Biology 325: Introductory Microbiology Laboratory Manual

Centre for Science
Athabasca University

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Introduction

Laboratory practice is an important component of a microbiology course. As is evident in many areas of science, there are specific techniques that are essential to acquiring applied skills. For example, examination of form and function of microorganisms, and the ability to develop pure culture techniques are two of the basic skills required in microbiology. As you proceed through the laboratory component of the course, you will acquire essential applied skills in this field of study. In addition, you will use these skills to develop a critical understanding of microbiology.

It is the objective of this laboratory manual to provide you with the necessary materials that will assist you in practicing basic microbiological techniques. As you proceed through the various exercises, you will find that there are specific skills that are unique to this area of science. However, there are also techniques that you can take with you, should you proceed in other fields of study in science.

It is important that you take the time to read your laboratory manual carefully before proceeding through the exercises. This will allow you to consider each exercise in advance, thereby avoiding the possibility of performing exercises incorrectly. Also, it is your responsibility to ensure that you are aware of safety issues in the laboratory. Please refer to the Laboratory Safety Guide before attending the laboratory.

In addition to the lab manual you may find it useful to bring the following items: the Biology 325 required textbook, the Colour Atlas, and a calculator. You are not required to bring a lab coat as we will provide this for you.

Laboratory Safety Guide

Because of their versatility to grow and proliferate in different environments, microorganisms can easily contaminate materials or people. It is therefore imperative that when you work with microorganisms, you conduct yourself in the laboratory safely and wisely. This will ensure that risk of potential infection or contamination will not occur.

Please read the following laboratory safety guidelines below.

1. Do not smoke, eat, or drink in the laboratory, or place any object on the laboratory bench which you could later transfer to your mouth. Keep your books, laboratory manual, and workbook at a reasonable distance from your work area. Keep cell phones away from your work area. If you use your cell phone please make sure you decontaminate it before leaving the laboratory.
2. Practice good aseptic techniques by performing the following before starting each class:
    a. Wash your hands thoroughly with soap and water before starting your exercises.
    b. Long hair should be tied back.
    c. Wear closed footwear to protect the feet.
    d. Wear a protective laboratory coat.
    e. Never place any instruments or materials into your mouth.
    f. Do not under any circumstances pipette by mouth. Use the mechanical apparatus provided.
    g. Use disposable latex gloves when handling blood products (eg. whole blood, plasma, serum, etc.).
    h. Decontaminate your work bench by applying the antiseptic wash provided for you.
3. Wear protective eyeglasses when dealing with chemicals that are heated.
4. Contain contaminated spills by placing a paper towel soaked in 70% ethanol immediately. Keep the towel on the spill for 20 minutes. Inform your instructor of the spill. Place the towel in the autoclave bag provided. Ensure that you wash your hands immediately after dealing with the spill.
5. Note the location of the first aid kit, fire extinguishers, emergency showers, and eye wash apparatus. Note the emergency exits in the laboratory.
6. Place pipettes that are used during class immediately after use into labeled containers of disinfectant.
7. Place glass slides that you have made yourself in the dishes or disinfectant. Do not discard any demonstration slides.

8. Collect used bottles, tubes, and cultures and put them in the designated area for autoclaving.
9. Place contaminated waste (culture plates, gloves, swabs) into the autoclave bags for disposal. All other waste can be disposed of in appropriately labeled containers.
10. Do not place any hazardous or infectious materials in the sink. Do not dispose of solid material in the sink.
11. All materials required for incubation or refrigeration should be appropriately labeled and placed in the relevant containers or trays provided.
12. At the conclusion of each class clean the microscope provided for you. Use lens tissue and cleaner to clean the oil immersion lens (100X) and leave the microscope standing with the low power lens in place. Avoid bumping the microscope.
13. At the conclusion of each session, tidy your laboratory bench and disinfect the work area.
14. Before leaving the laboratory, take off your lab coat and wash your hands thoroughly with soap and water.

Note: If you require any clarification on these guidelines do not hesitate to consult your instructor.

Practical Class Schedule

Day	Exercise	Topic
1	1	Introduction to the microscope.
	2	Microscopic slide techniques
	3	Streak plate method of bacterial isolation
	4	Aseptic technique
	Project	Bacterial identification project
2	5	Selective, enrichment, and differential media
	6	Differential tests
	7	Differential staining
	Project	Bacterial identification project
3	8	Determination of bacterial growth
	9	Sterilization
	10	Chemical control of bacterial growth
	11	Bacterial transformation
	Project	Bacterial identification project
4	12	Environmental and food microbiology
	13	Medical microbiology
	14	Immunology
	Project	Bacterial identification project

Grading

Your final laboratory mark constitutes 20% of your final grade in the course. Your laboratory report constitutes 75% of your final lab grade. Your laboratory report will consist of your bacterial identification project, i.e. the tests you performed, the results of these tests, and your conclusions, including which bacterial species you identified. For the other 25% you will be marked on participation, completion of the exercises in your lab manual (10 marks), and lab skills. The lab skills you will be marked on will be: Gram stain technique (5 marks), streak plate technique (5 marks), and Kohler illumination (5 marks) of your microscope.

Bacterial Identification Project

The next few pages contain information needed to complete your bacterial identification project and write your lab report. The lab report consists of your bacterial identification project and the results you obtained. Students will receive a tube of two unknown bacteria on Day 1 (one Gram positive species and one Gram negative species) which they will identify over the course of the 4 days using a number of selective and differential media and biochemical tests.

Bacterial Identification Project (Lab Report)

This project involves the isolation and identification of a mixed culture containing two unknown species of bacteria to the genus level. You will be required to go through the process of performing basic laboratory techniques in order to isolate a pure culture of each bacterium, and to use the key according to the principles of Bergey's Manual in order to identify each bacterial genus. Differential tests and media will be provided for you upon consultation with the lab instructor. You will continue with the project until the final day of the lab session.

The total mark for the report comprises 75% of your final lab mark and will be marked out of 100. The lab report should contain the following criteria:

1. **Objective:** A brief introduction that clearly states the objective of your study. **/5 marks**
2. **Materials and Methods:** Outline the methods used to isolate each species and obtain a pure culture of each. List the methods used for biochemical testing of your organisms and the methods used for testing your organisms on differential media and selective media. List all reagents used in staining and biochemical testing. **/10 marks**
3. **Results:** A written results section that outlines the Gram stain reaction of each species, the streak plate results, colony morphology on the plate, microscope morphology, and the results of all of the differential tests and selective media that you obtained. **/50 marks**

   Use figures and tables where possible to illustrate the results. Figures may be hand drawn or taken from online resources provided that they are cited and referenced. Figures and tables should be numbered and titled and referred to in the text where they are described.
4. **Discussion:** A conclusion stating the names (genus, and species, if possible) of the two bacteria identified, a brief summary of important results according to the flow chart, and a brief description of each bacteria and their relevance or applications (i.e., medical, industrial, etc.) **/15 marks**
5. **References:** A reference list according to the American Society for Microbiology (ASM) format and citing of your sources in the text. Students are required to use a minimum of 3 references (lab manual, Color Atlas, and textbook). Additional

references may be used and are particularly helpful when writing about the relevance of each species identified. **/10 marks**

6. **Grammar:** Students will be marked on scientific language, spelling, sentence and paragraph structure, and using italics to indicate bacterial genus and species names. **/10 marks**

You may wish to consult the following website for information on how to write a laboratory report:

http://www.studygs.net/labreports.htm

Examples of how to reference using ASM format:

http://journals.asm.org/
(Click on any of the journals listed and have a look at how they cite and reference sources. For example, click on Applied and Environmental Microbiology, and then Select an Issue from the Archive. Click on "Select an Issue" and then click on an issue that is at least one year old so that you have access. Each journal also has a link with "Instructions to Authors," which explains how reference lists and citations should be formatted. Click on "Authors" and then "Instructions to Authors")

The lab manual should be referenced as:

Zenteno, S.L. and L. Carter. 2018. Biology 325 Laboratory Manual. Centre for Science. Athabasca University. Athabasca, Alberta, Canada.

The lab report will be due 2 weeks following the lab. At the lab session, you will be informed of the due date. Lab reports are to be uploaded onto the Biology 325 Course Moodle Site. This site accepts a maximum of 10 MB so any reports larger will not upload. Please try to compress any images or not include images of all results so that your file is not too large. If you are unable to upload the lab report please contact your Academic Expert (AE) or the course professor by emailing:

fst_success@athabascau.ca

Bacterial Identification Project (Procedure)

The purpose of this project is to provide you with the skills to follow a bacterial identification key to classify the species of two unknown bacteria. You will be provided with a mixture of two different microorganisms, one Gram positive organism, and one Gram negative organism. You will follow through the process logically and come to conclusions based on your results and the information provided in the identification key. You will perform streak plate techniques, Gram staining, differential staining, and biochemical testing to identify your bacteria. You should record your observations and results each day. This information will be required for the identification process as well as for your lab report. Please note there will be time set aside in the afternoon of each day to work on your project.

Materials

Suspension of two unknown microorganisms (note: one organism is Gram positive and the other is Gram negative)
Nutrient agar plates (NA) and tryptic soy agar plates (TSA)
Slides
Gram stain reagents
Selective and differential media (MAC, EMB, SCA, SS, PIA, MSA)
Enteropluri tube
Biochemical tests and reagents

Procedure

Day 1

1. Gently mix the tube containing the unknown culture. Make a Gram stain of the culture and describe the two different types of organisms visible in the stain. Record the microscopic morphology for each organism, which includes the Gram reaction, cell shape, and cell arrangement.
2. Streak the culture onto one NA and one TSA plate and incubate at 37°C overnight. Make sure you clearly label the plates.
3. Some students may be required to streak an extra set of plates for incubation at 24°C (room temperature overnight).

Day 2

1. Examine the colony morphology on the representative plates. Describe the colour, diameter, shape, and edges of each colony type (see your text or **Colour Atlas** for assistance).
2. Select likely colonies that may be different for Gram stain determination and plating (you should have two separate colony types on your plates). **Please consult a lab instructor to verify that you have two different colony types.**

3. Perform Gram stains on a pure colony of each type or a portion of a colony type if large enough. Record the microscopic morphology for each organism.
4. Streak a pure colony of each type or use the other portion of the colony to streak onto 1 NA or 1 TSA plate (total 2 NA or TSA plates, 1 plate for each colony type) and incubate at the required temperature overnight.
5. Save your culture containing your unknowns in case you require them again. They will be refrigerated overnight.

Note: You will observe a mixed population of Gram negative and Gram positive organisms on your plates, therefore, try to look at single colonies located at the final streak line of your plate to isolate individual colonies. Please ask your lab instructor if you have trouble identifying two colony types. You may be required to re-streak your plates. The main objective for Day 2 is to try to determine which colony type corresponds to which Gram stain result (keep track of your results) and to be able to isolate a pure culture of each organism by streaking them out onto separate plates.

Day 3

1. Examine the plates which were incubated overnight. You should have a pure culture of each organism. Perform Gram stains on each culture, and streak each one onto 1 NA plate or 1 TSA plate again to confirm that you have a pure culture of each (total of 2 plates). Your Gram stains should confirm the presence of a pure culture of each. Record the microscopic and colony morphology.
2. Examine the flow chart (Figure 1.) provided in this section to select likely tests that may be able to be performed on the pure culture isolates. You may be able to begin some of these tests.
3. Streak your Gram positive organism onto the following plate: Mannitol Salt agar.
4. Streak your Gram negative organism onto the following plates: McConkey (MAC) agar, Eosin Methylene Blue (EMB) agar, Simmon's Citrate agar, and inoculate an Enteropluri tube. Depending on your unknown number you will either streak onto *Pseudomonas* isolation (PIA) agar or *Salmonella-Shigella* (SS) agar (your lab instructor will indicate which agar to use and this may change depending on the lab session).
5. If you have a Gram positive rod you may wish to save an older plate for your spore stain.
6. Please consult your lab instructor to make sure you are on the right track.

Day 4

1. Examine your NA and TSA plates to confirm you have isolated a pure culture of each organism. Gram stain each organism once again to confirm the reaction and purity of each. If you haven't already make sure you record your microscopic and colony morphologies.
2. Examine all of your differential and selective media to see the results for each of your organisms. Use Table 1., Table 2., and the flow chart (Figure 1.) provided in

this section to make your conclusions. Make sure you describe all of your plate results.

3. Perform any other tests relevant to the organism you may have (see flow chart). Make sure you record your results and describe them. You should be able to identify each of your two organisms by this point. Please consult your instructor for assistance.
4. You may take photos with your cell phone of your results for the lab report.

At this stage you should have finalized your results and identified your organisms. Please make sure that you have been marked on your lab work and your lab manual. Make sure you have all of the information you require to write your lab report.

Students that leave their microscope clean, without any residual oil, will receive 1 overall bonus mark for the lab component.

