Chapter 9

Koch's Crickets: A Study in Etiology

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Introduction

This lab is the result of a desire to make my microbiology labs both more challenging and investigative in nature. So many labs in the undergraduate setting are "cookbook" type experiences…i.e. Make smear, add stain, wash off stain, observe. I wanted to combine several of the numerous techniques that the students had learned throughout the semester and allow the students to use them in a "real life" situation. I also wanted to introduce my students to the concept of animals being used as test subjects. Due to the voluminous amounts of paperwork required, not to mention facilities needed for housing the organisms used for vertebrate research, I decided to use an invertebrate system.

In the south, crickets are a popular bait item and are thus available year-round without having to breed them in house. Also, since the majority of my students have used bait crickets before, they would be much less squeamish toward procedures involving the organism.

Whenever possible, I modeled this exercise to follow the description of Robert Koch's founding work in etiology as described in the book *Microbe Hunters* by Paul de Kruif (1926). I feel this book is almost mandatory reading for any student in any Microbiology class. This lab is suitable for any undergraduate microbiology class for sophomore through senior level, or for graduate level students when there is no distinct Pathogenic Microbiology course available.

At my school, my microbiology lab is the only section taught in that particular room the entire semester. Therefore, student preps can remain set up for multiple days without hindering further use of the room. Under these situations this lab can be easily completed in a week to ten days. Under other conditions the lab can be done in weekly "installments". This stretches out the time required for the completion of the exercise, but these installments can be run concomitantly with other experiments and exercises since each week's time requirement is less than one hour. General guidelines for running the lab under both sets of conditions (straight through and in installments) are provided in the Notes section at the end of the chapter.

Student Objectives

1. To become familiar with Koch's Postulates in a practical application.
2. To understand the steps required to identify the etiologic agent for a disease.
3. To understand how invertebrates may be used as test organisms.
4. To reinforce the techniques of sterile (aseptic) techniques, serial dilution, colony isolation and Gram staining in a practical application setting.
5. To provide students with the experience of working independently of the instructor in a multi-day exercise.
6. To review the use of controls in a scientific experiment.
7. To practice scientific record keeping and scientific writing.

Before beginning this exercise make sure you have *recently* read Chapter Four (Koch: The Death Fighter) in *Microbe Hunters*, by Paul deKruif, Harcourt Brace & Co., San Diego, 1926.

Theory and Background

Etiology is the study of the causative agents of disease. The first etiologist was Robert Koch, a nineteenth century German physician who is credited with discovering the pathogenic bacteria responsible for tuberculosis (*Mycobacterium tuberculosis)*.

The groundbreaking work of Dr. Koch demonstrated that a particular microorganism causes each particular disease. This is one of the most important concepts in the field of medical microbiology. Dr. Koch set forth the idea that to be considered to be the causative (etiologic) agent of a disease an organism had to fulfill certain criteria and to pass certain tests. These tests and criteria have been codified over the years and are now known as Koch's Postulates in honor of this important microbiologist.

The fulfillment of all of Koch's postulates is now considered absolute proof that an organism is the cause of a particular disease. Thus confirmed, that organism is then said to be the etiologic agent for the disease in question.

Koch's Postulates

In order to be considered the etiologic agent for a particular disease:

1. The causative agent (microbe) must be present in every individual with the disease.
2. The causative agent must be isolated and grown in pure culture.
3. The pure culture must cause the disease when inoculated into an experimental animal.

After Koch's original three postulates, a fourth postulate has been added.

4. The causative agent must be reisolated from the experimental animal and reidentified in pure culture.

In this laboratory exercise you will attempt to apply Koch's postulates to a particular situation in order to identify the microorganism responsible for the "Pink Plague" in the House cricket, *Acheta domestica*, which is commonly sold as fish bait and animal food.

Pink Plague

You are an entomological veterinarian specializing in infectious diseases. A business that raises crickets to be sold as bait has just contacted you. In the past two weeks they have lost practically all of the crickets in two of their breeding houses. All of the dead crickets exhibit an unusual coloration, they have turned a pale pink. The company has sent you a number of surviving crickets from their infected breeding houses. You have purchased a number of crickets of the same species from a local supplier to serve as controls.

