Enumeration and Identification of Enteric and Pseudomonad Proteobacteria from Agriculture-Impacted Rivers and an Exploration of Fecal Viruses and Protozoa

Resource Type: Curriculum: Laboratory

Publication Date: 10/27/2006

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Abstract

In this exercise, students analyze water samples from two local rivers, emphasizing MacConkey-selected gram-negative proteobacteria. They use lactose and oxidase testing to sort observed colonies into Enterics (both lactose positive and lactose negative) and Pseudomonads. While Enterics are typically associated with transient fecal contamination, Pseudomonads represent natural soil and water dwellers that play important roles in decomposition and bioremediation. Each student subcultures and identifies river-derived and provided enteric controls using a standard array of biochemical tests. Each student also analyzes waterborne and fecal-associated protozoa and viruses, using provided slides and online Centers for Disease Control and Prevention resources.

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.

INTRODUCTION

Learning Objectives.
Upon completion of this activity, students will be able to:
(1) understand direct counting methods, having applied these techniques to a local river habitat;
(2) recognize and sort enteric and pseudomonad colonies, including understanding implications of their relative numbers;
(3) identify provided enteric controls and river-derived isolates using standard biochemical tests and keys, demonstrating the power and limitations of culture-dependent approaches when dealing with unknown populations; and
(4) view and research viruses and protozoa associated with feces and waterborne illness.

Background.

This laboratory exercise has been carried out at Western Oregon University. A portion of it was included as one component of our poster entitled "A General Microbiology Lab Curriculum Featuring Culture-Dependent and -Independent Approaches and Computer-Based Project Presentations" at the 2004 American Society for Microbiology General Meeting. This exercise applies many fundamental microbiology principles and techniques (e.g., direct count enumeration, selective and differential media, identification using phenotypic tests and keys) to river habitats that are impacted by agriculture via fecal contamination and/or fertilizer run-off. After collecting and plating water samples from two local rivers, students sort and enumerate MacConkey-selected colonies into Enterics (both lactose positive and lactose negative) and Pseudomonads. Each student then uses a standard battery of enteric-based phenotypic tests to identify two river-derived lactose positive enterics and two assigned enteric controls. The prelab lecture for this unit emphasizes a detailed comparison of enteric versus pseudomonad proteobacteria in terms of phenotypic traits and roles in medicine, ecology, and bioremediation. Since 2002, all class coverage of enteric pathogens has been moved to lab, freeing up more lecture time later for other disease topics. Additional microscopy and online assignments were developed in 2003 to include protozoal and viral agents associated with fecal and waterborne transmission.

This curriculum was first implemented in 1999 for the laboratory component of General Microbiology (Biology 331), a course that all Biology majors are required to take. The typical annual impact is 32 students per year. Students complete this laboratory exercise during weeks four and five of this ten-week course, concurrent with the beginning of lectures about indigenous flora and bacterial pathogens. By this point in the lab, they have also mastered several microbiological skills, including aseptic technique, dilution series, direct counting, Gram staining, and various environmental enrichment techniques. The course requires *Brock Biology of Microorganisms* (4), an invaluable resource for bacterial diversity, water quality issues, and bioremediation. The same instructor (Boomer) who has developed and delivered lectures also runs the laboratory (which meets Tuesdays and Thursdays for 2 hours); first-person portions of this report refer to Boomer. Students receive all instructional materials at the beginning of the term.

PROCEDURE

Materials.
This exercise should be run with students working individually, although team set-up for initial collection, plating, and counting is appropriate. All prepared slides and cultures below are available through commercial venders. Although control cultures are available through the American Type Culture Collection (ATCC), the cost of these materials is highly prohibitive in comparison with biological supply companies like Wards (30 to $200 versus 7 to $15 per culture); thus, catalog numbers for media below refer to this source. I rely on a lab preparator who makes all media over the course of 6 to 8 hours (spread over 2 days) using premixed base reagents from Fisher Scientific; catalog numbers for media below refer to this source, with specific subbrands being indicated (e.g., Difco, Remel). Media recipes are also available in most standard microbiology lab manuals (2). All media can be made 4 to 6 weeks in advance and should be wrapped in plastic and refrigerated until use. The media-independent lab set-up time for this lab is 3 to 5 hours per session over 2 to 3 days. This approximation assumes instructors have all control cultures actively growing; initial set-up and maintenance of controls will add about 1 week of set-up time, albeit only 2 to 3 hours total during that time. Each student needs:

Session One

- 5 ml of river water (collected within 12 hours of lab, ideally assessed for temperature and pH)
- 5 MacConkey agar plates (Difco MacConkey agar base, DF0818-17-3)
- 5 sterile disposable pipettes
- Dally rod and alcohol or flame for sterilization
- Internet access (although assignments can be completed as homework elsewhere)
- Slides: *Entamoeba histolytica, Cryptosporidium parvum, Giardia lamblia, Balantidium coli**
- Microscopes and oil immersion supplies

*Slides should be the trophozoite form; one slide for every four students is adequate.