Observations

Observations

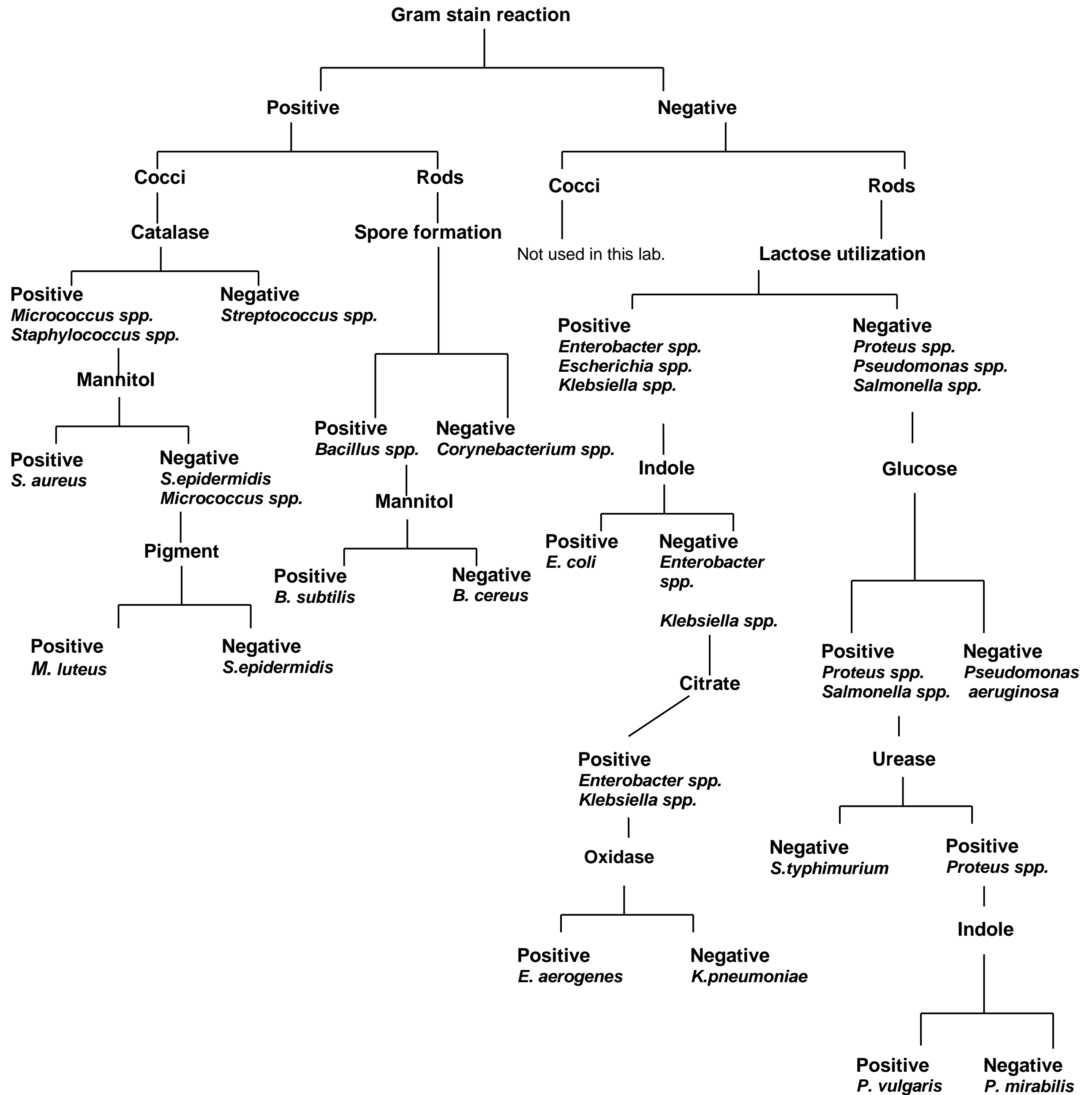
Table 1. Major characteristics of organisms encountered in the Biology 325 laboratory.

Organism	Gram stain	Colony morphology	Oxygen requirement	Motility	Spore formation
Micrococcus roseus	+ cocci	Smooth, raised, pink	Aerobic	-	-
Micrococcus luteus	+ cocci	Smooth, raised, yellow	Aerobic	-	-
Staphylococcus aureus	+ cocci	Smooth, raised, golden	Aerobic	-	-
Staphylococcus epidermidis	+ cocci	Smooth, raised, cream	Aerobic	-	-
Bacillus cereus	+ rods	Opaque, rough, waxy	Aerobic	-	+
Bacillus subtilis	+ rods	Opaque, rough, waxy	Aerobic	-	+
Escherichia coli	- rods	Smooth, raised, cream	Facultative anaerobe	+	-
Salmonella typhimurium	- rods	Smooth, small, grey	Facultative anaerobe	+	-
Salmonella enteritidis	- rods	Smooth, small, grey	Facultative anaerobe	+	-
Proteus vulgaris	- rods	Spreading, grey	Facultative anaerobe	+	-
Enterobacter aerogenes	- rods	Smooth, raised, white	Facultative anaerobe	+	-
Klebsiella pneumoniae	- rods	Slimy, white, translucent	Facultative anaerobe	+	-
Pseudomonas aeruginosa	- rods	Smooth, cream, green sheen	Aerobic	+	-

Table 2. Biochemical characteristics of organisms encountered in the Biology 325 laboratory.

Organism	H_2S	Indole	MR/VP	Citrate	Urease	Oxidase	Catalase	Gelatinase	Amylase	Glucose
Micrococcus roseus	-	-	NA	-	+	-	+	+(slow)	-	-
Micrococcus luteus	-	-	NA	-	+	-	+	+(slow)	-	-
Staphylococcus aureus	-	-	NA	-	-	-	+	+	-	+
Staphylococcus epidermidis	-	-	NA	-	-	-	+	-	-	+
Bacillus cereus	-	-	NA	-	-	-	+	+	+	+
Bacillus subtilis	-	-	NA	-	-	-	+	+	+	+
Escherichia coli	-	+	+/-	-	-	-	+	-	-	+
Salmonella typhimurium	+	-	+/-	+	-	-	+	-	-	+
Salmonella enteritidis	+	-	+/-	+	-	-	+	-	-	+
Proteus vulgaris	+	+	+/-	+	+	-	+	+	-	+
Proteus mirabilis	+	-	+/-	-	+	-	+	+	-	+
Enterobacter aerogenes	-	-	-/+	+	-	+	+	-	-	+
Klebsiella pneumoniae	-	-	+/- or -/+	+	+	-	+	-	-	+
Pseudomonas aeruginosa	-	-	-/-	+	-	+	+	+	-	+

Guide to Identification of Bacteria Used in this Course



Day 1

Objectives

At the conclusion of this session you should be able to:

1. Identify all major components of a compound light microscope and recognize their function in microscopy.
2. Set up the microscope using Kohler illumination
3. Examine specimens using low power, high power, and oil immersion magnification.
4. Interpret patterns of growth for different colonies.
5. Perform and identify a Gram stain of bacteria.
6. Perform a bacterial streak plate.
7. Aseptically transfer a sterile suspension using a loop to broth cultures or agar plates.

Exercise 1: Introduction to the Microscope

The study of microbiology requires the correct use of a light microscope. You should be able to recognize all of the major components of the microscope and identify their function. Proper understanding of function and setting is essential for maximum resolution of bacterial smears. Read the notes below before proceeding with this exercise.

Guidelines

1. The microscope should always be carried using both hands, one hand holding the arm of the microscope, and the other supporting the base.
2. The microscope is a precision instrument. Treat all of its components with care and ensure that it is kept free of dust. Clean objectives, condenser, and ocular lenses using quality lens paper. Ensure that oil is cleaned from the oil immersion objective after use.

1.1 Microscope orientation and Kohler illumination.

Materials

Compound light microscope
Glass slides
Marker
Immersion oil

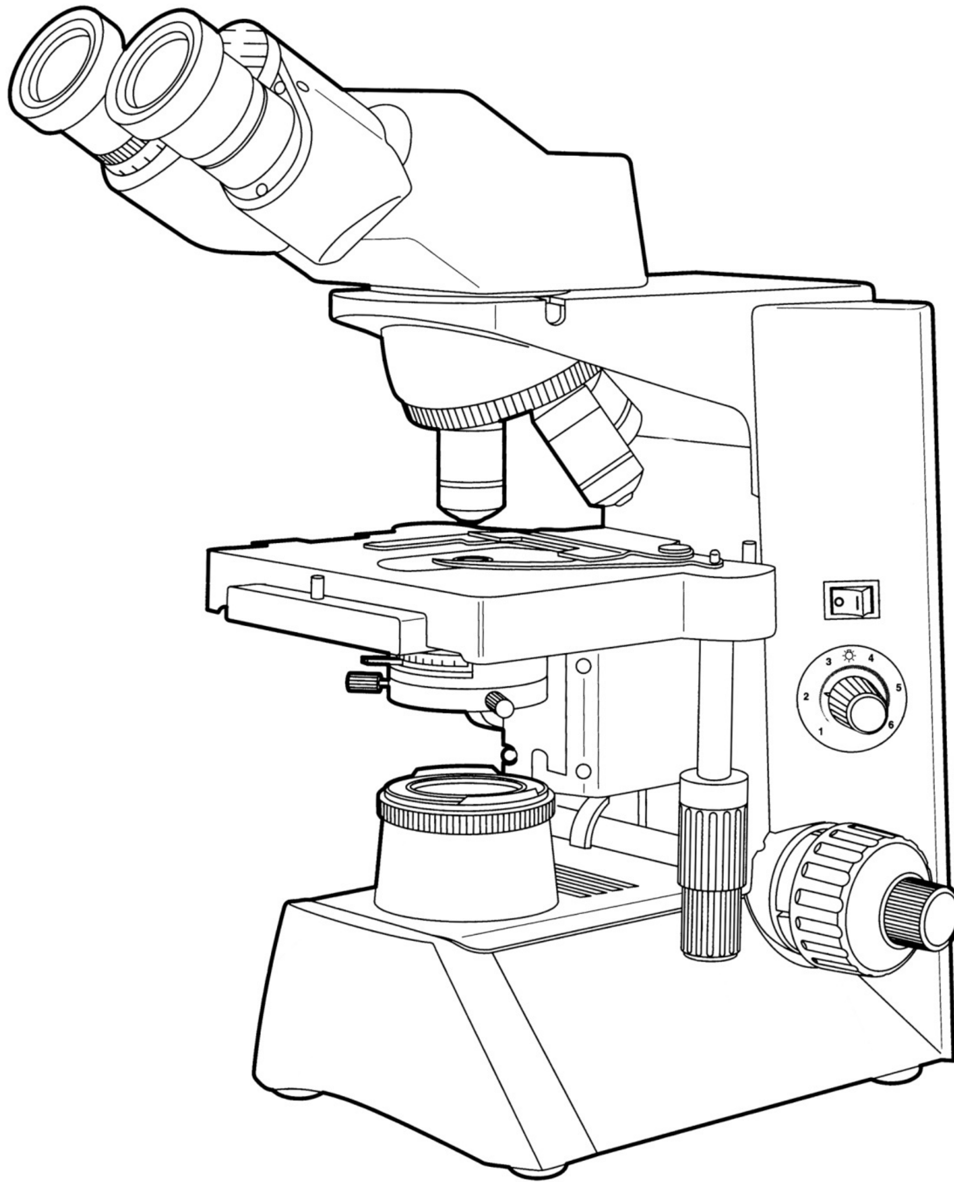
Procedure

1. Gently out your microscope, plug it in, and turn on the light. You should be able to see light coming through the stage.
2. Using the diagram provided on the next page, identify and label all of the components shown on your microscope:

   Ocular lens(es)
   Arm
   Base
   On/off switch
   Light adjustment
   Stage
   Condenser
   Aperture (iris diaphragm)
   Lamp
   Coarse focus knob
   Fine focus knob
   Slide adjustment knob
   Objective lenses
   Field diaphragm (field iris)

3. Fill in the following table with the function of each of the microscope components listed:

Component	**Function**
Ocular lenses	
Light adjustment	
Stage	
Condenser	
Aperture (iris diaphragm or condenser diaphragm)	
Coarse focus knob	
Fine focus knob	
Slide adjustment	
10X objective	
100X objective	



4. Take a clean slide and use a marker to make a line on the slide. Use this slide to set up Kohler illumination. Once you have mastered Kohler illumination, your technique may be marked by your instructor.

Setting up Kohler Illumination

1. Open the field diaphragm to its maximum setting and move the condenser up so that it is as near to the stage as possible.
2. Ensure that the lowest power objective is in position (this can be either 4X or 10X, depending on the microscope).
3. Turn on the lamp of the microscope and adjust the light intensity to its maximum.
4. Using a colour marker, draw a line on a glass slide and place it on the stage, securing it with the slide holder.
5. If the microscope is binocular, adjust the position of the eyepieces so that they comfortably correspond with the position of your eyes.
6. Center the slide and while observing through the ocular, slowly raise the stage using the coarse focus adjustment knob. This process allows you to focus on the specimen (in this case it is the line drawn on your slide). Raise the stage until the line visible through the eyepiece appears clearly focused. This will mean that the edges of the line will appear sharp.
7. Close both diaphragms of the light (field and iris diaphragms). You will now see a small circle or hexagon at the center of your slide. The remaining area will appear black. Using the condenser adjustment knob, focus until the edges of this circle (or hexagon) appear sharp.
8. If the hexagon or circle of light is not in the center of the field of view you will need to center the light. This is achieved by using the adjustment knobs (silver knobs under the stage and attached to the condenser. Center the circle of light in your field of view using these knobs, which will ensure the light is focused properly through your sample.
9. Once the light has been centered, slowly open the aperture (iris) diaphragm until a halo appears (should be around 0.5 or halfway point). Slowly open the field diaphragm until the light illuminates the entire field of view.
10. You may now lower the intensity of the light until it is about 50% of its maximum setting or it is comfortable for viewing.
11. When preparing specimens for microscopy, first focus on the specimen with low (10X) magnification using the coarse adjustment knob. Then carefully move the objective to high power (40X) and focus using the fine adjustment knob. In some cases, larger specimens (eg. algae) require this magnification. However, bacterial smears require the use of the oil immersion lens (100X). In order to do this, follow the steps above and once the bacteria have been seen under 40X, gently move the 40X objective to the right, stopping between the 40X and the 100X. Add one drop of oil to your slide. You can then move the 100X objective into place and focus with the fine adjustment knob.

