Project 4: Identification of Laboratory Stock Bacterial Cultures

This lab will take two weeks to complete. The procedures are divided into multiple lab sessions.

Readings:

See our class website for additional online and reading materials related to this lab.

Purpose:

The purpose of this laboratory is to provide opportunities for student (1) practice the techniques of pouring and inoculating media (2) interpret growth of bacterial culture on media (3) apply the characteristics and uses of a small selection of media and (4) characterize a bacteria using available testing methods.

Outcomes:

### After you complete this lab, you will be able to:

* Identify the controls in an experiment.
* Define negative and positive control.
* Apply the definitions of controls to determine the validity of an experiment.
* List two functions of controls.
* Employ controls in all experimental designs.
* Consistently inoculate media appropriately.
* Describe and employ the basics of aseptic media, culture and specimen transfer.
* Describe the impact of normal body flora and environmental sources of bacteria relative to aseptic technique.
* Understand the ubiquitous nature of bacteria.
* Perform aseptic technique, streak plate techniques, plate labeling and incubation.
* Describe how ingredients of media influence what you see on the plate.
* Describe the use of differential and selective media.
* Interpret growth on and/or use of the differential media and selective media used in the laboratory exercises.
* Describe how the characteristics of media might allow you to identify bacteria.
* List the ingredients in a particular medium that make it differential or selective.
* Explain how media ingredients select for or suppress groups of bacteria or differentiate among groups of bacteria.
* Use the characteristics observed in stock bacterial cultures to develop a dichotomous key to aid in the identification of unknown bacteria
* Collect and interpret data regarding the identity of an “‘unknown” bacterial culture.
* Develop scientific writing style.
* Write hypotheses
* Record results in a table and use a results narrative to report your findings

Terms to Know:

|  |  |  |  |
| --- | --- | --- | --- |
| Agar | *Enterococcus* | [normal flora](#_Hlk79213117) | TSI |
| alpha, beta and gamma hemolysis | group A ß-hemolytic strep | pH indicator | pH indicator |
| aseptic technique  | hemolysin | positive control | IMViC |
| autoclave | inoculate | selective media | Decarboxylases |
| blood agar | isolated colony | *Staphylococcus aureus* | API 20 e |
| Broth | MacConkey’s | *Staphylococcus epidermidis* |  |
| clinical sample | mannitol salt | streak plate |  |
| colony morphology | medium/media | sterilization |  |
| differential media | mesophile | *Streptococcus pyogenes* |  |
| Enterobacteriaceae | negative control | TSY |  |

### Be able to define these terms and apply them in the laboratory.

Introduction:

*The Goals of the Exercise*

The goal of this lab exercise is to give you an opportunity to characterize the stock cultures. In this way you are like the early investigators in microbiology: determining the characteristics of bacteria in order to determine the genus and species. You will work with a partner to determine the characteristics of ONE of the stock culture bacteria. The two of you will be experts for your assigned bacteria. You will use this information to formulate a key for identifying the bacteria should you ever encounter it again (you will likely encounter it in labs 5,6, or 7 as one of the unknowns).

All of the tests available to you are described below. You can add these tests to the arsenal of tests you already know (i.e. differential staining). You will set up the tests on your assigned bacteria during week 1 and read the results of the test during week 2. Once we have our results, we will convene a ‘mini conference’ for the experts (you all). All of the students who worked on a particular microbe will meet and determine the tests needed to identify the microbe. Then, you will come together with investigators who identified other microbes. Together you will develop a key for identifying the 8 stock cultures. This key will be what you will use to identify the bacterial unknowns you will receive in labs 5, 6 and 7.

*Background Information*

In clinical environments the medical microbiologist may a range of 100-150 different microbes involved in infectious diseases. When a microbial disease is suspected, a clinical specimen is collected and the specimen is inoculated onto media.

Bacteria and other microbes have particular requirements for growth. In order to successfully grow bacteria in lab so that we can stain and identify them, we must provide an environment that is suitable for growth. Growth media (singular: **medium)** are used to cultivate bacteria. Media are mixtures of nutrients that the microbes need to live. They also provide the necessary moisture and pH to support microbial growth. The medium that we use most often is designated "TSY" (**t**ryptic **s**o**y** agar). It isa ***complex*** ***nutrient medium that supports the growth of a wide variety of microbes*** (NOTE: this means TSY is neither selective nor differential!).

