HOST DEFENSE, INFECTION
AND PATHOGENESIS
MED 48801

Laboratory Manual
Spring, 2013
TABLE OF CONTENTS

Laboratory safety rules 1-2
Laboratory records and Reports 3
M & I Laboratory I 4-9
M & I Laboratory II 10-16
M & I Laboratory III 17-25
M & I Laboratory IV 26-30
M & I Laboratory V (A B C D) Diagnostic Unknowns 31-32
M & I Laboratory VI Parasitology 33-34
M & I Laboratory VII Mycology 35-38
Appendix 39-47
The main objectives of the laboratory portion of the course familiarize students with basic clinical immunological and microbiological procedures, particularly those used for diagnostic purposes. The pathology laboratories (in a separately provided Laboratory Manual) provide critical examples of materials to illustrate cellular response to injury and the pathophysiology of disease.

LABORATORY SAFETY RULES

It is important that you understand that the laboratory portion of this course can be a hazardous undertaking! Appropriate precautions at appropriate times must be observed.

In the interest of your own safety, eating and smoking in the laboratory are strictly forbidden. Do not put pencils, fingers or anything else near your mouth or eyes while working.

It is critical that you learn to work competently and safely. It is essential that you scrupulously observe the following:

- All cultures and specimens should be regarded as dangerous.
- Never pipette by mouth for obvious reasons. Always use a rubber bulb.
- An unobstructed working area is essential to safety. Keep nothing on your working space except the material being used in the immediate laboratory procedure. Take care to not contaminate your microscope, laboratory manual, or other personal property.
- Rigid aseptic technique must be followed at all times. The importance of this cannot be over-emphasized. Aseptic procedures will be a matter of daily concern to you for the rest of your medical career, and should be developed --starting with this course-- to the point where they become instinctive.
- To dispose of used equipment:
  - Place used bacteriological slides in the jars.
  - Place used pipettes in the containers.
  - Place any other specimens wherever instructed to do so.
  - Glassware is sterilized, washed, and reused. The plastic items are simply incinerated.
• Never remove infectious material from the laboratories.

• Clearly label each culture with your name, the number of the experiment and the date.

• When you are not actually using your burner turn it off.

• At the end of each laboratory period, clear the bench top of everything and wipe it down with disinfectant.

• Before leaving the laboratory, wash your hands carefully with soap provided.

• In any case where you are unsure, ASK BEFORE YOU ACT.

ACCIDENTS

In case of breakage of a tube or any other potentially dangerous occurrence:

• Make certain that those in the area are aware of the situation so that they do not accidentally become contaminated.

• Notify an instructor at once.

• Disinfect the contaminated area and objects under the supervision of the instructor.
LABORATORY RECORDS AND REPORTS

Records

In any course in experimental science, the accumulation of specific knowledge of the subject being treated is only one of the objectives of laboratory work.

Of equal importance is learning to observe closely and to record exactly your experimental results, to evaluate critically the design of experiments, and to assess intelligently both the validity and significance of results. Uncomprehending performance of instructions is of little value to you in this course or later.

A major reason for you to develop accurate and automatic methods of recording observations is the obviously vital importance of records in the care of patients. Without complete documentation of your observations of a patient today, you may miss a diagnosis or treat erroneously when referring back to the records in the future.

To this end, you should keep complete and concise records of results, conclusions, and evaluations for each experiment. Specific assignments to communicate your laboratory work to the instructor will made in the appropriate laboratory sessions.
M & I LABORATORY I

Host Defense
Clinical Immunology – Diagnosis of HIV Infection

INTRODUCTION

The purpose of this lab is to acquaint you with laboratory tests that are routinely performed in diagnostic clinical immunology labs. Use your knowledge of antibody specificity and antibody-antigen reactions to perform and/or interpret the following assays:

I. ELISA Screening of Serum Samples

Assay serum samples (patients A, B and C) for the presence of an antibody using Enzyme Linked Immunoabsorbent Assay (ELISA). Note that these are fictitious serum samples. They do not contain virus and therefore are not hazardous. However, for the sake of argument, assume the samples are being tested for human anti-gp120 antibody. Therefore, your task will be to:

1. Run positive & negative controls to ascertain validity of the assay.
2. Assess whether the serum sample results are positive or negative (i.e. HIV sero-positive or -negative)
3. Write-up your results and comment on the need to run confirmatory experiments. For example, based on your results, should the sample be re-tested? Should a new sample be collected? If so, when?