12. Comment on what you observe of the mark on your slide when you perform the following activities described in the table below:

Activity	**Effect on sample or line on the slide**
Kohler illumination	
Use of the 10X objective	
Use of the 40X objective	
Moving the stage back and forth	

Exercise 2: Microscope Slide Techniques

2.1 Wet mount preparation of a specimen for light microscopy.

Wet mount preparations are normally used to examine the structure of larger organisms such as fungi, protozoa, and algae. They are normally performed using water as the suspending medium. However, in order that internal structures can be better discerned, a suspending medium usually containing methyl blue stain is used.

Materials

Plate culture of *Rhizopus spp*. or *Penicillium spp*.
Pond water containing protozoa and algae
Methyl blue stain suspension
Glass slides
Coverslips
Plastic pipettes
Cotton swabs
Microscope

Procedure

1. Using a clean slide in each case make a wet mount preparation of each sample provided.
2. For the fungal preparation use a sterile wooden stick to gently remove a small amount of the top of the fungal growth on the plate and apply this to a drop of methyl blue stain on your glass slide and gently mix. Cover the sample with a cover slip by placing one side of the cover slip down onto the edge of the suspension at a 45° angle and slowly lowering the coverslip, avoiding the accumulation of air bubbles. View the sample under the microscope at 10X and 40X. Draw a labeled picture of what you see (spores, hyphae, etc.).
3. For the pond water simply apply a drop of the pond water to a clean glass slide and cover with a cover slip as described above.
4. When preparing specimens for microscopy, first focus on the specimen with low (10X) magnification using the coarse adjustment knob. Then carefully move the

objective to high power (40X) and focus using the fine adjustment knob. Draw what you see in your sample and try to identify the organisms.

Pond water:

Fungi:

2.2 Examination of Gram stained bacteria.

A major tool that aids in the classification of bacteria is the Gram stain. Bacteria are too small to be characterized by wet mounts techniques. By using specific dyes and knowledge of the principles underlying cell wall components of bacteria one can identify certain bacterial characteristics according to their reaction to a Gram stain. There are typically two reactions: Gram postitive (purple) and Gram negative (pink) organisms. You can refer to your **Colour Atlas** and textbook for the principles of the reaction. This exercise will enable you to visualize the size, shape, arrangement, and gram reaction of representative bacteria.

Materials

Prepared slides of Gram positive and Gram negative bacteria
Microscope
Immersion oil
Lens tissue

Procedure

1. Place the slide containing the stained bacterial smear onto the microscope stage.
2. Focus on the slide as outlined in Step 10 of Exercise 1.1. First focus on the sample using 10X power magnification and then focus on the sample using 40X magnification.
3. Move the objective away from the slide. Place a small drop of immersion oil onto the slide at the position the objective would normally be placed.
4. Carefully move the 100X objective to the center of the stage. Slowly move the fine adjustment forward and backward until the lens is coated evenly with oil. Focus on the specimen until a sharp image of the smear is evident.
5. Upon completion of examination of the slide, remove the slide and wipe the objective with lens tissue.
6. Observe 2 Gram positive and 2 Gram negative organisms using the slides provided for you.
7. Record your observations of each Gram stain according to Gram reaction (positive or negative), shape of the organisms (cocci or rods), and arrangement (clusters, single, paired, chains) in the table below. You may refer to your **Colour Atlas** for assistance. Try to visualize an example of each: a gram positive coccus, a gram positive bacillus, a gram negative coccus, and a gram negative bacillus species.

Results

Organism	**Gram reaction**	**Shape**	**Arrangement**	**Drawing**

2.3 Bacterial Gram stain technique.

This exercise will teach you to perform a Gram stain. You will note that it requires some practice in order to produce consistent results with your samples. Please refer to your textbook and the **Colour Atlas** to review the principles of the Gram reaction.

Materials

Broth culture of a mixture of *Escherichia coli* and *Micrococcus roseus*
Plate culture of *Bacillus subtilis*
Plate culture of *E. coli*
Forceps
Glass slides
Gram reagents
Bunsen burner
Loop
Sterile water
Microscope, immersion oil, lens paper

Procedure

1. Prepare Gram stains for all three samples provided.
2. Examine slides first at 10X, then 40X and then 100X (oil immersion). Draw diagrams of what you see for each sample and note the specific Gram reaction, shape, and arrangement of the bacteria.

Preparation of a cell smear

1. Obtain a clean glass slide. Handle the slide only by the edges so that grease and fingerprints are not transferred to its surface.
2. Sterilize the slide by passing it through the flame of the Bunsen burner. In order to do this, turn on the Bunsen burner and ensure that the flame is at its hottest setting. This means that the central flame will appear blue and its center will be golden-red. The junction where the gold-red is replaced by blue at the center is the hottest part of the flame. Hold the edge of the slide with a slide holder or forceps and quickly pass it through the Bunsen flame three times. Allow the slide to cool on a clean surface.
3. Using a marker or wax pencil draw a large circle on the slide and flip the slide over.
4. Sterilize a loop by placing its wire at the hottest part of the flame of the Bunsen burner. Allow the loop to turn red (this takes several seconds) and remove from the flame, but do not set down on the bench as this will contaminate the loop. You may set the loop upright on the stand provided.
5. To make a bacterial cell smear from a broth culture, first gently swirl the flask containing the bacterial culture. Remove the cap from the tube (as demonstrated

by your instructor), and sterilize the lip of the tube by passing through the flame of the Bunsen burner. Insert the loop into the broth, and remove a sample of the suspension. Return the lid of the tube before proceeding further.

Note: The loop itself will look like the surface of a drum when it contains the sample. If there does not appear to be fluid in the loop, repeat the above process.

Keeping the loop flat along the surface of the slide, make a uniform smear by spreading the contents of the loop back and forth within the circle you have drawn on the slide. You should see an opaque smear. This will ensure that a sufficient number of organisms are present for microscopic viewing. If you do not see an opaque smear, repeat this process.

6. If a smear is taken from an agar plate, immerse the loop first into some sterile water, and place the flat of the loop at the center of the slide (within the circle) so that a droplet of water forms on the surface of the slide. Sterilize the loop again, allow it to cool and then pick up a small portion of a bacterial colony with the side of the loop. Note that only a very small amount is necessary, as each colony represents millions of bacteria. If you apply too much, it will be difficult to discern single bacteria on the slide. Gently mix the bacteria in the water droplet to make the same bacteria smear described above.
7. Sterilize the loop and allow it to rest on the support.
8. Allow the slide to air-dry by placing it on a clean surface of the bench. Do not speed up this process by attempting to dry the slide over the flame as this will distort the cell wall components and give an inconsistent Gram reaction.
9. Once the slide has dried, the smear must be heat fixed by quickly passing it through the flame two or three times as demonstrated by your instructor. The slide is now ready for staining.

Gram stain technique

1. Prepare a fixed cell smear as described above.
2. Place the smear (cell side up) on a staining rack over the sink. Flood the smear with crystal violet by applying sufficient stain to completely cover the entire slide. Leave for 1 minute.
3. Tip the slide with forceps to allow the stain to run off. Turn the cold water tap on and gently pass the water hose over the slide to rinse it. Gently shake off the excess water from the slide.
4. Add iodine solution to cover the smear, tip the slide to drain the iodine, and then reapply with another application of iodine. Allow this to react 1 minute. Note: iodine serves as a mordant in this reaction. This means that the type of reaction occurring on the bacterial cells will prevent excessive decolourization due to the insoluable binding of the iodine with the crystal violet stain.
5. Pour off the iodine, gently rinse with water, and flood the slide with decolourizing solution (90% ethanol), while at the same time rocking the slide back and forth. This action allows the stain to decolourize uniformly. Note that as the slide decolourizes, granules from the stain will be removed from the cell smear.

Continue to add the decolourizer drop by drop, and slowly rock the slide. Decolourize for approximately 20 seconds or until you observe that the stain no longer leaches from the smear. Wash the slide thoroughly with a gentle stream of water.

6. Flood the slide with safranin and leave for 30 seconds. This provides a counterstain for the slide preparation.
7. Blot dry the smear by applying blotting paper or paper towel. You may also rinse the slide with water before blotting.

Note: Bacterial smears that have been stained for routine identification do not require a coverslip. After examining the slide the oil may be wiped off with a soft tissue. The slide can be stored in a clean, dust-free box for later viewing.

Results

Draw what you observe on your slides.

Exercise 3: Streak Plate Method of Bacterial Isolation

The aim of this technique in microbiology is to obtain single colonies without achieving contamination of organisms from extraneous sources (eg. your fingers, mouth, surface bacteria). This technique is important in the initial stages of bacterial identification. Individual colonies from mixed cultures of bacteria may be separated, and their characteristics, such as shape, size, colour, and distinguishing features may be observed. The streak plate method also allows the microbiologist to confirm and maintain pure cultures of bacteria.

3.1 Performing a streak plate from a plate culture and a broth culture.

Materials

Nutrient agar plates (3)
Plate culture of *E. coli*
Broth cultures of:
E. coli
M. roseus or M. luteus

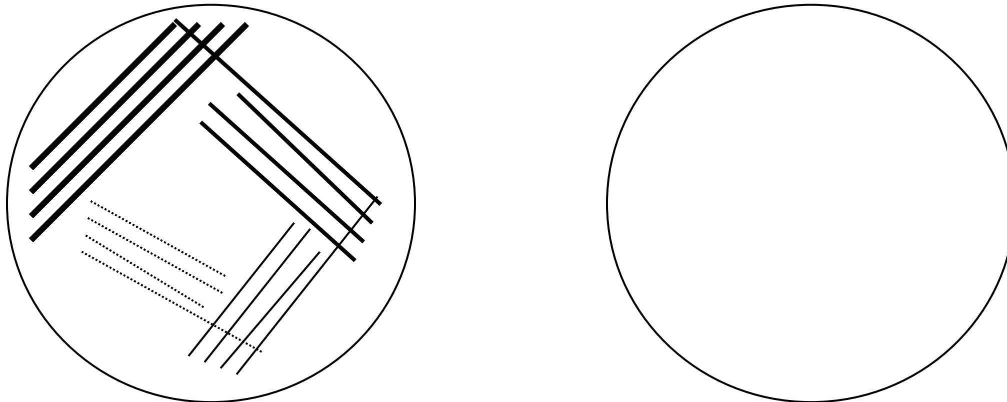
Procedure

1. Sterilize the inoculation loop, and sterilize the lip of the tube containing the broth culture as demonstrated by your instructor. Ensure that the culture is suspended uniformly by swirling the tube gently. Insert the loop into the tube and remove a sample of the culture. Again, ensure that the loop contains the broth culture. Sometimes when loops are too hot and are immersed in broth they will evaporate the suspension removed from the tube. To prevent this you may cool your loop on the inside of the tube before you take out the broth.
2. In order to begin streaking an agar plate place the nutrient agar plate provided for you with the base of the plate facing up. With the nutrient agar plate now inverted remove the base with one hand and have the agar surface facing you at an angle of 45°. Ensure that the plate is kept within the vicinity of the Bunsen flame. This will protect the plate from possible contamination.
3. Place the loop at the uppermost end of the plate, and apply a concentrated streak to the plate by spreading the side of the loop back and forth four or five times along the length of the uppermost part of the plate. Following this, place the plate onto its lid and rotate it 90°. Sterilize the loop and at this new position, streak back and forth again using the side of the loop. Repeat this process again. ALWAYS MAKE SURE YOU STERILIZE YOUR LOOP BETWEEN EACH STEP!

Note: The purpose of sterilizing the loop before streaking each new sector is to ensure that you will not be transferring the same numbers of bacteria to each sector of your plate. This will enable the formation of isolated colonies, which is the objective of this exercise. Thus, the microbiologist may examine the characteristics of individual

colonies, and also separate colonies which may exist in a mixed group of bacteria. If the loop is not sterilized at each point, there will be a confluence of bacteria with colonies running into one another, thereby making identification processes very difficult.

A diagram of a typical streak plate. Using the other circle provided, practice with a pencil.



4. Label the base of the plate with your name, the date, and exercise number, invert it, and place it into the container that will be collected for incubation.
5. Repeat the same process for the plate cultures of bacteria. In this case, sterilize the loop as described above. Remove a fraction of one colony from the plate and streak on a fresh agar plate as described above.
6. Invert all plates and store in the container provided for incubation. Incubate overnight at 37°C.