When the lab personnel make a medium, they measure out a designated quantity of dry powdered medium, add a designated amount of water and check the pH. They dispense the medium into bottles, cap it and autoclave it. Autoclaving is a process similar to home canning techniques of food preservation. Once the medium is **autoclaved** (or pressure cooked), it is considered sterile. The autoclave exposes the medium to high temperature (121°C) and pressure (15 psi) for 20 minutes. This exposure has been demonstrated to result in **sterilization.** (Sterilization is the process of killing *all* life forms.)

Many normal-flora microorganisms and clinically important microbes can be grown either in liquid medium (sometimes called **broth**) or on Petri dishes (also called Petri plates, or just plates). When microbes are grown on plates, **agar** is added to the liquid medium so that, when cool, the medium has the consistency of very stiff gelatin. Agar is an inert seaweed extract that solidifies at room temperature.

Onc the media has been inoculated, the bacterial cultures are incubated at temperatures known to be best for their growth. The bacteria that live on your body surfaces are mesophiles – they grow at temperatures between 25° and 45°C. Our cultures are incubated at 37° for 24 hours and then put in a holding cabinet until you return the following week.

The incubated cultures are evaluated at the end appropriate incubation period. It may be necessary at this time to sub-culture a suspect colony to obtain isolated colonies.

You will, once again, practice making **streak plates** in today’s lab. The streak-plate method is essentially a **dilution** technique that systematically spreads the bacterial cells over the surface of the medium to achieve growth of isolated colonies. Streaking a sample of bacteria on a plate as described in this lab results in dilution and separation of bacterial cells. When a plate is incubated after the sample has been streaked, each cell divides many, many times by binary fission and forms a **colony**. A colony that is not touching other colonies is said to be **isolated**, and all the cells in that colony are assumed to have arisen by division from a single cell. (FYI, a colony that is just barely visible to your eye contains at least a million cells!)

When a potential culprit has been isolated the ‘work up’ in the microbiology lab involves two things: 1. Performing the tests to determine the genus and species identification of the microbe and 2. Determining the antibiotic sensitivity.

To identify the bacteria in a sample, the isolation streak plate will make appearance of the colonies (i.e., from the **colony morphology**) obvious. If only one colony morphology is present on the plate, then all the colonies will look the same (same color, shape, elevation, etc.). If a plate contains many colonies of only one colony morphology, then the plate is said to be a pure culture of bacteria. Colonies that appear different are, in fact, different genera and/or species of bacteria. If many colony morphologies are present, then the specimen contained a variety of microbes. If a sample contains multiple kinds of bacteria, making a streak plate is the first step in obtaining pure cultures of each so they can be identified.

**Tests Used to Identify Bacteria**

*Special Media Plates*

![MPj04093970000[1]]()Finding and identifying the culprit causing a bacterial infection in a patient is sometimes like finding a needle in a haystack. Sometimes, microbiologists employ a culture medium that provides a comfy cozy growth environment for one type of microbe while making others miserable. This type of medium is known as a **selective medium.** There are many different types of selective media. Each contains substances (e.g., dyes or salts) that inhibit the growth of some bacteria while allowing others to thrive. In this way, if disease-causing bacteria are in a clinical sample, they can be “selected” to grow on this medium while the normal flora caught up in the dragnet of the specimen collection is selected against and does not grow on the selective medium.

Another tool in the microbiologist’s toolbox is the **differential medium**. A differential medium distinguishes groups of bacteria *as they grow* on the medium – you can see a difference. The different groups are often distinguished, or differentiated, on the basis of colony color on the differential medium or by some other characteristic that is rather obvious. Sometimes the characteristics of selective and differential media are combined in one medium. Such is the case for the **MacConkey’s** andthe **mannitol salt** media used in this lab — both are differential **and** selective.