Session Two

- 3 MacConkey agar plates (plus 1 to 2 extras for accidents and failures)
- 5 to 8 oxidase test disks provided in empty petri dishes with forceps*
- Oxidase controls on nutrient agar (I use *Escherichia coli* and *Pseudomonas putida*)
- 10 to 20 sterile toothpicks

*Review the Safety and Handling section for important additional information.

Sessions Three and Four

- 4 citrate agar slants (Difco Simmons citrate agar base, DF0950-17-1)
- 4 glucose ferment tubes (Remel phenol red broth base, R062202, plus 1% glucose)
- 4 sulfur/indole/motility (SIM) tubes (Remel SIM media, R454412)
- Any two enteric controls or unknowns, nearly all of which are pathogenic*

*Recommended culture information based on provided identification key (Appendix One).

Culture	Wards catalog #	Kind of culture
Escherichia coli	85 W 0400	Live tube
Citrobacter freundii[a]	85 W 1848	Freeze-dried pellet
Salmonella typhimurium[a]	85 W 1956	Freeze-dried pellet
Klebsiella pneumoniae[a]	85 W 1880	Freeze-dried pellet
Proteus mirabilis[a]	85 W 1895	Freeze-dried pellet
Shigella flexneri[a]	85 W 1932	Freeze-dried pellet
Pseudomonas putida	85 W 0710	Live tube

[a] Known pathogens; handle as biosafety level 2 agents. Additional information about BSL2 agents and facility requirements is described in the Safety and Handling section and Appendix One.

Student Version.
Appendix 1. Lab procedures
Appendix 2. River proteobacteria assignment
Appendix 3. Virus/protozoa assignment
Appendix 4. Partial key for virus/protozoa assignment

Instructor Version.
This class lab extends over four sessions that should be run back to back.

Session One
In session one, students are provided with an extensive prelab lecture about enterics and pseudomonads, emphasizing key

identification traits and respective roles in medicine, ecology, and bioremediation. Specifically, major reportable fecal-associated proteobacteria are described: *Escherichia coli* (toxigenic hemorrhagic strains), *Salmonella (typhimurium, enteritidis*, and *typhoid* species), *Shigella*, and *Campylobacter* (only the latter is not a true enteric and would not be retrieved using these lab methods). Lecture materials have not been provided in this presentation as all information and images were derived from the course text (4), *Sherris Medical Microbiology. an Introduction to Infectious Diseases* (6), and the Centers for Disease Control and Prevention (CDC) website.

Following lecture, I review the protozoa and virus assignment, emphasizing that students need to carefully manage their time during all sessions in such a way that enables them to complete all project components. Protozoa were selected to demonstrate all four motility-based subgroups: amoeboid *Entamoeba histolytica*, sporozoa *Cryptosporidium parvum*, flagellate *Giardia lamblia,* and ciliate *Balantidium coli.* Students should review their text and the CDC website prior to examining slides so they can distinguish pathogens from host tissues. In addition to simple scientific drawings, I require that students physically setup and show me one of the slides during session four, one of two times students are required to demonstrate physical skill using a microscope (the other being Gram staining earlier in the term).

Following lecture, each student prepares five replicates of river water (0.2 ml per plate) collected earlier that morning by student volunteers, as shown in Fig. 1 (see additional information in Safety and Handling section). Alternatively, instructors may collect these samples without students up to 12 hours before class, refrigerating samples between collection and class plating. My class samples and compares Gentle Creek, a small stream that runs through several miles of farmland, with the Willamette River, a large river that runs through a few hundred miles of industrial and agricultural areas. Both areas are publicly accessible, popular for fishing, swimming, and boating. Water samples, collected in October and April, have been observed to range from 9 to 15°C and 4.5 to 6.5 pH. In general, students retrieve 5 to 30 colonies per MacConkey agar plate (a representative plate is shown in Fig. 1), which typically includes 3 to 10 morphologically different lactose-positive and lactose-negative variants (anecdotal observations suggest that counts vary according to the amount of recent rainfall). To achieve comparable numbers and variation, instructors may have to adjust student replicate numbers or select different river systems in their area. Given no other procedural obligations during session one, students are able to begin protozoa and virus assignments. Depending on the lag time between sessions, instructors or students should refrigerate plates after 18 to 24 hours.

Session Two

During this session, each student enumerates his/her plates in terms of lactose-positive versus lactose-negative colonies, which many students have a difficult time judging. If this is the first time they are working with this medium, they should be provided either with plated controls (e.g., lactose-positive *Escherchia* versus lactose-negative *Salmonella*) or review control images, available in most standard lab texts (e.g., Wistreich, 7). Following enumeration, each student then completes two tasks: coliform subculture and oxidase-pseudomonad testing. For the former, each student selects two morphologically distinct lactose-positive enterics and streaks to isolate each onto new MacConkey agar plates. Students are warned that they must come in to check and refrigerate their plates within 24 hours, repeating streak plating as needed until they have two pure isolates by session three (in our case, 4 days away). Although students have had to perform streak plating almost ten times already, 10% of my students fail to produce proper plates on the first attempt because they have forgotten some basic step in streak plating (e.g., used the wrong pattern or forgot to flame between sectors). Thus, they are reminded what a “good” streak plate should look like in their procedures: homogenous colony types with some pure colonies that are well-isolated.