REMEMBER TO PROPERLY LABEL THE *BASE* OF EACH PLATE WITH YOUR NAME AND DESCRIPTION OF EXERCISE.

Results

1. The next day, describe in your own words what you see on all plates. Measure the approximate diameter of colonies from each plate, and describe the colour, shape, and edges of typical isolated colonies.

Exercise 4: Aseptic Technique

This exercise will involve the transfer of substances in broth culture or agar plates without introducing contaminants to the system. This procedure is known as "aseptic technique". For this exercise you will first perform the aseptic transfer of sterile broth into tubes of sterile broth. You will then transfer sterile broth to a nutrient agar plate and transfer material to two other plates.

Aseptic transfer involves a systematic delivery of material from one receptacle to another in an organized procedure whereby the transferring apparatus (in this case a loop, but may involve the use of sterile pipettes) is sterilized between each transfer. If you follow the procedure correctly, you will be able to ensure that you are transferring nothing but what is contained in the original tube.

Materials

Nutrient agar plates (2)
Tubes of sterile nutrient broth (4)

Procedure

4.1 Transfer of nutrient broth to tubes

1. Label 4 nutrient broth tubes #1 to #4. Tube #4 will serve as the control. This tube will not be opened, and will be used to compare the presence or absence of bacterial growth in other tubes. Light the Bunsen burner and sterilize the loop. If you are right handed, pick up the first tubes with your left hand. Remove the cap of the tube with your fourth and fifth fingers without touching the underside of the cap. Hold the cap by wrapping your fourth and fifth fingers around it while you are working.
2. Sweep the lip of the tube through the flame two or three times to ensure sterility of the tube.
3. Taking the loop (which has been sterilized) into your right hand (remember you are still holding the cap), and carefully insert it into the broth. Remove the loop, pass the lip of the tube through the Bunsen flame again, and then place the cap back onto the tube. ENSURE THAT YOU HAVE A NOTABLE BROTH SUSPENSION CONTAINED WITHIN THE LOOP.
4. Pick up the second tube containing sterile nutrient broth, and repeat the process of uncapping and sterilizing the tube as before. In this case you are holding the loop containing the broth from the first tube. Once you have sterilized the lip of the second tube, insert the loop into the second tube, give it a little shake, and remove the loop.
5. Sterilize the loop again, and insert it into Tube #2, and proceed to Tube #3 as described above.

Notes:

This procedure should be performed carefully and quickly, thereby avoiding the possibility of contamination.

In the case of cumbersome lids or caps, if the uppermost side is flat, you may rest it on the bench nearest the Bunsen flame. Do not place the cap with its interior resting on the bench, as it may become contaminated.

In some cases, caps may not be used. For example, you may be working with large flasks containing broth cultures. These may be sealed with non-absorbent cotton wool plugs. In this case, you MUST hold the plug between your fourth and fifth fingers while you are working. These plugs may easily become contaminated if placed on the work bench.

In some laboratories, the use of glassware has been dispensed with and replaced with sterile plastics. If sterile plastic tubes (eg. polystyrene) are used, then omit the procedure of sterilizing the lip of the tube. Otherwise the plastic will melt.

4.2 Transfer between agar plates

1. In preparation for this exercise, place Tube #3 of nutrient broth in front of you, and appropriately label two nutrient agar plates. Place the plates near you in the inverted position (lid side resting on the bench).
2. Sterilize the loop and proceed to remove a sample of broth culture as described in the previous exercise. Replace the cap on the tube, and pick up the base of the first nutrient agar plate, with agar side facing you (as described in the previous exercise). Proceed to streak the plate as described previously.
3. Streak a second plate using Tube #3.
4. Place all plates and tubes in the container for incubation.

Results

4.1 Transfer of nutrient broth to tubes

Complete the table below to record your results. Record the presence (+) or absence (-) of bacterial growth (as measured by the presence of turbidity as compared to the control tube).

Sample	Tube 1	Tube 2	Tube 3	Control tube
Growth				

4.2 Transfer between agar plates

Record your observations for original broth culture and plate cultures.

Sample	Tube 3	Plate 1	Plate 2	Control plate (Unopened NA plate)
Growth				

<u>Question</u>

What conclusions can you draw about your aseptic technique from the above two exercises?

Day 2

Objectives

At the conclusion of this session, you should be able to:

1. Identify differences in morphology of bacterial colonies.
2. Distinguish the type of bacteria and growth patterns that occur on differential, selective, and enrichment media.
3. Determine and perform differential stains and tests according to given properties of bacteria.

Exercise 5: Selective, Enrichment, and Differential Media

In order to definitively isolate specific microorganisms the growth of unwanted microorganisms can be restricted by addition or limitation of specific components or chemicals. This allows the promotion of growth of bacteria that you wish to propagate and the suppression of growth of bacteria you wish to discourage. The collective term for this kind of media is “selective” media. In order to examine the growth characteristics of bacteria on selective media, chemical indicators are added to the media. Thus, in addition to the media being selective for growth of certain organisms, one may also “differentiate” between those organisms that use the special components in the media from those that do not use these components. This media is classified as both selective and differential. In some cases the growth of certain bacteria may be enhanced by the addition of special components, such as blood or serum. This type of media is known as “enrichment” media. Please refer to your **Colour Atlas**, which described in detail the different types of selective, differential, and enrichment media that is available.

5.1 Isolation of bacteria on selective, enrichment, and differential media

Materials

Suspensions in sterile distilled water of:

Escherichia coli
Salmonella typhiumurium
Staphylococcus aureus
Staphylococcus epidermidis

Inoculation loop
Sheep blood agar plates (2)
Mannitol salt agar plates (2)
Nutrient agar plates (4)
MacConkey agar plates (2)
Eosin methylene blue agar plates (2)

Procedure

5.1.1 Blood agar as a differential enrichment medium

1. Using a marker, clearly label each plate with your name and date.
2. Label one plate *S. aureus* and the other *S. epidermidis*.
3. Using the broth cultures provided, streak organisms on a blood agar plate according to the protocols described in Exercise 4.
4. Invert each plate and store for incubation at 37°C overnight.

5.1.2 Mannitol salt agar as a selective and differential medium

1. Repeat the same procedure as described above using *S. aureus* and *S. epidermidis*.
2. Incubate plates.

5.1.3 MacConkey agar as a selective and differential medium

1. Follow the procedure described above, except streak one plate with *E. coli* and the other with *S. typhimurium*.
2. Incubate plates as described above.

5.1.4 Eosin methylene blue agar as a selective and differential medium

1. Streak one plate with *E. coli* and the other with *S. typhimurium* as described above.
2. Incubate plates.

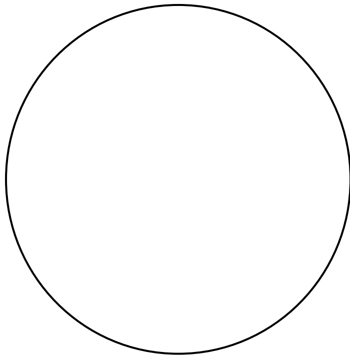
5.1.5 Nutrient agar as an all-purpose growth medium

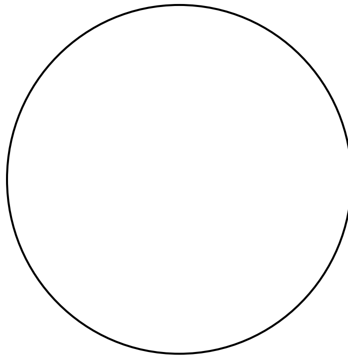
1. Repeat the same procedure using all four organisms provided.
2. Incubate plates.

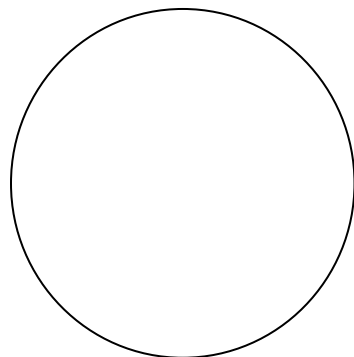
Results

5.1.1 Sheep blood agar

1. Draw the typical isolated colonies seen in both plates. Describe what you see in each case. Compared to the control plate, how has the agar changed? Are the results different for *S. aureus* compared to *S. epidermidis*?





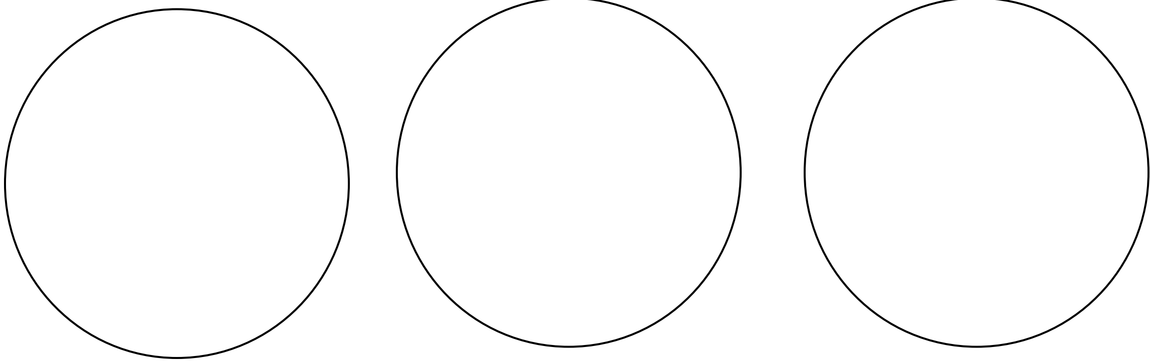


S. aureus ***S. epidermidis*** **Control**

2. Explain how the hemolysis differs in appearance. Name the two types of hemolysis observed in the plates.

5.1.2 Mannitol salt agar

1. Draw and label typical isolated colonies for each bacterial species streaked on the test plates.



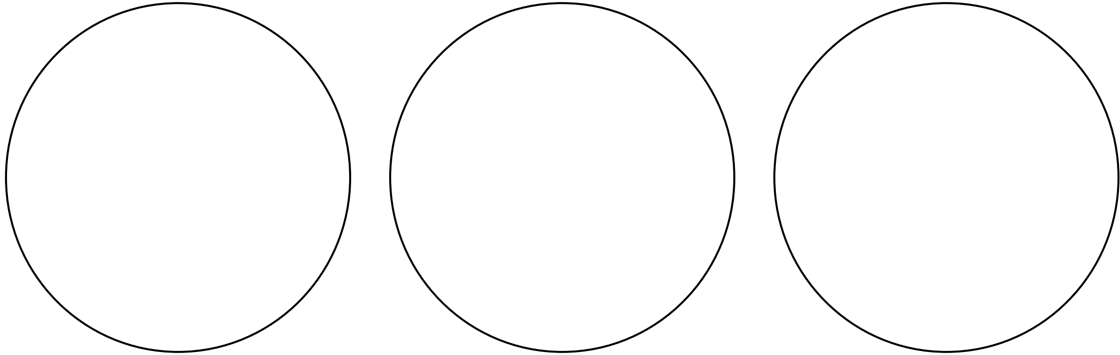
S. aureus ***S. epidermidis*** **Control**

2. How does the growth of each bacterium differ on this media?

3. Why are the colony colours different?

5.1.3 MacConkey agar

1. Describe the differences in growth patterns among the two bacteria streaked on the test plates.

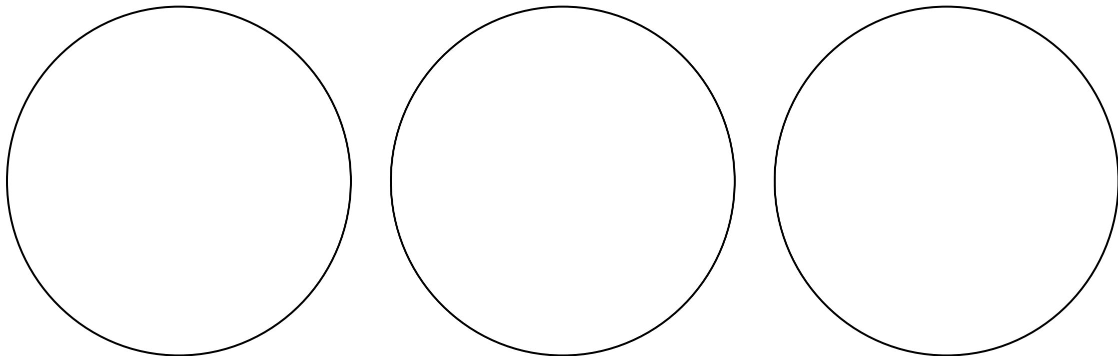


E. coli ***S. typhimurium*** **Control**

2. Do the organisms tested ferment lactose? How is this evident?

5.1.4 Eosin methylene blue agar

1. Describe the differences in growth patterns between the two types of bacteria streaked on this medium.

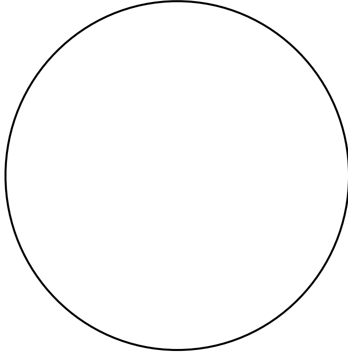


E. coli ***S. typhimurium*** **Control**

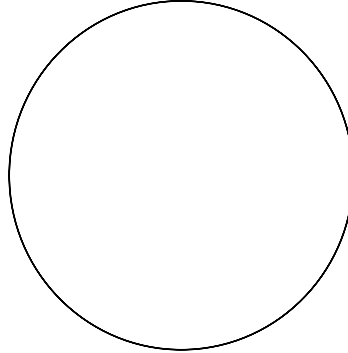
2. Which of the two organisms ferments lactose more vigorously? What colour indicates avid, or vigorous, lactose fermentation with this medium?

