Lab VII-4

Investigating Bacterial Antibiotic Sensitivity

Equipment and Materials

You'll need the following items to complete this lab session. (The standard kit for this book, available from *www.thehomescientist.com*, includes the items listed in the first group.) In addition to the items listed below, you'll also need the equipment and materials from lab session VI-2 for making up four nutrient agar Petri dishes and six nutrient broth tubes.

Materials from Kit

- □ Goggles
- □ Beaker, 250 mL
- □ Antibiotic capsule, amoxicillin (250 mg cap)
- □ Antibiotic powder, chlortetracycline (3.3%)
- □ Antibiotic powder, sulfadimethoxine (88%)
- □ Antibiotic solution, neomycin (200 mg/mL)
- □ Chromatography paper
- □ Forceps
- □ Petri dishes
- □ Pipettes
- □ Ruler
- □ Test tubes
- □ Test tube rack

Materials You Provide

- □ Gloves
- □ Aluminum foil
- □ Chlorine bleach container
- □ Hole punch (or scissors)
- □ Marking pen
- □ Microwave oven
- □ Nutrient agar (see Lab VI-2)
- □ Nutrient broth tubes (see Lab VI-2)
- □ Pure cultures (from Lab VI-3)
- □ Refrigerator
- □ Sanitized work area
- □ Soda bottles
- □ Water, distilled

Background

If you or any member of your family is immuno-suppressed, do not do this session unless your physician tells you that it is safe to do so. The three bacteria species we use in this session are widely used for high-school biology labs, and are generally considered to be non-pathogenic. Regardless, you should exercise extreme caution

in dealing with and disposing of them, particularly in the second and third procedures.

In bacteria and other organisms that reproduce quickly, genetic mutations are frequent. Most of these mutations are harmful, leading to the death of the individual bacterium. The end of the line, so to speak. Some mutations are neither harmful nor helpful, so that bacterium and its descendants simply continue reproducing. A few mutations are helpful, at least in specific circumstances, by giving that bacterium and its descendants a selective advantage.

For example, in a large population of a particular species of bacteria, the vast majority of those bacteria may be vulnerable to a specific antibiotic. In the presence of that antibiotic, nearly all of the bacteria die off quickly, or at least stop reproducing. But “nearly all” is not “all.” A tiny percentage of the original population may have genetic mutations that provide partial or complete resistance to that antibiotic.

This, incidentally, is why it's a very good idea to finish the full course of an antibiotic rather than stop taking it once you feel better. Discontinuing use of the antibiotic prematurely allows the small surviving numbers of bacteria to breed quickly and the infection comes roaring back, this time resistant to the antibiotic.

Antibiotics fall into one of two broad classes (with some blurring, depending on the specific bacterium in question). *Bactericidal* (also called *bacteriocidal* or *bcidal*) antibiotics—including amoxicillin and neomycin—actually kill bacteria via one or more mechanisms. *Bacteristatic* (also called *bacteriostatic* or *bstatic*) antibiotics—including chlortetracycline and sulfadimethoxine—do not kill bacteria directly, but greatly inhibit their reproduction.

In either case, antibiotics do not eradicate the bacteria present. Instead, they simply reduce the population to a small enough number that your own immune system can deal with them.

So, let's say we start with a population of one billion bacteria, all but one of which is susceptible to the bactericidal antibiotic we introduce into the culture. In short order, 999,999,999 of the bacteria drop dead, leaving only one survivor. From the point of view of the 999,999,999 bacteria, this is an end-of-the-world scenario, but from the point of view of the sole survivor, this is very good news indeed. It now has the resources of the entire “world” available to it. Plenty of food, plenty of room to grow, and no interfering neighbors. Time to have lots of children. Hurray!

That surviving bacterium immediately gets to work. After 20 minutes, there are two of it. After 40 minutes, there are four. And so on. After 30 generations—only 10 hours—there are again one billion bacteria present, and every one of them is resistant to the antibiotic. (This is actually a gross oversimplification of bacterial resistance to antibiotics, but it hits the high points.)