The second task is to sort 10 lactose colonies using the oxidase test (see additional comments in Safety and Handling section). Oxidase testing effectively distinguishes pseudomonads from enterics (a positive and negative control are shown in Fig. 1); it is also worth mentioning to students that this test is medically important because *Neisseria* (i.e., causative genera for gonorrhea and meningococcal meningitis) are also distinctively oxidase positive. While oxidase is a strong indicator of *Pseudomonas*, there are a few pseudomonads that will use lactose, albeit at low levels. In researching and developing this exercise, I actually could not find this information in basic sources like the course text and *Shorter Bergey's Manual* (1). I eventually located a useful table in the *Manual of Clinical Microbiology* (5), which reported that three of ten clinically relevant pseudomonads variably used lactose at rates of 25% (versus 100% for true lactose users like *Escherichia*). Given this information, it is possible that this exercise misses some lactose-positive pseudomonads, and I make students aware of this fact while discussing the procedures. However, lactose-positive MacConkey colonies are challenging to interpret using oxidase tests because they are already dark purple. Given that students have not done or seen oxidase testing before, they run controls prior to assessing river colonies.

Since initiating this lab, class data suggests that 70 to 100% of all lactose isolates are oxidase-positive pseudomonads. Anecdotally, most students seem at first relieved to discover this information, which implies to them that there are fewer fecal-associated bacteria in the water. The more they come to understand pseudomonads, however, the more they develop new and equal concerns about (a) the significance of these data in terms of potential industrial pollution selection; and (b) health issues concerning contact pseudomonad skin infections. Instructors should thus be prepared to discuss these kinds of follow-up questions and may even want to consider additional extensions, some of which are described in the Possible Modification section.

Sessions Three and Four

During these two sessions, each student's primary goal is to use a basic array of enteric-focused medical tests to identify two river lactose-positive enterics and two assigned controls. Specific tests and media are extensively described in virtually all course texts and laboratory manuals (e.g., Wistreich, 7); thus, detailed descriptions of these procedures (sulfur, indole, motility, lactose, glucose, and citrate) are not included in this paper. Indeed, this set of activities represents nothing more than a simple variation of standard gram-negative identification exercises, emphasizing the use of phenotypic keys and illustrating alternative metabolic substrates and products. While I have, over the years, elected to pare down my array of class tests, other instructors could readily expand testing (e.g., adding urea, Voges-Proskauer, methyl red, a larger battery of carbon utilization tests, etc.). After presenting an overview of methods during a brief prelab lecture, students set up all tests using all samples during session three and then read them during session four. It should be emphasized that river lactose-positive enterics seldom match anything on the key I provide or even in more extended resources (e.g., Bergey's, 1), a likely product of performing too limited testing and the fact that "natural" isolates may, in fact, represent new strains, species, or genera. As an environmental microbiologist trained in culture-independent methods, I believe it is important that

students wrestle with unknown diversity, the implications of which are important for microbial ecology, applied microbiology, and emerging disease. Nevertheless, it is also important that students experience the satisfaction of identifying assigned controls that will match the table so as not to leave them entirely frustrated. While I grade controls all-or-nothing, I am flexible in my evaluation of river isolates given a logical defense. The final 30 minutes of class are devoted to the microscopy practical, which most students take less than 5 minutes to complete.

Safety and Handling Issues.

Local rivers contain, minimally, enterics from untreated agricultural run-off (typically, students observe 10^3 to 10^4 lactose-positive enterics per liter). River samples also contain pseudomonads (on the order of 10^3 to 10^4/liter) and lactose-negative enterics (on the order of 10^1 to 10^2/liter). It is also possible that water samples contain protozoa or viruses but we do not actively amplify or assay for these microbes. Consequently, students are required to wear gloves (we use nonlatex given allergy issues) and clean hands while sampling and handling raw river samples. Following initial plating, extra river water and materials contaminated with river water are immediately autoclaved. MacConkey-retrieved isolates are handled as though they are pathogens and students are fully informed that provided controls include pathogenic genera.

Although all controls recommended in this procedure are available commercially, most are defined as biosafety level 2 (BSL2) pathogens and additional precautions must be taken. Instructors should review specific information about these agents and BSL2 facility definitions; an appropriate source is the CDC/NIH handbook "Biosafety in Microbiological and Biomedical Laboratories" (available online at http://www.cdc.gov/od/ohs/biosfty/bmbl4/bmbl4toc.htm). Although BSL2 agents are moderate pathogens, their main mode of transmission is via contact, not air. Consequently students should use gloves, goggles, and lab jackets when handling these agents. A specific citation of the CDC/NIH description of BSL2 agents and facilities is included in the student procedures (Appendix One).