5.1.5 Nutrient agar

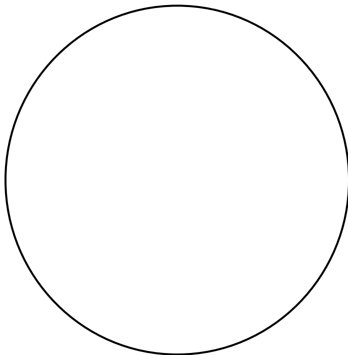
1. Describe or draw the growth of each of the four organisms on this media.



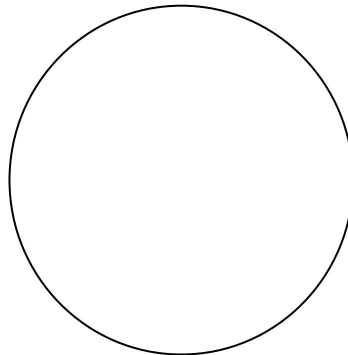
S. aureus



S. epidermidis



E. coli



S. typhimurium

5.2 Demonstration of selective and differential media

Materials

Prepared plates of bacteria grown on:

- Eosin methylene blue (EMB) agar
- Mannitol salt agar
- Sheep blood agar
- Salmonella-Shigella (SS) agar
- MacConkey (MAC) agar
- Simmons citrate agar
- Nutrient agar

Procedure

1. Examine the plates and describe the types of colonies observed according to the colour (and include diameter for the nutrient agar plates as this may help you with your unknown project). Note the reaction of each species for each type of media. Enter your observations into the tables provided.

Results

EMB agar

	S. aureus	***E. coli***	***S. typhimurium***
Colony colour			
Media colour			
Reaction (positive or negative for lactose)			

MacConkey agar

	E. coli	***Shigella spp.***	***S. typhimurium***
Colony colour			
Media colour			
Reaction (positive or negative for lactose)			

Mannitol salt agar

	S. aureus	***S. epidermidis***
Colony colour		
Media colour		
Reaction (positive or negative mannitol)		

Sheep blood agar

	S. aureus	*S. epidermidis*	*S. pneumoniae*
Colony colour			
Media colour			
Reaction (positive or negative for hemolysis)			

SS agar

	S. sonnei	*S. typhimurium*
Colony colour		
Media colour		
Reaction (positive or negative H_2S)		

Simmons citrate agar

	S. typhimurium	*E.coli*	*Enterobacter spp.*
Colony colour			
Media colour			
Reaction (positive or negative for citrate)			

Nutrient agar

	S. aureus	*S. epidermidis*	*S. pyogenes*
Colony diameter			
Colony colour			
Reaction (positive or negative for growth)			

	E. coli	*S. typhimurium*	*S. enteritidis*
Colony diameter			
Colony colour			
Reaction (positive or negative for growth)			

	S. sonnei	*Enterobacter spp.*
Colony diameter		

Colony colour		
Reaction (positive or negative for growth)		

2. For the following agar media used, list their **significant components** and explain how they react. Provide one example of a bacterium that will react to the components (positive), and one example of a bacterium that will not react (negative). Consult your **Colour Atlas** for assistance.

Agar Medium	**Components**	**Function/Reaction**	**Positive Example**	**Negative Example**
Nutrient agar				
Mannitol salt agar				
MacConkey agar				
EMB agar				
Simmons citrate agar				
SS agar				
Sheep blood agar				

Exercise 6: Differential Tests

Microbiologists use specific identification tools to separate bacteria according to knowledge of how bacteria utilize a number of different metabolites. Biochemical testing is a critical step in the classification of microorganisms. This is used in conjunction with bacterial growth on differential and/or selective media as shown in Exercise 5. Taken together, these procedures form the basis of differentiation between species according to Bergey's Manual. As you proceed through the following tests please consult your **Colour Atlas** for interpretation.

6.1 Tests that demonstrate the activity of some extracellular enzymes

6.1.1 Starch agar

Materials

Plate culture of starch agar of:
E. coli
B. subtilis
Iodine solution

Procedure

1. Examine the plates provided for you. Flood each plate with iodine solution.
2. Describe your observations in the table below. Indicate in the table if there was a colour change to the colonies or the media and if the organism is positive or negative for the ability to degrade starch.

Results

Iodine	***E. coli***	***B. subtilis***
Before adding iodine		
After adding iodine		

1. Describe the basis of this test: What enzyme does the organism possess if it is positive for starch utilization?

6.1.2 Gelatin hydrolysis

Materials

Nutrient gelatin stab tubes of:
Staphylococcus aureus
Staphylococcus epidermidis
Bacillus subtilis
Proteus vulgaris
Sterile stab (negative control)

Procedure and Results

1. Examine the tubes provided for you. Record you observations in the table.

Sterile stab (nil)	***S. aureus***	***S. epidermidis***	***B. cereus***	***P. vulgaris***

2. What enzyme does this test for? What does this enzyme hydrolyze? How is this reflected in the test?

6.2 Tests that demonstrate the effects of some intracellular enzymes

6.2.1 Carbohydrate fermentation

Materials

Semisolid agar tubes containing glucose and a green-yellow pH indicator, inoculated with:
Escherichia coli
Salmonella typhimurium
Staphylococcus aureus

For each sample two tubes were inoculated. One tube was incubated 18 hours and the other for 48 hours. Two uninoculated controls have been included as controls. Your **Colour Atlas** does not describe exactly the same test, however the principle behind the other fermentation tests (purple broth and phenol red broth) is the same as the glucose fermentation test that you will observe.

Procedure and Results

1. Examine each sample and record your observations in the table below. (Yellow indicates a lowered pH).

Organism	**Observations 18 h**	**Observations 48h**	**Acid/Gas formation 18h**	**Acid/Gas formation 48h**
Nil				
E. coli				
S. typhimurium				
S. aureus				

2. What conclusions can be made about the biochemical pathways utilized by organisms that caused the media to turn yellow in the main tube and yellow in the inner tube and produced gas in the inner tube? What are the oxygen requirements of these organisms?

3. What is happening in the tubes that turned yellow in the main tube, but remained green in the inner tube and did not produce gas? What metabolic pathway are these organisms using? What are the oxygen requirements of these organisms?

6.2.2 Kligler's Iron Agar (Hydrogen Sulfide Test)

Materials

Tube slants incubated at 37ºC for 24 and 48 hours inoculated with:

Escherichia coli
Shigella flexneri
Proteus vulgaris
Salmonella enteritidis

Procedure and Results

18 hours

Organism	**Colour of slant**	**Colour of butt**	**Gas production**	**H_2S production**
Nil				
E. coli				
S. flexneri				
P. vulgaris				
S. enteritidis				

48 hours

Organism	**Colour of slant**	**Colour of butt**	**Gas production**	**H_2S production**
Nil				
E. coli				
S. flexneri				
P. vulgaris				
S. enteritidis				

6.2.3 Catalase reaction

Materials

Nutrient agar plates inoculated with the following organisms and incubated at 37ºC overnight:

Staphylococcus aureus
Micrococcus luteus
Enterococcus spp.

Bacillus subtilis
Staphylococcus epidermidis
Streptococcus spp.
3% hydrogen peroxide
Pipette

Procedure and Results

1. Place a drop of 3% hydrogen peroxide directly onto a small area of bacterial growth on an agar plate.
2. Observe the results. The production of bubbles indicates catalase production.
3. Record your observations in the table.

Catalase production

Organism	Catalase production (+) or (-)
S. aureus	
M. luteus	
Enterococcus spp.	
B. subtilis	
S. epidermidis	
Streptococcus spp.	

1. What metabolic function does the catalase test represent?

2. What is the reaction that this enzyme catalyzes?

3. Can anaerobic bacteria perform positive catalase reactions? Explain your answer.

6.2.4 Urease test

Materials

Overnight cultures of urea broth containing:
Escherichia coli

Proteus vulgaris
Salmonella enteritidis

Procedure and Results

1. Examine the broth cultures and record your observations in the table.

Urease

Organism	Observation	Urease (+) or (-)
Nil		
E. coli		
P. vulgaris		
S. enteritidis		

1. Describe the metabolic process involved in the urease test.

2. What indicator is used in the urease broth?

6.2.5 Oxidase reaction

Materials

Nutrient agar plates incubated at 37ºC for 48 hours previously with the following organisms:
Salmonella enteritidis
Escherichia coli
Pseudomonas aeruginosa
Oxidase reagent
Inoculation loop or sterile cotton swab
Filter paper

Procedure and Results

1. Using a sterile cotton swab, pick lots of colonies of bacteria from an agar plate.
2. Add one or two drops of oxidase reagent to the bacterial sample on the swab.
3. Observe whether a colour change occurs (if the sample turns purple).

4. Record your observations in the table.

Oxidase

Organism	**Observations**	**Oxidase (+) or (-)**
S. enteritidis		
E. coli		
P. aeruginosa		

1. What metabolic process does the oxidase reaction demonstrate?

6.2.6 Identification of *Enterobacteriaceae* (IMVic test)

The term "IMVic" denotes the four key tests used to identify enteric microorganisms. The letters abbreviated above represent the following tests: indole reaction, methyl red test, Vogues-Proskauer, and citrate utilization.

These tests are incorporated as part of the Enteropluri tube test and therefore will be demonstrated and observed as part of this test. Please consult your **Colour Atlas** for the principles behind these reactions. Your instructor will explain these tests to you.

6.2.7 Identification of *Enterobacteriaceae* (Enteropluri Test)

As you have seen with the previous tests, each can be performed individually with specific media and chemicals. However, given that the Family *Enterobacteriaceae* is so large and diverse, that tests to rapidly identify these organisms have been developed. The Enterotube is one of the most common tests for the comprehensive and rapid determination of enteric organisms. Rather than carrying out each reaction individually, this test compartmentalizes all of the necessary reactions that can be performed and then observed at the same time. Please read your **Colour Atlas** for a description and model of the Enterotube.

Materials

Enteropluri tubes containing one culture of the following organisms that were previously incubated at 37ºC for 24 hours:

Pseudomonas aeruginosa
Proteus vulgaris
Escherichia coli
Salmonella typhimurium

Procedure and Results

1. Your instructor will direct one member from the class to perform the indole test in the appropriate chamber of the Enteropluritube.
2. Observe the reactions in each of the chambers of the tube and record the results below. You may consult the **Colour Atlas** to see how the positive and negative reactions for the MR/VP tests should appear. You will use a different indole reagent (cinnemaldehyde) than that described in the **Colour Atlas**. The principle of the test is the same, but a positive indole test is green, not red.

Enteropluri Test results

	Glucose	Gas	Lysine	Ornithine	H_2S	Indole	Adonitol	Lactose	Arabinose	Sorbitol	Vogues Proskauer	Dulcitol	Phenylalanine Deaminase	Urea	Citrate
P.a.															
P.v.															
E.c.															
S.t.															

Exercise 7: Differential Stains

The ability to stain bacteria is extremely important in assisting their classification and identification. As demonstrated in Exercise 2, the Gram stain enables one to categorize bacteria according to the specific characteristics of their cell wall components. In addition, properties such as shape and arrangement of bacteria on the slide preparation also help with the first steps of identification and classification of bacteria. Because bacteria come with a myriad of different characteristics, the use of dyes can enable detection of specific features that aids in providing clues to their classification.

This exercise will draw from knowledge of special features of bacteria, and some stains will be used to examine these features. Please note that the staining techniques you will be undertaking only represent a fraction of the diverse stains that exist in microbiology.

7.1 Gram stain

You have already been introduced to this staining technique. While the Gram stain enables you to discern bacteria that comprise differences in their cell wall components (Gram positive versus Gram negative), there are also some additional clues that can be used to determine special features in bacteria. In this section you will compare the morphological characteristics of several bacteria using the Gram stain technique.

Materials

Nutrient agar plate cultures of the following:

- *S. aureus*
- *B. subtilis* (18-24 hour culture)
- *B. subtilis* (4 day culture)
- *Corynebacterium spp.* (18 hour culture)
- *Corynebacterium spp.* (4 day culture)

Gram stain reagents
Inoculation loop
Glass slides

Procedure

1. Prepare slides of each sample and heat fix as described in Exercise 3.
2. Label each slide to ensure identification of each sample.
3. Prepare Gram stains of each.

Results

1. Record the microscopic observations in the table below.

	S. aureus	*B. subtilis* (24h)	*B. subtilis* (4 day)	*Corynebacterium spp.* (24h)	*Corynebacterium spp.* (4 day)
Drawing of organisms observed under microscope					
Cell arrangement					
Gram reaction					
Are spores visible?					
Other features?					