Materials: (per group of 2-4 students)

Animal:	"Infected" crickets (3-4) Control crickets (10)
Animal Maintenance:	Dry dog food pellets (6-10) Raw carrot cross sections (one carrot per group)
Microbiological Media:	Nutrient broth dilution blanks (9.9mL in sterile test tubes; 10-14 plus extras) Nutrient agar plates *(6-10 plus extras) Melted Nutrient agar blanks* 25 mL (maintained at 60^{o}C) for pour plates *either plates or blanks required, not both
Microbiological Supplies/ Equipment:	Petri plates (disposable, 15x100mm) (one sleeve) Incubator (set at 30^{o}C) (1 per class, depending on size) Gram staining kit (1) [From Carolina Biological or Fisher] Inoculating loop (1) Spreader for dilution plating (plate count) (1) Microscope slides (3-4) Microscope with oil immersion lens and immersion oil (1) Dissecting microscope (1) Bunsen burner or alcohol lamp (1)
General Supplies/ Equipment:	Insulin syringes (calibrated to 30 units) (5-6) Vortex mixer (1-2 per class) Dissecting pins (4-5) Small dissecting scissors (optional) (1) Sterile 1.0 mL pipettes (10) and pipette aids (1) *or* Digital pipettor with sterile tips (1) Styrofoam trays (available from meat section of supermarket) (2-4) Sharps Container (Autoclavable) (1 per group)

General Supplies/ Equipment:	Insulin syringes (calibrated to 30 units) Vortex mixer Dissecting pins Small dissecting scissors (optional) Sterile 1.0mL pipettes and aids or Digital pipettor with sterile tips Styrofoam trays (available from meat section of supermarket) Sharps Container (Autoclavable)

Experimental Procedure

Stage 1: Isolation of suspected pathogen

1. Observe both the infected and control sets of crickets for signs of disease. Record all of your observations in your project notebook. Place several of your control crickets in a Petri plate. Wrap the plate with several layers of plastic wrap. Place this plate in the freezer to humanely euthanize these specimens.

2. Examine any dead crickets from the infected group under the dissecting microscope. Record your observations in your notebook.

3. Carefully dissect one of the dead infected crickets. Record your observations in your notebook. **Wear gloves during this procedure.**

4. Transfer the internal organs of the dead cricket to a tube of nutrient broth. Vortex the tube vigorously for 30 seconds.

5. Perform the same procedure on one of your frozen control crickets. Allow the cricket to thaw to room temperature before you begin your dissection.

6. Take 0.1 mL of media from the tube containing the organ of the dead cricket and prepare a dilution series to 10^{-8}. Plate 0.1mL of solution from each dilution onto the surface of a nutrient agar plate. Label each plate with the dilution used in plating. Incubate the plate for thirty-six hours at 30^{o}C. If you are preparing pour plates of these samples, add 0.1mL of the sample to a sterile Petri plate. When you have prepared plates from each dilution, pour some of the melted agar (now cooled until it can be comfortably held in the hand for at least ten seconds) into the plate while *gently* swirling the plate in a clockwise motion to distribute your sample within the agar. When your plates have completely hardened, invert them and store them in the incubator.

7. Repeat step 6 with the tube containing the control cricket's organs.

8. Incubate both sets of plates for thirty-six hours at 30^{o}C.

9. Examine the plates from the diseased cricket for colonies that are likely candidates to be the causative agent of the "Pink Plague." (Remember your observations of the dead cricket's body!). Do you observe similar colonies on the plates of the control cricket? On

the plate containing the mathematically appropriate dilution (the one giving between 30 and 300 colonies) count the number of candidate colonies. Compare this number with the number of non-candidate colonies. Record this data in you project notebook. Carefully select isolated colonies of your suspected pathogen. Streak these colonies onto fresh nutrient agar plates. Allow these plates to incubate overnight at 30^{o}. This culture will be used for tomorrow's Gram staining. *It is important that cultures used for Gram staining be no more than 24 hours old!*

10. Perform a Gram stain on the suspected pathogen. In addition to its Gram properties, make a note of its size, shape, and arrangement of bacterial cells in your project notebook.

11. Inoculate a nutrient broth tube with the remainder of the colony that you used for the Gram stain. Allow this culture to grow for 48 hours at 30^{o}C. Gently vortex the culture for 15-20 seconds 4-5 times daily to oxygenate the media and insure maximum growth. If a shaker is available - shake at a moderate rate for the entire period.

Stage 2: Inoculation of the experimental animals

1. Gently wrap an uninfected cricket in plastic wrap. The wrap should be tight enough to immobilize the cricket, but not so tight as to harm it. Place the wrapped cricket on its dorsal side (*on its back*) in one of your Styrofoam trays.

2. Fill one of your insulin syringes with the 24-48 hour culture of your suspected pathogen. Insulin syringes are calibrated in units. Carefully inject 1.0-2.0 units of this culture into the abdomen of the cricket. Place the cricket into a sterile, disposable Petri plate. You may inject all of your experimental crickets with the same syringe.

3. Repeat step 2 until at least six crickets have been injected. You may inject all or your experimental crickets with the same syringe. Distribute the crickets between at least two Petri dishes so that each dish contains no more than three crickets. Place two dog-food pellets and a slice of carrot in each dish.

4. Dispose of the syringe in the Sharps container. DO NOT RECAP THE SYRINGE!!!

5. Repeat steps 1-3 with each of six control treatment (CT) crickets. Each of these crickets will receive an injection of 1.0-1.5 units of sterile nutrient broth.

