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Lesson

Assessing *in vivo* Antimicrobial Activity Through the Analysis of *Galleria mellonella* Kaplan-Meier Plots

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Abstract

Antimicrobials and their use in the treatment of bacterial infections are a fundamental lesson in all microbiology courses. Typically, cultivation-based methods are used to teach students about antimicrobial testing. A complementary approach relies on the use of *in vivo* models to assess the effectiveness of a drug within a living organism. In particular, *Galleria mellonella* (Greater Wax Moth) an invertebrate commonly used as an experimental model to study bacterial virulence and antimicrobial drug susceptibility. To provide students with an opportunity to learn about *in vivo* antimicrobial testing without the need of a hands-on laboratory experience, we designed a lesson in the form of a collection of case studies using *G. mellonella* survival curves. Through the analysis of hypothetical *in vitro* and *in vivo* experiments, students learn how living systems are beneficial for drug development and testing. In this lesson, students are given hypothetical data for an antimicrobial compound tested using multiple approaches. Working in pairs, students analyze the data derived from *in vitro* and *in vivo* tests to determine the compound's activity against a microbe. In their analysis they determine whether or not the compound could be used as a therapeutic. Their findings are summarized in the form of a short paper. With this exercise, students learn to evaluate drug testing beyond the traditional cultivation-dependent methods. The lesson provides students with an opportunity to understand how animal models are used for antimicrobial screening while strengthening their ability to analyze and interpret quantitative data.

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Supporting Materials: Supporting Files S1. Antimicrobial Testing – Microbes and compounds; S2. Antimicrobial Testing – *Galleria mellonella* papers; S3. Antimicrobial Testing – *Galleria mellonella in vivo* testing; S4. Antimicrobial Testing – Analyzing Kaplan-Meier plots; S5. Antimicrobial Testing – Case study introduction; S6. Antimicrobial Testing – Case study handouts with answers; S7. Antimicrobial Testing – Final paper assignment handout; S8. Antimicrobial Testing – Final paper rubric; S9. Antimicrobial Testing – Case study answers; and S10. Antimicrobial Testing – Case study data slides.

Learning Goals

Students will:

- Learn how invertebrate animal models can be used for antimicrobial drug testing.
- Learn how survival curves are used to assess the effectiveness of an antimicrobial in a living organism.

Learning Objectives

Students will be able to:

- Describe how *G. mellonella* can be used in antimicrobial testing.
- Evaluate *in vivo* survival data from publications.
- Compare and contrast the data derived from *in vitro* and *in vivo* testing methods.
- Interpret survival curve data for specific microorganism in the presence of an antimicrobial compound both *in vivo* and *in vitro*.
- Analyze quantitative data in the form of a Kaplan-Meier Plot (survival curve) to determine the effectiveness of an antimicrobial drug in the form of a Case Study.
- Synthesize ideas in the form of a short lab report.

INTRODUCTION

Antimicrobials are agents that kill or stop the growth of microorganisms. With the fortuitous discovery of penicillin by Alexander Fleming, antimicrobials changed the approach used for the treatment of bacterial infections (1). Although antimicrobials are still an effective treatment option today, the emergence of antibiotic resistance has pushed the need to discover novel antimicrobials for clinical use. Novel antimicrobials are subject to a variety of testing methods prior to their use. To determine if a drug is able to kill or inhibit a microbe, *in vitro* or culture-based approaches are typically used in the laboratory. Methods such as the Minimum Inhibitory Concentration (MIC) Assay and the Kirby-Bauer Susceptibility Test (2) are staples in the study of antimicrobial activity *in vitro*. These experiments allow us to assess the parameters under which the drug works best against a given microbe. One of the most desirable characteristics of an antimicrobial is its selective toxicity. Antimicrobials should target the microbe but not affect the host. The antimicrobial's effect on the host and its physiology cannot be determined using culture-based approaches, as this experimental strategy only measures killing or inhibition of the microbe in a synthetic environment (cultivation media). It is for this reason that *in vivo* testing is important for proper antimicrobial research. *In vivo* antimicrobial testing refers to the use of model organisms as testing environments to determine if a drug is effective within a living system. By using a living organism, you can also acquire information on dosage and toxicity, important characteristics for any drug meant for human use. Model organisms are routinely used to assess host-pathogen interactions, which are essential for the development of an effective antimicrobial. Mice and primates are the gold-standard models; however more recently, invertebrates are also being used for *in vivo* work (3). As vertebrate animal models are costly and require permits for their use, an invertebrate system can provide preliminary, low-cost information to justify research with other models. *Galleria mellonella* (Greater Wax Moth) has been proven as a reliable invertebrate model to study bacterial infections and antimicrobial drug susceptibility (4,5). *G. mellonella* has been shown to have high correlation to murine models for infectious studies (6). Additionally, *G. mellonella* contains immune responses similar to those of mammalian innate immunity, making it an experimental system that considers the interplay between pathogen and host. The use of this organism in microbiology research specifically focused on antimicrobial studies has significantly increased in the past years (7), indicating that its use is translatable in the field. *G. mellonella* survival curves can be used as a tool for students to learn about the effectiveness of a drug when administered within a living system (8). In a typical experiment, *G. mellonella* larvae are injected with a microbe, followed by treatment with the experimental drug. Survival of the larvae over time reflect whether or not the drug is effective in its ability to clear the bacterial infection. The survival data is represented in the form of a graph called a Kaplan-Meier plot. These graphs are used for longitudinal studies where the experimental group is tracked from the beginning of the experiment until the time of event.