II. Western Assay Confirmation of ELISA Results

A Western assay (also called immunoblotting) was set up for you using the samples you tested in part I. You will need to interpret these results and comment on them in your laboratory report. No additional lab work will be needed – we are simply providing you with data.

III. FACS Analysis to Measure CD4+ & CD8+ Cells:

Examine results of FACS analysis of peripheral blood samples taken from the three patients. You will need to interpret these results and comment on them in your laboratory report. No additional lab work will be needed – we are simply providing you with data.
PROCEDURES

I. ELISA to Test for the Presence of Antibody to an HIV Glycoprotein (gp120) in Serum Samples

Materials

- ELISA plate -“gp120”-sensitized 96-well plates containing phosphate-buffered saline (PBS)
- Serum samples A, B, C
- Positive control sample (D) and negative control sample (E)
- Squirt bottles containing PBS (phosphate buffered saline)
- Alkaline phosphatase (AP)-conjugated monoclonal (Mab) mouse anti-human IgG
- Substrate for AP – p-nitrophenylphosphatase

Method

The following diagram and summary outline steps already performed and those which you will do. This is followed by specific instructions for completion of the ELISA in the laboratory.

Work in groups of 4. Each group will work with one ELISA plate. The set-up of the plate is illustrated in the following diagram.

**ELISA Plate Set-up**

```
<table>
<thead>
<tr>
<th>A1</th>
<th>A2</th>
<th>A3</th>
<th>A4</th>
<th>A5</th>
<th>A6</th>
<th>A7</th>
<th>A8</th>
<th>A9</th>
<th>A10</th>
<th>A11</th>
<th>A12</th>
</tr>
</thead>
<tbody>
<tr>
<td>blank</td>
<td>1:2</td>
<td>1:4</td>
<td>1:8</td>
<td>1:16</td>
<td>1:32</td>
<td>1:64</td>
<td>1:128</td>
<td>1:256</td>
<td>1:512</td>
<td>1:1024</td>
<td>1:2048</td>
</tr>
<tr>
<td>B1</td>
<td>B2</td>
<td>B3</td>
<td>B4</td>
<td>B5</td>
<td>B6</td>
<td>B7</td>
<td>B8</td>
<td>B9</td>
<td>B10</td>
<td>B11</td>
<td>B12</td>
</tr>
<tr>
<td>blank</td>
<td>1:2</td>
<td>1:4</td>
<td>1:8</td>
<td>1:16</td>
<td>1:32</td>
<td>1:64</td>
<td>1:128</td>
<td>1:256</td>
<td>1:512</td>
<td>1:1024</td>
<td>1:2048</td>
</tr>
<tr>
<td>C1</td>
<td>C2</td>
<td>C3</td>
<td>C4</td>
<td>C5</td>
<td>C6</td>
<td>C7</td>
<td>C8</td>
<td>C9</td>
<td>C10</td>
<td>C11</td>
<td>C12</td>
</tr>
<tr>
<td>blank</td>
<td>1:2</td>
<td>1:4</td>
<td>1:8</td>
<td>1:16</td>
<td>1:32</td>
<td>1:64</td>
<td>1:128</td>
<td>1:256</td>
<td>1:512</td>
<td>1:1024</td>
<td>1:2048</td>
</tr>
<tr>
<td>D1</td>
<td>D2</td>
<td>+ cont.</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>blank</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>E1</td>
<td>E2</td>
<td>- cont.</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>blank</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
```

areas shaded gray represent empty wells that are not used for this ELISA
**ELISA Protocol Summary**