Finally, all oxidase test methods use tetramethyl-p-phenylenediamine dihydrochloride, a moderately toxic agent that should not contact skin. After comparing several different testing formats, I prefer oxidase disks (BBL Taxo N Discs) because they have a long shelf life and are easy to control in terms of safety and contact issues, particularly relative to the liquid-filter approach. The disks are also significantly more cost-effective than DrySlides. After discussing safety issues with the class, I distribute preassembled petri dishes with oxidase disks and forceps to each student.

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.

ASSESSMENT and OUTCOMES

Suggestions for Assessment.
Individual students each turn in completed copies of the River Proteobacteria Lab Analysis Worksheet (20 pts). Student teams turn in the completed Waterborne Viruses and Protozoa Worksheet, which includes the microscopy practical (20 pts). Additionally, 20% of the lab midterm exam (20/100 total points) covers this lab exercise. The combined value of this lab unit is 20% of the lab assignment grade (60/300 total points).

Field Testing.
Since being developed in 1997, approximately 160 junior- or senior-level undergraduate Biology majors have completed this curriculum. Most students (50 to 60%) were pursuing careers in the health sciences. The remaining students sought careers in secondary education and research (academic, government, or biotechnology).

Student Data.
We have completed assessment of lab curricula in General Microbiology, which serves a maximum of 16 students per term. Thirty-eight students rated this curriculum on a 10-point scale (10 = best; 1 = worst) in fall 2003, spring 2004, and fall 2005, as summarized below:

Please rate the statement: This lab…	Average rating
Made connections beyond microbiology	8.7
Improved my awareness of microbial diversity	9.4

Enhanced my interest in scientific research	8.6
Exposed me to new technology	7.9
Enhanced my organizational skills	7.9
Enhanced my writing skills	7.0
My overall rating of this lab is	8.7

SUPPLEMENTARY MATERIALS

Possible Modification.
Given that this activity is an inquiry-driven adaptation of standard unknown identification exercises, there are many ways that this lab could be modified, three of which are presented here.

Modification One–Different or Expanded Sampling Approaches

In my current version of this laboratory exercise (run since 2003), I have pared down my sampling sites to two local river locations, comparing enterics and pseudomonads at a small and mostly agriculture-impacted river with those populations at a larger, agriculture- and industrial pollution-impacted river. When initially troubleshooting this exercise (2000 to 2002), I allowed each student team to design and execute its own river sampling procedures, my only requirements being that each team sample and compare at least three locations (notably on their own time and using their own transportation) and develop a reasonable hypothesis based on their selected sites. Student teams were extremely creative when given this opportunity, with the most elaborate and memorable project being a study wherein a student team selected and compared four locations along a single river—from its pristine headwaters all the way to its impacted terminus. Although I was very tempted to use this worthwhile and interesting study for my current class, there were two strong deterrents: (i) sampling required nearly 8 hours, over half of which was driving, and (ii) headwater samples contained only 5 to 10 colonies using these retrieval methods, which would have been inadequate for subsequent individual student subculture analyses. Nevertheless, allowing student teams to design their own experiments is strongly encouraged, provided that instructors make efforts to assess plans and ensure that adequate MacConkey-derived colonies will likely be retrieved if dependent analyses and identification are to be carried out.

Modification Two–Antimicrobial Sensitivity Testing

Given the extensive agricultural use of antimicrobial compounds, testing river isolates for drug resistance is an interesting and relevant follow-up lab. This extension involves propagating colony isolates in nutrient broth overnight and then preparing lawns for disk-based testing. However, a key problem I have encountered is the fact that many river isolates (in some classes, as many as 75%) fail to grow in nutrient broth. Consequently, I recommend heavily streaking isolates directly onto new MacConkey agar plates and then placing antimicrobial disks directly thereon to assess for relative sensitivity. To retrieve more accurate and comparable data, instructors should attempt traditional Kirby-Bauer sensitivity testing (2), experimenting with other nonselective media (e.g., tryptic soy broth) prior to assessment on Mueller-Hinton agar.

Modification Three–Pseudomonas Identification and Bioremediation Assay

As with enterics, it is also possible to identify river pseudomonads and controls to the species level via subculture purification and an array of biochemical tests (e.g., commercially available pseudomonad species include *aeruginosa*, *fluorescens*, *fragi*, and *putida*). Some recommended differentiation tests are summarized below, with more in the Bergey's Manual (1). Finally, an indirect bioremediation assay can be performed, as adapted from Luu et al. (3). After streaking isolates on minimal or nutrient agar and incubating 12 to 18 hours, 5 mg of indole crystals are placed in the petri dish lid where they volatilize up to the media/growth zone. Degradation of this aromatic yields a variety of colored products —typically red, blue, or brown—and has been shown to correlate with the ability to break down chemically similar compound (e.g., trichloroethylene, chlorinated and/or aromatic compounds). A representative image of this assay is shown in Fig. 1, although these isolates were enriched from hydrocarbon-contaminated soil, not river water.