The majority of microbiology laboratory courses focus on cultivation-based methods as way to teach students about antimicrobial activity. We designed a series of case studies, applicable to both remote and in-person instruction, that allow students to learn about a complementary approach to traditional antimicrobial susceptibility testing through the analysis of data. While the majority of lessons focus on the analysis of data derived from *in vitro* approaches, our exercise uses problem-based learning to expose students to the use of *in vivo* testing approaches. After learning about *in vivo* antimicrobial testing methods, students expand their understanding of antimicrobial susceptibility by analyzing *G. mellonella* survival data from one of seven possible case studies. Each of the case studies is designed to present possible outcomes and scenarios encountered when testing a drug *in vitro* and *in vivo*. By comparing and contrasting the data provided from both approaches, students can identify the advantages and disadvantages of each approach and use their collective analysis of the data to determine if a drug works.

In this lesson, students work to complete a case study with the goal of answering two main questions:

- Preliminary Testing: Is the compound effective in inhibiting microbial growth *in vitro*?
- Transferability to living system: Is the compound still effective *in vivo* when used within a living system?

Working in pairs students are given one of two possible silver-cyanoximate compounds that have been previously demonstrated to have antimicrobial properties within indwelling medical devices (9). Students then select a microbe from a list of organisms, which in our course ranged from well-studied *E. coli* to select ESKAPE (*Enterococcus, Klebsiella, Actinobacter, Pseudomonas and Enterobacter*) pathogens (10). ESKAPE pathogens are widely studied due to their high virulence and their antibiotic resistance. In the case study, students critically evaluate provided hypothetical data from both *in vitro* (MIC) and *in vivo* (*G. mellonella* survival curves) tests to determine the antimicrobial effectiveness of their assigned compound. After interpreting their data, students produce a short lab report where they evaluate the compounds potential as an antimicrobial. This lesson provides students with the opportunity to use case studies as a means to build upon knowledge in the discipline of microbiology and antimicrobial research. It also provides students the opportunity to learn about invertebrate model systems and their use in microbiology research.

Intended Audience

This lesson is intended for intermediate to upper-level life science/microbiology students in a microbiology course. We have used this lesson with biology and marine biology majors in an undergraduate institution.

Required Learning Time

This lesson was taught as part of a microbiology course for a period of approximately three weeks. The microbe/compound assignments were given at the start of the semester. Lectures providing background for the case study were integrated into the course material. The lessons themselves were completed in two 50-minute synchronous virtual lecture periods. The case study was then assigned, and students were given three weeks to analyze and write their short paper.

Prerequisite Student Knowledge

Students participating in this lesson should be enrolled in a course that has discussed material such as bacterial enumeration (Colony forming units; CFU/mL), Minimum Inhibitory Concentration (MIC), antibiotics and *in vitro* methods of antimicrobial testing (MIC, Kirby-Bauer). MIC and Kirby Bauer methods are commonly described in Microbiology textbooks. Open-source textbooks such as OpenStax Microbiology provide the necessary background on these methods (11). It is preferable that these topics are discussed prior to the case study being provided to students.

Prerequisite Teacher Knowledge

Instructors should be familiar with techniques used in antimicrobial testing such as MIC Assays and Kirby-Bauer tests. They should also be able to explain how survival plots (Kaplan-Meier plots) can be used to assess bacterial virulence and drug susceptibility testing. The *G. mellonella* model for antimicrobial susceptibility testing can be easily learned with enough detail to instruct the class from reading the literature on the subject (8,12-14). A list of manuscripts that can be used to prepare for the lesson is provided in Supporting File S2. Antimicrobial Testing – *Galleria mellonella* papers. Additionally, background on the model and the experimental design are described in the following paper (15).