2. Can you note the difference between the 18-24 hour and 4 day cultures of *B. subtilis* and *Corynebacterium spp.*? If so, describe what you see microscopically.

7.2 Schaeffer-Fulton spore stain

When conditions are ideal for bacteria they will metabolize nutrients and exist as vegetative cells. However, as a mechanism of survival, some bacteria have the capability to remain dormant during extreme conditions that are not conducive to their growth. Thus, in order to retain their capacity to grow when conditions become optimal, these bacteria will form endospores, which remain as intracellular structures within the cells. Should conditions become even more hostile to the bacteria, they will release these structures, which in their free form are called spores. In either case, these structures remain as an impermeable armor, retaining all the genetic material required to proliferate at a more favorable time. These endospores are capable of resisting the damaging effects of heat, cold, radiation, chemicals, and dessication.

Materials

24 hour and 4 day nutrient agar plate cultures of
Bacillus subtilis
Malachite green stain
Safranin stain
Hot plate
Glass slides
Microscope and immersion oil

Procedure

1. Make a new smear of each sample, air dry, and heat fix the slides.
2. Flood smears with malachite green and steam slides by placing on a hot plate. Note: do not boil the stain, as this will destroy the bacteria. Ensure that stain is present at all times during this period. You may need to add more stain if it appears to be evaporating from the surface of the slide.
3. Steam the slides for 5 minutes.
4. Remove the slide from the hot plate, cool, and rinse in running tap water.
5. Counterstain with safranin for 30 seconds.
6. Blot dry and examine microscopic characteristics using oil immersion.

Results

1. Record the microscopic observations of each slide as described below. Compare this with the Gram stain slides processed earlier. Indicate the location of spores as terminal, central, or subterminal.

	Spore stain *B. subtilis* (24h)	Spore stain *B. subtilis* (4 day)
Drawing of organisms observed under microscope		
Are spores visible?		
Colour of spores		
Vegetative cell colour		
Location of endospore		

1. Why is it necessary to use heat during the preparation of the spore stain?

2. What are the major differences between the vegetative cell and a spore?

3. Is the Gram stain a reliable stain that shows the presence of spores? Why or why not?

Day 3

Objectives

At the conclusion of this session, you should be able to:

1. Perform and understand the methodology for determining microbial growth.
2. Construct a growth curve for *E. coli*.
3. Understand the principles of various sterilization techniques.
4. Perform minimal inhibitory concentration (MIC) testing.
5. Perform genetic manipulation of bacteria through transformation.

Exercise 8: Quantifying Bacterial Growth

There are many areas in microbiology where quantifying microorganisms is essential. For example, in the food industry, testing of possible microbial content in foods is a necessary component of quality control. Also, in research settings, such as pharmaceutical laboratories, the efficacy of antimicrobial agents on growth of microorganisms is commonly tested. Thus, accuracy in determining exact microbial numbers is of importance to the outcome of scientific investigations. The experiment described below will demonstrate one of the most common methods for determining the growth and viability of a bacterial culture.

8.1 Bacterial growth curve

Materials

Nutrient broth containing a pure culture of *E. coli* previously incubated for 18 hours at 37°C
Conical flask containing 100 ml of nutrient broth
Shaking water bath adjusted to 37°C
Spectrophotometer and cuvettes
Sterile test tubes for dilutions
Sterile water for dilution
1 ml, 5 ml, and 10 ml pipettes
Eppendorf pipettes and tips
Nutrient agar plates
70 % ethanol in beakers
Glass spreader (hockey stick)
Vortex

Procedure

1. For the two techniques used in this exercise you will be required to follow the growth of *E. coli* in nutrient broth. **You will work in groups**.
2. Using aseptic technique, remove 3 ml of the overnight culture and use this to inoculate 100 ml of the sterile nutrient broth.
3. Gently swirl the flask and place it in a shaking water bath. It is from this flask that you will remove a sample at each time point for turbidity measurement and quantitation of viable bacteria.

8.1.1 Turbidometric determination of bacterial growth

The spectrophotometer is an apparatus that is used to determine the turbidity of a liquid solution. For the microbiologist, this method is extremely useful, as it can be related to cell numbers in a particular suspension. This equipment is useful in determining the rate of growth of different microorganisms.

1. Remove aseptically a 5 ml sample of your suspension for determination of turbidity or **optical density**. Your instructor will demonstrate the use of the spectrophotometer.
2. Before you read your sample, set the spectrophotometer to a wavelength of 600 nm. Pipette 5 ml of nutrient broth only (no *E. coli*) into a cuvette and insert into the appropriate chamber in the spectrophotometer (this is called a blank). Adjust the spectrophotometer so that the reading will be zero. You have now calibrated the apparatus so you can accurately read your *E. coli* sample.
3. Place the 5 ml sample into a cuvette and measure the optical density of the suspension.
4. Record the optical density measurement of your sample for each time point. The time points will be at 0, 1, 2, 3, and 4 hours.
5. Once you have read your sample, you may discard the sample into a container of bleach and rinse the cuvette for further use. Do not discard the cuvettes!!!

Results

1. Record the optical density readings for your group in the table.

Time (hours)	**Optical Density OD_{600} (nm)**
0	
1	
2	
3	
4	

2. Using the graph paper provided draw a growth curve based on the turbidity readings. Clearly label the axis, and indicate the phases of growth (lag, log, stationary, death), where applicable.

8.1.2 Determination of viable cell numbers using spread plate technique

Although the presence of microorganisms in a solution may contribute to the optical density, or physical viewing of total numbers, this does not give an indication of whether the cells are alive or dead. Thus, a precise measure of viability is determination using the plate count technique.

Refer to your Colour Atlas for details on serial dilutions and spread plate technique.

For this technique, use sterile water to make ten-fold dilutions of each sample as follows:

Time 0: dilute samples in distilled water from 10^{-1} to 10^{-3}, plate 10^{-1} to 10^{-3} dilutions.
Time 1: dilute samples in distilled water from 10^{-1} to 10^{-4}, plate 10^{-2} to 10^{-4} dilutions
Time 2: dilute samples in distilled water from 10^{-1} to 10^{-5}, plate 10^{-3} to 10^{-5} dilutions
Times 3 and 4 hours: dilute samples in distilled water from 10^{-1} to 10^{-6}, plate 10^{-4} to 10^{-6} dilutions.

At each timepoint, aseptically transfer 0.5 ml of suspension into 4.5 ml sterile distilled water (10^{-1}). Except for the zero timepoint, continue to make ten-fold dilutions as follows:

1. Mix the tube containing the 10^{-1} dilution and remove the cap using your pinky finger. You may hold the cap in this position to ensure that it does not get contaminated.
2. Take a sterile 1 ml pipette, and remove 0.5 ml of the sample and directly place into 4.5 ml of sterile water (this will now be a 10^{-2} dilution). Discard the pipette immediately after use into a waster bucket of bleach. Mix the tube by vortexing, and remove a 0.5 ml sample using another sterile 1 ml pipette, and place into a sterile tube containing 4.5 ml of water (10^{-3}).

Note: if you do not use a clean pipette for each of these dilutions, you shall be transferring the original suspension each time, thereby carrying organisms over from the previous dilution, which results in inaccurate counts.

3. Repeat serial dilutions of samples until the final (10^{-6}) dilution.
4. Beginning with the final dilution (10^{-6}), briefly mix the sample by vortexing, and aseptically remove 0.1 ml (100 μl) of the sample into the middle of a previously labeled nutrient agar plate.
5. Flame the spreader to burn off the ethanol. Then use the spreader to spread the sample of bacteria around the agar plate until it is dry.
6. Repeat this for all of the dilutions in the series.

7. Allow the agar to solidify, invert plates, and incubate at 37°C overnight.
8. The next day, count all colonies observed on the agar. Note count the dilutions that are countable, ie. plates with 30-300 colonies.

Results

1. Tabulate the number of viable cells and record in the table below. Remember that each colony is equivalent to 1 colony forming unit (CFU) or 1 cell. Because you plated only 0.1 mL of sample per plate, you must also take into account a plating factor (PF) of 10. To calculate the number of cells per ml use this formula:

colonies on plate X 1/dilution X PF = #cells/ml

Eg. 33 cells on the 10^{-5} plate would be:

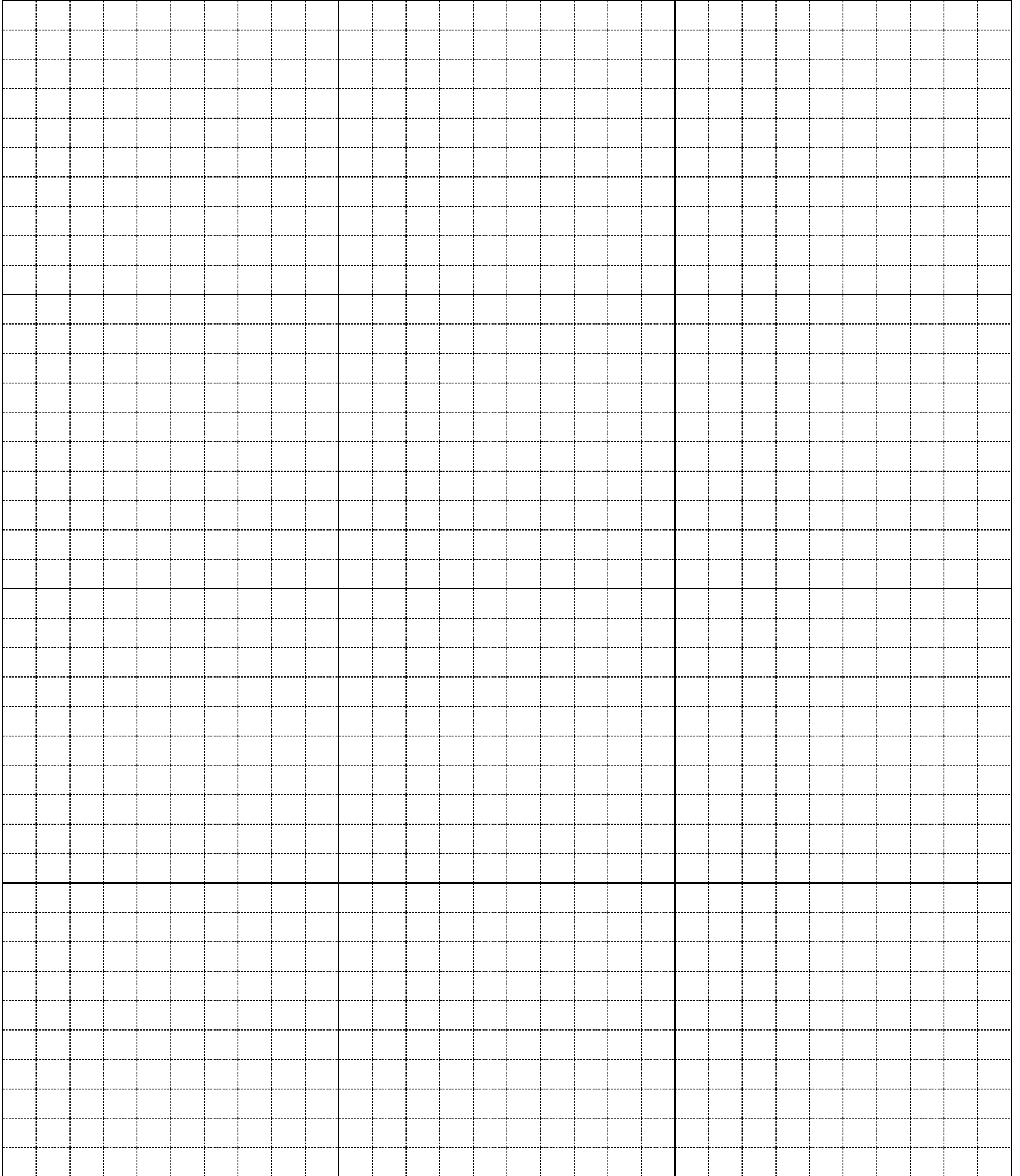
$33 \times 10^5 \times 10 = 3.30 \times 10^7$ cells/ml (33 000 000 cells/ml)

Time (h)	**# Colonies on plate**	**Dilution used**	**# cells/ml**	**Log #cells/ml**
0				
1				
2				
3				
4				

2. Using the original graph of turbidity, plot the log number of viable cells/ml for each time point.
3. Determine the generation time by using the following formula:

$$\text{Generation time} = \frac{\Delta t \log 2}{\text{Log } n - \log N}$$

Where: N = number of bacteria at a particular time point during log phase
n = number of bacteria at a second time point during log phase
Δt = time



Questions

1. Describe the differences between methods determining total and viable cell numbers.

2. Would you expect the exponential (log) phase of growth to vary if twice the number of organisms were used to originally inoculate the broth culture?

3. How would the growth curve vary if a second microorganism were added to the broth culture?

Exercise 9: Sterilization

The purpose of sterilization is to ensure that materials are free of microbial contamination. In addition, it is preferred that materials are not deformed or altered as a result of the sterilization process (unless they are discard materials). The demonstrations below highlight some of the important principles of sterilization techniques. You can consult your textbook regarding these procedures.