Species	Denitrification	Gelatin hydrolysis	Fluorescence	Pigment
aeruginosa	+	+	+	+ on King B only
fluorescens	+	+	+	-
putida	-	-	+	-
stutzeri	+	-	-	-
mendocina	+	-	-	+ on nutrient
malei	+	+	-	-

FIG. 1. Miscellaneous Images

Acknowledgments.
Water assessment equipment was supported by a National Science Foundation Microbial Observatories/Research at Undergraduate Institute grant (NSF-MO/RUI 0237167). I wish to thank Mary Bicknell and Denise Anderson for introducing me to *Pseudomonas* identification methods when I was a graduate teaching assistant in their undergraduate microbiology course at the University of Washington in 1989-1990. I also researched and adapted the bioremediation assays when I was supported by the Waksman Foundation for Microbiology (2000-2001) to develop and teach a special course for secondary science educators called "Microbes and Biotechnology." Finally, I wish to acknowledge the Biology Lab Preparator, Piper Mueller-Warrant, for providing specific media ordering and preparation information during the revision of this manuscript.

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APPENDIX 1: Lab Protocol, All Sessions

Introduction to Pseudomonads and Enteric Proteobacteria

Many proteobacteria can be found in local river habitats, two subgroups of which provide the focus of this unit: enterics and pseudomonads. Enterics are oxidase-negative rods that metabolize simple sugars. Coliforms, sometimes called fecal indicators, typically refer to lactose-positive enterics. Although coliforms include important gastrointestinal bacteria such as *Escherichia* (both pathogenic strains and normal flora), there are disease-relevant lactose-negative enterics (e.g., *Salmonella*). Pseudomonads are oxidase-positive rods that utilize both simple sugars as well as a polymeric carbon sources. Some pseudomonads are distinguished by their ability to oxidize hydrocarbons and xenobiotics (e.g., PAH and PCB) and thus may be selected in industrially-polluted habitats. While enterics often represent transient river contaminants, pseudomonads thrive naturally in soil, mud, and water, with some causing inhaled or contact diseases. In this lab unit, you will compare proteobacteria from local rivers using MacConkey agar, a selective and differential medium used to assess water quality. In addition to counting and identifying river lactose-positive isolates, you will also sort and count lactose-negative isolates using the oxidase test.

Overview—Individual Waterborne Protozoa and Virus Assignments

Many protozoa and viruses can also be found naturally occurring or as contaminants in rivers. Given the complexities of isolating and describing these agents, each person will explore several important fecal-associated and/or waterborne protozoa (*Entamoeba histolytica*, *Cryptosporidium parvum*, *Giardia lamblia*) and viruses (poliovirus, Norwalk agent/Norovirus, hepatitis A) using prepared slides, your textbook, and the Centers for Disease Control and Prevention website. During the final hour of this lab unit, each student will set up one assigned protozoa slide, center a representative agent in focus, and show your instructor. You should have all previous questions done so you will be able to perform this task.

ACTIVITIES - SESSION ONE

Collection and Plating—Team and Individual

At least two students from different teams will accompany me to Independence Park at 7:30 a.m.
On site: Collect 10 ml of water, performing pH and temperature assessments on-site as directed.
In lab: Gently swirl or invert river water before removing every sample to avoid settling.
Each person will spread 0.2 ml of water onto each of five MacConkey agar plates; incubate plates at 37°C.

ACTIVITIES - SESSION TWO

Individual Subculture and Assessment

Lactose-positive colonies—coliforms
Select three different lactose-positive colonies; perfectly streak onto MacConkey agar plates; incubate at 37°C.
Check the plates within 24 hours. If the plate is good, store it in the refrigerator; if the plate is bad, repeat streak plating until perfect by NEXT LAB!

"Good" is a properly streaked plate that contains uniform colony types and single, pure colonies; the latter of which are needed and will be used for upcoming identification testing.

Lactose-negativecolonies oxidase sorting—pseudomonads or enterics?
Handle oxidase discs with forceps in empty petri dishes to avoid contact with skin
Sort 10 lactose-negative colonies into oxidase-positive or oxidase-negative categories. Why? Significance?
Press colonies into oxidase test disc using toothpick; perform one at a time to avoid confusion.

ACTIVITIES - SESSIONS THREE and FOUR

Individual Identification Projects

Each person will set up and use the following tests to make a best-guess identification of two river-derived lactose-positive enterics and fully identify two assigned enteric controls from the identification table. The latter set will be graded all-or-nothing. When recording data, describe what you see (colors, cloudiness, etc.), do not just indicate whether it is positive or negative.

Citrate: heavily inoculate one isolate per slant tube. After 2 days of growth, blue indicates positive and green indicates negative. Some organisms do not grow on this medium so make sure to distinguish between growth green and no growth which is also green.

Glucose: inoculate one isolate per broth tube. After 2 days of growth, yellow indicates glucose to acid; gas indicates a bubble in Durham tube.

Sulfur-indole: inoculate one isolate per golden tube by loop-plunging into semi-solid agar. After 2 days of growth, black indicates sulfur positive and golden indicates sulfur negative. For indole production, add 10 to15 drops of Kovács and wait 2 minutes. If Kovács turns red, it is indole positive; if not, it is indole negative.