SCIENTIFIC TEACHING THEMES

Active Learning

Outside of class students are required to complete assigned textbook and primary literature readings. Prompts for assigned reading material are included in the lessons. In lecture, students are first introduced to the use of *G. mellonella* and *in vivo* antimicrobial studies. Application-style questions are included for students to test their understanding of the concepts discussed. Application-style questions are asked to the entire class as well as in smaller groups (breakout sessions). The questions are used to gauge students' perceptions on antimicrobial activity, the testing of antimicrobials in the experimental setting, as well as their understanding of how animal model data provides context for a therapeutic. Prior to the discussion of invertebrates in antimicrobial testing and the assignment of the case study, students are asked to read a manuscript where this model has been used. In class, students practice the analysis and interpretation of *G. mellonella* survival plots by using Kaplan-Meier plots from published work. Students interpret and analyze Kaplan-Meier plots as an exercise in problem based-learning (16). For the entirety of the case study students work in collaborative groups of two in the analysis of the provided data. As they work on their case study, they can discuss their ideas with each other, their classmates and the instructor, who can consistently provide feedback through virtual or in-person meetings.

Assessment

Lectures include application-style questions that allow students to quickly assess their understanding of the discussed concepts. Students are asked questions specific to an assigned manuscript as part of the lesson. At the end of the lecture students demonstrate their ability to interpret a Kaplan-Meier plot by analyzing a provided survival curve derived from the literature. This final practice opportunity uses data from a different manuscript than the one assigned. The case study is then provided to students for them to work on in assigned pairs. Weekly one-hour open sessions were used to formatively assess students in their ability to interpret their assigned data. After the completion of the case study, students write a short report. Papers are assessed on the students' ability to provide proper background on the assigned microbe and compound, accurate interpretation of the data, and the use of literature to support the analysis in their discussion. The paper is the summative assessment for the assignment. As an alternative assignment to the paper, students can present their case study to the class if the assignment is used as part of an in-class activity.

Inclusive Teaching

Students do not have to pursue a career in the life sciences or allied health studies in order to benefit from the thematic nature of the exercise. The lesson is presented using a variety of modalities that benefit a variety of learners (lecture and case study). To provide representation, the selected manuscripts highlight scientist of diverse backgrounds in antimicrobial research. Student choice is supported by allowing students to choose their microbe and or compound. The format of the lesson encourages students to discuss their ideas with their peers allowing students to see different perspectives on the topics discussed. This lesson also emphasizes critical thinking, writing, and provides students with an opportunity to practice data analysis. More importantly, this is an exercise that does not require technical experience, expensive specialized laboratory equipment for its execution, and can be completed in person or online, making it readily accessible for a variety of instructional modalities, especially within resource-limited institutions.

LESSON PLAN

The lesson is designed to encourage discussion and collaboration between the students and the faculty teaching the lesson. Students are allowed to choose a microbe and compound to research independently at the start of the semester. To complement their understanding of *in vitro* testing methods, students are introduced to *in vivo* testing of antimicrobials as part of the course curriculum. After learning about *in vivo* antimicrobial testing methods, students are assigned a case study where they can collaborate to interpret data from hypothetical *in vitro* and *in vivo* experiments. By analyzing provided data and applying the concepts learned in class, students evaluate the effectiveness of the drug against a specific pathogen. They report their findings in the form of a short paper. A summary of the different scenarios and microorganisms used in the Case Study assignment can be found in Table 2. Case Study Scenarios.

Classroom Environment

This lesson was taught online (due to COVID) and included both synchronous and asynchronous teaching approaches. However, its design is meant to be applicable to a variety of teaching modalities, including in-person instruction. This lesson was taught in a classroom with 14 students working in pairs. Student pairs were assigned at random on the first day of class. After pair assignments, students were given a choice of microbe and a compound from a predetermined list (Supporting File S1. Antimicrobial Testing – Microbes and compounds). The assigned microbe and compound would be used for their case study later in the semester. A research prompt is also included Supporting File S1. Antimicrobial Testing – Microbes and compounds to facilitate the student's independent research of their assignments in preparation for the case study. For class meetings, students participated in online, synchronous, discussion-based lectures and completed activities to reinforce the topics discussed. Whole class and small group (breakout sessions) discussions were used during lecture to assess students understanding of the material. In-class practice was also used to reinforce students' ability to interpret data presented in the case study. Once the case study was provided, students were given three weeks to work outside of class time to analyze the data and complete the final short paper assignment. The instructor met with students to answer questions related to the case study virtually during open 1hr sessions. Students were encouraged to meet regularly with the instructor to discuss their progress on the assignment. This exercise assignment was the final project for our course.

Overall Instructor Preparation for Classes

The majority of the class preparation for the instructor involves reviewing reading assignments and preparing course materials used in the lectures. It is up to the discretion of the instructor to choose how they want to deliver the lesson. PowerPoint and handouts were used by this instructor for synchronous teaching. Pre-recorded lectures were also used for asynchronous instruction when needed. All course materials were made available online. Different forms of presenting the class material may be used based on instructor preference and the teaching modality.