6. Place 3 completely untreated (no injections whatsoever) crickets into each of two Petri dishes. Provide these crickets with dog food and a carrot slice.

7. Observe these crickets AT LEAST three times daily until two of the newly infected crickets have died.
8. Observe the dead crickets. Compare their appearance with the original infected crickets.

9. Examine both groups of control crickets for signs of the disease. If any have died, compare their appearance to that of the dead experimental crickets.

Stage 3: Reisolation of the suspected pathogen

1. Dissect out the abdominal organs of one of newly deceased crickets.

2. Transfer the internal organs of the dead cricket to a tube of nutrient broth. Vortex the tube vigorously for 30 sec.

3. Perform the same procedure on one of your frozen control crickets.

4. Perform an identical dilution series as you did in the previous stage of the experiment.

5. Select a likely candidate colony and perform another Gram stain. Also note size, shape, and arrangement of bacterial cells.

Stage 4: Comparison of original and subsequent bacterial isolates

1. Compare these results to your original isolate. If they match, you have found the etiologic agent of Pink Plague!

2. (Optional) Write up your experiments in the form of a journal article suitable for publication in a scientific journal. Your instructor will provide with a style guide for this paper.

3. Dispose of all laboratory materials as directed by your instructor.

Questions:

1. Why did you need to plate the material from the uninfected crickets in the first phase of this study?

2. Why did you inject sterile nutrient broth into one of your sets of control crickets?

3. What percentage of pathogen colonies did you find in the infected crickets? What would have been the ramifications of finding similar colonies in your control organisms?

4. Using your notes and *Bergey's Manual* try to identify the bacterium responsible for the pink plague.

5. What difficulties do researchers have in applying Koch's postulates to viral diseases. How do they deal with these limitations?

Instructor's Notes: Koch's Crickets (The Standard Lab)

First and foremost: I recommend that students read the chapter (#4) on Robert Koch in the text Microbe Hunters by Paul deKruif (Harcourt Brace Publisher, ISBN 0-15-600262-0 for the paperback edition). This adds a *much greater* dimension to this exercise.

I also recommend that this experiment be performed by groups of three students working as a team. This is a multi-day experiment and requires that students have reasonable access to the laboratory during non-lab hours. This standard exercise is only suitable for students who are capable of working independently, without direct faculty supervision. In an AP biology-type setting this lab can be done with junior/senior level students with teacher supervision at each stage.

Safety issues are covered in Appendix A, animal care in B, and report format(s) in C.

All media, etc. are prepared using standard methods. I have the students prepare some of their own media.

I use the time line below for preparation for this lab. Day –2 = two days prior to lab, etc.

Day -2

Start cultures of *Serratia marcescens* in 9.9mL of nutrient broth (TSB may be substituted, but does not work as well). This is when I usually make up the rest of my dilution blanks. I store these extra blanks in the lab refrigerator. Set incubator to 30^{o}C.

Day -1

Purchase crickets (~26/group). Place them in a commercial, plastic cricket habitat from a bait store. Provide the crickets with dog food, carrots, and bedding (pieces of torn brown paper toweling).

Day of Lab (3 or so hours prior to lab)

Inject 2-3 crickets/group with 2-3 insulin units of *Serratia*. Place in Petri plates with one slice of carrot. These serve as the infected specimens from the "business". Set out dilution blanks, etc.

Other notes:

In my experience, the infected crickets die within 12-30 hours of injection. The most notable thing about the dead crickets is the pink color that is visible under the abdominal exoskeleton (hence the name "Pink Plague" as opposed to "systemic serratiosis").

The student will hopefully remember this color and pick the reddish pigmented colonies from their plates.

S. marcescens is a gram-negative rod.

Instructor's Notes: Koch's Crickets (The Alternate Lab)

Since I have my own teaching lab that is not used by any other faculty, I am able to provide daily access to my students who, in turn, can leave their lab material "set up". I realize that this is a somewhat rare occurrence so I have worked out a way for this lab to be done in weekly components. This does require more work for the lab instructor, but this additional time is mainly moving prep materials from incubator to refrigerator and vice versa. Most weeks' work requires less than 30-45 minutes of time so that one can easily work on other exercises during the same laboratory period.

These are the suggested changes:

Prior to lab: Inject crickets several days before the start of lab. As the crickets die, place them in groups (the ones that died since you last checked them) in Saran™ wrapped Petri plates. Keep these plates in the back corner (coldest part) of a refrigerator.

First Laboratory Meeting: Give students dead infected crickets along with several live, uninfected ones. Have them make observations and do the initial dissection and sample taking (internal organs into nutrient broth). Students then do their dilutions and plate them. Each group's plates are then well-wrapped in Saran™ wrap and placed in the incubator.

