The Antibiotic Resistance Phenomenon: Use of Minimal Inhibitory Concentration (MIC) Determination for Inquiry Based Experimentation

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Abstract. The antibiotic resistance phenomenon in bacteria is reported regularly in the popular press and is a well recognized problem. This mini-workshop presents a multi-week module that provides a hands-on opportunity for students to investigate this problem. It introduces the students to Minimal Inhibitory Concentration (MIC) determination, use of sub-MIC levels to induce bacteria, and the possible development of cross resistance; that is resistance to an antibiotic different from the inducing antibiotic. Macrolide antibiotics (particularly tylosin, erythromycin, azithromycin, and roxithromycin) will be used in this study. Tylosin, an antibiotic with widespread use in veterinary medicine, will be used as the inducing antibiotic. Students will investigate whether increased resistance to tylosin will result in increased resistance to other macrolides. This is significant as other macrolides, particularly erythromycin and azithromycin, have widespread use in medicine. Inherent in this module is flexibility for student design to select: (1) sub-MIC [tylosin], (2) use two drugs together, or (3 ask a related question. The students can discuss the implications of the data collected probing the effect of antibiotic usage in veterinary medicine. This workshop will provide protocols needed for introductory experiments (serial dilutions), the MIC and the induction study. Participants will have the opportunity to perform MIC simulations to understand the techniques necessary to successfully complete this investigation. Finally, group discussions will explore the utility of the module and how it can be adapted for different levels of college undergraduates. Development for this course was made possible by funds from the Associated Colleges of the South Keck Science Reform Mini-grant program.

Introduction

A new course, Emerging Infectious Diseases, was developed to fulfill the science requirement for non-sciences students at the University of Richmond (UR). One major goal of this course is to pique and stimulate the interests of this population. To accomplish this, laboratory experiments were designed to be hands on and engaging.

This workshop presented a multi-week experiment that involves the students in: (1)understanding biological questions that can be asked, (2) assisting in designing an open-ended

project, (3) using equipment and procedures previously taught, (4) collecting and analyzing data, (5) understanding the significance of the data, and (6) written communication of the investigation. This laboratory module was designed to allow students some experience with independent research

Macrolides (erythromycin for example) are commonly used to treat a variety of infections, in humans, particularly respiratory and soft tissue infections. The antimicrobial spectrum of this class of drugs is somewhat larger than penicillin leading to their frequent use. Prescribing of these drugs is watched to avoid overuse. However, tylosin, a macrolide, is used exclusively in veterinary medicine to treat respiratory or skin infections in livestock. In addition, this drug is used in small animals due to its anti-inflammatory and antibiotic properties to treat colitis. It has also been used as a growth promoter in feed animals.

 Since macrolide use is widespread, the question posed investigates whether exposure to tylosin (at a level that will not kill the microbe) over a defined period (2 weeks) will lead to not only increased resistance to tylosin but to other macrolides as well.

In order for this investigation to be successful students must perform and understand what is meant by the Minimal Inhibitory Concentration (MIC). The MIC tells the experimenter what the minimal concentration of an antibiotic is needed that results in no growth of the bacteria.   MIC's are determined for tylosin, erythromycin, azithromycin, and roxithromycin   After the MIC's have been established, a class discussion ensues and the appropriate sub-MIC antibiotic concentration (tylosin) to be used for induction can be selected (students can try different concentrations to investigate what level may be needed for successful induction). Test tubes are set up that contain a volume of growth medium (generally 1 -2 ml). Students pipette the volume of the antibiotic needed to achieve the sub-MIC value they will use for their induction study (these tubes can be stored in the refrigerator). The first tube is inoculated with the selected strain and incubated overnight. Each day, for 14 days, 5 µl of the overnight culture is used to inoculate a fresh tube and incubated. At the end of the induction period the determination of the MIC's to the selected antibiotics is repeated.