Part I. Introduction to In Vivo Testing: G. mellonella and the Analysis of Kaplan-Meier Plots

Instructor Preparation for Class Part I

Before class, the instructor should review the literature for *G. mellonella* regarding antimicrobial testing. Suggested papers are included in Supporting File S2. Antimicrobial Testing – *Galleria mellonella* papers. The instructor should choose one manuscript from the list that is appropriate for students based on their proficiency level. This paper should be assigned at least one week prior to the discussion of the material in class. Videos demonstrating *G. mellonella* assays are also available at Jove.com (17). These are beneficial if the instructor needs a visual aid to explain this methodology to students. The PowerPoint used for the instruction summarizes the key aspects of the topic the instructor should discuss (Supporting File S3. Antimicrobial Testing – *Galleria mellonella in vivo* testing). The instructor should also go over Supporting File S4. Antimicrobial Testing – Analyzing Kaplan-Meier plots in preparation for the discussion of how Kaplan-Meier plots can be analyzed.

Student Preparation for Class Part I

Students were assigned a paper demonstrating the use of *G. mellonella* for antimicrobial research. A list of suggested papers can be found in Supporting File S2. Antimicrobial Testing – *Galleria mellonella* papers. Students were expected to complete the reading prior to attending class.

Class Part I. Introduction to *In vivo* Testing: *G. mellonella* and the Analysis of Kaplan-Meier Plots

The material in this part of the lesson was discussed using Supporting File S3. Antimicrobial Testing – *Galleria mellonella in vivo* testing. To begin the discussion, the instructor asked questions related to the characteristics of antimicrobials. For example, "What are some important characteristics of a promising antimicrobial?" This question allows students to consider the following: mode of action, toxicity, range and dosage. Following a discussion on how antimicrobials work and what they are, the instructor introduced animal models in the study of antimicrobials; specifically, *G. mellonella*. As part of the lecture students were asked to discuss the benefits of this model system. For example, an instructor could ask: "What is *G. mellonella* and how is it used in microbiology research?", "Why is this invertebrate a good model system?", or "What are the benefits of using this organism in comparison to a mouse?" The purpose of these questions is to address the usefulness and validity of the model system.

Once students had the opportunity to answer, the instructor proceeded to explain how *G. mellonella* can be used experimentally for antimicrobial testing. The instructor took time to answer any student questions. The instructor continued the presentation and discussed Kaplan-Meier plots to the class in the context of *G. mellonella* studies. In the presentation, the instructor explained how these plots are generated using a hypothetical scenario to demonstrate how the data is collected and graphed. Following this discussion, the instructor asked questions related to the assigned reading material (these questions will vary based on the instructor's paper assignment/choice). Questions specific to the assigned paper's methodology/data should be aimed to help students understand the application of the model system in an experiment.

Using figures from the assigned paper, the instructor proceeded to demonstrate how to interpret this type of data. The instructor used guide questions to develop class discussion and to highlight key points using Supporting File S4. Antimicrobial Testing – Analyzing Kaplan-Meier plots. Afterwards, students were given a *G. mellonella* Kaplan-Meier plot to interpret as a group. At the end of class all students were once again given an additional Kaplan-Meier plot to analyze. Student questions were answered.

Part II. Case Study Assignment

Instructor Preparation for Class Part I

Before class, the instructor should review the presentation found in Supporting File S5. Antimicrobial Testing – Case study introduction. This PowerPoint was used to introduce and explain the Case Study Assignment to students in the course. The instructor should also prepare individual Handouts for each Case study (Supporting File S6. Antimicrobial Testing – Case study handouts with answerss), the final paper assignment (Supporting File S7. Antimicrobial Testing – Final paper assignment handout) and its rubric (Supporting File S8. Antimicrobial Testing – Final paper rubric). In our lesson, we provided all of these material as electronic files.

Student Preparation for Class Part II

Students were assigned a microbe and a compound at the start of the semester, at this point in the lesson students were expected to have researched their assigned microbe and compound independently. A research prompt included in Supporting File S1. Antimicrobial Testing – Microbes and compounds was provided to students to aid in their research prior to this lesson at the start of the semester. Students were also taught how to analyze of Kaplan-Meier plots from the previous lesson. Students should come to class prepared to share background information on their microbe/ compound assignments.