*Steps done for you: 1-3*

*Steps you will complete: 3-11*

1. Coat each well of the plate with “gp120.”
2. After coating is complete (usually overnight), wash away any unbound gp120 using PBS and then add a non-specific blocking protein to block any sites on the plastic well that have not been bound to protein. This prevents non-specific sticking of the serum samples to be added later.
3. Wash away any unbound blocking protein with PBS.
4. Add serially diluted samples (1:2, 1:4, 1:8 etc.) to rows as indicated in the diagram above. In this case serially diluted serum sample A is added to row A, B to row B and C to row C, beginning with a 1:2 dilution in column 2. Incubate for 1 hr.
5. To well D2 add a positive control sample (i.e. a sample that is known to have anti-gp120 antibody and therefore will react with the gp120 in the well). Incubate for 1 hr.
6. To well E2 add a negative control serum sample (i.e. a sample that does NOT have any anti-gp120 antibody.) Incubate for 1 hr.
7. Wash away unbound serum with PBS — repeat three-times.
8. Add AP-conjugated monoclonal (Mab) mouse anti-human IgG and incubate 1 hr.
9. Wash all wells three times with PBS to remove unbound Mab.
10. Add AP substrate. Incubate and observe color reaction as it develops.
11. Record results visually and using an ELISA reader, a spectrophotometric device that measures absorbance in each well and subtracts background absorbance automatically.

**Detailed Protocol for Steps 3-11**

1. The plate provided to you has been coated overnight with gp120 and blocked. You will begin at step 3 (see above).

2. Decant blocking solution and wash the plate three times in PBS. Hold the 96-well plate inverted over the sink and “flick it” to remove the contents of the wells. Then, blot the plate onto some paper towels to remove residual buffer. Fill the wells with PBS and then invert plate over sink again. Wash plate three more times in PBS. It’s crucial that you remove all of the PBS otherwise you will further dilute the samples prepared in step 3 when they are added to the wells.
3. Serial dilute the samples (1:2, 1:4, 1:8 up to 1:2048) as indicated in chart above. First label tubes (A1-A12, B1-B12, C1-C12). Pipet 150 \( \mu \)l of PBS into each of the tubes. Pipet 150 \( \mu \)l of samples A, B, and C into tubes, A1, B1, and C1 respectively. Mix the tubes very well by gently shaking them or lightly vortexing. Remove 150 \( \mu \)l from tube A1 and add to tube A2 and mix well. Do twofold dilution from A2 to A3 and so forth until A12. Do similar dilutions of B1 -B12 and C1-C12. After dilutions are complete pipet 100 \( \mu \)l of each diluted sample into appropriate wells as indicated above. Controls, D2 and E2 will be provided to you. Incubate plates at room temperature on benchtop for 45 minutes.

4. Flick contents of wells in sink and wash plate five times with PBS using squirt bottle. Since these are not real clinical samples, this is an acceptable procedure. If you were testing clinical specimens, the contents of the wells would be decanted into an appropriate biosafety container. You would also be wearing protective gloves.

5. Pipet 100 \( \mu \)l of AP-mouse anti-human IgG Mab into each well, rows A,B,C and into control wells D2 and E2. Incubate 1hr. on your benchtop.

6. Following the 1 hour incubation period, remove the contents of the wells by flicking into the sink. Then, wash plate five times with PBS.

7. Add 100ul of the AP-substrate to each well (rows A B C and controls). Incubate plates for several minutes at 37\(^\circ\)C until color develops and record results.

8. As color develops, usually beginning with the positive control, you can record results visually based on color intensity using a scale (-, +, ++, ++++, +++++). In addition, each group will also be allowed to read their plate in ELISA reader which provides a more quantitative measure of the color intensity in each well.

II. Western Assay to Confirm the Presence of Antibody to HIV Proteins in Serum Samples

When serum samples test positive for HIV antibody by ELISA, confirmatory tests must be performed. Currently, many labs continue to use a Western blot assay to confirm such results. It should be noted, however, that PCR assays for evidence of viral nucleic acid are rapidly replacing these Western assays. The data supplied to
you is to be used in your interpretation of the results of HIV tests for patients A, B and C. The protocol summary below is merely provided to indicate how such data is generated.

**Western Protocol Summary**  
(*Assays performed for you – see data supplied*)

1. Electrophorese a commercially purified standard preparation of HIV proteins in 5 lanes of an SDS polyacrylamide gel. This procedure will separate the different HIV proteins in the preparation according to their molecular weight.
2. Transfer proteins on the gel to a nitrocellulose membrane by electrophoretic blot transfer overnight.
3. Cut nitrocellulose into strips corresponding to lanes 1-5 of the gel.
4. Incubate strips in a blocking solution containing non-specific blocking proteins to block any sites on the membrane that have not been bound by HIV proteins.
5. Incubate each strip in a separate chamber with the solutions indicated.
   a. a cocktail of mouse Mab against each HIV protein (+ control)
   b. serum sample from patient A
   c. serum sample from patient B
   d. serum sample from patient C
   e. serum sample from a healthy control patient (negative control)
1. Wash strips extensively to remove unbound serum proteins.
2. Incubate strips for 1 hour with secondary anti-IgG antibody conjugated to alkaline phosphatase (AP)
   a. strip 1 with AP-goat anti mouse IgG antibody
   b. strip 2-5 with AP-goat anti human IgG antibody
1. Wash strips extensively to remove unbound AP-conjugated antibody.