ID Table For Enterics - from text p. 353 and Bergey's Manual

	Sulfur	Indole	Lactose	Glucose-acid/gas	Citrate
Escherichia coli	-	+	+	+/+	-
Citrobacter freundii	+	-	+	+/+	+
Citrobacter intermedius	-	+	+	+/+	+
Salmonella typhimurium	+	-	-	+/+	+
Klebsiella pneumoniae	-	-	+	+/+	+
Proteus mirabilis	+	+	-	+/+	NG[b]
Shigella flexneri	-	var[a]	-	+/-	-

[a] Var.,varies by strain.
[b] NG, no or limited growth usually obtained using this medium.

SAFETY AND HANDLING SECTION

Some controls listed above are classified as mild to moderate pathogens. Until this lab, you have only worked with mild pathogens that are classified as biosafety level 1 (BSL1) agents, appropriate for basic and introductory teaching labs. During these lab activities, you may encounter BSL2 agents, appropriate for advanced teaching labs but requiring more precautions and experience. To ensure your safety and familiarize you with federally-defined lab safety standards, you are being provided with relevant excerpts from the Centers for Disease Control and Prevention/National Institutes of Health "Biosafety in Microbiological and Biomedical Laboratories" handbook, the entire contents (including definitions of higher BSL facility definitions and a list of pathogens for each category) of which can be found online at:
http://www.cdc.gov/od/ohs/biosfty/bmbl4/bmbl4toc.htm

Excerpts from the Biosafety in Microbiological and Biomedical Laboratories Handbook:

Biosafety Level 1 practices, safety equipment, and facility design and construction are appropriate for undergraduate and secondary educational training and teaching laboratories, and for other laboratories in which work is done with defined and characterized strains of viable microorganisms not known to consistently cause disease in healthy adult humans. *Bacillus subtilis, Naegleria gruberi,* infectious canine hepatitis virus, and exempt organisms under the NIH Recombinant DNA Guidelines are representative of microorganisms meeting these criteria. Many agents not ordinarily associated with disease processes in humans are, however, opportunistic pathogens and may cause infection in the young, the aged, and immunodeficient or immunosuppressed individuals. Vaccine strains that have undergone multiple in vivo passages should not be considered avirulent simply because they are vaccine strains.

Biosafety Level 1 represents a basic level of containment that relies on standard microbiological practices with no special primary or secondary barriers recommended, other than a sink for handwashing.

Biosafety Level 2 practices, equipment, and facility design and construction are applicable to clinical, diagnostic, teaching, and other laboratories in which work is done with the broad spectrum of indigenous moderate-risk agents that are present in the community and associated with human disease of varying severity. With good microbiological techniques, these agents can be used safely in activities conducted on the open bench, provided the potential for producing splashes or aerosols is low. Hepatitis B virus, HIV, the salmonellae, and *Toxoplasma* spp. are representative of microorganisms assigned to this containment level. Biosafety Level 2 is appropriate when work is done with any human-derived blood, body fluids, tissues, or primary human cell lines where the presence of an infectious agent may be unknown. (Laboratory personnel working with human-derived materials should refer to the OSHA *Bloodborne Pathogen Standard* for specific required precautions.)

Primary hazards to personnel working with these agents relate to accidental percutaneous or mucous membrane exposures, or ingestion of infectious materials. Extreme caution should be taken with contaminated needles or sharp instruments. Even though organisms routinely manipulated at Biosafety Level 2 are not known to be transmissible by the aerosol route, procedures with aerosol or high splash potential that may increase the risk of such personnel exposure must be conducted in primary containment equipment, or in devices such as a BSC or safety centrifuge cups. Other primary barriers should be used as appropriate, such as splash shields, face protection, gowns, and gloves. Secondary barriers such as handwashing sinks and waste decontamination facilities must be available to reduce potential environmental contamination.

APPENDIX 2: Individual Student Worksheet - River Proteobacteria Lab Analysis

Name: _____

1. Record your assigned river, its temperature, and its pH based on information provided by the collectors during the first session of class.

2. Record your MacConkey agar plate counts, including sorting by the colors and lactose activity of each.

	# Purple/pink Lactose (+)	# Light/white Lactose (-)
Plate replicate 1		
Plate replicate 2		
Plate replicate 3		
Plate replicate 4		
Plate replicate 5		
Average		

3. Of the lactose-negative colonies tested for oxidase, how many were positive?

4. Using answers from questions 2 and 3, calculate the following in terms of cells/liter in the original river sample. Be careful with pseudomonad value as it requires calculating and correctly applying the percentages observed in question 3.

Total enterics/liter in original river sample

Lactose-positive enterics/liter in original river sample

Pseudomonads/liter in original river sample

5. What do the above distributions of proteobacteria suggest to you about this site in terms of possible pollution impact? Is the water safe to drink? Why or why not?

6. Using instructor-provided controls and river lactose-positive isolates, complete the following phenotypic identification chart. Make sure you indicate the letter or number of your controls as indicated below.