Class Part II. Case study Assignment

A short lecture was used to introduce the scope of the Case Study assignment (Supporting File S5. Antimicrobial Testing – Case study introduction). The instructor briefly reviewed the information discussed in the course that would help them work through their case study. This was an opportunity to provide the rationale behind the exercise. It is important for the instructor to clearly state the overarching goal of the project, which is for them determine if their assigned compound works against a given pathogen. This is accomplished by interpreting data collected *in vitro* and *in vivo*. The instructor should also remind students that they have already researched their assigned microbe and compound. It is helpful to take a few minutes to have students share with the class what they know about their microbe and compound. They can also make predictions on whether or not they think their compound could work. Following this introduction of the assignment, the instructor provided each student pair with their assigned case study (Supporting File S6. Antimicrobial Testing – Case study handouts with answers). Each student pair received a different data set to analyze. Case studies were identified with the assigned microbe name. Students were also provided with the instructions describing the criteria for the paper (Supporting File S7. Antimicrobial Testing – Final paper assignment handout). Students were given the remainder of the class period to discuss their assignment and ask the instructor questions.

Class Part III. Independent Study Work and Paper Writing

Student used the provided handouts as a guide for the analysis of their data. Periodically the instructor held weekly one-hour open sessions for student pairs to check in and ask questions pertaining their case study and project. Originally our course was scheduled to have two 1.5 hour laboratory sessions a week. As we were working remotely, this time was then allotted for students to work on their case studies virtually on a weekly basis. Supporting File S6. Antimicrobial Testing – Case study handouts with answers for the questions provided in the assignment. This can be used to help instructors answer questions directly related to the data analysis. Instructors are encouraged to become familiar with the data found in Supporting File S9. Antimicrobial Testing – Case study answers prior to starting the open session. To facilitate discussion of data with students, the instructor used a guide to help students interpret their data (Supporting File S9. Antimicrobial Testing – Case study answers). Students were not given this file and it was only used by the instructor. An objective of the assignment is for students to reach conclusions about the data on their own. To assist in the writing of the paper, students followed a Departmental Writing Guide used in our program for all our Biology courses. For students who have little to no experience writing a scientific paper, it is recommended that the instructor schedule a required additional class meeting (ideally before the case study assignment) to go over the sections of this specific assignment such as Introduction, Results, and Discussion. In our program, these are exercises done as part of our Introductory Biology curriculum. We reviewed these briefly at the start of the semester for this course. Students also used the open office hours to ask the instructor questions regarding the paper and its writing. Additionally, our students made use of the campus tutoring and writing center to get feedback on their manuscripts. Papers were collected 3 weeks after the assignment was given and graded following the Final paper Rubric found in Supporting File S8. Antimicrobial Testing – Final paper rubric. A PowerPoint with the data slides for all case studies can be found in Supporting File S10. Antimicrobial Testing – Case study data slides. This file may be useful if the instructor wishes to use the data as part of a class exercises/discussion instead of the lab paper assignment.

Table 1. describes the recommended timeline for this lesson.

TEACHING DISCUSSION

The introduction of antimicrobial testing methods, a fundamental topic in microbiology education, provides students with the opportunity to learn how antimicrobials are used to control bacterial growth (18). Because antimicrobial resistance is a relevant topic, it should be discussed in microbiology courses using a problem-solving approach. Microbiology education benefits from the study of antimicrobial research as students can actively engage in the topic by using research as a means to learn (19). To complement the topic of *in vitro* antimicrobial testing, case studies focused on data analysis can be used as a way to reinforce how antimicrobials are tested in the research setting. Case-based learning (CBL) has been demonstrated to be an effective means for students to better retain and apply knowledge (20). Case studies have also been shown to be more effective than other methods of content delivery by increasing student performance in courses (16). This lesson provides a unique way of introducing students to the use of animal models with data analysis as an approach to understanding antimicrobial drug testing within a virtual environment. One of the most challenging aspects of adapting work to the virtual setting is overcoming the perception that online laboratories are not as effective as in person exercises (21). However, if students see value in what they are learning with these lab experiences, they can facilitate learning even within complex biology topics (22).

Following a blended style approach, we set to design a lesson for students to learn about antimicrobial testing without the need for a practical (wet lab) laboratory. To accomplish this, we created a data analysis assignment as a tool to introduce animal models in antimicrobial testing. In microbiology courses, students traditionally perform Kirby-Bauer Tests or Minimum Inhibitory Assays (MIC). These exercises, although critical for testing, fall short of demonstrating how we can determine the safety and effectiveness of a drug in a living system. Host-microbe interactions are pivotal in drug development, and by introducing *in vivo* testing methods, students can identify and consider variables that influence the effectiveness of the drug. Although the data used in this exercise was hypothetical in nature, the scenarios reflected real outcomes encountered when experimenting with new antimicrobials (Table 2. Case Study Scenarios). By studying and comparing different methods of antimicrobial testing, students learned to determine the benefits and drawbacks of the methods and generated conclusions on what methods were better suited for a given approach. For example, when students go over MIC data, they usually conclude that a lack of growth is equivalent to death of the microbe which is translated as "the drug being effective." In our case study, when students were given MIC data, they were also asked to interpret complementary *in vivo* data. The goal was for them to recognize the limitations of these assays, specifically, the need for additional testing methods when the results were not recapitulated using the two different approaches.

