Antibiotic Susceptibility of *E. coli* Isolates

Resource Type: Curriculum: Laboratory

Publication Date: 6/25/1999

Authors

Christopher Woolverton
Kent State University
Kent, Ohio 44242
USA
Email: cwoolver@kent.edu

Heidi Hawkins
College of Southern Idaho
Twin Falls, Idaho
USA
Email: hhawkins@shields1.csi.cc.id.us

Abstract

Students work in cooperative teams of mixed abilities to design and complete an experiment to isolate and characterize *E. coli* from different sources and to evaluate the differences in antibiotic susceptibility of their strains. This activity does not replace regular laboratory activities but is integrated into the normal lab to augment discussions on diversity, mutation, and antibiotic resistance. A formal report is used to assess the project. Because of potential risks of isolating multiply-antibiotic resistant bacteria, this experiment should be carried out with students who have proficiency in aseptic technique and appropriate regard for laboratory safety.

Activity

Invitation for User Feedback. If you have used the activity and would like to provide feedback, please send an e-mail to MicrobeLibrary@asmusa.org. Feedback can include ideas which complement the activity and new approaches for implementing the activity. Your comments will be added to the activity under a separate section labeled "Feedback." Comments may be edited.

Editor's Note (2008): This Curriculum Resource was published prior to establishment of current criteria of submission, and as such, does not contain all criteria required of current publications. However, the Editorial Committee felt that the activity itself remained worthwhile and relevant, and encourages potential users to contact the authors for clarification as needed. If you do update this activity for use with your students, and are interested in updating the resource for distribution in the library, please contact ASM at MicrobeLibrary@asmusa.org.

INTRODUCTION

Time Required.
Laboratory work - several hours spread out over at least 4 days (e.g., at minimum, all or parts of 4 lab periods); additionally, sample collection will require some time prior to the first lab period. Overall, the design, data collection, and report writing may take 5 weeks.

Pedagogical Function.
This activity was designed to teach about antibiotic resistance and strain diversity. Additionally, students will gain experience in experimental design, data analysis, and scientific writing. Successful students will also improve technical lab skills, time management skills, and their ability to work as part of a team.

Background.
Before beginning the project, all students should have demonstrated proficiency in laboratory safety. Basic techniques such as aseptic handling of organisms, bacterial isolation procedures, and general principles of differential and selective media should be mastered.

The key objective of this laboratory exercise is for teams of students to apply the knowledge obtained in lecture and lab to designing a method for answering this question: "Does the antibiotic sensitivity pattern vary between different natural populations of *E. coli*?"

Each team must 1) design an appropriate *E. coli* isolation and identification procedure, 2) design an appropriate method for characterization of antibiotic sensitivity, and 3) conduct all experiments, which may include preparation of their own media, if indicated by the instructor. In order to carry out these three steps, students need to consider the following:

a) Where is *E. coli* found?

b) What is its ecological niche and role in nature? c) How can enrichment and selective/differential media be used to isolate *E. coli* from its niche? d) How can it be proven than a given isolate is indeed *E. coli*? e) Why might it be anticipated that different *E. coli* isolates would exhibit different antibiotic resistance profiles?

f) What techniques are used to determine the resistance or susceptibility of an organism to antibiotics?

g) What are the mechanisms by which different antibiotics inhibit bacterial growth, and how do bacteria become resistant to these antibiotics?

h) How many samples, from how many different niches, need to be tested in order to establish a pattern of resistance?

All procedures are readily found in various parts of texts and lab manuals, so the initial design does not require library research. Students work cooperatively in all parts of the design and completion of the project. The initial isolation and antibiogram analysis typically take up to 72 hours. Students will have five weeks to complete the entire assignment.