	Control _____	Control _____	River lactose (+) 1	River lactose (+) 2
Sulfur				
Indole				
Lactose				
Glucose acid/gas				
Citrate				

7. Identify your instructor-provided controls; remember, these are graded all-or-nothing.

Control _____ is: Control _____ is:

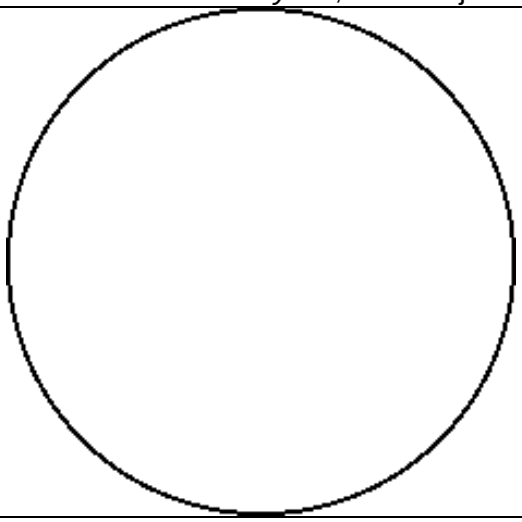
8. For each river isolate, prepare a flow chart and identify your organisms as closely as possible using the available tests. Use your text or other resources to find at least two other tests that could improve your identification.

APPENDIX 3: Individual Student Worksheet—Waterborne Viruses and Protozoa

Name: _____

A. Protozoa: Review your text and/or the Centers for Disease Control and Prevention (CDC) website before tackling the real slides.

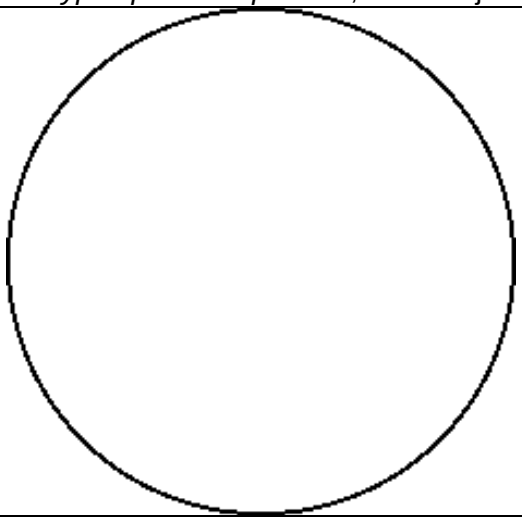
Entamoeba histolytica, 100X obj.



Entamoeba concept check

Motility group	
Mitochondria?	
Makes cysts?	
Any two unique disease features	
Epidemiology (one example)	

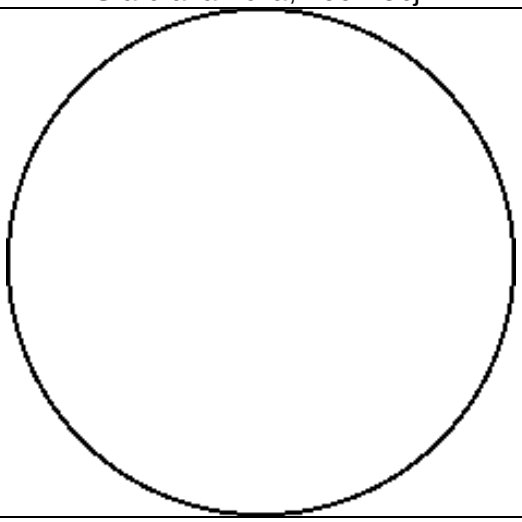
Cryptosporidium parvum, 100X obj.



Cryptosporidium concept check

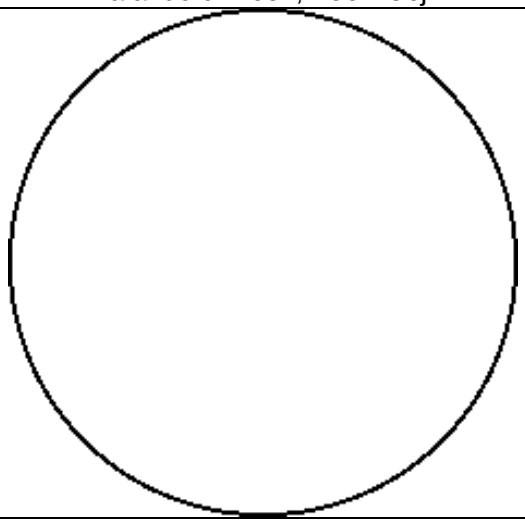
Motility group	
Mitochondria?	
Makes cysts?	
Any two unique disease deatures	
Epidemiology (one example)	

Giardia lamblia, 100X obj.



Giardia concept check

Motility group	
Mitochondria?	
Makes cysts?	
Any two unique disease features	
Epidemiology (one example)	

Balantidium coli, 100X obj.	*Balantidium* concept check
	

Motility group	
Mitochondria?	
Makes cysts?	
Any two unique disease features	
Epidemiology (one example)	

B. Microscopy practical: Instructor's initials _____.

C. Viruses:

(1) Electron micrographs. Using the CDC website (search for PHIL, Public Health Image Library section), locate and print one image of each virus; label and staple to this worksheet.

(2) Using your text and the CDC website (A-Z Index), complete the following table about each virus.