9.1 Sterilization demonstration

There will be a display of materials that have been sterilized by different techniques. The table below lists a number of different items that can be sterilized. Complete this table by checking the appropriate box or boxes that correspond to a sterilization technique.

	Autoclave-Liquid Cycle	Autoclave-Dry Cycle	Filtration	Gamma rays
Plastic syringes				
Glass bottles				
Gauze bandage				
Glucose solution				
Agar broth				
Antibiotic disc				
Plastic Petri dishes				

9.2 Sterilization by boiling

Materials

Nutrient broth cultures (3 per group) containing:

Staphylococcus aureus
Escherichia coli
Bacillus subtilis

Nutrient agar plates (3)
Sterile microcentrifuge or Eppendorf tubes for aliquoting cultures
1000 ml beaker or smaller for a water bath
Thermometer
Hotplate
Bunsen burner
Inoculation loop

Procedure

1. You will use one nutrient agar plate per organism. Divide each plate into four sections. Label each section with one of the four temperatures that will be used:

25, 50, 75, and 100°C. Also label the plates with the individual names of the organisms.

2. Pipette 1 ml of each overnight culture into 1 Eppendorf tube for a total of 3 tubes, one for each organism. These tubes are well suited for use in the water baths.
3. Inoculate the three plates in the 25°C section with each of the above bacteria.
4. To create a 50°C water bath, heat the beaker filled with water and monitor the temperature until it reaches 50°C. Place the three Eppendorf tubes into a floating holder and into the water and maintain this temperature for 10 minutes.
5. Carefully remove each tube and inoculate the plate labeled 50°C with each of the three organisms.
6. Repeat steps 3 and 4 for the remaining temperatures.
7. Incubate the plates for 24 hours at 37°C.

Results

Tabulate the amount of growth observed on each plate according to the following denotation:
+++ (confluent growth)
++ (moderate growth)
+ (poor growth)
- (no growth)

Organism	**25°C**	**50°C**	**75°C**	**100°C**
S. aureus				
E. coli				
B. subtilis				

Questions

1. Which of the above organisms showed the greatest resistance to boiling? Which showed the least? Why?

2. Which organism was the most temperature sensitive? Suggest an explanation for this result.

Exercise 10: Chemical Control of Bacterial Growth

Antimicrobial agents can also be used to kill or inhibit the growth of bacteria. These agents are usually chemicals that have profound effects on microbial growth. While bacteriostatic agents are chemicals that will stop the growth of bacteria without actually killing them, bactericidal agents can kill bacteria. Bacteriolytic agents will not only kill bacteria, but will also lyse them. The experiments outlined below feature some of the effects of these agents.

10.1 Agar diffusion technique

Materials

Sterile absorbent discs
Forceps
95% alcohol
Overnight nutrient broth cultures of:
- *Staphylococcus aureus*
- *Bacillus cereus*
- *Escherichia coli*

Nutrient agar plates (3)
Sterile cotton swabs
Beakers containing the following disinfectants:
- tea tree oil
- 10% H_2O_2
- Dettol®

Procedure

1. Divide the plate into three sections, one for each chemical and label. Label the plates, each with one of the three organisms.
2. Immerse a sterile cotton swab into a nutrient broth culture of *S. aureus*. Make a lawn plate culture by spreading this culture horizontally on the agar plate, followed by vertical strokes.
3. Divide the plate into three sections, one for each chemical.
4. Sterilize forceps by dipping ends into 95% alcohol and passing through a Bunsen flame.
5. Carefully pick up a disc with sterile forceps, immerse in tea tree oil, and blot gently on absorbent paper. Place disc onto the plate approximately 2.5 cm from the perimeter of the plate and gently press down.
6. Repeat the above procedure for the remaining discs and test organisms. Ensure that the discs are sufficiently separated so as not to interfere with each other.
7. Invert plates and incubate at 37ºC for 24 hours.

8. The next day, use a ruler to determine the zone of inhibition around each disc. This can be done by measuring the radius, the distance from the middle of the disc to the edge of where the bacteria have stopped growing (area of clearing).

Results

Measure the zones of inhibition. Record your results in the table below.

Organism	**Tea tree oil**	**10% H_2O_2**	**Dettol®**
S. aureus			
B. cereus			
E. coli			

Questions

1. Which of the above test organisms showed greatest sensitivity to the antimicrobial agents? Which showed the least?

2. Describe an experiment that would use the above technique to test the minimum inhibitory concentration of an antibiotic.

3. What factors should one consider for testing of antimicrobial agents?

10.2 Measurement of antimicrobial activity (minimum inhibitory concentration)

Materials

10 tubes containing 4 ml nutrient broth tubes
10 sterile test tubes
Sterile distilled water (for making dilutions)
Nutrient broth culture of *Staphylococcus aureus* incubated at 37ºC 24 hours previously
Disinfectant solutions:

vinegar, household cleaner, Presept, benzalkonium chloride
(one disinfectant per group so results can be compared between groups)

Pipettes for dilutions

Procedure

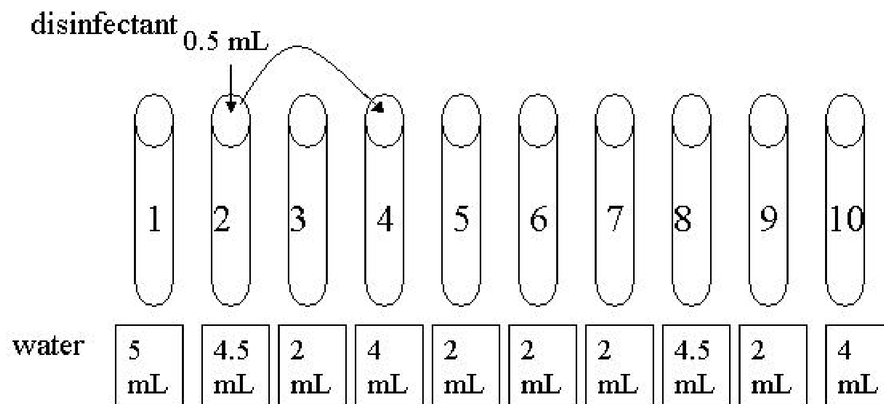
1. Label 10 nutrient broth tubes with following concentrations: Tube 1(nil), Tube 2(1:10), Tube 3(1:20), Tube 4(1:50), Tube 5(1:100), Tube 6(1:200), Tube 7(1:300), Tube 8(1:500), Tube 9(1:1000), Tube 10(1:1500). Label the other set of 10 tubes (empty) with the same values. These values will denote the concentration of disinfectant added to the tube.
2. Use a glass pipette to add sterile water to the empty tubes as follows:

   Tube 1: add 5 ml of water

   Tubes 2 and 8: add 4.5 mL of water

   Tubes 3, 5, 6, 7, and 9: add 2 mL of water

   Tubes 4 and 10: add 4 mL of water



3. Each group will use one disinfectant. You will dilute the disinfectant in the tubes of sterile water that you prepared in step 2. Before you do this, add arrows on the diagram above to show how you will make the dilutions (describe below).

   Tube 1 (nil): nothing (5 ml of water only) (nil)

   Tube 2: add 0.5 ml disinfectant to 4.5 ml water (1:10)

   Tube 3: remove 2 ml from tube 2 and add to the 2 ml water (1:20)

Tube 4: remove 1 ml from tube 2 and add to the 4 ml water (1:50)

Tube 5: remove 2 ml from tube 4 and add to the 2 ml water (1:100)

Tube 6: remove 2 ml from tube 5 and add to the 2 ml water (1:200)

Tube 7: remove 1 ml from tube 5 and add to the 2 ml water (1:300)

Tube 8: remove 0.5 ml from tube 4 and add to the 4.5 ml water (1:500)

Tube 9: remove 2 ml from tube 8 and add to the 2 ml water (1:1000)

Tube 10: remove 1 ml from tube 7 and add to the 4 ml water (1:1500)

4. Remove 1 ml of the diluted disinfectant solution from the tubes above and add to the corresponding tubes containing 4 ml of nutrient broth labeled in step 1.
5. Aseptically add 20 µl of bacterial culture to the 10 tubes from step 4. Your final set of tubes should contain 4 ml of nutrient broth, 1 ml of your diluted disinfectant, and 20 µl of *S. aureus*.
6. Incubate tubes overnight at 37°C.

Results

1. Record your observations in the table below according to the presence (+), or absence (-) of growth. Vortex the tubes before observing them.

Disinfectant Used:

Disinfectant Dilution	Nil	1:10	1:20	1:50	1:100	1:200	1:300	1:500	1:1000	1:1500
Growth (+ or -)										

2. Determine the minimal inhibitory concentration (MIC), as judged by the first tube to show no detectable growth.

3. Does the above experiment provide information as to whether the disinfectant is bactericidal or bacteriostatic? Explain your response.

4. How would you determine minimal bactericidal concentration (MBC)?

Exercise 11: Bacterial Transformation

Transformation is defined as the horizontal transfer of "naked DNA" from one bacterial species to another. In nature this often occurs when bacteria die and fragments of their genetic material are taken up by other bacterial species in the environment and incorporated into the chromosome. Natural competence is the ability of an organism to take up foreign DNA. Only a few bacterial species are naturally competent, such as *Haemophilus*, *Neisseria*, and some *Streptococcus spp*.

Transformation can be utilized in the laboratory for the process of cloning. A typical plasmid is a small, circular segment of DNA that replicates independently of bacterial chromosomal DNA. A DNA fragment of interest (that encodes for a protein of interest) can be ligated to a plasmid and then introduced into *E. coli* in order to produce many copies of the gene, or to produce the protein of interest. Since *E. coli* is not naturally competent, one of two procedures can be used to transfer the plasmid: electroporation and chemical transformation using $CaCl_2$. We will follow a chemical transformation protocol in order to introduce a plasmid into *E. coli*.

11.1 Transformation of a *GFP*-containing plasmid into *E. coli*

The jellyfish, *Aequorea victoria*, is capable of producing light due to its gene for green fluorescent protein (GFP). In this experiment we will transform pGLO, a plasmid containing the GFP gene, into *E. coli*. This plasmid also contains a promoter that is stimulated in the presence of the sugar, arabinose. We will plate potential transformants on agar containing arabinose to "turn on" transcription of the GFP gene. We will then be able to determine if the transformation was successful by looking for light production in the transformed colonies (transformants).

Materials

Purified plasmid DNA (pGLO)
E. coli culture on an LB plate
Transformation solution (contains $CaCl_2$)
LB broth
LB agar plates with ampicillin (2)
LB agar plate with ampicillin and arabinose (1)
LB agar plate (1)
Sterile microcentrifuge tubes (2)
Microcentrifuge tube racks
Pipettes and pipette tips
Sterile microcentrifuge tubes
Sterile plastic loops
Sterile spreaders
Water bath
Ice
UV light box

Procedure[1]

1. Your instructor will provide you with two microcentrifuge tubes, labeled +pGLO and –pGLO. Each tube will contain 250 μl of transformation solution. Keep the tubes on ice until step 5.
2. Use a sterile plastic loop to pick up a ½ loop of *E. coli* from an LB plate. Dip the loop in the +pGLO tube to transfer bacteria into the tube. Repeat this step to add bacteria to the –pGLO tube. Put the tubes back on ice.
3. Add 10 μl of pGLO to the +pGLO tube. Mix tubes well. Incubate both tubes on ice for 10 min.
4. Label four LB plates as follows:

   +pGLO/amp, +pGLO/amp/ara, -pGLO/amp, -pGLO
5. Heat shock the cells by placing both tubes at 42ºC for 30 seconds.
6. Place cells on ice for 2 min.
7. Add 250 μl of LB broth to each tube and incubate cells for 20 min to 1 hour at 37˚C. This is the "recovery" period.
8. Plate 200 μl of the +pGLO sample onto each of the LB plates labeled +pGLO/amp and +pGLO/amp/ara.
9. Plate 200 μl of the -pGLO sample onto each of the LB plates labeled -pGLO/amp and –pGLO. Incubate all 4 of the plates overnight at 37˚C.
10. The next day, examine the colonies for light production under a UV light box. If the plasmid was taken up by the *E. coli* and the GFP gene expressed, the colonies will produce light.

Results

1. **Record your observations for each of the four plates that you have.**

Questions

1. Why was arabinose included in the growth medium?

[1] Adapted from Biotechnology Explorer™, pGLO™ Bacterial Transformation Kit

2. Why must antibiotics be incorporated into the growth medium?

3. What was the purpose of the –pGLO samples plated on LB agar with ampicillin and LB agar without ampicillin?

4. What are the two other methods of genetic transfer in bacteria?

5. Name one application of genetic engineering.

Day 4

Exercise 12: Environmental and Food Microbiology

Microorganisms are used in a variety of ways in industrial applications. For example, they may be harvested to produce a specific product, such as a protein. Or they may be used to make certain foods and beverages, such as yogurt, cheeses, and beer. On the negative side, some microorganisms may have deleterious effects where, for example, they may contribute to the spoilage of food products. Sometimes, these organisms may cause changes to the taste or consistency of the food without having ill effects on human consumption. At other times, contaminating bacteria may be pathogenic in nature, and can produce toxins, which may be responsible for causing fever, diarrhea, and vomiting. The experiment described in this exercise will explore some of the effects microorganisms may have on food.