PROCEDURE

Materials.
Laboratory materials normally available in a microbiology teaching laboratory are required. These include: Bunsen burners, inoculating loops, sterile cotton swabs, specimen containers, test tubes, Durham tubes, microscopes, staining reagents, incubators, and millimeter rulers. Bacteriological media such as lactose peptone broth, Eosin-Methylene Blue (EMB) agar (or MacConkey agar or other differential/selective media for enterics), Mueller-Hinton agar, and all equipment to prepare and sterilize these media are also necessary. Students will be informed which of the dozen or so common media for characterizing *E. coli* isolates will be available for their use.

Antibiotic impregnated paper disks (available through various scientific supply companies as "susceptibility tests" of 50 disks per cartridge) and Barium sulfate turbidity standards will also be required.

Useful resource materials include a reference copy of Bergey's manual and the package inserts from the susceptibility test disks (which describe interpretive standards for disk diffusion antibiotic testing).

Instructor Version.

1. During the first lab period: students in each team should bring to the laboratory various materials that they have determined are likely to yield *E. coli*. If the material is solid, use a sterile cotton swab to sample a bit of the material and transfer it to enrichment media
   If the material is liquid, add a few drops to enrichment media
2. During the second lab period: streak samples from all positive enrichment tubes onto your selective and differential media
3. During the third lab period: pick colonies that have the typical *E. coli* appearance and inoculate these onto media for antibiotic testing. If desired, streak these colonies onto additional media for further characterization.
4. During the fourth lab period: determine the antibiotic resistance profiles for each isolate and carry out any additional characterizations.

Safety Issues.

Because of the potential for isolating multiply resistant strains of *E. coli*, this experiment is not recommended for use except by microbiology students who have had several weeks or months of laboratory experience and who are mature enough to focus meticulous attention on their aseptic technique. Lab coats and appropriate footwear are highly recommended. All cultures and contaminated materials must be discarded in appropriate receptacles with autoclaving. In addition, meticulous care will be needed around open flames and any broken glass.

Students may wish to collect specimens from sewage treatment plants or livestock feedlots. While these are excellent sources of *E. coli*, instructors should weigh carefully the risks in isolating enteric pathogens carrying multiple resistance plasmids from these sites. A blanket policy forbidding use of sewerage or feedlot specimens may be advisable.

ML Safety Statement regarding Environmental Isolates

The Curriculum Resources Committee recognizes that isolated organisms can be a powerful learning tool as well as a potential biological hazard. We strongly recommend that:

- Environmental enrichment laboratories should only be performed in classes in which students have been trained to work at a BSL2.
- Direct environmental samples (eg. soil, water) which are known to contain infectious organisms should be handled according to the biosafety level of that infectious agent.
- Cultures of enriched microorganisms, derived from environmental samples, should be handled using Biosafety Level 2 precautions.
- Mixed, enriched or pure cultures of microorganisms from environmental samples with a significant probability of containing infectious agents should be manipulated in a biosafety cabinet if available.
- Where possible, media used for the enrichment of environmental isolates should contain an appropriate anti-fungal agent.
- Instructors should be aware if they are teaching in regions with endemic fungi capable of causing systemic infections, and should avoid environmental isolations.

ASSESSMENTS and OUTCOMES

Suggestions for Assessment.
A likely protocol that students may derive from reading their text and lab manual is as follows:

a. To obtain *E. coli*, use animal feces, pond water (especially any pond with ducks), raw meat, or students' own rectums. Swab the feces, meat, or rectum and insert the tip end into a tube of lactose-peptone broth with Durham tube insert. Break off the handle of the swab if it interferes with closing the cap. For pond waters and other liquid samples, add a few drops to

a lactose-peptone broth (with Durham tube).

b. Incubate the lactose-peptone broth tubes at 44° C for 24 to 48 hours. This temperature inhibits many mesophilic bacteria, but *E. coli* should just be able to grow. Growth and gas production should be evident.

c. If gas is present, streak the culture onto an EMB or MacConkey plate (or other) and incubate at 37^{o}C. After 24 to 36 hours, colonies with a characteristic coliform appearance should be apparent (e.g., greenish sheen on EMB, or dark pink on MacConkey agar).

