.03 ± 0.44 mm with gentamicin and 28.22 ± 0.48 mm, 26.54 ± 0.61 mm, and 24.64 ± 0.55 mm with trimethoprim-sulfamethoxazole, respectively. Based on the *t* test, the diameters of the zones of inhibition with the culture adjusted to the 0.5 McFarland turbidity standard were significantly greater with both antibiotics than the zones produced with either of the denser cultures.

Plating. The sets of 30 plates used to examine the effect of cell concentration were inoculated, 10 plates each, by three of the authors. ANOVA revealed that there was no significant difference between the diameters of the zones of

inhibition within each set of 30. Therefore, it did not make a difference if one person or several different people inoculated the plates.

Class activity. Based on the results described above, a lab exercise was developed to show the importance of adhering to a standardized protocol and to introduce elementary statistics in a practical setting. The efficacy of gentamicin and trimethoprim-sulfamethoxazole with different cell concentrations was examined by comparing diameters of zones of inhibition on plates inoculated with *E. coli* suspensions adjusted to 1, 2, 3, and 4 McFarland turbidity standards with plates inoculated with a culture adjusted to

TABLE 2. Screening for antibiotic activity^a

Antibiotic	Diameter of zone of inhibition (mm)		
	<i>E. coli</i>	<i>S. aureus</i> 6358	<i>S. aureus</i> 25923
Ampicillin	19	38	37
Chloramphenicol	20	24	20
Ciprofloxacin	29	22	21
Nitrofurantoin	17	ND ^b	ND
Gentamicin	22	20	20
Tetracycline	20	32	25
Trimethoprim-Sulfamethoxazole	22	27	26
Oxacillin	ND	25	21
Vancomycin	ND	16	15

^aDuplicate plates were inoculated with overnight cultures and incubated overnight at 37°C.

^bND, not done.

the 0.5 McFarland turbidity standard recommended in the NCCLS method (Table 4). Based on the *t* test, a significant difference was observed between the diameters of the zones of inhibition for each antibiotic with each culture density compared to the culture adjusted to the 0.5 McFarland turbidity standard.

For use in class, it was decided that the activity would be performed in groups of four students. Each group inoculated two sets of ten plates, one set with an *E. coli* suspension adjusted to the 0.5 McFarland turbidity standard and the other set with a cell suspension adjusted to the 1, 2, or 4 McFarland turbidity standard. After incubation, the diameters of the zones of inhibition around the gentamicin and trimethoprim-sulfamethoxazole disks were compared between the two sets of plates using the *t* test (Fig. 1). In addition, each member of the group measured each zone independently, and the four sets of data were compared using ANOVA (Fig. 2). Means and standard deviations were calculated, as well. The complete protocol is available (4, or from the authors). Overall, the activity exposed students to new laboratory techniques (e.g., using McFarland turbidity standards, making spread plates with a swab) and increased their statistical literacy through discussing the importance and use of statistics with subsequent hands-on application where they analyzed their data and used the outcome of the statistical analyses to evaluate the null hypotheses regarding the effects of inocula concentrations and different individuals making measurements of the zones of inhibition.

Assessment. The activity was used in a sophomore-level course on laboratory procedures, the second in our major's sequence, with 26 students. A pre- and posttest (Table 1) revealed substantial improvement in understanding when the *t* test (31% to 88%) and ANOVA (8% to 85%) are appropriate analysis tools, as well as the importance of following a standardized protocol. In an open-ended

question, most of the students (67%) stated that the best part of the activity was learning how to analyze data with statistics. Students continued to perform well on questions focused on statistics that appeared in quizzes and examinations throughout the semester. In addition, 83% of student groups (10 out of 12) incorporated statistical analysis in the design of independent projects within the class. No group in six previous classes ever designed their experiments with statistics in mind.

DISCUSSION

The development of laboratory activities that incorporate statistical analyses is essential for educating undergraduates with the tools of the professional microbiologist. The American Society for Microbiology has specific recommendations regarding the basic laboratory skills microbiology students should acquire (<http://www.asm.org/Education/index.asp?bid=10051>). Most of the bench skills, that is, the laboratory skills specific to microbiology, are encompassed by the activity described here and are amply developed using activities present in microbiology lab manuals. More importantly, the activity described here will help develop laboratory thinking skills, specifically cognitive processes and analysis skills. Since the importance of these skills is widely recognized, the absence from microbiology lab manuals of activities that promote them implies that a greater effort to develop them be put forth. Hopefully, this manuscript illustrates how a common laboratory exercise can be modified to include statistical analyses that contribute to hypothesis testing, experimental design, and data analysis. The disk diffusion assay allowed students to use basic laboratory skills to introduce variables to a standardized protocol and then determine if the effects of those variables were significant by using appropriate statistical tests.

