A Simple Lab Exercise Demonstrating Koch's Postulates

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Virtually every textbook of microbiology and pathology includes a discussion of Koch's Postulates, a set of demonstrative criteria for the identification of a pathogen as the causal agent for a particular disease. The laboratory exercise discussed below has enabled my students to apply this principle to a real and important plant disease. It also develops several microbiological techniques, yet requires only very basic lab equipment.

Koch's Postulates

While Pasteur demonstrated the power of vaccination to prevent anthrax in sheep, a German physician, Robert Koch (1843-1910) was successful in identifying bacteria in diseased sheep and in causing anthrax in mice and other animals by injecting them with blood from the affected sheep.

Koch devoted his life to the study of bacteria. He developed staining techniques to enhance the usefulness of the microscope. He also developed solid growth media which enabled him to separate and isolate individual bacterial cells, and made possible the production of pure strains. These methods are still in use today (Baldry 1976).

Koch is best known, however, for the discovery of the bacterium which causes tuberculosis and for the principles by which he proved the causative relationship between the bacterium and the disease.

The modern expression of Koch's Postulates can be traced to the American bacteriologist Erwin Smith (1854-1927) (Smith 1905) and can be expressed as:

1. The causative agent must be present in the diseased organism;
2. The causative agent must be isolated from the diseased organism and grown in pure culture;
3. The isolated causative agent must produce the disease when inoculated into a susceptible healthy organism; and
4. The causative agent must then be re-isolated from the experimentally diseased organism, grown in pure culture, and be found identical to the original isolate.

FIGURE 1. Symptoms of tomato speck on tomato leaves.

The Disease and the Pathogen

Bacterial speck is a disease of tomato which occurs worldwide (Good and Sasser 1980). The early symptoms are dark, water-soaked lesions 3-5 mm in diameter with distinct yellow halos (fig. 1). Large areas of the leaflets may become chlorotic and, with further advancement, leaves may become tattered. Specks also appear on the fruit, resulting in a loss of quality and in significant economic losses.

The causative agent of bacterial speck of tomato is *Pseudomonas tomato* (Okabe) Altstatt. The pathogen may remain viable in the soil for long periods of time in the absence of the tomato (Schneider and Grogan 1977). In the field, plants are inoculated by splashing rain or irrigation water as well as by wind-driven soil. Penetration is through stomates or through injuries from transplanting, cultivation, wind-blown soil, etc.

Infected plants produce large amounts of inoculum which can spread rapidly through a densely populated crop. The disease progresses most rapidly during rainy and windy weather. In the laboratory, symptoms are apparent 4-6 days after inoculation.

Equipment and Preparation

Cultures of *P. tomato* are easily maintained on petri dishes or agar

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slants containing King's Medium B (table 1). The pathogen can also be kept dormant for at least one year by transferring a colony to 1 ml of sterile water, capping, and storing at room temperature. Streaking from the water to a petri dish and incubating at room temperature readily produces an active culture. The identity of the culture can be checked by its fluorescence under ultraviolet light and by its ability to produce the characteristic symptoms on tomato.

TABLE 1. King's Medium B

Proteose Peptone No. 3	2.0%
Glycerol, C.P.	1.0%
K_2HPO_4 (anhydrous)	0.15%
$MgSO_4$ 7 H_2O	0.15%
Agar	1.5%
pH 7.2	

(King, *et al.*, 1954)

We have successfully used several cultivars of tomato as laboratory hosts and believe that most commonly available tomatoes are suitable. Plants having four true leaves, four to eight weeks old, growing in two- or four-inch pots have worked satisfactorily. We have a greenhouse available for production and maintenance of our plants, but any environment with sufficient light, temperature, and water will work. The disease does not spread readily on the greenhouse bench, and there appears to be no risk to other plants (Schneider and Grogan 1977).

The remaining materials and equipment needed for the exercise are common laboratory supplies.

Class Exercise

Table 2 lists the equipment and supplies to be provided at each work station. Plates may be pre-poured or prepared by the students depending on the time and equipment available.

As written, the exercise involves about one hour the first week, one-half hour the second, and fifteen minutes observation time the third week.