d. Pick an isolated coliform colony and transfer to a tube of sterile saline. Bring the turbidity of the tube to that of the appropriate barium sulfate standard (that is, approximately 5 X 10^{7} cells per ml). Swab a sample from this tube onto a Mueller-Hinton plate, and apply antibiotic disks.

e. If desired, streak a sample from this tube onto additional media to check for culture purity, Gram stain, and other biochemical characteristics.

f. Measure the diameter of the zone of inhibition around each antibiotic disk (in mm), and determine whether this diameter corresponds to "susceptible", "intermediate", or "resistant" status, according to the information supplied with the antibiotic susceptibility tests.

Instructors should plan time for discussion of likely *E. coli* habitats prior to the first lab period. In addition, time for the students to organize their teams (and discuss who will bring samples from which sources) may be allotted before the first lab session of this experiment. Alternatively, instructors can provide the students with number of likely *E. coli*-bearing materials in order to minimize the time needed to introduce and discuss this exercise.

Instructors will need to provide appropriate review and guidance for each teams' protocols and all other issues introduced in section II. B, above. The activity can be made more directed (less student designed) by providing to the students part or all of the experimental steps needed.

Feedback and review of Gram staining results (*E. coli* should be a Gram negative, non-spore forming rod), streak plate technique, and interpretation of differential media results will be needed for each team during the course of the experiment.

Each student completes a formal research report in the format of a scientific paper. Assessment is based on 1) experimental design, 2) ability to follow format guidelines, 3) quality of data evaluation, and 4) quality of discussion of rationale for differences observed in antibiotic sensitivity patterns. Questions addressed in the discussion should include: "Why use EMB (MacConkey) medium to isolate coliforms?" "Why use Mueller-Hinton agar for the agar disk diffusion technique?" "What are the mechanisms of antibiotic resistance?" The first draft of a student's report is evaluated without grading. Constructive comments are made for the purpose of giving guidelines for revision of the paper with the goal of helping students improve their writing. Final drafts are evaluated and graded.

Problems and Caveats.
Laboratory strategies may include:

1. Comparison of specific antibiotic batteries (cell wall synthesis inhibitors, protein synthesis inhibitors, for example) to evaluate the biological effects of structural analogs or altered targets. This will focus on antibiotic mechanisms.
2. Correlation between environmental source of isolates and antibiotic sensitivity patterns. This will focus on selective pressures, diversity and evolutionary theory.
3. Comparison of bacterial isolates to evaluate multi-drug resistance. This will focus on genetics, gene transfer and gene frequency between populations.

SUPPLEMENTARY MATERIALS

Possible Modifications.

1. Responses to other antibacterial agents (disinfectants, soaps, etc.) may be used to determine whether strains of *E. coli* can survive under these environmental pressures.
2. Wells can be cut into the agar hydrogel to create a place to apply viscous liquids or suspensions (see Appendix for antibiotic solubilities).

References.
Murray, Baron, Pfaller, Tenover and Yolken (eds), 1995. Manual of Clinical Microbiology. ASM Press, Washington, DC.

Appendices.
Table of Antibiotic Solubilities.

Recipes for Media.
See DIFCO Manual, DIFCO Laboratories, Detroit, MI or online media recipes.
McFarland Barium Sulfate turbidity standard is made in clean 15 x 150-mm glass tube as 0.05 mL of 1% aqueous barium chloride and 0.95 mL 1% aqueous sulfuric acid.

Media for Isolation and Enumeration

Curriculum Resources

Appendix. Table of Antibiotic Solubilities

SOLUBILITY (mg/ml)

ANTIBIOTIC	WATER	OTHER
Ampicillin	~1	
Cefoxitin	~2	10-acetone
Methicillin sodium	>300	40-ethanol
Penicillin G potassium	>100	100-ethanol
Vancomycin	>100	1-methanol
Chloramphenicol	2.5	10-acetone
Clindamycin	~10	~10-ethanol
Erythromycin	2	~10-ethanol
Gentamicin	>100	~10-ethanol
Streptomycin	>20	>20-methanol
Tetracycline hydrochloride	>20	>20-methanol
Norfloxacin	0.28	5.1-acetone
Rifampin	~1	~10-methanol
Sulfamethoxazole	0.85	2.3-methanol
Trimethaprim	0.04	7.3-benzyl alcohol

Curriculum Resources

Media for Isolation and Enumeration

Note: Media marked with an * indicate they are readily available commercially.