**III. FACS Analysis to Measure Frequencies and Ratios of CD4+ & CD8+ Cells**

Fluorescence Activated Cell Sorters (FACS) can separate cells based on differences in size, granularity and presence of cell surface molecules. By gating the machine properly and labeling cells with fluorescent antibodies specific to cell surface molecules unique to specific cell populations, one is able to quantitate numbers of cells in various lymphocyte populations and thus also the ratio of one cell type to another. For instance the ratio of CD4+ to CD8+ T cells has long been
an important indicator of immune system health in persons with HIV disease. The data supplied to you is to be used in your interpretation of the overall immune system health of patients A, B and C. The protocol summary below is merely provided to indicate how such data is generated.

**FACS Analysis Protocol Summary**  
*(Assays performed for you – see data supplied)*

1. Patient’s blood is drawn and peripheral blood lymphocytes (PBLs) isolated.
2. PBLs are immunostained with fluorescent antibody to cell surface markers.
3. Cells are washed to remove non-specifically bound antibody.
4. Cells are centrifuged and resuspended in PBS.
5. Cells are then analyzed for the presence of bound fluorescent antibody by flow cytometry using a FACS. Cells individually pass through a laser beam and cells bound to fluorescent antibody are excited and fluoresce. A photomultiplier detects information on the size, granularity and fluorescent emissions of the cells.
6. Data collected is displayed by the machine in several formats such as a histogram, a dot blot or contour graph. You have been provided with dot blots for lymphocyte analyses of patients A, B, and C.

**LAB REPORTS**

Each individual in the group should complete their own lab report. Lab reports should take the form of a scientific paper with an introduction, a protocol section, a results section and a discussion. In your discussion make sure you interpret the data and discuss the following:
- the validity of the results based on your positive and negative controls
- In light of the Western Blot results, should any samples be retested? If so when?
- Interpret the FACS data. What does this tell you about the overall immune health of each patient?
M & I LABORATORY II
Introduction to Microbiology

INTRODUCTION

The purpose of this and the following two laboratory sessions is to provide an introduction to some of the basic techniques used in the diagnostic laboratory for the identification of infectious agents, bacteria, parasites, and fungi. You will also learn to perform throat cultures and will examine the results of your own throat cultures.

The identification of bacteria with regard to their genera and species is based on a system which classifies bacteria according to certain physical and chemical properties. Increasingly in the clinical laboratory, many of these tests are automated. Additionally, physio-chemical tests are being replaced more and more with DNA-based and immunologically-based tests. This trend will undoubtedly continue. However, learning the procedures demonstrated here provides a strong conceptual framework for interpreting and understanding laboratory results of all types.

The properties commonly used for identifying bacteria include:

1. Colonial morphology and associated features such as pigmentation, zones of hemolysis on blood-agar media, presence of capsular material.
2. Microscopic morphology - cellular shape, size and arrangement.
3. Staining properties of the cell envelope - Gram positive or negative, acid fastness.
4. Nutritional requirements and capacity to utilize specific carbohydrates.
5. Production of characteristic metabolic end products.
7. Oxygen requirements i.e., obligate or facultative aerobe or anaerobe.

This laboratory session will be used to illustrate how some of the above properties are actually applied in microbial identification and to instruct you in the methodology of the Gram stain. Most bacterial species can be classified by this stain into one of two large groups, Gram positive or negative. The stain also facilitates the identification of bacterial cell's morphology by light microscopy.
PROCEDURES

Materials - Plates, Cultures and Samples

A. Sterile blood agar plates will be used to perform throat cultures.

B. In addition, you will be given blood agar cultures of microbes listed below. Blood agar is a basal nutrient medium to which is added 5% sheep blood for enrichment so as to grow as many different kinds of bacteria as possible. Also, the effect of microbial growth on the erythrocytes is often helpful in identification.

