Antibiotic Resistance

Prelab

Study this Antibiotic Resistance lab description, the Genereux and Bergstrom paper on the Readings page of the course website, and textbook sections 26.4-26.6; then do the Lab 4 Prelab linked to the course website. The next week's prelab link is posted each Thursday afternoon. See "Submitting Catalyst Exercises" in this course manual; prelabs are due by Tuesday at 8:00 AM. (2 points)

Learning Objectives

At the end of this three-week lab, you should be able to:

- Describe how researchers estimate the frequency of antibiotic-resistant cells in natural populations of bacteria
- Understand how gene flow, mutation, genetic drift, and natural selection affect the frequency and spread of resistant alleles in bacteria populations
- Begin to explore the use and analysis of large data sets in biology
- Find and explain a pattern in the antibiotic resistance of skin bacteria in the student population, using chi-squared analysis and graphing

This lab is also designed to help you review other concepts introduced in lecture, including evolutionary arms race and host-parasite interactions. The skills you will practice include culturing bacteria and using statistical tests and graphs to analyze a large data set.

Introduction

Antibiotics are molecules produced by bacteria and fungi that kill bacterial cells. Biomedical researchers have isolated hundreds of different antibiotics, and many have proven to be extremely effective in the treatment of bacterial infections in humans. The antibiotic penicillin, for example, from the fungus *Penicillium chrysogenum*, has been credited with saving hundreds of thousands of lives.

To be medically useful, an antibiotic must affect the bacterial invader but not harm the human host. Penicillin is toxic to bacteria because it inhibits the formation of a material called peptidoglycan, which is the main structural element in bacterial cell walls. The molecule is harmless to humans, however, because human cells do not contain peptidoglycan.

Unfortunately, bacteria can evolve resistance to antibiotics. For example, if a mutation allows a fungus or bacterium to synthesize an antibiotic that is effective against a bacterial species with which it competes, then natural selection begins to favor members of the competitor species that exhibit resistance to the compound. Over time, the percentage of individuals in the competitor population that are resistant to the antibiotic will increase. In response to the evolution of these resistant forms of bacteria, natural selection will begin to favor members of the antibiotic-producing species that synthesize modified compounds or new antibiotics that are effective against the resistant competitors. When this occurs, the competitor population experiences natural selection favoring resistance to the novel antibiotic, and so on. Biologists describe a series of reciprocal interactions such as this as a **coevolutionary arms race**.

A similar arms race is now underway between biomedical researchers and disease-causing microorganisms or **pathogens**. *Streptococcus pneumoniae*, for example, is a common bacterial pathogen in humans and a leading cause of pneumonia and meningitis. Just 25 years ago this organism was readily controlled by penicillin. Now many countries report that over 30% of all patients infected with this pathogen do not respond to treatment with penicillin. The unresponsive patients are infected with *S. pneumoniae* cells that are resistant to this antibiotic. Unless alternative antibiotics are available, physicians may be able to do very little to combat the infection. The evolution of antibiotic resistance presents one of the great medical challenges of our time.

How do bacteria become resistant to a particular drug? Several mechanisms have been documented.

- Some individuals have uncommon versions of the molecule targeted by the drug. If the chemical structure of the target molecule is different enough, the drug may not bind and disrupt its function.
- Some individuals have enzymes that break down the drug before it can poison the cell.
- Some individuals have molecules called pumps that eliminate the drug from the cell interior, before the drug can affect the cell's function.

Although the genes that confer antibiotic resistance can and do arise via mutation, they are often acquired directly from another bacterium via a special form of gene flow. Transfer of genes between bacterial cells is possible because some bacterial cells contain small, chromosome-like loops of genes called **plasmids**. Most of the genes involved in antibiotic resistance are found on plasmids; some plasmids contain genes that confer resistance to several different antibiotics. Plasmids that contain resistance genes can be transferred from one bacterial cell to another even if the receiving bacterium belongs to another species. Gene flow also occurs when organisms (in this case, whole bacteria cells) migrate from one population to another (think "sneeze").

The goal of this laboratory is to introduce you to how biomedical researchers study the evolution of antibiotic resistance. You'll begin by investigating the frequency of antibiotic resistance in *Staphylococcus epidermidis*, a non-pathogenic bacterium commonly found on human skin. You will do this by collecting a sample of these cells from your own skin, and then using this sample to inoculate plates. The plates contain a medium that is conducive to the growth of *S. epidermidis* but detrimental to the growth of other bacteria. One week later you'll use the isolated colonies of *S. epidermidis* that have grown to inoculate new plates. By adding disks containing several different antibiotics to these new plates, you will be able to determine whether antibiotic-resistant cells are found among the *S. epidermidis* on your body. In the third and final session in this lab, you will estimate the frequency of antibiotic resistant *S. epidermidis* in the Biology 180 student population, and then use the combined data from many years of Biology 180 to investigate patterns of antibiotic resistance in a healthy student population.