Non-selective or general purpose media

1. ***PCA – Plate Count Agar** (a.k.a. Standard Methods agar). A medium used for the routine enumeration of bacteria in food.
2. ***TSA – T-soy agar** (a.k.a. Tryptic Soy Agar or Trypticase Soy Agar). A widely used medium for the cultivation of many heterotrophic bacteria.
3. ***R2A – R2 Agar**. A low nutrient medium devised for the recovery of bacteria from treated potable water. Energy sources range from pyruvate to starch.
4. ***NA – Nutrient Agar**. A classic medium for growth of bacteria. Beef extract provides a complex mixture of minerals, vitamins and some carbohydrates.

Differential Media

1. ***King's Medium B** (a.k.a. Pseudomonas F or Flo agar). A differential medium for the detection and isolation of fluorescent *Pseudomonas spp.* Enhances the elaboration of fluorescent pigments. View colonies under UV light to observe fluorescence.
2. ***Sheep Blood Agar** (5% sheep blood in T-soy agar). A differential medium for the detection of hemolytic bacteria. A standard medium for isolating bacteria from clinical specimens.

Selective Media

1. ***Eosin Methylene Blue (EMB) Agar**. A differential plating medium for detection and isolation of gram-negative coliforms. Eosin inhibits the growth of gram-positive bacteria. Differentiates lactose-fermenters from lactose-nonfermenters. Fecal coliforms, such as *E. coli* are blue-black with a greenish metallic sheen; *Enterobacter* forms large pink, mucoid colonies; lactose nonfermenters such as *Salmonella* and *Shigella* are translucent and amber or colorless; *Acinetobacter* spp. turns bright blue.
2. ***Mannitol Salt Agar**. Selective and differential medium for isolation and differentiation of *Staphylococcus*. Contains mannitol, 7.5% sodium chloride and phenol red indicator. The NaCl inhibits organisms other than staphylococci. A yellow color indicates fermentation of mannitol.
3. ***PEA Agar**. Phenolethanol Agar is a selective medium for isolating staphylococci and streptococci in the presence of gram-negative organisms such as *Proteus* spp. or *Escherichia coli*.
4. ***Sabouraud Glucose (Dextrose) Agar**. This medium is for the isolation and cultivation of fungi (yeasts and molds). The selective property of this medium is the relatively low pH (about 5.6). To make the medium more selective for fungi, 0.05 g/l chloramphenicol can be added. The Emmon's formulation of this medium is buffered to a pH of 7; it will not be as inhibitory to bacteria.
5. **Actinomycete Isolation Agar**.

   There are several approaches to selection of actinomycete type organisms. Two examples are given below.

   Glycerol Arginine Agar. This medium is somewhat selective for actinomycetes (particularly streptomycetes). It is suggested that cycloheximide (50 µg/ml) and nystatin (50 µg/ml) be added to prevent growth of fungi. The basis for selectivity appears to be the high carbon/nitrogen ratio,

which favors actinomycetes over the unicellular bacteria. Per liter of distilled water: glycerol 12.5 g, arginine 1.0 g, NaCl 1.0 g, K_2HPO_4 1.0 g, $MgSO_4 \cdot 7H_2O$ 0.5 g, $Fe_2(SO_4)_3 \cdot 6H_2O$ 0.01 g, $CuSO_4 \cdot 5H_2O$ 0.001 g, $ZnSO_4 \cdot 7H_2O$ 0.001 g, $MnSO_4 \cdot H_2O$ 0.001 g, agar 15 g.