1. *Staphylococcus aureus* - a Gram-positive coccus normally found in the naso-pharynx.
2. *Streptococcus viridans* - a Gram-positive coccus normally found in the pharynx.
3. *Escherichia coli* - a small Gram-negative bacillus normally found in the large bowel.
4. *Bacillus subtilis* - a large Gram-positive bacillus normally found in soil and frequently found as a contaminant in clinical specimens.
5. *Klebsiella pneumoniae* - a small Gram-negative bacillus that may be found as part of the normal bowel flora. It generally produces a large polysaccharide capsule.
6. *Candida albicans*. A yeast-like fungus frequently isolated from many sites on the body. Corn meal agar plates of *C. albicans* are also available for your inspection.

C. You will also be given Sabouraud-Dextrose culture of *Aspergillus niger* - a saprophytic mold-like fungus which is a common cause of pulmonary disease in immunologically compromised patients. Sabouraud agar is designed especially for the growth of yeast and fungi while minimizing the growth of the usually far more numerous bacteria.

D. Each group will receive a slide of *Trichuris trichuria* ova. *T. trichuria* is a typical nematode (round worm). The ova are presented here for your examination.
Methods

1. **Throat Cultures** - Using a sterile cotton swab, take a throat sample from a fellow student then streak a blood agar plate (Your lab instructor will demonstrate this. Work in pairs so that everyone will have an opportunity to participate). Label plates with the name of the person being tested. Place plates (inverted) in the 37°C incubator located in the laboratory. You will examine the cultures at the next lab session.

2. **Bacterial Colonial Morphology** – Observe the various culture plates for the morphology of the colonies. Pay attention to the characteristics listed below. Compare different species in regard to these characteristics.

**Colony types** are generally described as being mucoid (M), smooth (S), or rough (R).

- **Mucoid** colonies have a watery, glistening appearance which usually indicate the presence of large amounts of capsular material.

- **Smooth** colonies have a creamy, homogeneous texture. Freshly isolated clinical specimens of Gram-negative bacteria, which produce a "wild type" lipopolysaccharide O antigen, generally have this appearance.

- **Rough** colonies are drier in appearance than smooth colonies. Repeated subculturing in the laboratory frequently selects for mutants which have lost the ability to synthesize complete surface antigens and these mutants often have a rough-type colonial morphology. Rough mutants of a species are generally less virulent than the wild type strains of that same species because the mutants generally are more readily phagocytized.

**Size of colonies** of a specific species on a specific media tends to be a stable characteristic ranging from pin-point to 2 or even 3 millimeters. Note the size relationship of bacteria and yeast colonies.

**Shape of colonies** tends to be circular. The colonies of some species may spread irregularly or have jagged edges.

**Color of colonies** is most often non-pigmented and white to beige (some are nearly transparent). A few produce distinctive pigments either within the cells (manifested throughout the colonies) or in the underlying medium.
3. **Fungal Colonial Morphology** -

- **Yeast**s are somewhat slower growing than most bacteria. The colonies are often smaller and of a creamy consistency.

- **Molds** are slow growing (several days to weeks) and produce large colonies that eventually will fill the entire plate. They have a felt-like texture due to their entangled filamentous nature.

Compare the colonies produced by typical yeast, *C. albicans*, and typical mold, *A. niger*.

4. **Hemolysis** - A number of clinically important bacterial species excrete enzymes that lyse red blood cells and others produce metabolic end products that cause a discoloration of the blood agar medium. These are both described as types of hemolysis for the purpose of identification on blood agar plates. Types of hemolysis include:

- **Alpha hemolysis**: This is merely discoloration of hemoglobin from bright red to a dirty gray-green. The RBCs are not destroyed.

- **Beta hemolysis**: Here both the hemoglobin and the cell membranes are totally destroyed so that clear transparent rings surround each colony of beta-hemolytic organisms.

- When bacteria have no effect on the RBCs, they are called non-hemolytic

5. **Microscopic Examination - The Gram stain**

This is the fundamental dichotomy in bacteriology. Bacterial cells, for the most part, either retain the Gram stain (appear purple) or are easily decolorized and then counterstained (appear pink). This is a reflection of major structural differences in the two groups of bacteria and is quite useful as a first step in species identification. Each group should Gram stain examples of all species and each person within each group must practice and learn this skill.