One of the objectives of the lesson was to introduce the use of animal models in antimicrobial research. The *G. mellonella* case studies provided students with the opportunity to learn how invertebrates can be used to test antimicrobials (4). By evaluating the benefits of the larval model, students gained a better understating of how animal models are useful in antimicrobial research. Comments such as "*the animal model added an invaluable level of complexity to the experiment, demonstrating how animals are used in research and how we need to treat them*" suggest that the exercise helped students understand the complexity and usefulness of animal models in antimicrobial research.

The lesson also focused on data analysis with the use of Kaplan-Meier plots. This provided an opportunity for students to interpret quantitative data. Survival patterns provided information on the effectiveness of the tested drug. An intentional aspect of the exercise was to present experimental data where culture-based data did not match *in vivo* data. Scenarios like these gave students the opportunity to consider the complexity of a living system and the variables that could affect the outcome of whether a drug was effective. For example, in one of the case studies (Supporting File S6. Antimicrobial Testing – Case study handouts with answers; *S. epidermidis*), the *in vitro* data supported the compounds antibacterial activity in killing the microbe. However, when the compound was used to treat the infected larvae, it was unsuccessful in clearing the infection. This allowed students to see how a drug that works *in vitro* does not necessarily also work *in vivo*. This lesson objective was achieved as students clearly articulated that "*the project highlighted how in vitro, and in vivo models differ and the benefits each has in interpreting data. I think the most important finding was how the success of an antimicrobial in vitro did not necessarily perform the same in vivo.*" This approach also allowed students to hypothesize possible reasons why the drug failed to clear the infection when it worked well in the culture-based test. Examples of these interpretations could include proper dosage and clearance of the drug by the host's metabolism. In each specific case study students were able identify the differences between culture-based tests and the *in vivo* (larval survival) tests. Students presented good discussion points in their papers by successfully identifying limitations of *in vitro* testing, including how *in vitro* tests do not provide information with regards to toxicity or effects the drug may have on the host. For example, a student mentioned that "*the project gave them first-hand experience in lab the process behind determining an antimicrobial's clinical efficacy and safety.*"

The survival data also highlighted the importance of controls in testing to validate one's results. In the analysis and discussion, students were able to point out how results were inconclusive unless proper controls we used as a comparison point. In one case study (Supporting File S6. Antimicrobial Testing – Case study handouts with answers; *S. mutans*) the compound was ineffective in killing bacteria when compared to the antibiotic control. This prompted the discussion of how the drug was not a better alternative than the antibiotic for treatment based on these results. They were also able to identify drug toxicity issues, an important aspect of drug safety. Students were assigned two case studies with this scenario (Supporting File S6. Antimicrobial Testing – Case study handouts with answers; B. subtilis and *E. coli*). Students successfully identified compounds that had toxic effects on the larvae and concluded that this drug was likely unsafe to use in a human patient. As the paper was the summative assessment of the lesson, successful interpretation of each case study required students to review the literature to support their conclusions. Overall, student groups were able to determine whether the drug worked based on the interpretation of the provided combined data and were able to articulate their conclusions in the written form.

This lesson was very well received by the students enrolled in our course. Students regularly attended the open sessions to discuss their data and showed interest for the relevance of the work they were conducting for the project. In many cases they took time to discuss their data with their classmates during these sessions or brought up their projects during class lectures. Students also appreciated the simplicity of the model system to better understand *in vivo* testing. For example, one student said, "*our lessons and investigations for the antimicrobial properties of silver compounds were very simple to understand with G. mellonella as our model organism.*" Students showed an interest to learn because they were motivated by the subject.

Improvements

The instructor can choose to use data from published work in the exercise instead of the hypothetical data provided. The instructor can also generate their own hypothetical Kaplan-Meier plots to reflect other scenarios such as antagonism or synergy between antimicrobials tested *in vivo*. The instructor may also choose to assign more than one case study per student group by combining data from the several scenarios provided. As the data provided is hypothetical and interchangeable the instructor can combine multiple case studies and apply them to one microorganism and compound. If given more time, students can then compare multiple scenarios and outcomes in antimicrobial testing at the same time for a given microbe.