	Poliovirus	Hepatitis A	Norwalk
Genetic material			
Enveloped?			
Any two unique disease features			
Epidemiology (one example)			

(3) Current research: For one of these viruses (your choice), locate a primary research article (less than 5 years old) about this agent. For this assignment, go to www.asm.org and publications/journals and select from *Journal of Clinical Microbiology, Journal of Virology*, or *Clinical & Diagnostic Laboratory Immunology.* Answer the following questions about the selected article:

a) Which virus is this article about?

b) Briefly describe three research procedures used in this article (i.e., one sentence per procedure).

c) Briefly describe three conclusions made by the authors of this article (i.e., one sentence per conclusion).

APPENDIX 4: TABLE KEY. Waterborne Viruses and Protozoa Worksheet

All answers are taken from a combination of the course textbook and Centers for Disease Control and Prevention Health Topics A-Z website. The disease features and epidemiology answers will vary considerably.

A. Protozoa:

*Entamoeba c*oncept check

Motility group	Sarcodina/amoeba
Mitochondria?	No anaerobic metabolism
Makes cysts?	Yes
Any two unique disease features	Causes true dysentery, invasion of intestinal wall and inflammation
	Also invades liver and brain if untreated
Epidemiology (one example)	About 100,000 people die/year worldwide

Cryptosporidium concept check

Motility group	Apicomplexa/sporozoa; nonmotile in adult stages
Mitochondria?	Yes
Makes cysts?	Yes, highly chlorine resistant
Any two unique disease features	Mild, self-limiting in immune-competent people
	Serious, possibly fatal for immune-compromised, HIV/AIDS
Epidemiology (one example)	Largest U.S. waterborne epidemic; 403,000 affected in Wisconsin in 1993.

Giardia concept check

Motility group	Mastigophora/flagella
Mitochondria?	No, true diplomonad
Makes cysts?	Yes, moderately chlorine resistant
Any two unique disease features	Acute forms: explosive, foul, watery diarrhea: no blood
	Can be acute, chronic, or present in asyptomatic carriers
Epidemiology (one example)	Largest U.S. waterborne epidemic; 403,000 affected in Wisconsin in 1993.

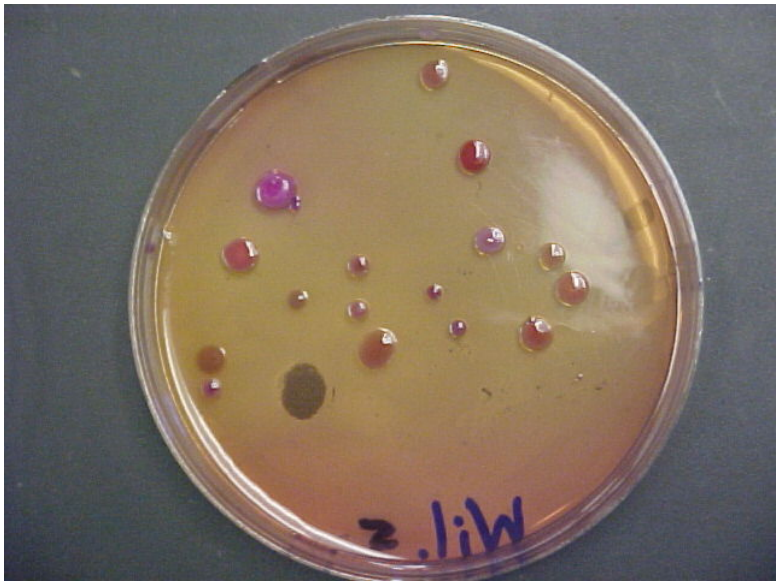
Balantidium concept check

Motility group	Ciliophora/ciliate
Mitochondria?	Yes, and also have micronuclei and endosymbiotic bacteria
Makes cysts?	Yes
Any two unique disease features	Causes dysentery, invasion of intestinal wall and inflammation
	Less invasive beyond GI tract than *Entamoeba*
Epidemiology (one example)	Poor reporting, most significant reservoir is pigs

B. Viruses

	Poliovirus	Hepatitis A	Norwalk
Genetic material	RNA, single stranded (+)	RNA, single stranded (+)	RNA, single stranded (+)
Enveloped?	No	No	No
Any two unique disease features	Most symptoms in the GI tract - diarrhea	Symptoms - nausea, fever, dark urine, jaundice	Mild to moderate diarrhea, Usually self-limiting
	CNS infection and paralysis relatively minor outcome	Average symptoms last 28 days (range is 15 to 50 days)	Incubation 24 to 48 hours, symptoms 24 to 60 hours
Epidemiology (one example)	1952 was peak year in U.S. 52,000 paralysis cases	100,000 U.S. cases/year (despite vaccine)	50% of all gastroenteritis!! 23 million U.S. cases/year

Willamette River in Independence, Oregon



MacConkey Plate, Willamette River

Oxidase Disk Testing: *Escherichia* (left), *Pseudomonas* (right)

Bioremediation/Indole Assay

FIG. 1. Miscellaneous Images