Goals for this Experiment. Conceptually students will have a hands-on opportunity to demonstrate the generation of increased antibiotic resistance in selected bacterial strains.   They will also investigate the real possibility of cross resistance; that is the development of increased resistance to an antibiotic not used as the inducing agent. Technically, students will use equipment (micropipettors and microcentrifuge tubes) and "put into practice" their abilities to perform dilutions. This is a timely investigation, is suited well for non-science students (and biology majors), and provides them with a research experience.

Student procedures

For this investigation to be successful, the induction study should be preceded by introductory experiments providing the students with the skills and abilities to understand and accurately complete the necessary protocols. Preliminary procedures need to introduce the students to micropipettors (and pipetting), and serial dilutions. This laboratory equipment and procedure are pivotal to a successful induction study.

Materials
- A selected bacterial strain pre-tested to determine the MIC for the selected macrolides
- A panel of macrolides: erythromycin, azithromycin, tylosin as examples
- Luria-Bertani broth (LB) for bacterial growth and induction
- Microtiter plates (and a plate reader) or test tubes and a spectrophotometer

Preliminary experiments: Determine the baseline sensitivity/resistance to the selected bacterial strain against a panel of macrolide antibiotics: Perform MIC's

1. Inoculate the selected bacterial strain to grow overnight.
2. Have the students dilute (using serial dilutions) the overnight culture to approximately 10^3 organisms/ml. This is the bacterial sample to be evaluated for antibiotic sensitivity or resistance.
3. Aliquot the seeded broth into 12 wells in a microtiter plate (125 µl per well) or use 12 test tubes (1-2 ml LB broth).
4. Add each macrolide to the first well (or tube) of one set of 12 bacterial samples (either the 12 wells in a microtiter plate or the 12 test tubes). For the microtiter plates add 125µl of the selected antibiotic (to well #1) or add 1-2 ml of the antibiotic to the first test tube (the volume of antibiotic added should be equivalent to the seeded broth volume added in the tube). Mix well.
5. Serially dilute the antibiotic. <u>For the microtiter plates</u>, remove 125 µl of the antibiotic-seeded LB broth mixture (from well #1) and add it to well #2. Mix well, remove 125 µl and add to well #3. Keep diluting the mixture, in this manner, through well #11. Do not add any antibiotic-seeded LB broth mixture to well #12.The last well (#12) serves as a growth control. Be sure there is one well on the plate with sterile growth medium added. This serves to blank the spectrophotometer. <u>Using test tubes,</u> remove the volume of the antibiotic-seeded LB broth mixture (equal to the volume of antibiotic added) and add it to test tube #2. For example, if you are using 1 ml of seeded LB broth per tube, you will add 1 ml of the appropriate macrolide. To serially dilute, 1 ml aliquots will be removed from each tube. Remove the selected volume and add it to tube #2.   Mix well, remove the same volume (as used previously) and add to test tube #3. Keep diluting the mixture, in this manner, through test tube #11. Do not add any antibiotic-seeded LB broth mixture to test tube #12.The last test tube (#12) serves as a growth control.
6. Repeat the above process for each macrolide drug to be evaluated.
7. Representative antibiotic concentrations.

Figure 1. Representative macrolide antibiotics at concentrations appropriate for the bacterial strain we used.

Tylosin   100 ug/ml   Erythromycin   10 ug/ml
Axithromycin 10 ug/ml   Roxithromycin   100ug/ml

Well #	1	2	3	4	5	6	7	8	9	10	11	12
T	50	25	12.5	6.25	3.125	1.56	0.78	0.39	0.195	0.099	0.049	Control
E	5	2.5	1.25	0.6255	0.312	0.156	0.078	0.039	0.019	0.009	0.0049	Control
A	5	2.5	1.25	0.6255	0.312	0.156	0.078	0.039	0.019	0.009	0.0049	Control
R	50	25	12.5	6.25	3.125	1.56	0.78	0.39	0.195	0.099	0.049	Control
	Blank											

Representative microtiter plate: T = tylosin, E = erythromycin, A = azithromycin, R = roxithromycin, Blank= broth only

8. Incubate the microtiter plate or test tubes at 37° C overnight.
9. Determine the MIC results. The results can be determined by using an ELISA reader (see Figure 2) for the microtiter plate. When using test tubes, growth can be assessed by visual observation or recording optical density using a spectrophotometer.