* **MacConkey’s** has a TSY-like composition to which a carbohydrate (lactose) and other ingredients (crystal violet, bile salts and a pH indicator) have been added. It is ***selective***, because its crystal violet and bile salts inhibit the growth of some organisms (Gram-positive bacteria). It is also ***differential*** because of the addition of lactose and the pH indicator. Lactose fermenters (bacteria that metabolize lactose and produce acidic metabolites that cause the pH indicator neutral red to turn pink/red) will grow in pink/red colonies, while non-lactose fermenters will be colorless and clear. Members of the family Enterobacteriaceae (Gram-negative bacilli) are commonly isolated from urine cultures, because the Enterobacteriaceae are members of the normal flora in your intestinal tract. They are most commonly lactose fermenters. MacConkey’s is a tool in the medical technologist’s tool box; it enables her to suspect the culprit in a UTI after a preliminary observation (Bauman p. 181, Fig. 6.14).
* **Mannitol salt** is ***selective***, because it has a high concentration of NaCl (7.5%). Most bacteria cannot survive in this high-salt environment, but species in the genus *Staphylococcus* grow well in this medium. Mannitol salt is also ***differential*** in that it contains a pH indicator that changes from red to yellow in the presence of acid. *Staphylococcus aureus* is able to ferment the sugar mannitol, producing acid as a product, which turns the pH indicator from red to yellow. This ability to ferment mannitol distinguishes *Staphylococcus aureus* from *Staphylococcus epidermidis.*

The two examples above may lead you to think that all special media are both differential and selective. This is not true. An example of a medium that is differential, but not selective is blood agar. Many specimens received in a clinical microbiology lab are plated onto **blood agar**, because it supports the growth of a wide range of medically important organisms. It is even richer than TSY, and more organisms will grow on blood agar than on TSY. Remember, TSY is not selective because it does not suppress the growth of any microbes, but rather supports the growth of a broad range of bacteria and fungi. By the same token, blood agar is not selective and because it is an enriched medium, it supports a more broad range of microbial growth. Blood agar is ***differential***, and microbiologists can easily distinguish some clinically significant bacteria by their appearance, or hemolysis pattern, when cultured on blood agar. Blood agar contains 5% sheep’s blood. Certain bacteria produce extracellular enzymes called **hemolysins** that lyse the red cells completely (**beta-hemolysis**), producing a clear zone around the colony. Other bacteria produce hemolysins that produce a greenish discoloration around the colony – indicating incomplete hemolysis (**alpha-hemolysis**). Still other bacteria have no effect on the red cells (**gamma-hemolysis**). Blood agar is often inoculated from a patient’s throat swab. (This swab is an example of a **clinical sample**. A clinical sample is a sample of a tissue taken from a patient for diagnostic purposes.) The microbiologist is trying to detect the presence of group A beta-hemolytic streptococci (Gram-positive cocci that cause beta-hemolysis on blood agar). The major human pathogen in this group is *Streptococcus pyogenes,* the causative agent of strep throat. Normal throat flora will exhibit alpha- or gamma-hemolysis, but not beta-hemolysis.

*The Bacitracin Test*

Bacitracin is an antibiotic. The Bacitracin Test is a based on the disc diffusion Kirby-Bauer technique utilized for antibiotic sensitivity testing. The test helps identify *Micrococcus luteus* because *Micrococcus* is sensitive to Bacitracin; the zone of inhibition is greater than 30 millimeters. Other bacteria in the laboratory stock cultures have zones of inhibition less than 30 millimeters.

*Tests Used to Identify Enterobacteraceae*

Gram negative bacteria in the family Enterobacteraceae are often identified using a series of four biochemical tests known by the acronym IMViC which stands for **I**ndole, **M**ethyl-Red, **V**oges-Proskauer, and **C**itrate tests. Each is described below:

* The **I**ndole test determines if bacteria can convert tryptophan to indole. The bacteria are inoculated into media and allowed to grow. At the end of the incubation period, you will add a reagent known as Kovac’s reagent to the test tube containing the media and the bacteria growing in the media. A positive result occurs when a red ring or layer forms at the top of the tube. This reaction occurs because the bacteria growing in the media make an enzyme known as tryptophanase. If they cannot make the enzyme then they cannot convert tryptophan to indole and no red ring will form (negative result).
* The **M**ethyl-Red test determines if bacteria can ferment glucose to a variety of different acids, which drops the pH of the media. The bacteria are inoculated into the media, allowed to grow, and reagents are added after the growth phase to detect the presence of these mixed acids. Methyl red, a pH indicator added to the media tube containing the bacteria, will turn red at pH levels less than 4. This red color is considered a positive methyl-red test. A negative test is indicated by no color change.
* **V**oges-Proskauer is a test performed to determine if the bacteria makes acetoin from glucose. Alpha-naphthol and sodium hydroxide are added to the broth culture of bacteria. The development of a red color indicates a positive test (i.e. acetoin production) while no change in color is a negative test indicating the bacteria cannot produce acetoin.
* **C**itrate media is a differential medium. Bacteria, growing on the media, capable of utilizing citrate as a carbon and energy source because they make citritase, will cause in increase in pH. The alkaline pH causes the color indicator in the media to change from green to blue. Thus, a blue color indicates a positive test (i.e. bacteria make citritase can use citrate) and a green color indicates a negative test (i.e. bacteria do not make citritase) (Rao, n.d.; Tankeshwar, 2013; American Society for Microbiology, 2010)

Another medium used to evaluate the a number metabolic capabilities of enteric bacteria is called the Triple Sugar Iron (TSI) media. This medium also has a pH indicator which turns the media from pink to yellow in acidic pHs. Acid is produced by bacteria when they ferment sugars. These bacteria can also produce gas in this metabolic process which might be sufficient enough to crack the media or lift the media from the butt of the tube. The media also detects bacteria that can reduce sulfate to hydrogen sulfide. The hydrogen sulfide combines with the iron in the media to form a black color. Given all this, there are a number of observations to make of bacteria inoculated into this media:

* Tubes containing bacteria that ferment only glucose causing an acid pH will have a yellow butt and a pink slant indicating a small amount of acid production.
* Bacteria that ferment glucose, sucrose and/or lactose (the three sugars indicated by the name Triple Sugar) will generate significant amount of acids causing both the butt and slant on the tube to turn yellow. Additionally, if gas is produced it may be enough to form a crack in the media or lift the media from the butt of the tube.
* Bacteria that do not ferment the sugars may grow on the media, but there will be no color change. Additionally, such non-fermenting (obligate aerobes) bacteria may accumulate acids only on the slant, so the butt of the tube would be pink and the slant yellow.
* Bacteria that reduce sulfates will grow in the media and the tube will turn black.

 (Fankhauser, 2015)

Controls

For most of the experiments that you perform in this course, you will include controls. **Controls** are samples whose identity you already know that are subjected to the same procedures as your unknown sample. A **positive control** is a known sample submitted to the procedures that will show you a positive answer. In this exercise you inoculate sectors on the control medial plates with laboratory stock bacteria because you know (initially, by looking up the organism in your text, lab manual or on-line; later, you’ll know this from the experience of working with the organism and various media) how these bacteria are supposed to behave on a particular medium.

For instance, in this exercise you inoculated mannitol salt media with *Staphylococcus aureus* (*S. aureus*) and *Staphylococcus epidermidis* (*S. epi*). These are both positive controls for the selective nature of the mannitol salt agar because they will both grow on the media. Only *S. aureus*, however, is a positive control for the differential nature of mannitol salt because it will ferment the mannitol, drop the pH of the surrounding media and cause a color change to occur. The media will change from pink to yellow.

A **negative control** is a known sample that should show you a negative result. In the example (above) of the mannitol salt media *S. epi* is an example of a negative control for the differential nature of the media. *S. epi* does not ferment the mannitol and so no media color change occurs*. E. coli* might be considered a negative control on the selective nature of mannitol salt media.

PROCEDURES for Project 4 - Session 1:

Each lab group of 2 students will inoculate one of the stock bacteria into all of the media described above, and perform differential staining techniques. The media techniques include isolation streak plates, inoculation of slant tubes and inoculation of broth (liquid) media. These inoculated media will be incubated and stored until the second week of the lab. In Session 2, the team will record the results and establish a key to help identify unknown bacteria in future lab exercises.

The stock cultures are identified by Gram stain reaction and morphology in the table below. You might also use the endospore and acid-fast stain results in the key you develop.

*Instructions for Setting Up Tests:*

|  |  |
| --- | --- |
| Test | Stock Bacteria |
| Stock Bacteria | *E. coli* | *Salmonellapullorum* | *Ent.aerogenes* | *Ent.cloacae* | *Staph.aureus* | *Staph.epidermidis* | *Micro.luetus* | *Bacillussubtilis* | *Myco.smeg.* |
| Gram Stain | As previously performed |
| Acid Fast Stain | As previously performed |
| Endospore Stain | As previously performed |
| TSY/ colony morphology | Isolation streak plates are inoculated |
| Mac Conkeys | Isolation streak plates are inoculated.  |
| MannitolSalt  | Isolation streak plates are inoculated |
| Blood Agar | Isolation streak plates are inoculated. |
| Bacitracin | Plates are inoculated using a cotton swab dipped in a solution containing bacteria. The cotton swab is swabbed over the entire surface of the media plate and a bacitracin disc is dropped in the center of the plate. The disc is little tapped using a sterile inoculation loop to make certain it is firmly affixed to the media (as demonstrated). Plates are incubated upside down in the green bin until next week |
| Indole | Inoculate the media slant and stab the slant one time, inserting bacteria deep into the media tube: Sterilize an inoculating loop in the bactoincinerator. Use the cooled loop to touch the culture slant and transfer *E. coli* to the slant by gently dragging the loop in a zig-zag pattern over the surface of the slant. Using the same inoculating loop, stab the loop about an inch into the media slant surface. Repeat the technique for each stock culture. |
| Methyl Red | Inoculate the media broth: Sterilize an inoculating loop in the bactoincinerator. Use the cooled loop to touch the culture slant and transfer *E. coli* to the methyl red broth media. Use a twisting motion and the side of the tube to make certain the transfer is complete (as demonstrated). Repeat the technique for each stock culture. |
| Voges-Proskauer | Inoculate the media broth: Sterilize an inoculating loop in the bactoincinerator. Use the cooled loop to touch the culture slant and transfer *E. coli* to the VP broth media. Use a twisting motion and the side of the tube to make certain the transfer is complete (as demonstrated). Repeat the technique for each stock culture. |
| Citrate | Inoculate the media slant only: Sterilize an inoculating loop in the bactoincinerator. Use the cooled loop to touch the culture slant and transfer *E. coli* to the slant by gently dragging the loop in a zig-zag pattern over the surface of the slant. Repeat the technique for each stock culture. |
| TSI | Glucose | Inoculate the media slant and stab the slant one time, inserting bacteria deep into the media tube: Sterilize an inoculating loop in the bactoincinerator. Use the cooled loop to touch the culture slant and transfer *E. coli* to the slant by gently dragging the loop in a zig-zag pattern over the surface of the slant. Using the same inoculating loop, stab the loop about an inch into the media slant surface. Repeat the technique for each stock culture. |
| GlucoseLactoseSucrose |
| Gas |
| Sulfate reduction |

All plates and tubes are labeled with initials from your team, the date, the name of the test, and the name of the bacterial sample. All tests are stored in the ‘SAVE’ bins on the back bench. They will be incubated at 37o C for 24 hours and then stored until you return next week to ‘read the results’. It is your responsibility to make certain your tests have been stored in the correct place as directed by your instructor.

PROCEDURES for Project 4 - Session 2:

This week you will look at the tests you set up last week and *read the results*. To do this you will:

* Practice observing colonies for characteristics that distinguish different colony types
* Gain experience interpreting selective and differential media.
* Develop a key for distinguishing the stock bacteria.