In the last lab session, we produced pure cultures of *Bacillus subtilis*, *Micrococcus luteus*, and *Rhodospirillum rubrum*. In this session, we'll test those three species for susceptibility to four antibiotics: amoxicillin, chortetracycline, neomycin, and sulfadimethoxine. We will then develop a strain of one of the species that is resistant to one of the antibiotics.

Procedure VII-4-1: Testing antibiotic sensitivity

In the preceding lab session, we produced pure broth cultures of three species of bacteria. In this procedure, we'll inoculate Petri dishes with those pure broth cultures, flooding the agar surfaces of the dishes to grow a bacterial “lawn” in each dish. After inoculating the dishes, we'll place paper discs infused with various antibiotics on the agar surfaces and incubate the Petri dishes until the

bacterial lawn appears. If a particular species of bacteria is sensitive to one or more of the antibiotics, an area free of bacterial growth (called an *inhibition zone*) will appear around the disc or discs. We'll measure the sizes of those zones, which indicate the relative sensitivity of the various bacteria to the various antibiotics.

1. If you have not already done so, put on your goggles and gloves.
2. Sterilize several plastic pipettes. To so so, fill the 250 mL beaker with tap water and fill each of the pipettes with that tap water. Put the beaker in the microwave and bring the water to a boil. Allow the water to boil gently for a minute or two, and then carefully remove the beaker from the microwave, cover it loosely with aluminum foil to prevent airborne contamination, place the beaker in your sanitized work area, and allow it to cool.
3. Loosen the cap on the 125 mL bottle of sterile nutrient agar you made up in session VI-2 and place the bottle in the microwave. Heat it carefully, 15 or 30 seconds at a time and swirling the bottle between heatings, until the agar is warm enough to flow freely. Carefully remove the bottle from the microwave oven and place it in your sanitized work area.
4. Carefully remove a sterile Petri dish from its packaging. Keep the base and lid in tight contact to avoid contaminating the dish. Place the Petri dish base (smaller) side up on your sanitized work surface and use the marking pen to label it. Write a tiny “1” at the center of the dish (to indicate the first bacteria type, *Bacillus subtilis*). Designate the divider in the Petri dish as pointing to the noon and 6:00 positions on the circumference. Around the edge of the dish surface write a small “A” at about 2:00, an “C” at 4:00, an “N” at 8:00, and a “S” at 10:00 (for amoxicillin, chlortetracycline, neomycin, and sulfadimethoxine, respectively).
5. Repeat step 4 with a second and third Petri dish, labeled “2” and “3” for *Micrococcus luteus* and *Rhodospirillum rubrum*, respectively. (Retain the fourth Petri dish for use in the following procedure.)
6. Working aseptically, lift the lid of the first Petri dish just enough to transfer sufficient warm liquid agar gel to each half of the dish to fill it to a depth of a few mm. Tilt the dish back and forth to spread the liquid agar, and then place the dish on a clean, flat surface to cool. Fill the other two dishes using the same procedure. When you have filled all three dishes, place them in the refrigerator and allow them to cool for several minutes, until the agar gels sets completely.

In the following steps, which should be performed in your sanitized work area, we'll flood the agar surfaces of the three Petri dishes with a few mL each of the corresponding pure broth cultures. The goal is to produce an even growth of the bacteria across the entire surface of the agar, called a *bacterial lawn*. Having even coverage makes it easier to determine the relative effectiveness of different antibiotics in retarding bacterial growth.

Before proceeding, prepare 1 mg/mL antibiotic solutions, as follows:

- Amoxicillin – dissolve one 250 mg capsule in 250 mL of distilled water in a clean, labeled soda bottle.
- Chlortetracycline – This powder contains 3.3% active chlortetracycline, or 33 mg/g.) Dissolve 3.0 g of the chlortetracycline powder in 100 mL of distilled water in a clean, labeled soda bottle. If you do not have a balance, ???
- Neomycin – use a clean graduated pipette to transfer 1.25 mL of the stock 200 mg/mL neomycin solution from the kit to a clean, labeled soda bottle and add distilled water to bring the volume to 250 mL.