IMPORTANT PRECAUTIONS FOR THIS LAB!

1. **No food or drink in the lab.**
2. **Avoid long, flowing sleeves, scarves and similar apparel that could dislodge items from the bench tops. If you have long hair, be prepared to tie it back during lab.**
3. **Wash and dry hands at the beginning and end of each session.**
4. **Leave the cover on your plate at all times except when instructed to do otherwise.**
5. **Do not place any contaminated material on bench tops.**
6. **Discard all contaminated material in the waste containers provided.**

EXERCISE 1: Isolation of *Staphylococcus epidermidis*

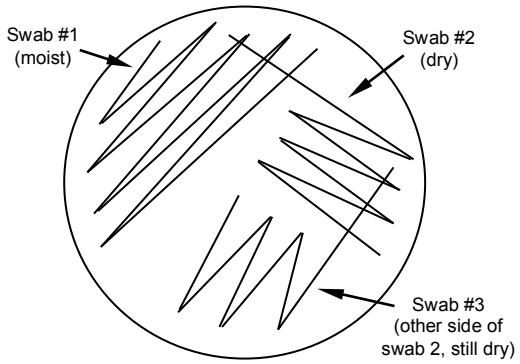
Two weeks before the data analysis portion of this lab, you will "plate out" a sample of bacteria from your skin in a way that will allow you to isolate colonies of a benign bacterium called *Staphylococcus epidermidis*. ("Benign" means that it does not cause disease.) Other types of bacteria in addition to *S. epidermidis* live on your skin, but we want to examine just one species.

You will be provided with the following supplies to begin this part of the lab:

- 1 mannitol salt agar plate
- 2 sterile swabs
- 1 tube of sterile saline

PROCEDURE

1. **Label** your plate (the base with the pink agar, not the lid) with your name and lab section.
2. **Moisten** a sterile swab in saline. Roll the swab against the inside of the tube to remove any excess.
3. Collect a sample of cells from the crease between your cheek and the **outside** of your nose.
4. Lightly roll the swab over 1/3 of the plate, rubbing all sides of the swab against the surface of the agar (as shown for "swab #1" in the diagram at right).
5. **Discard** the swab in the receptacle provided and cap the tube of saline.
6. Use a new **dry** sterile swab to streak material from the first area onto the adjacent area, then turn the swab over and streak material from the second area to the third area (as shown for "swab #2 and "swab #3" in the diagram to the right).
7. **Discard** the swab in the receptacle provided.
8. **Invert** your plate and give it to your TA.



EXERCISE 2: Antibiotic Susceptibility Testing

One week before the data analysis portion of this lab, you will create a plate that contains only *Staphylococcus epidermis* and expose the bacteria to four different antibiotics that physicians use to fight bacterial infections.

You will be provided with the following supplies for this part of the lab:

1 Mueller-Hinton agar plate

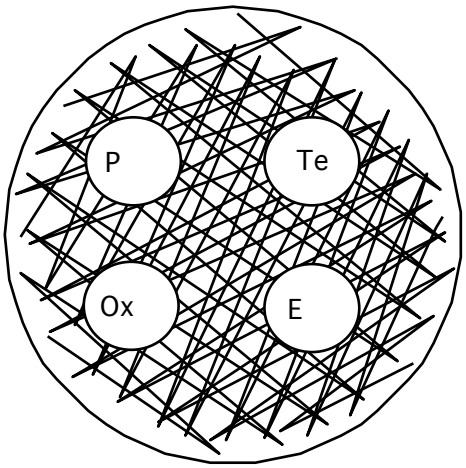
1 sterile swab

1 tube sterile saline

4 types of antibiotic disks

PROCEDURE

1. **Label** the agar plate (the base, not the cover) with your name and lab section.
2. Find the mannitol plate that you inoculated in the first lab period and look for an isolated, **pink** colony on your plate. Choose the colony that is the most uniform in color and texture. **Use only one colony!!**
3. Use a sterile swab to pick up the isolated colony. Stir this sample into a tube of sterile saline to make a solution of bacteria.
4. Use the swab to streak your new plate in 3 directions. Try to obtain an even spread across the plate.
5. **Discard** the used swab and your mannitol plate in the waste container provided.
6. Use the disc dispenser to place 4 discs impregnated with antibiotics on your plate.
7. **Invert** the plate (the discs will stick to the moist agar) and put it in the tray for your lab section.