Day after Lab. #1: Instructor removes plates from incubator and places them in refrigerator. The cultures will keep for at least two weeks in this state. Check on them periodically. Sometimes if the media is very moist, the colonies will "run" and smear all over the plate.

Day before Lab Meeting #2: Remove cultures from refrigerator, unwrap, and place in incubator. I recommend that this be done at least 30 hours prior to beginning of lab.

Second Laboratory Meeting: Students count plates. Make subcultures of suspect colonies (hopefully the "pink" ones). Cultures need to be made onto solid media (for Gram staining) and into liquid media (for injection). Students then place their cultures in the incubator. This doesn't take more than 45-60 minutes. There is plenty of time to get in another laboratory exercise into this period (I have three hour labs).

Day after Lab Meeting #2: Early the next morning, remove the cultures from the incubator, wrap tubes with Saran™ wrap or seal caps with Parafilm™ and place in refrigerator.

Day before Lab. Meeting #3: Late in the afternoon remove the cultures from the refrigerator, unwrap and leave in incubator overnight.
Third Laboratory Meeting: Students perform the Gram stain. Students inject crickets with broth culture.

Now comes the tricky part! You will need to monitor the treated (injected with pathogen) crickets over the next several days. As they die they can be placed in either the refrigerator or freezer (in Saran™ wrapped Petri dishes). The other crickets will *probably* live the entire week provided the are fed and "watered" with fresh carrot slices every day or two.

Morning of Laboratory #4 (at least 4 hours before lab): Place frozen/ refrigerated crickets in the incubator. Remove the crickets from the incubator to let them cool down to room temperature prior to being distributed to the students.

Fourth Laboratory Period: Students essentially perform the dissection/dilution procedure from Lab. #1. From this point the laboratory is basically a repeat of the prior three labs.

If you have any questions about this laboratory please do not hesitate to contact me!

Appendix A Safety Issues

This is definitely NOT an introductory microbiology lab! If you have room for only one microbiology lab in your syllabus, do a Gram stain and a streak plate. I teach this lab during the last month of the semester, so I expect that the students have learned (and learned to follow) standard safety techniques appropriate to the microbiology laboratory. I realize that at larger schools there are already standard procedures in place for the precautions required for each procedure (staining, etc.) so I will address only those issues that are unique to this particular lab.

The foremost concern, of course, is the use of the syringes to inject the crickets. Gloves, of course, are no protection against a sharp needle so I do not require that they be used. I am much more afraid of a student melting a glove to their hand with a Bunsen burner than sticking themselves with a *Serratia* contaminated needle.

The main concern with the syringes is that:

1. They should not be recapped, and
2. They should be disposed of in a sharps container immediately instead of being kept around until the regular lab cleanup.

Through the years I have only had two students stick themselves with a *Serratia* contaminated needle. Neither one developed even a local point-of-entry sore. Through the years I have not only stuck myself with syringes containing *Serratia*, but on one memorable occasion (in a rush to get the crickets injected before lab) I actually injected about two units of the *Serratia* into the palm of my hand. Again, I did not develop any signs of infection at all.

Serratia cultures can be obtained from any supplier of bacterial stocks. I use Carolina Biological Supply, Burlington, NC, 800-334-5551, or www.carolina.com.

Appendix B Animal Care

I do not purchase the crickets until the last possible minute. Where I am located they are available year-round in bait shops. If I had to order them from a supplier I would arrange for them to come in not earlier than the end of the week before this lab was to be taught.

Crickets are cannibals! Overcrowding stimulates cannibalism. That is the reason for placing no more than three crickets per Petri plate. The longer you are planning on keeping crickets, the more containers you will need. I would not keep more than 50 crickets in a standard sized bait holder for more than a day or two at most. Provide them with hiding places by placing multiple sheets of paper towels in their containers (they like to hide between them). They must be provided with food (dry dog or cat food pellets, the cheaper the better) and a moisture source. I like carrot slices rather than potato bits because I find that carrots remain fungus free for longer periods. These slices need to be removed and replaced with fresh slices every day or two.

Putting crickets in a refrigerator for 10-15 minutes before injecting them slows them down and makes them easier to handle. I do this for the ones I personally inject since I am doing 50 or so at a time. I let the student enjoy the thrill of the hunt by having them grab and subdue fully active specimens! Be prepared for numerous escapees until the students get the hang of things!

Appendix C Student Report Formats

I have tried a number of variations through the years, from fill in the blank data sheets to very free-form reports. I prefer the latter rather than the former. All of my microbiology students keep a lab notebook throughout the semester. They are expected to record procedures and results for all of their experiments. This is exercise is not different. My favorite form and the one that I am currently using is having the student prepare (as a group) a journal article (3-5 pages with references) on the results of their research. I recently purchased a digital camera so that these publications can now be illustrated.