- Sulfadimethoxine – This powder contains 89+% active sulfadimethoxine, or about 890 mg/g. Transfer 0.28 g of the sulfadimethoxime powder from the kit to a clean, labeled soda bottle and add distilled water to bring the volume to 250 mL. If you do not have a balance, ???

These dilute antibiotic solutions are unstable, and should be prepared as soon as possible before use, ideally during the lab session itself. If you must store them for more than an hour before use, keep them refrigerated. We have successfully used all of these solutions after 72 hours of refrigeration, but the antibiotics degrade unpredictably, as do activity levels.

If you incubate your cultures at 37 °C overnight, the refrigerated solutions should be fine. If you incubate at room temperature for several days, mix up fresh solutions for the following procedures.

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7. Set up a beaker or similar container filled with one part of chlorine laundry bleach to four parts water. This serves as your disposal vessel during the following steps.
8. Remove the three Petri dishes and the three pure broth cultures from the refrigerator and place them on your sanitized work surface.
9. Remove a sterile pipette from the beaker and expel all of the water from it back into the beaker.
10. Squeeze the pipette bulb to expel as much air as possible, uncap the *Bacillus subtilis* broth tube, insert the tip of the pipette into the tube, and draw up a full pipette of *Bacillus subtilis*. Recap the tube and replace it in the rack.
11. Lift the lid of Petri dish #1 just enough to allow you to insert the tip of the pipette. Expel all of the liquid in the pipette into one section of the Petri dish and tilt the dish back and forth to distribute the *Bacillus subtilis* broth culture across the entire surface of the agar.
12. Tilt the dish to collect the remaining liquid along the edge and draw that liquid back up into the pipette.
13. Expel the liquid into the other section of the Petri dish, tilt the dish back and forth to distribute the liquid across the entire agar surface, and again draw up the excess liquid into the pipette.
14. Replace the lid on the Petri dish. Immerse the tip of the pipette into the chlorine bleach solution in the disposal vessel, expel the liquid in the pipette into the disposal vessel, and draw up a full pipette of the bleach solution. Allow the pipette to remain in the disposal vessel.
15. Repeat steps 9 through 14 to inoculate a bacterial lawn of *Micrococcus luteus* in Petri dish #2 and *Rhodospirillum rubrum* in Petri dish #3.
16. Allow all three dishes to remain undisturbed for several minutes
17. While you're waiting, cut or punch 16 discs (or squares) of chromatography paper 0.5 to 1 cm in diameter (or on a side). We'll use 12 of those in this procedure and the remaining four in the next procedure.
18. Flame-sterilize the forceps, and use it to transfer four of the paper discs to Petri dish #1, positioning them two per section and spaced so as to allow the maximum possible distance between each disc and other discs and the side of the Petri dish. Use the forceps tip to press down gently on each disc to cause it to adhere to the agar surface. Lift the dish lid as little as possible during this procedure, and replace it immediately after you complete this step.

These discs are not sterile, but we're about to saturate them with relatively concentrated solutions of antibiotics, which should kill or suppress any incidental microorganisms present on the paper.

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19. Repeat step 18 for the other two Petri dishes. When you finish, you should have three Petri dishes, each with four paper discs spaced widely on the agar surface.
20. Use a sterile pipette to draw up 0.5 mL or so of the metronidazole solution. Lift the lid of Petri dish #1 just enough to introduce the tip of the pipette, touch the tip of the pipette to the paper disc that corresponds to the “A” label on the dish, and expel just enough of the amoxicillin solution to dampen the paper disc. Do not allow the pipette to touch anything in the dish other than the surface of the paper disc. Carefully withdraw the pipette and replace the lid on the Petri dish.
21. Repeat step 20 to moisten the “A” discs in Petri dishes #2 and #3.
22. Repeat steps 20 and 21 to moisten the other nine discs in all three Petri dishes with the corresponding solutions of chlortetracycline, neomycin, and sulfadimethoxine.
23. Allow all three Petri dishes to sit undisturbed on a flat surface for one hour or more. The goal is to allow the paper discs to dry out and adhere to the agar surface.
24. Invert the Petri dishes (agar-side on top) and allow them to incubate in a dark area. At body temperature (37 °C), noticeable growth should occur overnight. If you're incubating at room temperature it may require two days or more for growth to become evident.