In order to develop statistical thinking with a focus on asking appropriate questions, collecting data efficiently, summarizing and interpreting data, and recognizing the

TABLE 3. The effect of medium volume on antibiotic activity^a

Agar volume (ml)	Diameter of zone of inhibition (mm)		
	Gentamicin	Ciprofloxacin	Trimethoprim-sulfamethoxazole
20	24.69 ± 0.82	38.57 ± 1.04	29.78 ± 0.76
25	24.42 ± 0.75	37.42 ± 0.79	29.31 ± 0.54
30	24.54 ± 0.66	36.17 ± 1.04	29.43 ± 0.61

^aPlates were prepared with different volumes of Mueller-Hinton agar. Zones of inhibition were measured on sets of 30 plates.

TABLE 4. Effect of cell concentration on antibiotic activity^a

Density (McFarland turbidity standard)	Gentamicin			Density (McFarland turbidity standard)	Trimethoprim-sulfamethoxazole		
	Mean	Standard deviation	<i>t</i> calculated (vs. 0.5)		Mean	Standard deviation	<i>t</i> calculated (vs 0.5)
0.5	24.8	0.45		0.5	29.2	0.45	
1	23.6	0.55	3.79	1	28.4	0.55	2.53
2	23.4	0.55	4.43	2	27.2	0.84	4.71
3	22.4	0.55	7.59	3	26.0	0.71	8.55
4	21.4	0.55	10.7	4	25.2	0.45	14.1

^aPlates of Mueller-Hinton agar were inoculated with cultures adjusted to different densities (0.5, 1, 2, 3, and 4 McFarland turbidity standards) and the diameters of zones of inhibition were measured. The means and standard deviations for sets of five plates were calculated. Values of the *t* statistic were calculated between the culture with the standard density of 0.5 McFarland and the other cultures to compare with the *t* critical value of 1.89.

Disk Diffusion Assay					
GM10, 0.5	GM10, 1			t-Test: Two-Sample Assuming Equal Variances	
24	24				
25	26				GM10, 0.5
24	25			Mean	24.4
25	24			Variance	0.266667
25	25			Observations	10
24	26			Pooled Variance	0.405556
24	25			Hypothesized Mean Difference	0
25	26			df	18
24	25			t Stat	-2.457864
24	25			P(T<=t) one-tail	0.012172
				t Critical one-tail	1.734063
24.4	25.1 = mean			P(T<=t) two-tail	0.024345
0.516398	0.737865 = s.d.			t Critical two-tail	2.100924

FIG. 1. Typical student data for diameters of zones of inhibition from gentamicin for *E. coli* cultures adjusted to 0.5 and 1 McFarland standards. Results of the *t* test, performed using Microsoft® Excel, indicate that the zones with the greater cell density were significantly smaller than the zones with the less dense culture.

Disk Diffusion										
SXT, 0.5					Anova: Single Factor					
A	B	C	D	= student	SUMMARY					
29	29	30	27		Groups	Count	Sum	Average	Variance	
32	29	31	31		A	10	298	29.8	0.844444	
29	29	30	27		B	10	302	30.2	1.511111	
30	32	31	31		C	10	304	30.4	0.266667	
30	31	31	30		D	10	298	29.8	2.4	
30	32	30	31							
30	31	31	30							
30	30	30	30							
29	29	30	31		ANOVA					
29	30	30	30		Source of Variation	SS	df	MS	F	P-value
29.8	30.2	30.4	29.8 = mean		Between Groups	2.7	3	0.9	0.716814	0.548441
0.918937	1.229273	0.516398	1.549193 = s.d.		Within Groups	45.2	36	1.255556		2.866265
					Total	47.9	39			

FIG. 2. Typical student data for measurements of the diameters of zones of inhibition from gentamicin for an *E. coli* culture adjusted to a 0.5 McFarland standard, made separately by four different students. ANOVA (using Microsoft Excel) suggests no significant difference between the sets of measurements.

limitations of inferential statistics, Hogg (3) suggested specific objectives. These include (i) emphasizing the importance of data and their concomitant variability, (ii) incorporating more statistical concepts using computers for calculations instead of machine formulas, and (iii) fostering active learning through more hands-on activities, including laboratory exercises and projects. The activity developed here (4) addresses these points. The pre- and posttests demonstrated that students learned appropriate use of the t test and ANOVA. Furthermore, they learned through experience and determining statistical significance that adherence to standard protocols decreases the introduction of variations that can skew results. The most exciting outcome was having 83% of the students incorporate statistics in the design of independent projects, the first time any group incorporated statistics in the seven times the class has done independent projects. These results are tremendously encouraging, convincing the authors that similar exercises should be designed, incorporating additional statistical methods such as linear regression, chi-square test, and descriptive statistics.

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