12.1 Food microbiology

Materials

Sample of contaminated food
Gram reagents
Prepared plates from food sample
Fixed yogurt smears for examination

Procedure

1. Examine a fixed yogurt smear and draw a few representative cells.
2. Prepare and examine a Gram stain of the contaminated food sample.
3. Examine samples of the contaminated food on agar plates and Gram stain one of the colonies.

Results

1. Record your observations:

   Yogurt smear:

Contaminated food:

2. Can you identify the species present in the contaminated food? If yes, what characteristics allowed you to conclude what this organism is?

3. Is this organism a food pathogen? If so, what disease does it cause?

4. How are bacteria used in making yogurt? What are the benefits of eating yogurt?

12.2 Bacterial flora in the environment

This experiment will be performed on Day 2 or 3.

Materials

Nutrient agar plates (2)
Sterile cotton wool swabs
Sterile distilled water

Procedure

1. Wet 1 sterile cotton swab in sterile water. Obtain a swab sample of a surface (eg. telephone handle, doorknob, pen, computer keyboard). Streak one nutrient agar plates by spreading the swab across the plate horizontally and vertically.
2. For the remaining plate (1), place the lid of the nutrient agar plate on the bottom half of the plate, and leave the plate exposed to the air at a particular location (eg. bathroom, laboratory, refrigerator, for at least three hours). After three hours, cover the plate with the lid.
3. Incubate all plates at 37^oC for 24 hours.

Results

1. Record your observations in the table that follows of the types of colonies, and appearance of predominant cells by performing wet mounts and Gram stains if applicable. Draw two to three of the typical organisms observed in each location (use the table on the next page).

Location	Observation of colonies	Wet mount	Gram reaction

Questions

1. Can you predict which types of microorganisms are present at the different localities? Is there an abundance of one type over others in any one particular location?

2. In terms of the concept of nosocomial contamination, what types of organisms would you expect to find if this experiment was performed in a hospital setting?

12.3 Biosensors to monitor pollution levels

Bacteria may be engineered to measure the levels of a particular pollutant or chemical. This is an example of what is referred to as a biosensor. Biosensors allow us to measure many different types of chemicals in the environment and can be very helpful in determining pollution levels as they are quite sensitive in the levels they can detect. One way to engineer a biosensor is to fuse the

Exercise 13: Medical Microbiology

Objectives

At the conclusion of this session, you should be able to:

1. Recognize normal bacterial flora of the body.
2. Understand the principles of nosocomial infections.
3. Examine medically important bacteria.
4. Determine antibiotic sensitivity of some microorganisms.

Bacteria play a critical role in medical microbiology. The exercises in this section will demonstrate some of the important properties of medically relevant bacteria. The role of bacteria in disease will be considered.

13.1 Nosocomial infections: the effects of washing hands on bacterial load

Materials

Lawn culture of *Micrococcus roseus*
Nutrient agar plate (1)
Choice of the following treatments or alternatives:
Hand soap
70% ethanol
Purell
Antibacterial hand soap

Procedure

1. Draw a line down the center of the bottom half of the nutrient agar plate. Label one half "control" and the second half "washed".
2. Press down the fingertips of your hand onto the plate of *M. roseus*.
3. Press these fingers onto the "control" section of your nutrient agar plate.
4. Wash your hands with one of the treatments available and press fingertips onto the section of the plate labeled "washed".
5. Incubate your plate in inverted position at 37°C for 24 hours.

Results

Compare your results with others and record the observations in the table below.

Treatment	Growth	Comments
Hand soap		
70% ethanol		
Antibacterial soap		
Purell		

Questions

1. Was there any treatment that appeared to be more effective?

2. Is proper hand washing technique as important as the disinfectant that is used?

3. Suggest reasons why hand washing is important in a clinical situation.

13.2 Normal bacterial flora

This experiment will be performed on Day 2 or 3.

Materials

Nutrient agar (NA) plates (2)
Sheep's blood agar (SBA) plates (2)
Sterile cotton swabs

Procedure

1. Immerse one swab in sterile water and thoroughly swab one part of your anatomy (eg. scalp, behind the ears, armpit, foot). Prepare a lawn plate on one NA plate and one SBA plate as described previously.
2. Use a *new* cotton swab to swab inside of your mouth and prepare a lawn plate on one NA and one SBA plate.
3. Incubate plates for 24-48 hours.
4. Examine the plates for morphological appearance of colonies, and perform Gram stains and wet mounts (if applicable) of selected colonies. You may also perform a catalase test to confirm catalase positive organisms.

Results
Record your observations in the table below:

Site	Appearance of colonies	Microscopic Appearance

1. What is the role of normal flora in human health?

13.3 Sensitivity to Antibiotics

Demonstration of the E-test for determination of MIC (minimal inhibitory concentration).

The E-test strip is a rapid and accurate method for the determination of MIC of a particular organism for an antibiotic and yields an exact MIC value without having to calculate the concentration of the antibiotic or measure the zone of inhibition.

Materials

Overnight broth cultures of the following organisms:

Staphylococcus aureus
Escherichia coli
Pseudomonas aeruginosa

E-test strips of antibiotics from 4 major groups:

Ampicillin (Am) - β lactam, semi-synthetic penicillin, inhibitor of cell wall synthesis
Chloramphenicol (Cl) – inhibitor of protein synthesis
Tetracycline (Tc) – inhibitor of protein synthesis
Gentamicin (Gm) – aminoglycoside, inhibitor of protein synthesis
Clarythromycin (Ch) – macrolide, inhibitor of protein synthesis

Procedure

Each organism was swabbed onto an agar plate (Mueller Hinton) to form a lawn of confluent growth. An E-test strip was then added aseptically onto the surface of the plate and the plates were inverted and incubated at 37°C for 18-24 hours. The MIC value is considered to be the point at which growth around the E-test strip stops. Sensitive organisms will have a zone of inhibition around the strip, resistant organisms will not.

Results

1. Record the MIC values in the table below.

Organism	**Am**	**Cl**	**Tc**	**Gm**	**Ch**
S. aureus					
E. coli					
P. aeruginosa					

2. Describe the resistances of the different organisms (i.e. which organisms were resistant to which antibiotic)?

3. Were there any differences between the Gram positive and the Gram negative organisms in MIC values for each antibiotic?

4. Suggest why some organisms are more resistant than others to various antibiotics.

13.4 Microbial Virulence Factors

Microorganisms possess a number of cellular structures or enzymes that contribute to their ability to colonize a host and/or cause disease. These structures maybe be cell-associated or secreted and may allow the bacteria to adhere, be mobile, degrade tissues, or prevent phagocytosis by host cells.

Materials

Prepared slides of flagella, capsule.

Procedure

View images of flagella and capsule on the microscope under the 100X oil immersion objective and draw what you see in the space below. Then answer the questions that follow.

Results

1. What is the function of flagella and capsule respectively? Are these structures cell-associated or secreted?

2. Name two other bacterial virulence factors, outline their function, and indicate if they are cell-associated or secreted.

13.5 Diagnosis of Infection

Infection diagnosis involves obtaining a patient sample, depending on the type of infection suspected. The sample is then plated to culture the organism and a number of biochemical tests and selective and differential media are used to identify the organism. In addition the sample may be stained to observe the organism within the body tissues, which is an important part of diagnosis of a particular disease.

Materials

Prepared images of gram-stained patient samples.

Procedure

View images of gram-stained patient samples which were obtained on the microscope under a 100X oil immersion objective. Answer the questions below.

Questions

1. What are the four patient samples represented here?

2. What are the four infections represented and the causative agents?

3. What human cell types are commonly found in the patient samples, in particular slides 1-3? Why is this cell type missing from slide 4?

Exercise 14: Introduction to Immunology

Immunology is the study of the immune response. This involves the interaction between antigens, antibodies, and cells. Immune responses are generated by the body to material it considers to be "foreign". Depending on the type of recognition, the immune system will usually react in a particular sequence of events, culminating in the removal of the foreign material.

Microorganisms can generate different immune responses. Depending on the species and their particular characteristics, the numbers present, and their location on the human body, the immune system will mobilize an active process of removal and destruction of these organisms. This exercise will explore some of the basic principles of immunology.

14.1 Antigens and antibodies

Direct ELISA to identify an unknown antibody

Materials

96-well microtitre plate
Antigens to three known intestinal pathogens
Primary antibody: patient sera
Secondary antibody: linked with horse radish peroxidase enzyme
Blocking solution
Coating solution
Wash solution
Substrate for horse radish peroxidase

Solution Preparation:

- Coating Solution: Antigen or antibody are diluted in coating solution to immobilize them to the microplate. Commonly used coating solutions are: 50 mM sodium carbonate, pH 9.6; 20 mM Tris-HCl, pH 8.5; or 10 mM PBS, pH 7.2. A protein concentration of 1-10 µg/ml is usually sufficient. This lab will use PBS-Tween.
- Blocking Solution: Commonly used blocking agents are: BSA, nonfat dry milk, casein, gelatin, etc. Different assay systems may require different blocking agents. This lab will use PBS-Tween.
- Primary/Secondary Antibody Solution: Primary/secondary antibody should be diluted in 1X blocking solution to help prevent non-specific binding. A concentration of 0.1-1.0 µg/ml is usually sufficient.
- Wash Solution: Typically 0.1 M Phosphate-buffered saline or Tris-buffered saline (pH 7.4) with a detergent such as Tween 20 (0.02%-0.05% v/v).

Scenario

A young child was admitted to emergency with the following symptoms: diarrhea, vomiting, nausea, and fever. The child had attended a picnic earlier that day. Although biochemical testing and plating on differential media can be used to help identify the cause of the girl's infection, an ELISA using antigens of known intestinal pathogens will be conducted immediately to more rapidly identify what the child has contracted, so that physicians can initiate treatment.

You will be provided with a sample of serum from the patient and antigens of three known intestinal pathogens: *Shigella sonnei*, *Escherichia coli*, and *Salmonella typhimurium*.

Protocol[2] [3]

Apply Antigen (This will be completed for you)

1. Add 50 µl antigen from the known pathogens diluted in coating solution to rows A-C and columns 1-12 in the microtitre plate
2. Incubate up to 1 hour at room temperature.
3. Empty plate and tap out residual liquid.

Block Plate

1. Add 300 µl blocking solution (PBS-Tween) to each well.
2. Incubate 5 min, empty plate and tap out residual liquid.

Steps 1 and 2 were performed in advance; please proceed from this step on.

Dilute and React Primary Antibody

1. Add 50 µl of PBS/Tween to rows A-C and columns 1-12 in the microtitre plate.
2. Add 50 µl of the patient serum to row A, column 2 only. Similarly, add 50 µl of patient serum to row B, column 2, and 50 µl of patient serum to row C, column 2. (Do not add patient serum to column 1, as it will be a negative control). Dilute the patient serum for each row by transferring 50 µl from column 2 into column 3, and then 50 µl from column 3 into column 4 and so on. The dilutions are indicated in the following table.

[2] This procedure is adapted from http://www.chemicon.com/techsupp/Protocol/indirectELISA.asp and the Biotechnology Explorer™ ELISA Immuno Explorer™ Kit Instruction Manual
[3] For a simulated ELISA experiment please see: http://www-micro.msb.le.ac.uk/labwork/LabWork.htm

		1	2	3	4	5	6	7	8	9	10	11	12
Shigella sonnei	**A**		1/2	1/4	1/8	1/16	1/32	Etc.					
Escherichia coli	**B**												
Salmonella typhimurium	**C**												

3. Incubate for 10 min to 1 hour at room temperature.
4. Empty the plate, tapping out residual liquid on a stack of paper towels.

Wash Procedure

1. Fill each well with wash solution (PBS-Tween).
2. Invert plate to empty, and tap out residual liquid.
3. Repeat 3 to 5 times.

Add Secondary Antibody Solution

1. Add 50 µl diluted secondary antibody to each well.
2. Incubate for 10 min to 1 hour at room temperature.
3. Empty plate, tap out residual liquid and wash (using PBS-Tween) as before.

React Substrate

1. Dispense 50 µl substrate into each well.
2. If desired, after sufficient color development add 100 µl of the appropriate stop solution to each well. (We will not do this step.)
3. The microtitire plate could be read with an automated reader. However, the colour change may also be detected with the naked eye.

Results

1. Which species of bacteria was the girl infected with? How do you know?

2. At what dilution of primary antibody was the reaction no longer visible (refer to the column number)?

3. How could diluting the primary antibody in series be helpful in a clinical setting?

14.2 Cells of the immune system (demonstration)

Materials

Chart describing human leukocytes
Prepared slides of human blood smears
Microscope

Procedure

1. Examine the smears under low and high power magnification.
2. Study the chart provided that described different types of white blood cells or leukocytes.
3. Using these materials, identify and draw the following cell types: a polymorphonuclear leukocyte (PMN or neutrophil), a monocyte, a lymphocyte. These can be drawn in the table below.

	PMN	**Lymphocyte**	**Monocyte**
Appearance			
Function			

END OF LAB!

PLEASE MAKE SURE YOUR INSTRUCTOR HAS MARKED YOUR:

1. **Streak plate**
2. **Kohler illumination**
3. **Gram Stain**
4. **Lab book**