EXERCISE 3: Scoring plates for antibiotic sensitivity

In this exercise you will determine whether the *Staphylococcus epidermidis* growing on your skin are resistant to four different antibiotics. You will also assess the frequency of resistant forms in the class as a whole and consider whether variation in degree of resistance exists. Note that you might still have resistant staph cells growing on your face even if you have never used a topical antibiotic (one applied to your skin). Resistant staph cells can be favored by natural selection because antibiotics that are administered via pill or injection are transported to the skin surface.

PROCEDURE

1. **KEEPING THE COVER ON YOUR PLATE AT ALL TIMES**, look for zones of inhibition around each antibiotic disk. Using a ruler, measure the diameter (in millimeters) of the zone of inhibition. If there are small colonies within a zone of inhibition, measure the zone **inside** those resistant colonies – an antibiotic must inhibit even the most resistant bacteria. If bacteria are growing in contact with a disk, enter zero (0, no inhibition); if results for a disk are unreadable, leave that table cell blank.
2. Use the table below to interpret your results. The diameter of the zone of inhibition is influenced by the bacterium's sensitivity to the antibiotic and by the concentration and molecular weight and structure of the antibiotic.
3. Set your plate with cover aside until the TA collects it.

	Zone diameter—nearest whole mm.			Your results	
Antibiotic	**Resistant**	**Intermediate**	**Sensitive**	**Diameter (mm)**	**Interpretation** (resistant, intermediate, or sensitive)
Penicillin	≤ 28		≥ 29		
Tetracycline	≤ 14	15-18	≥ 19		
Erythromycin	≤ 13	14-22	≥ 23		
Oxacillin	≤ 10	11-12	≥ 13		

4. Your TA will tally the results for your entire lab section. Enter those results below:

Antibiotic	Resistant	Intermediate	Sensitive
Penicillin			
Tetracycline			
Erythromycin			
Oxacillin			

5. Discuss the lab section results and complete the Exercise 3 Report.

EXERCISE 4: Data analysis of population-level resistance

In this exercise, you will explore a large dataset from many previous years of Biology 180 students. First, you will enter your own resistance data for the use of future lab participants.

1) Enter your own personal resistance data using the online questionnaire. Your answers will be entirely anonymous and cannot be traced back to you in any way, even by the course instructors.

2) Your TA will provide an Excel file containing data from a similar questionnaire collected in previous years. Drug resistance is indicated by a "1" in the column corresponding to each drug; drug sensitivity is indicated by a zero. The numerical scores associated with each column reflect the answers to the other questions on the questionnaire. Use the laminated chart on your bench to interpret the numbers in the spreadsheet.

3) Your job is to explore these data and look for interesting patterns.

   Start by examining the laminated legend. Each question in the survey yields one or more factors that you could investigate, and the categories can be aggregated in many different ways. For example, the "living situation" categories are:

   1. Live alone
   2. Dorm or group living involving *more than 5* unrelated adults
   3. Dorm or group living involving *5 or fewer* unrelated adults
   4. Family with children *over 6 years of age*
   5. Family with children *6 years of age or younger*

   You could leave the five categories separate, but the results might be difficult to interpret. So, instead, you could add together categories 2 through 5 to create the category "lives with others," and compare it to "lives alone." Or add together categories 2 and 3 ("live with people unrelated to you") and compare to "live with people related to you" (categories 4 and 5).

   The other survey questions can be similarly analyzed, and other possible questions can be asked, such as a) is resistance to one antibiotic associated with resistance to another? or b) have there been changes in resistance over time?

4) With your lab partners, think of a question that interests you, then explore the data using *chi-squared analysis* (see below). Complete the Exercise 4 Report, and include your chi-squared analysis results and a bar chart.

Using the Chi-squared Test

Introduction

Biologists often measure the frequencies of data that occur as categories, and then need to assess whether the observed frequencies are consistent with the expectations of the relevant null hypothesis. For example, in genetics you might perform an experimental cross under the hypothesis that the two traits you are interested in are autosomal and unlinked. In this case, you'd expect to observe four phenotypes (the phenotypes are categories) in a 9:3:3:1 ratio—meaning, in frequencies of 9/16, 3/16, 3/16, and 1/16. But it's extremely unlikely that the phenotypes you observe in the experiment will be in *exactly* these frequencies. Are the genes really autosomal and unlinked, meaning that the difference from the expected frequencies is just due to chance? Or is the difference from the expected frequency large enough to indicate that something else is going on—meaning that your hypothesis is probably not correct?

One of the standard statistical approaches to answer questions like this is called the chi-squared test. The basic approach is to calculate a test statistic that *summarizes the differences between the observed and expected values*, for all of the categories measured. The test statistic is called χ^2 (χ is the Greek letter chi) and is calculated as:

$$\chi^2 = \sum \frac{(\text{observed-expected})^2}{\text{expected}}$$

The logic here is to calculate the difference between the observed and expected frequencies for a particular category (like one of the phenotypes in a cross), and square the result so that all of the data are positive. You then divide by the expected frequency, to scale the difference you calculated. For example, if the numerator is 4 and the expected frequency is 6, then the squared difference between observed and expected is very large. But if the expected frequency is 100, then the squared difference is actually small. The summation sign (Σ, the Greek letter sigma) means that you sum up all of these values for all of the categories in the study.