*Instructions for Interpreting Results*

|  |  |
| --- | --- |
| Test | Stock Bacteria |
|  | Gram negative bacilli | Gram positive cocci | Gram positive bacilli |
|  | *E. coli* | *Salmonellapullorum* | *Ent.aerogenes* | *Ent.cloacae* | *Staph.aureus* | *Staph.epidermidis* | *Micro.luetus* | *Bacillussubtilis* | *Myco.smeg.* |
| Gram stain | Observe controls first, and if they are in control, observe your test. Record the stain reaction and cell morphology. |
| Acid Fast | Observe controls first, and if they are in control, observe your test. Record the stain reaction and cell morphology. |
| Endospore | Observe controls first, and if they are in control, observe your test. Record the stain reaction and cell morphology. |
| TSY | Observe and record your observations regarding the colony morphology. |
| Mac Conkeys | Your observations should determine if the bacteria can grow on MacConkeys or not. If it does grow, did it ferment the lactose causing a deep pink color in the colony (positive) or not (negative). |
| MannitolSalt  | Your observations should determine if the bacteria can grow on mannitol salt agar or not. If it does grow, did it ferment the mannitol causing the media to turn yellow (+) or not (-) |
| Blood Agar | Observe and record the colony morphology and hemolysis pattern of the bacteria. Using your observations, classify the bacteria as alpha, beta, or gamma hemolytic. |
| Bacitracin | Measure the zone of inhibition, if it exists, around the bacitracin disc using a metric ruler (as demonstrated). Record the millimeters of the size of the zone of inhibition. Zones greater than 30 mm are considered sensitive (positive) to bacitracin while zones less than 30 mm are considered resistant (negative). |
| Indole | Add 5 drops of Kovac’s reagent to the incubated test tube. A red/pink ring at the interface of the media and air indicates a positive test; no pink/red ring in a yellow or straw colored medium indicates a negative test. |
| Methyl Red | Add 5 drops of methyl red reagent to the tube and record the color. A pink/red color is positive and no color change/yellow straw colored media is a negative results. |
| Voges-Proskauer | Add about 10 drops of alpha naphthol to the remaining culture in the tube. Follow the alpha naphthol with 5 drops of potassium hydroxide. Gently ‘tickle’ the tube. Observe for the formation of a pink ring near the top of the culture broth within 1 minute of adding the reagents. The pink ring is a positive result; no color change is a negative result. |
| Citrate | Observe for growth or no growth on the citrate slate. If there is culture growth record the color the media. A change to blue is a positive result; no color change or green media is a negative result. |
| TSI | Glucose | If the slant if pink (alkaline) and the butt is yellow (acid) glucose was fermented. This is recorded K/A and indicates only glucose was fermented.  |
| GlucoseLactoseSucrose | If the slant and the butt are yellow, record A/A. This indicates all the sugars were fermented. Some bacteria may yield alkaline (pink) slants and butts (K/K) or alkaline slants and no color change in the butts (K/NC).  |
| Gas | Record gas production as positive if the media has been cracked or the media in the butt of the tube has lifted from the bottom of the tube. Record no gas production if you do not make these observations. |
| Sulfate reduction | Record sulfate reduction if the media in the tube appears black. |

*Record Your Results:*

|  |  |
| --- | --- |
| Test | Stock Bacteria |
|  | Gram negative bacilli | Gram positive cocci | Gram positive bacilli |
|  | *E. coli* | *Salmonellapullorum* | *Ent.aerogenes* |  | *Staph.aureus* | *Staph.epidermidis* | *Micro.luetus* | *Bacillussubtilis* | *Myco.smeg.* |
| Gram Stain |  |  |  |  |  |  |  |  |  |
| Acid Fast Stain |  |  |  |  |  |  |  |  |  |
| Endospore Stain |  |  |  |  |  |  |  |  |  |
| TSY |  |  |  |  |  |  |  |  |  |
| MacConkeys |  |  |  |  |  |  |  |  |  |
| Mannitol Salt |  |  |  |  |  |  |  |  |  |
| Blood Agar |  |  |  |  |  |  |  |  |  |
| Bacitracin |  |  |  |  |  |  |  |  |  |
| Indole |  |  |  |  |  |  |  |  |  |
| Methyl Red |  |  |  |  |  |  |  |  |  |
| Voges-Proskauer |  |  |  |  |  |  |  |  |  |
| Citrate |  |  |  |  |  |  |  |  |  |
| TSI | Glucose |  |  |  |  |  |  |  |  |  |
| GlucoseLactoseSucrose |  |  |  |  |  |  |  |  |  |
| Gas |  |  |  |  |  |  |  |  |  |
| Sulfate reduction |  |  |  |  |  |  |  |  |  |
| Arginine |  |  |  |  |  |  |  |  |  |
| Ornithine |  |  |  |  |  |  |  |  |  |