Adaptations

Although our lessons were delivered online (fully virtual), this lesson is also intended to be used in-person. The lesson is designed to be amenable to a variety of instruction modalities. Instead of working in pairs, larger group assignments (groups of 4 or 5) can be used to accommodate larger class sizes. Larger group sizes than these are not recommended because it can make it difficult for students to coordinate meetings outside of class time, especially if the course is being instructed virtually. As an alternative, the case study can be incorporated into a lecture as an in-class exercise and does not have to span a three-week period. Additionally, in place of the paper assignment students can analyze the data and present their findings to the class as an alternative assignment. This can allow all the students in the course to consider various possible scenarios encountered when testing a novel compound. The instructor can change the content of the case study (microbe and compounds preference) based on their teaching preference.

SUPPORTING MATERIALS

- Supporting File S1. Antimicrobial Testing – Microbes and compounds. Sample microbes and compounds for case study.
- Supporting File S2. Antimicrobial Testing – *Galleria mellonella* papers. List of paper suggestions for assignment.
- Supporting File S3. Antimicrobial Testing – *Galleria mellonella in vivo* testing. Lecture presentation slides.
- Supporting File S4. Antimicrobial Testing – Analyzing Kaplan-Meier plots. Guide questions for lecture discussion.
- Supporting File S5. Antimicrobial Testing – Case study introduction. Presentation to introduce case study.
- Supporting File S6. Antimicrobial Testing – Case study handouts with answers.
- Supporting File S7. Antimicrobial Testing – Final paper assignment handout.
- Supporting File S8. Antimicrobial Testing – Final paper rubric. Rubric for assessment of final paper.
- Supporting File S9. Antimicrobial Testing – Case study answers. Data table interpreting/ summarizing case studies.
- Supporting File S10. Antimicrobial Testing – Case study data slides. PowerPoint containing individual data slides for instructor use.

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Table 1. Lesson plan for *Galleria mellonella* antimicrobial case study*.

Activity	Description	Estimated Time	Notes
Assignment of Case Study Microbes and Compounds			
Prepare microbe and compound assignments for each student pair	Create a list of student /microbe and compound assignments to bring with you to class or create a word document to post online. Provide each student pair with the complete name of the microbe (Genus and species) and the structure of the assigned compound.	5-10 minutes	Microbe and Compound lists are found in Supporting File S1. Antimicrobial Testing – Microbes and compounds. The instructor may choose any compound or antimicrobial they wish for the case study as the data presented is hypothetical in nature and it not identified with a specific compound/ microbe. The instructor may also choose to use other microorganism(s) other than those used in our lesson. Students should be assigned the compound and microbe at the start of the semester. This allows for students to independently research their assigned microbe and compound outside of class time. Supporting File S1. Antimicrobial Testing – Microbes and compounds also includes an optional assignment prompt to aid students in their research.
Preparation for Class Part I. **Introduction to *in vivo* testing methods: *G. mellonella* and the Analysis of Kaplan-Meier Plots**			
Select one primary literature article on *G. mellonella* and assign it to students.		Variable	This paper should be provided to students at least 1 week prior to the discussion of this topic in class. Supporting File S2. Antimicrobial Testing – *Galleria mellonella* papers provides a list of papers that can be assigned.
Prepare lecture slides for introduction to *in vivo* testing methods and Kaplan-Meier Plot Analysis.	Include specific questions from the assigned *G. mellonella* paper.	Variable	This lesson should be taught after *in vitro* antimicrobial testing methods have been discussed in lecture. Presentation slides are found in Supporting File S3. Antimicrobial Testing – *Galleria mellonella in vivo* testing.
Print Kaplan-Meier Analysis guide questions to use in class discussion		Variable	Supporting File S4. Antimicrobial Testing – Analyzing Kaplan-Meier plots contains guide questions that can be used to discuss the Analysis of Kaplan-Meier Plots in class.
Class. Part I. Introduction to In-vivo testing methods using G. mellonella and the Analysis of Kaplan-Meier Plots (50 minutes)			
Lecture on *G. mellonella* and its use in antimicrobial screening	Interactive lecture with application style questions. Answer student questions on the model system.	15 minutes	Include questions related to assigned paper in slides. Sample lecture slides are found in Supporting File S3. Antimicrobial Testing – *Galleria mellonella in vivo* testing.
Optional: Present to the class a video on experimental design of the assay		12 minutes-variable	Instructor used Jove video(13), This content was made available for free when the course was taught. This is optional. To save class time for discussion, the instructor can assign the video alongside the paper so that students can watch outside of class time.
Mini-lecture explaining Kaplan-Meier plots	Interactive lecture with representative data from publications. Include Kaplan-Meier plot figures from literature for students to practice the analysis in class.	20 minutes	The instructor can choose any figure from a paper where *G. mellonella* was used for antimicrobial testing. Supporting File S4. Antimicrobial Testing – Analyzing Kaplan-Meier plots contains guide questions that can be used to help with the Analysis of Kaplan-Meier Plots in antimicrobial testing.