TABLE 2. Equipment and Supplies Needed

First Week	*Second Week*
Infected tomato plant	UV viewer
Disinfectant for lab benches	Q-tips
Bunsen burner, matches	Bunsen burner, matches
Transfer loop	Transfer loop
Sponge	Test tube
Test tubes with 1 ml sterile water (2)	Distilled water wash bottle
Glass stirring rod	Healthy tomato plant
Cork borer	Pot marker
Forceps	
Petri dishes with King's B agar (2)	
Distilled water wash bottle	
Marking pen	

First Week:

1. Present the students with diseased plants and observe and discuss the symptoms. One plant can provide for four or five students.
2. Remove a leaflet with speck symptoms and cut out a circle of leaf tissue with a medium-sized cork borer.
3. Take up disc with forceps, rinse first with tap water then with distilled water, and place in a test tube with 1 ml sterile water.
4. Thoroughly macerate the leaf disc with a glass stirring rod which has been sterilized by passing through a flame.
5. Using aseptic technique, streak a petri dish containing King's Medium B from the water-leaf suspension. Care must be exercised in flaming and cooling the inoculating loop during the streaking process since growth is profuse and individual colonies are desired (fig. 2).
6. Label the plates with the name of the medium, the inoculum, the date, and the initials of the worker. Incubate the cultures upside down for one week at room temperature. Inspect the plates daily and refrigerate as needed to prevent overgrowth. Cultures should be removed from the refrigerator 24 hours prior to continuation of the exercise. Plates should be stored upside down to prevent condensation water from running across the agar surface.

Second Week:

1. Examine the cultures. A properly streaked plate will have individual cream-colored bacterial colonies available and will be free of contaminants. Check for fluorescence under ultraviolet light. (A standard "black light" will work.)
2. Prepare inoculum by removing a single colony of bacteria with an inoculating loop and transferring it to 3 ml of distilled water in a test tube. Disperse the bacteria into the water by vigorous thumping and swirling.
3. Inoculate healthy plants by dipping a cotton swab into the inoculum and rubbing it gently onto the upper leaf surface. Caution should be exercised not to tear the epidermis;

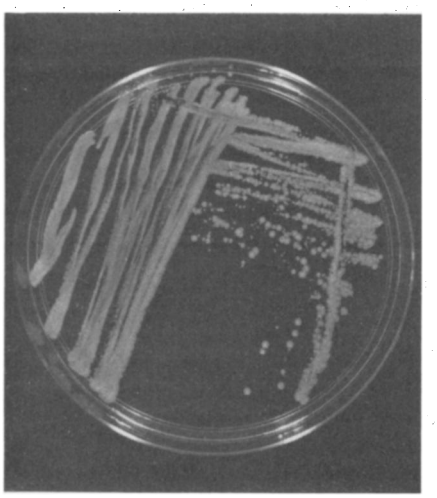

FIGURE 2. Agar plate properly streaked making individual colonies available.

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an important point can be made that bacteria can enter through the natural stomatal openings. Inoculate all leaflets of one or two leaves.

4. Using the same procedure, inoculate control plants with distilled water only.

5. Use a wooden pot marker to record the date, treatment, and student initials, and return to the growing area.

Third Week:

1. Examine the newly inoculated plants for symptoms of tomato speck. Compare these symptoms with the original plants. Compare inoculated leaves with those not inoculated.

2. Discuss how Koch's Postulates have been demonstrated (and what remains to be done to satisfy the fourth requirement.)

Variations on the Basic Exercise

The steps outlined above can be broadened slightly to produce a more systematic approach to the demonstration. Leaves from a healthy plant could also be sampled for the presence of the pathogen and a "blank" or control sample carried through. Healthy plants could be inoculated with non-pathogenic bacteria or with contaminants from the agar plates (although we have experienced surprisingly infrequent contamination).

The most obvious addition to the exercise is to re-isolate the pathogen from the newly infected tomato and compare it with the initial isolate, thereby satisfying the fourth postulate.

I have found, however, that the more basic steps detailed above are of sufficient length to adequately demonstrate the principles in question within a reasonable framework of time.

Conclusion

Working with tomatoes infected with *Pseudomonas tomato*, I have provided a laboratory exercise which can easily and in a reasonable period of time demonstrate the workings of Koch's Postulates. The exercise also provides an opportunity to practice aseptic techniques used in microbiological laboratories.

I would be happy to provide a bacterial culture to anyone who would like to try the procedure.

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Letters

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or "models," one sees immediately why creationism cannot be part of science. The fundamental postulate of all creationists' "models" is a supernatural creative force. For more than 300 years, scientists have agreed that assumptions about the supernatural were not admissable as scientific postulates. All the postulates of the three major theories in evolution are assumptions about nature. The attempts to bring the supernatural back into science stem from a failure to understand the limited realm of science, and to sense vast realms of human experience outside of science. The proper teaching of science as systems of theories —hypothetico-deductive systems— could help remove some of this failure.

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Chicken Embryos

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