Figure 2. Representative optical density recordings as detected by an ELISA plate reader. The MIC is the first well (lowest antibiotic concentration) where growth of bacteria is inhibited.

T	0.001	0.002	0.002	0.046	0.089	0.224	0.345	0.347	0.359	0.382	0.399	0.389
E	0.002	0.009	0.066	0.068	0.056	0.049	0.055	0.068	0.345	0.347	0.359	0.378
A	0.002	0.009	0.066	0.068	0.056	0.049	0.055	0.068	0.375	0.344	0.389	0.394
R	0.002	0.009	0.066	0.068	0.056	0.049	0.055	0.068	0.298	0.304	0.311	0.384

*One well on the plate should contain broth only to blank the spectrophotometer.
Key: T=tylosin, E=erythromycin, A=azithromycin, and R=roxithromycin.
Use these results to determine the MIC of the selected strain to each of four macrolides tested.

b. Would induction with other macrolides yield the same pattern?
c. Does induction to macrolides lead to increased resistance to other antibiotics that work at the level of the ribosome (tetracyclines for example)?
d. Does the concentration of the inducing antibiotic matter? Do you need the highest possible concentration of the antibiotic or will very low levels also induce?

e. How many days will result in induction? Will greater resistance develop over more days?

**This list is not exhaustive. It is just some examples.

12. Prepare your test tubes for the study. Aliquot 2 ml sterile LB per tube. The number of tubes set up is determined by the time line established for the study (14 tubes for a two-week study). Aseptically add the volume of the selected antibiotic (determined by the baseline MIC) to each tube. Each tube should be labeled with the student (or group name), lab section, and tube number. Tubes should be stored in the refrigerator until ready for use.

13. Leave one tube with no antibiotic added. Using a freshly growing overnight culture, dispense 5 μl of the overnight culture to tube #1 as well as the one tube with no antibiotic added (to guarantee that the culture is viable and can grow under the conditions).Incubate at 37° C overnight.

14. Each day transfer 5 μl of the overnight culture to a fresh tube (containing the determined amount of antibiotic). The number of transfers will depend on the study designed.

15. At the end of the induction series perform post-induction MIC's. Follow the same procedure as used to determine the pre-induction (steps 2-6, 8, 9 above).

16. Evaluate the data. Compare the post-induction MIC values with the pre-induction values.

17. Write up the data: This write up can take a variety of approaches depending on the desired outcome. For non-science students a formal lab report may not be helpful to the student.

18. Further studies:
    a. Kirby-Bauer assays can be preformed with other antibiotics and compare pre and post-induction samples. This could expand the number of antibiotics to be tested.
    b. Based on the data collected students can expand their study. Students can use the induced bacterial strain and investigate whether certain conditions (increased [antibiotic], or longer exposure to the inducing agent) will result in the development of greater resistance.
    c. Different microbes can be used. If the first sample was Gram positive cocci, the investigation can be performed with a different microbe, either a Gram positive rod or a Gram negative microbe.
    d. Investigations of very low concentrations (parts per million) can be used to assess the possibility of the development of resistance of microbes in the environment.
    e. Combinations of antibiotics can be used together to determine the effect on the induction of antibiotic resistance.

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About the Author

Paula Lessem obtained her Ph.D. from Rutgers University in 1994. She has been on the biology faculty at the University of Richmond since 1996. She is Director of Biological Laboratories where she is involved in design and implementation of laboratory experiments in genetics and cell and molecular biology labs. In addition, she has developed biology courses targeted at non-science students and has been involved in implementing these classes. She has been funded by the Associated Colleges of the South for the design and implementation of science courses aimed at non-science populations. One grant funded the development of the module presented in this Proceedings.