What do you do, once you have calculated the value of χ^2? A statistician named Karl Pearson, who invented the chi-squared test, found that χ^2 values have a distribution that can be calculated, based on the assumption that the observed and expected values differ by chance. This distribution specifies the probability, or *p*-value, that various values of χ^2 occur by chance. The distribution, though, depends on something called the degrees of freedom (abbreviated df). Degrees of freedom are a measure of how constrained the data are—how much they can vary, given the information available.

Doing the Calculation – Some Examples

When you set up a chi-square test, you routinely arrange the data in rows and columns, with the rows containing the data from each treatment and the columns containing the categories that you've measured. In the dihybrid cross example above, you'd just have a single row with one column for each of the phenotypes observed. You'd put in the number of individuals of each phenotype observed. Then you need to generate the expected values. In this case, the expected values are the 9/16, 3/16, 3/16, and 1/16 frequencies from the hypothesis you're testing. You'd generate the expected numbers—to compare with the observed numbers—by multiple each frequency by the total number of offspring observed. Then you'd simply crank through the formula above to calculate the value of χ^2.

In many cases, though, you have several rows of data (e.g. from different experimental treatments) and you want to test the hypothesis that the data are independent of each other—instead of in a specific set of frequencies. For example, consider the following dataset from a cross that R.C. Punnett published in 1923. The numbers indicate the number of offspring observed with each of 4 phenotypes.

	Purple	Red	**Totals**
Long	226	97	323
Short	95	1	96
Totals	321	98	419

Note that the chart includes "marginal totals" and the "grand total"—the number of individuals summed across each row (323 and 96) and down each column (321 and 98), along with the total sample size (419). To generate expected values under the hypothesis that the phenotypes are independent of each other, you take the marginal totals as given and ask what the numbers in the

internal cells would be if the two marginal values for each cell were combined independently. For example, the expected value for "Long-Purple" would be calculated as

(proportion of longs) x (proportion of purples) x (grand total), or

(323/419) x (321/419) x 419 = 247.45

You get the same number, using slightly different logic, by calculating the relative frequency of each cell in the hypothesized population (where there is no difference in proportions between rows) and multiplying it by the sample size. In the case of the Long-Purple cell, the calculation would be 321/419 x 323 = 247.45. (This is the frequency of purples times the total number of Longs.)

As before, you plug the observed and expected values into the equation and sum over all the cells (in this case, 4) in the study to get the value of χ^2.

Interpreting a χ^2 Value

Before you can determine the probability of getting a particular value of χ^2, you have to determine the number of degrees of freedom in the data. For data in this course, df is always calculated as (# rows -1) x (# columns – 1). If you only have one row of data, df is simply the # columns – 1. In the dihybrid cross example, the number of degrees of freedom is 3; in the long/short/purple/red example, df = 1.

Now that you have a value of χ^2 and the relevant degrees of freedom, you can use a chart or computer program to look up the *p*-value—the probability of getting the result under the null hypothesis that you are testing. Your TA can help you with this.

One final note: the chi-squared test only tells you the overall probability that the observed and expected values are the same. If there is a significant difference, it doesn't tell you which row or column is responsible.

Exercise 3 Report

Student Names_____

You have just done a test that is nearly identical to those ordered by physicians and performed in hospitals and clinics. Look at the data collected for your lab section and answer the following questions.

1. In this population of bacteria, resistance to which antibiotic is most common? Least common? Generate two hypotheses to explain why **bacterial populations** might have a higher frequency of resistance to some antibiotics than others.

   Most common: _____ Least common: _____

   - Hypothesis 1:

   - Hypothesis 2:

2. State a hypothesis to explain why different **host individuals** (your classmates) might have bacteria that differ in their resistance to certain antibiotics.

3. Predict the results that will be observed if this same lab is done with Biology 180 students 10 years from now. Explain the logic behind your prediction.

Exercise 4 Report

Student Names _____

To summarize your analysis of antibiotic resistance in bacteria colonizing Biology 180 students, your group should complete and hand in one copy of this report. **Print and attach your chi-squared test results and graph(s)**.

Question:

Hypothesis:

Which evolutionary mechanism is involved? Explain.

Null hypothesis:

Data used (which variables; how did you group the categories; what did you do with the "intermediates"?):

Prediction of your hypothesis:

Prediction of null hypothesis:

Results:

Conclusions: