

Unit 1. Welcome to the resistome

"We may be heading, from many directions, toward the inability to treat some patients," said Thomas O'Brien, MD, Brigham and Woman's Hospital, Boston MA.

The wonder drugs

Antibiotics are medications taken to fight infections caused by bacteria. When they first became available during World War II (1939-1945), antibiotics were called "wonder drugs" because of their impressive record for safety and effectiveness. Well-known antibiotics include penicillin (**Figure 1**), erythromycin, ciprofloxacin and tetracycline.

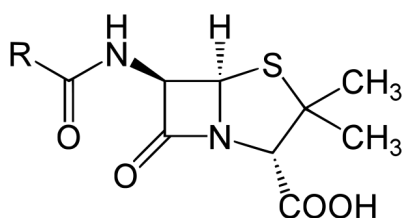


Figure 1. Penicillin, the first natural antibiotic discovered by Alexander Fleming in 1928.

Over the past sixty years, physicians have come to depend on antibiotics to treat many diseases caused by bacteria, from simple skin infections to life threatening sepsis (blood infections). Antibiotics are also crucial for treating patients who may get infections from surgery, chemotherapy and transplants. However, doctors are reporting increasing numbers of bacteria that fail to respond to traditional antibiotic treatment (Neu, 1992). **Figure 2** (adapted from Speller et al. 1997) illustrates the rapid increase in resistance levels observed for some of the more frequently employed antibiotics. We are now reaching a state where treatment for common infections will become limited, expensive, and in some cases, non-existent.

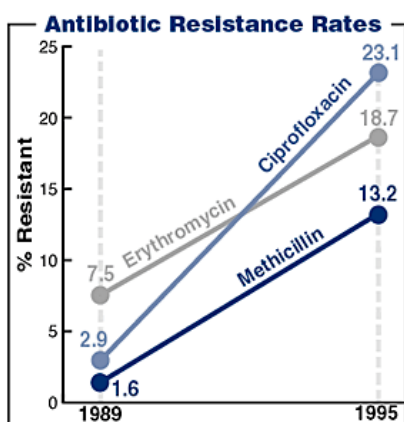


Figure 2. Comparison of the levels of antibiotic resistance to three commonly employed drugs from 1989 to 1995.

Although even the most careful use of antibiotics can result in the emergence of antibiotic-resistant bacteria, widespread and inappropriate use of these important drugs greatly accelerates the process. The more often bacteria are exposed to antibiotic, the more likely resistance will develop. It has been reported that in the USA alone over 3 million pounds of antibiotics are used every year on humans (Null et al. 2005). Even worse, over millions of pounds of antibiotics are given to farm animals, often administered as a prophylaxis, i.e. to maintain health, rather than treat disease. Equally troubling is the fact that doctors continue to prescribe antibiotics for diseases for which antibiotics have no efficacy, such as viral infections (Egger 2002). It is alarming to think

that many prescriptions are written each year for inappropriate reasons. Almost half of patients with upper respiratory tract infections in the U.S. still receive antibiotics from their doctor even though the CDC warns that 90% of upper respiratory infections, including children's ear infections, are viral and antibiotics don't treat viral infection (Egger 2002).

How the wonder drugs work

Each class of antibiotic kills or inhibits cell growth in a different fashion. **Figure 3** illustrates the three primary modes of action for some commonly employed antibiotics. The following list provides a more detailed description of modes of action for a sample of the more frequently used antibiotics:

1. **Aminoglycosides.** Aminoglycosides bind to the bacterial 30S ribosomal subunit, interfering with protein synthesis. By binding to the ribosome, aminoglycosides inhibit the translocation of tRNA during translation and the bacterium is unable to synthesize proteins necessary for growth.
2. **Beta-lactams.** Penicillins and cephalosporins work by interfering with inter-peptide linking of peptidoglycan, a structural molecule found specifically in bacterial cell walls. Cell walls without intact peptidoglycan cross-links are structurally weak and prone to collapse when the bacteria attempts to divide.
3. **Macrolides.** Macrolides exert their bacteriostatic effect by binding irreversibly to the 50S subunit of bacterial ribosomes and thus inhibit translocation of tRNA during translation.
4. **Quinolones.** Quinolones inhibit bacterial DNA replication, which permits them to

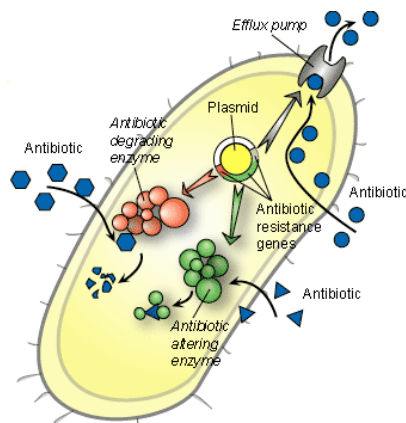


Figure 3. Illustration of the primary modes of action for antibiotics in a bacterial cell.

inhibit a broad range of bacteria.

5. **Tetracyclines.** Tetracyclines inhibit bacterial protein synthesis by preventing tRNA molecules from binding to the 30S ribosomal subunit.

Evolving resistance to antibiotics

The biological mechanisms resulting in antibiotic resistance are diverse, but they can be classified into three general categories (Hawkey 1998). First, bacterial enzymes can actively degrade the drug. Resistance to the beta-lactam antibiotic ampicillin, for instance, can be mediated by the enzyme beta-lactamase. Ampicillin normally functions as an irreversible inhibitor of transpeptidase enzymes, whose function is to cross-link structural components of the bacterial cell wall. Beta-lactamase enzymes hydrolyze beta-lactam rings and render such antibiotics incapable of reacting with bacterial transpeptidase enzymes. Another example is the aminoglycoside antibiotic kanamycin,

a bacterial protein synthesis inhibitor. Enzymes conferring the kanamycin-resistant phenotype inactivate the drug by covalent attachment of a phosphate or other group.

Second, bacteria can develop mutations that render the target molecule unable to interact with the antibiotic. Streptomycin is an aminoglycoside antibiotic effective against a wide variety of bacteria. Spontaneous streptomycin-resistant *E. coli* can be selected under laboratory conditions. Resistance to this protein synthesis inhibitor maps to the *rpsL* gene, which encodes a ribosomal protein. Another example is the DNA gyrase inhibitor nalidixic acid. DNA gyrase is a super-coiling enzyme, which modifies DNA topology, and is essential for DNA replication. Nalidixic acid inhibits gyrase action by stably binding a gyrase/DNA reaction intermediate. Nalidixic acid resistance mutations in *E. coli* map to the structural genes for both gyrase subunits. Mutants have presumably lost the ability to bind nalidixic acid.

Finally, changes in bacterial cell permeability to an antibiotic can confer resistance. Ampicillin resistance in Gram-negative bacteria can arise from mutations in outer membrane proteins, porin, which normally act as molecular sieves, allowing the diffusion of molecules into the cell according to charge and size. Ampicillin resistance mutants of this type have a reduced ability to transport the drug into the cell.

Resistance, resistance, everywhere!

The basic quantitative measure of the *in vitro* activity of antibiotics is the minimum inhibitory concentration (MIC), which is the lowest concentration of an antibiotic that results in inhibition of visible growth (e.g. turbidity in broth culture) under standard conditions. For an antibiotic to be effective the MIC must be achievable at the site of the infection.

In this lab we will sample the levels and types of antibiotic resistance in bacteria isolated from domesticated and farm animals and compare those levels to other studies (human and livestock). The basic protocol is quite straightforward. A sample of bacteria is obtained from an animal's feces and used to create a lawn of cell growth on a nutrient-rich plate. Antibiotic impregnated discs are applied to the surface of the plate and the antibiotic is allowed to diffuse into the adjacent medium, creating a gradient. Following incubation, a bacterial lawn appears on the plate (greenish haze in **Figure 4**). Zones of bacterial growth inhibition may be present around the antibiotic disk, corresponding to the sensitivity of the bacterium to that antibiotic. The size of the zone of inhibition is dependent on the diffusion rate of the antibiotic, the degree of sensitivity of the microorganism, and the growth rate of the bacterium. Note that the larger the zone of inhibition, the lower the MIC.



Figure 4. Testing the susceptibility of *Staphylococcus aureus* to antibiotics by the Kirby-Bauer disk diffusion method. Antibiotics diffuse out from antibiotic-containing disks and inhibit growth of *S. aureus* resulting in a zone of inhibition.

The bacterial culprits

You will be sampling a variety of Gram-negative bacteria that ferment lactose in this laboratory. It is not within the scope of this lab to identify all of the bacteria you sample – however we may know some of the species by sight! In any event, you will obtain a sample from the target animals that the TA assigns your group (more on that below) and then attempt to isolate one particular bacterial species that will very likely be abundant in your sample, *Escherichia coli* (the lab rat of the microbial world!). We will use selective plating methods to eliminate growth of other species and then use the ability of *E. coli* to utilize lactose as a sole food source to differentiate from non-lactose fermenter such as *Salmonella*. You will then employ a suite of commonly encountered antibiotics against the bacteria you have isolated and determine whether resistance to antibiotics is present in your sample of strains. You will then compare your data with the data generated by other groups in your lab section to determine which animal groups have the highest levels of resistance and how those levels compare to published data.

Lab Goals

Students will learn the following key points:

- that bacteria may harbor resistance to antibiotics
- how exposure to antibiotics can increase levels of resistance
- how such resistance is detected in numerous environmental settings
- how to record and analyze resistance data
- how to assess population-level (or epidemiological) data
- that resistance is an important public health issue that you can do something about

Lab Timeline

Although your experiment may require modifications to the following timeline, what follows is roughly what you should expect over the next three laboratory sessions.

Lab 1. Introduction to micro techniques and antibiotics

- Overview and logistics
- Discuss the Farm Animal Resistance article
- Go over basic bacterial sampling and identification techniques
- Observe basic microbiology techniques
- Practice these techniques
- Form groups; TA assigns host animal
- Go over next lab expectations

BEFORE next lab, check practice plates and record observations

Lab 2. Bacterial isolation

- Collect and label plates and review sampling protocol within group
- Isolate bacteria and place plates in incubator
- Go over next lab expectations and observe antibiotic sensitivity testing
- Discuss data entry and analysis

- ***BEFORE next lab, check plates and record observations. Also, ensure that you have a minimum of 25 well-isolated colonies (contact TA if not)***

Lab 3. Meet the resistome

- Identify and isolate colonies for antibiotic sensitivity testing
- Proceed with antibiotic sensitivity testing
- Discuss with group how to analyze your data, share your data with other groups, and present results in a lab report and to the class
- Lab report expectations

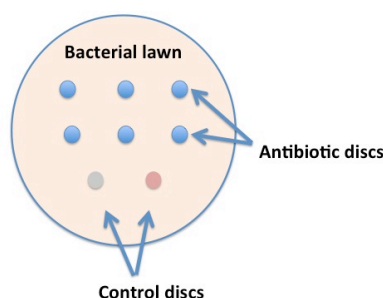
Materials and Methods

In this lab you will be introduced to techniques associated with clinical microbiology. You will learn how to sample bacteria, how to use selective growth conditions to eliminate certain species from a sample, how to create a bacterial lawn on a media plate, and how to use an antibiotic impregnated disc to screen for resistance. Your life will be far easier in the long run if you take time before you start the experiment to carefully read and/or design the protocol, including the number of samples used, the number and types of antibiotic discs, etc. Do not forget to include a positive and negative control in your experiments – which we will provide for you. The following is a guideline on how your experiment will proceed, but you may modify the method (seek approval from your TA for any modifications):

1. Samples. Your TA will determine your target animal group. Some will use samples from household pets, others will use samples horses, sheep or goats. Fecal samples will be used for this lab section and the TA will provide the samples to you. Be sure to record the sample tube numbers you use and label your plates accordingly.
2. Plating for bacteria (USE GLOVES!). Once you have your fecal samples you will proceed with bacterial isolation. USING THE TECHNIQUE PRESENTED AND PRACTICED IN CLASS, grab a cotton swab with sample and streak on one MacConkey agar plate then, using a new swab, collect cells from the agar plate and streak on a fresh plate. Repeat this for four additional samples for a total of 10 plates. Next, repeat this process with controls (see below). All plates should be labeled appropriately. Incubate your plates inverted for 15-24 hours at 37°C. RETURN TO CHECK YOUR PLATES. You may notice that some of your samples will have little bacterial growth, others will have more (you are looking for plates containing roughly 10 – 100 isolated colonies). Thus, you may need to repeat the process with a larger sample or make appropriate dilutions to obtain fewer bacteria on your plates. Your TA will help you with this process. It may take several attempts so factor that process into your experimental design. Make sure you record all results and photograph plates for your records.
 - Positive control: *E. coli* BZB1011. This strain will grow on your plates as pink/red colonies and should be sensitive to the antibiotics you test.
 - Negative control: Use sterile media as your negative control
 - You will want to see no growth on the negative control and roughly 50 colonies on the positive control. If you do not obtain this result see if you can figure out why and try again. If you still do not obtain this result after a second try contact your TA for help.
3. Create lawns and test for resistance. You will now isolate individual colonies and test for sensitivity to a number of antibiotics.

- First, label one new plate for each colony you will use from your original plate spreads. Choose 20 well-isolated and ample-sized colonies for this next step. You will end up using 21 (including control) plates with antibiotic discs.
- Using a sterile stick, grab the colony and then place it in a labeled 1 ml tube of isotonic solution and mix thoroughly to ensure the cells are transferred to the solution. Insert a cotton swab into the solution then swab across the entire plate to ensure that bacterial cells cover the plate. You will rotate $1/8$ of a turn and repeat this process. Do this once more for a total of three times to create a lawn of cells that cover the plate following the incubation period (see Figure 4). Let the plates sit (covered) while you prepare your antibiotics.
- Commonly employed antibiotics will be made available in the laboratory. They will be prepared at appropriate concentrations so that you can apply 25 μ l to a sterile disc.
 - Determine how many discs you need for your experiment. Lay the discs out on weighing boats for accessibility.
 - Use sterile forceps to place each disc on the appropriate plate in a predetermined location. Use a pipette to administer the appropriate volume of solution to each disc. Make sure that your plates are labeled to avoid confusion. See **Figure 5** for an example of how to arrange your discs on your plate.

Figure 5. An experimental plate with antibiotic discs



- After you apply the antibiotic discs to your plates, invert them and incubate them at 37°C for 16-18 hours. You must return to remove your plates from the incubator and either 1) store them in the refrigerator or 2) measure your zones and record your data. If you cannot record your results between 16-18 hours but can do so shortly after, you may leave your plates on your bench and record results later.
- Measure the resulting zones of growth inhibition surrounding each of the discs on your plates (**Figure 6**). This is done with a clear ruler. These measurements are then used to determine whether a strain is sensitive or resistant to an antibiotic – based upon standards provided by the antibiotic manufacturer (see **Table 1** for example). The TA will provide a zone diameter interpretive chart for that antibiotics used.

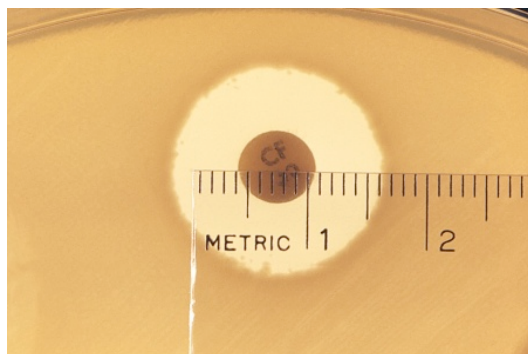


Figure 6. Measuring zone of inhibition on Mueller Hinton plate with antibiotic impregnated disc.

Table 1.

***E. coli* and other enteric Gram Negative Rods
(Zone Diameter, nearest whole mm)**

	Resistant	Intermediate	Susceptible
Amikacin (30 µg)	≤14	15-16	≥17
Ampicillin(10 µg)	≤13	14-16	≥17
Cefazolin (30 µg)	≤14	15-17	≥18
Gentamicin (10 µg)	≤12	13-14	≥15
Tetracycline (30 µg)	≤14	15-18	≥19
Ticarcillin (75 µg)	≤14	15-19	≥20
Trimethoprim (5 µg)	≤10	11-15	≥16
Tobramycin (10 µg)	≤12	13-14	≥15

Clean up

All waste material that involves bacteria is to be disposed of in the labeled waste bins. All contaminated materials (those in the labeled waste bin) used in this experiment must be sterilized before disposal. Your TA will ensure this procedure is carried out. However, just so that you know, the preferred method is sterilization by autoclave or, if there is no machine available, flood the tubes and plates with a disinfectant solution such as 10% bleach. After sufficient time in the autoclave (20 minutes) or exposure to the disinfectant solution for 20 to 30 minutes, the decontaminated materials are disposed of in the regular waste stream.

Data collection

You will record in your lab notebook all of the information required to repeat this experiment, along with your observations and conclusions. For example, you must note how/what samples were used, the number of plates you used, how you prepared antibiotic/antibacterial discs, what the plates looked like, etc.

Your resistance data will be entered into an excel spreadsheet to score the following results for each plate (at a minimum):

- plate ID
- growth/no growth of lawn
- zones of inhibition for each of the antibiotics tested
- whether strains are sensitive or resistant
- percentages of those resistant/ sensitive
- additional observations

When you create this spreadsheet, consider that you will want to determine the levels of resistance for different samples and different antibiotics. You will also want to include data from other lab members to increase your sample sizes. Consider how you will want to analyze the data you produce. For example, you may wish to compare the levels of resistance observed in the different animal groups. Or you may want to compare with published results. How would you compare these data on your spreadsheet?

Lab Report

You will be responsible for preparing a laboratory report. The format of the laboratory report is similar to a research publication, just much shorter and with far less data. You have already been provided with a template for the lab report – find it on the course website. Your TA will also cover lab report expectations.

Useful References – The ones posted below have been used in the past but there are MANY useful references out there....use the UMass database, Google scholar or NCBI to search for them!

General Audience Book – very informative

Levy, S. 2002. The antibiotic paradox: How the misuse of antibiotics destroys their curative powers. 2nd ed. Perseus Publishing, pp353.

Scientific Articles

Null, G, et al. Death by medicine. 2005. Journal of Orthomolecular Medicine, Vol 20: pp21-34.

Egger, W. Antibiotic resistance: unnatural selection in the office and on the farm. 2002. Wisconsin Medical Journal, Vol 56: pp34-39.

Neu H.C. The crisis in antibiotic resistance. 1992. Science, Vol 257: pp1064-73.

Hawkey, P. The origins and molecular basis of antibiotic resistance. 1998. British Medical Journal, Vol 317: pp657-660.

Speller DCE, Johnson AP, et al. Resistance to methicillin and other antibiotics in isolates of *Staphylococcus aureus* from blood and cerebrospinal fluid, England and Wales, 1989-95. *Lancet*, August 2, 1997;350, pp323-25