Starch Casein Nitrate Agar. This medium is somewhat selective for actinomycetes. It is suggested that cycloheximide (50 µg/ml) and nystatin (50 µg/ml) be added to prevent growth of fungi. Selectivity is partially based on use of complex substrates as well as the high carbon/nitrogen ratio. Per liter of distilled water: starch 10.0 g, casein 0.3 g, KNO_3 2.0 g, NaCl 2.0 g, K_2HPO_4 2.0 g, $MgSO_4 \cdot 7H_2O$ 0.05 g, $CaCO_3$ 0.02 g, $FeSO_4 \cdot 7H_2O$ 0.01 g, agar 18.0 g.

Note: Actinomycetes often are slow growing. Incubation should continue for at least one to two weeks. Also, since these media are not 100% selective, actinomycete colonies will have to be identified by colonial morphology or by microscopic examination.

6. **Medium BG-11 for Cyanobacteria**. $NaNO_3$ 1.5 g, K_2HPO_4 0.04 g, $MgSO_4 \cdot 7H_2O$ 0.075, $CaCl_2 \cdot 2H_2O$ 0.036 g, citric acid 0.006 g, ferric ammonium citrate 0.006 g, EDTA (disodium salt) 0.001 g, Na_2CO_3 0.02 g, trace metal mix A5 (see below) 1.0 ml, agar (if needed) 10.0 g, distilled water 1.0 L. The pH should be 7.1 after sterilization.
   Trace metal mix A5: H_3BO_3 2.86 g, $MnCl_2 \cdot 4H_2O$ 1.81 g, $ZnSO_4 \cdot 7H_2O$ 0.222 g, $Na_2MoO_4 \cdot 2H_2O$ 0.39 g, $CuSO_4 \cdot 5H_2O$ 0.079 g, $Co(NO_3)_2 \cdot 6H_2O$ 49.4mg, distilled water 1.0 L.
   Light is the energy source for cyanobacteria.

Other media suggestions

1. **To inhibit fungi.**

   To inhibit fungal growth, cycloheximide can be added to a medium at a concentration of 50 mg/ml. This is especially important for soils that may contain pathogenic fungi such as species of *Histoplasma* or *Coccidioides.*

2. **Variations of T-soy agar.**

   Used to select for organisms able to grow with fewer nutrients or in the presence of more sodium chloride.

   0.1x TSA – Tryptone 1.5 g, Soytone 0.5 g, NaCl 0.5 g, agar 15 g per liter. [Note that T-soy broth has glucose and dipotassium phosphate and different amounts of tryptone and soytone per liter than T-soy agar.]

   TSA + 5% NaCl – Tryptone 15 g, Soytone 5 g, NaCl 50 g, agar 15 g per liter.

3. **Variations on minimal agar**.

   Minimal agar is minimal with regard to organic compounds. Bacteria with the capacity to synthesize all organic compounds from a limited organic carbon source can grow on this medium. Bacteria that require specific growth factors will not grow by themselves but may form satellite colonies around other colonies that secrete such nutrients.

   Minimal agar – Dextrose 1 g, dipotassium phosphate 7 g, monopotassium phosphate 2g, sodium citrate 0.5 g, magnesium sulfate 0.1 g, ammonium sulfate 1 g, agar 15 g per liter.

Minimal agar + 0.1% benzoate – To minimal agar ingredients add 1 g sodium benzoate per liter.

Minimal agar medium + 0.1% selected fatty acid salt – To minimal agar ingredients add 1 g fatty acid salt (e.g. sodium palmitate).

Nitrogen-free minimal agar medium + 0.1% selected fatty acid salt – Minimal agar ingredients minus the ammonium sulfate (substitute sodium or potassium sulfate) plus 1 g of a selected fatty acid salt. This medium would require the bacteria to grow by fixing nitrogen from the air; a carbohydrate such as mannitol could be substituted for the fatty acid salt.