    If one or more of the paper discs falls off when you invert a Petri dish, don't be too concerned. By that time, the antibiotic solution has infused into the agar.

25. After the bacterial lawn has fully developed in all three of the Petri dishes, use the ruler to measure the diameter of the inhibition zone (the area in which the bacterial lawn is either absent or significantly less dense than the surrounding area), if any, that surrounds each antibiotic disc, and record those values in your lab notebook.

    In some cases, the boundary may be quite distinct, with a completely clear area abutting the unaffected bacterial lawn. In other cases, there may be a more or less gradual transition from clear through slightly cloudy to very cloudy to the unaffected bacterial lawn. In that case, do your best to measure the diameter of the area that includes all of the clear and cloudy portions to the edge of the unaffected lawn.

26. Sterilize the three used Petri dishes as described in the preceding lab session and dispose of them.

Procedure VII-4-2: Culturing an antibiotic-resistant bacteria strain

In the preceding procedure, we tested three species of bacteria for sensitivity to four antibiotics. In this procedure we'll choose one of those 12 combinations and culture a strain of that bacteria that is resistant to that antibiotic.

1. Based on your results in the last procedure, choose a bacteria/antibiotic combination in which the bacteria showed moderate susceptibility to the antibiotic, as indicated by a medium-size inhibition zone, ideally one with a gradual transition from clear to cloudy. For example, if you found that neomycin is moderately active against *Rhodospirillum rubrum*, you might choose that combination.
2. Prepare and sterilize six glass broth tubes, each about 3/4 full of nutrient broth, as described in lab session VI-2. Allow the tubes to cool and label them #1 through #6.
3. Observing aseptic procedure, inoculate each of the six tubes with the pure culture of the selected bacteria.

4. Observing aseptic procedure, use a sterile pipette to transfer two drops of the selected antibiotic solution to tube #1, four drops to tube #2, eight drops (0.25 mL) to tube #3, 0.5 mL to tube #4, and 1.0 mL to tube #5. Retain tube #6 unchanged as a control.
5. Incubate all six tubes in a dark area, observing them regularly until changes become apparent in tube #1. At 37 °C, changes may appear overnight. At room temperature, it may take two or three days for changes to become evident.
6. Once tube #1 shows a visible change, continue incubating the tubes, keeping a close eye on tubes #2 through #5 and comparing them for cloudiness against the control tube. Cloudiness indicates bacterial growth, and our goal is to determine the tube with the highest concentration of antibiotic in which growth of the bacteria is not completely inhibited.

Procedure VII-4-3: Retesting sensitivity of the resistant strain

Having developed a strain of bacteria known to be resistant to one of the antibiotics, our next step is to re-culture that strain on a fresh nutrient agar plate and to repeat the antibiotic sensitivity tests with each of the four antibiotics. Obviously, we expect the inhibition zone to be larger for the selected antibiotic, but we also want to determine what effect, if any, the changes in the resistant bacteria strain have on its sensitivity to the other three antibiotics.

1. Of the broth tubes you cultured in the last procedure, choose the tube with the highest antibiotic concentration that still shows significant growth (cloudiness).
2. Using the contents of that tube, repeat the first procedure to inoculate the entire surface of the fourth agar Petri dish, place four paper discs on the surface of the agar, and infuse those discs with the four antibiotics.
3. Incubate the Petri dish until the bacteria lawn appears. Measure the inhibition zones for each of the antibiotic discs and record your observations in your lab notebook.

Dispose of all cultures in your chlorine bleach container.

Review Questions

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1. Why did we label the Petri dishes on their bases rather than on their lids?

   The base contains the agar gel and antibiotic sensitivity discs, which remain in fixed position relative to the base. The lid of the Petri dish can be rotated, which would cause us to lose track of which disc contained which antibiotic.

2. In the second procedure, you cultured any one of the three bacteria species in the presence of different concentrations of any one of the four antibiotics, and then chose the tube with the highest concentration of antibiotic in which growth was evidenced by cloudiness. Why did we go through this process rather than simply adding the highest concentration of the antibiotic to one broth tube and incubating it until growth was evident?

   Answers will vary.

   The real answer is that high concentrations of a bactericidal antibiotic may in fact kill every bacterium present. The highest concentration we used was 1.0 mL of an antibiotic solution that contains 2.5 mg/mL, which the broth diluted to roughly 0.25 mg/mL or 250 mg/L. In an adult human, achieving and maintaining that serum level would typically require frequent dosages of about 1.5 g to 2.5 g of the antibiotic, which is on the very high end for most antibiotics. Accordingly, we have reason to suspect that the higher levels of antibiotic

present in our broth tubes may in fact eradicate the bacteria in the tubes. We don't want to kill (or suppress, in the case of tetracycline) all of them, just almost all of them.

Based on the information they have available, thoughtful students may propose that high levels of the antibiotic would leave such a tiny fraction of the bacteria alive that extended culturing time would be needed to allow the bacteria to develop in large enough numbers to provide a good bacterial lawn after re-culturing.

3. In the second procedure, why did we not exclude tetracycline from the trial candidates? Tetracycline is bacteristatic, which means that an individual bacterium that is sensitive to it is not killed, but only has reproduction suppressed. Since a broth tube has no immune system to kill those suppressed bacteria, they will be present after re-culturing. Why is this not a problem?

   The presence of tetracycline inhibits reproduction only of sensitive bacteria. During innoculation of the broth tube, only relatively small numbers of bacteria were introduced to the broth media, presumably some sensitive individuals and some resistant individuals. The presence of tetracycline prevents the former from reproducing, but has little or no effect on the latter. Accordingly, after incubation, the population of the broth tube is overwhelmingly resistant bacteria, with only a tiny percentage of sensitive individuals. When we inoculate the Petri dish with this culture, resistant individuals outnumber sensitive individuals by millions or more to one, so the bacterial lawn will be made up almost enclusively of resistant individual bacteria.

4. After doing the third procedure, you find (as expected) that the inhibition zone for the selected antibiotic with the resistant culture is smaller than it was with the original pure culture, but you also find that the inhibition zone for another of the antibiotics is smaller than it was with the original pure culture. Propose an explanation.

   Bacterial resistance to antibiotics is extremely complex and arises via several mechanisms. Based on what students know at this point, the most reasonable explanation is that the genetic mutation that conferred resistance to the selected antibiotic also conferred resistance to a second antibiotic. In fact, this actually occurs frequently in the real world, particularly among closely-related groups of antibiotics or those that function via similar mechanisms. For example, a bacterial species that develops resistance to one beta-lactam antibiotic, such as penicillin, may simultaneously develop resistance to other beta-lactam antibiotics, such as cephalosporins.

5. After doing the third procedure, you find (as expected) that the inhibition zone for the selected antibiotic with the resistant culture is smaller than it was with the original pure culture, but you also find that the inhibition zone for another of the antibiotics is larger than it was with the original pure culture. Propose an explanation.

   Based on what students know at this point, the most reasonable explanation is that genetic mutations can go both ways. In this case, it's not unreasonable to propose that the mutation that increased resistance to the selected antibiotic simultaneously decreased resistance to another antibiotic, particularly one that is not closely related or that operates via a different mechanism.