Activity	Description	Estimated Time	Notes
Preparation for Class Part II. Case Study Assignment			
Review the provided lecture to introduce Case Study assignment to class.		15- 30 minutes	Sample Presentation can be found in Supporting File S5. Antimicrobial Testing – Case study introduction..
Prepare slides with Case study data.	Make individual files for each Case study handout. It is helpful to identify each slide deck with the student pair names. Make sure you delete the answers/ instructor notes in red.	Variable	All Case study Handouts are found in Supporting File S6. Antimicrobial Testing – Case study handouts with answers. This file also contains instructor notes/ answers to questions in red font.
Print or post copies for final paper instructions		Variable	Final paper Instructions can be found in Supporting File S7. Antimicrobial Testing – Final paper assignment handout.
Class. Part II. Case study (25 – 50 minutes)			
Mini-lecture on Case study		15 minutes	7 different case studies were prepared for this class. Each student pair received a different case study. All case studies files are in Supporting File S6. Antimicrobial Testing – Case study handouts with answers. All case study files were provided as an electronic file.
Answer student questions on case study assignment	Hand out Case studies/ Pair		Students can be given the remaining class time to start working on the case. This can also help students organize their time for outside of class work with their partner
Handout instructions for Final paper assignment		10 minutes	Instructions for final lab paper are found in Supporting File S7. Antimicrobial Testing – Final paper assignment handout. Rubric used to grade final papers is found Supporting File S8. Antimicrobial Testing – Final paper rubric.
Class. Part II. Case Study Independent Work/Writing Paper			
Analysis of data and writing of final paper		2- 3 weeks	Students were given 3 weeks to complete this assignment. Students met as a pair with instructor to go over the data as needed. These were optional. For an abbreviated version of this lesson, students can be given the case study only (no paper assignment) as part of a class exercise. The time needed to complete the assignment can be adjusted by instructor. Notes and analysis of each case study are found in Supporting File S9. Antimicrobial Testing - Case study Answers. Supporting File S10. Antimicrobial Testing- Case study Data slides contain the individual. Graphs for instructor use. These can be used to discuss the data in class or in individual meetings with students. The slides also contain notes related to the data.

**For our course, all assignment handouts, guidelines and documents were provided online.*

Table 2. Summary of Scenarios presented in each case study.

Case Study Organism	Description/Medical Relevance of Microbe	Antimicrobial Testing Scenario	Description of Case Results/Student Gains
E. coli	Gram-negative Rod Human commensal of the gut Opportunistic/ enteric pathogen	Toxicity of compound	The compound is able to inhibit bacterial growth *in vitro* however, when tested *in vivo* it is toxic. Allows students to consider drug toxicity and the differences between *in vitro* and in- vivo results.
B. subtilis	Gram-positive rod Soil microorganism Non-pathogenic, may cause food poisoning Closely- related to pathogenic species B. cereus	Toxicity of compound and control antibiotic	The compound and antibiotic control were able to inhibit bacterial growth *in vitro*, however when tested *in vivo* they were both toxic. Allows students to consider drug toxicity and the differences between *in vitro* and in- vivo results. It also allows students to consider the importance of controls and the selection of the appropriate animal model system for drug screening. Presents the importance of validating models used in research.
S. epidermidis	Gram-positive cocci Human commensal Can colonize indwelling medical devices and prosthetics	Compound in only effective *in vitro*.	Allows students to compare the differences between *in vitro* and in- vivo results. Compound is only effective *in vitro*. A drug's effectiveness can be influenced by host where it is administered.
A. faecalis	Gram-negative rod Opportunistic pathogen Causes urinary tract infections (UTI)	Compound is more effective than antibiotic *in vivo*.	Compound works both *in vitro* and *in vivo*. Presents the scenario where a novel drug is shown to work better than a known antimicrobial. Validating further testing. Allows students to consider the importance of controls as a means to validate one's results.
K. aerogenes	Gram-negative rod Opportunistic pathogen Nosocomial pathogen associated with drug resistance and outbreaks in hospital setting.	Compound is only effective *in vitro*.	Allows students to consider drug toxicity and the differences between *in vitro* and in- vivo results. Compound is only effective *in vitro*. Novel drug does not work better than a prescribed antibiotic.
P. aeruginosa	Gram-negative rod Opportunistic pathogen Exhibits drug resistance as a biofilm former Colonizes lungs CF (cystic fibrosis) patients	Compound is equally effective than the antibiotic.	Compound works both *in vitro* and *in vivo*. Allows students to consider the importance of controls as a means to validate one's results.
S. mutans	Gram-positive cocci Human exclusive pathogen Causes dental cavities and endocarditis	Compound is effective *in vivo*.	Compound works both *in vitro* and *in vivo*. Although effective, novel compound does not work better than a prescribed antibiotic.