The first thing you will need to do is prepare satisfactory slides for staining:
Prepare Samples (Slides) for Gram Staining

1. Place a drop of water in the center of a glass microscope slide using a plastic, disposable loop.
2. Use the loop to obtain a bit of bacterial growth from one colony on the agar surface. *It is important that you take only the minutest amount - otherwise your smear will be far too dense. Suspend the growth in the drop of water by gently stirring the loop in it.*
3. Allow to air dry.
4. "Heat Fix" by passing the slide quickly through the flame of a Bunsen burner 2 or 3 times. *This will cause the organisms to adhere to the glass. Do not overheat or the integrity of the cells will be destroyed.*

After completing steps 1-4 above, proceed with the Gram stain described on the following page.

**Gram Staining**

1. Cover the smear (prepared as described above) with crystal violet. Let stand 30 seconds.
2. Wash the stain off gently with flowing water for about 5 seconds. Shake off excess water.
3. Cover the smear with Gram's iodine solution for 1 minute. Wash with water and shake off excess water.
4. Decolorize with 95% ethanol solution. Tilt the slide slightly and slowly drop ethanol above the smear on the slide, allowing the ethanol to run down across the smear. Do this until the purple color just ceases to flow away from the smear. *It is most important that the smear be neither over- nor under-decolorized. Excessive decolorization may remove enough dye to give false Gram-negatives; insufficient decolorization may give false Gram-positives.*
5. Wash with water for about 5 seconds. Shake off excess water.
6. Counterstain with safranin for 30 seconds.
7. Wash with water. Shake off excess water. Allow to air dry, or carefully blot with bibulous paper.
8. Examine the various organisms under oil immersion (900-1000x).
LAB REPORTS

Each individual in the group must complete their own report. Lab reports for M & I Labs II, III and IV are handed in as a single paper. Each of these labs concludes with a blank table to record the results of that lab session. These tables along with a short introduction and short discussion constitute the expected laboratory report.
1. **Bacterial, Colonial, and Microscopic Morphology**

<table>
<thead>
<tr>
<th>Colony type</th>
<th>Hemolysis</th>
<th>Gram stain</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. <em>S. aureus</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B. <em>S. viridans</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C. <em>E. coli</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>D. <em>B. subtilis</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>E. <em>K. pneumoniae</em></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

2. **Fungal Morphology**

A. *C. albicans*

B. *A. niger*

2. **Size Relationships**

   Compare colony sizes for various microbial species on blood agar:

   Compare cell sizes for various microbial species examined by Gram staining:
INTRODUCTION

In many instances it is not possible to identify bacteria solely on the basis of their colonial and microscopic morphologies and Gram staining characteristics. For example, all of the Enterobacteriaceae, including both normal bowel flora such as *Escherichia coli* and pathogenic species of the genera *Salmonella* and *Shigella*, are small Gram-negative bacilli which produce very similar colonies on blood agar. It also is frequently difficult to morphologically differentiate among some species of *Staphylococci*. Similarly, *Streptococcus viridans*, which is part of the normal pharyngeal flora, and *Streptococcus pneumoniae*, the most common cause of bacterial pneumonia, are often difficult to differentiate, especially if the pneumococcus does not produce much capsular material. In cases such as these, definitive identification is accomplished by including in the diagnostic work-up, tests for the following:

1. specific metabolic capabilities, such as the ability to utilize specific carbohydrates or other carbon sources
2. end products of metabolism such as organic acids and gas
3. specific enzyme activities, such as coagulase in the case of *S. aureus*
4. sensitivity to specific antibiotics such as optochin, which is used to differentiate between *S. viridans* and *S. pneumoniae*;
5. specific surface antigens, such as the H and O antigens of enterobacteria.

The purpose of this laboratory session is to illustrate how the type of testing alluded to above is used to identify various bacteria. Some of the cultures and tests can be interpreted today. In some other cases you will set up further testing that will be interpreted at the next lab session. You will also have the opportunity to examine your throat cultures for microbiological work-up.
PROCEDURES

Materials

Cultures:

1. Mixed cultures - Blood agar, Hektoen-Enteric (H-E) and MacConkey plates inoculated with the same mixed cultures of Staphylococcus aureus, S. epidermidis, Escherichia coli, and Salmonella enteritidis.
2. Blood agar plates inoculated separately with Streptococcus pneumoniae and S. viridans then overlayed with optochin discs.
3. Blood agar plates inoculated with Streptococcus pyogenes then overlayed with bacitracin discs.
4. H-E and MacConkey plates inoculated separately with the following enteric bacteria:
   a. Escherichia coli
   b. Enterobacter aerogenes
   c. Salmonella enteritidis
   d. Proteus mirabilis
5. Stock culture agar slant inoculated with Mycobacterium smegmatis.
6. Throat cultures (prepared in previous lab).

Plates and slants:

1. Blood agar, MacConkey, Hektoen Enteric Agar, Citrate slants, Urea slants, Motility-Indole-Ornithine (MIO) media, MRVP (glucose) broth.
2. Reagents for Acid-Fast Stain
3. API Strips
4. Reagents for IMViC Tests
   Definition: A series of tests to distinguish among some Gram-negative bacteria, particularly members of the Enterobacteriaceae. 
   I = Indole, M = Methyl red, V = Voges-Proskauer, C = Citrate. The second "i" is only added to make this mnemonic device pronounceable.
Methods

1. Examination of mixed cultures on blood agar (including your own throat culture), MacConkey and H-E plates
   All (with the exception of your unique throat cultures) plates contain the same mixture of microbial samples.

   A. Examine the 2 blood agar plates (one with your throat culture and the other which we will have prepared for you), MacConkey and H-E plates which were inoculated with mixed cultures of *S. aureus*, *S. epidermidis*, *E. coli*, and *S. enteritidis*. How many different colony types can be distinguished on the different media?

   B. Gram stain representative colonies. How many different bacterial types are found?
   *You should dispose of your throat culture plate at this point since no additional tests will be performed on the microorganisms that grew. Remember, plates to be disposed are put in designated areas to be later autoclaved prior to disposal.*

   C. Purify both Gram-positive and Gram-negative bacteria identified above. Streak onto new media from the same colony used in identification of each bacteria type. Gram positives should be streaked on blood agar and Gram- negatives on MacConkey media.

   *The laboratory instructors will demonstrate the procedure for picking and purifying bacteria by streaking on appropriate media. Save the original plates, in the cold room, for the next lab.*
2. Differential diagnostic methods:

A. Optochin Test

Examine the blood agar plates that were inoculated with *S. pneumoniae* and *S. viridans*. For many years it has been recognized that pneumococci differ from other streptococci in the marked fragility of the pneumococcal cell surface. Originally this was determined by a procedure in which "bile" (later sodium deoxycholate solutions) was used to try to dissolve suspect organisms. *S. pneumoniae* were "bile soluble" while other streptococci were not. The bile solubility tests correlate perfectly with sensitivity to optochin. Thus, *S. pneumoniae* is sensitive to optochin, whereas other streptococci are resistant.

To do the optochin test, a blood agar plate is heavily inoculated and then a paper disk impregnated with optochin (ethylhydrocuprein) is firmly pressed onto the agar surface. The plate is then incubated for 24 hours at 37°C. This works best if incubation is in an atmosphere of increased pCO₂. Examine the optochin test plate. Record your observations and describe what the test shows.

B. Bacitracin Test

This is valuable for the fast detection of the worrisome beta-hemolytic Group A streptococci, an organism most feared for the sequelae which may follow the initial infection.

To do this test, heavily streak a blood agar plate with the suspected streptococci to form a lawn of bacteria over the plate surface. Place a paper disk impregnated with the antibiotic bacitracin in the area inoculated with bacteria. Firmly press the disk onto the agar. Incubate for 24 hours at 37°C.

A zone of inhibition of any size around the bacitracin disk should be presumed to indicate Group A beta-hemolytic streptococci (*S. pyogenes*). The consequences of false positive results are trivial compared to those of false negative results as the identification of Group A strep is important so that appropriate antibiotic therapy can be begun. Examine the bacitracin test plate and record your observations.
C. Growth characteristics of representative enteric bacteria on H-E and MacConkey media:

Read culture media descriptions following the lab exercises in the appendices of this manual. Based on your reading and your examination of the cultures provided, record lactose plus or minus and H$_2$S plus or minus for each of the enteric bacteria provided for demonstration.

D. Differentiation between *S. enteritidis* and *P. mirabilis*

It generally is not possible to differentiate between most species of *Salmonella* and *Proteus* on the basis of their growth characteristics on H-E medium. These genera can be distinguished, however, on the basis of urease production. Urease is an enzyme which degrades urea liberating NH$_3$ which can be detected on urea slants. These slants contain a pH indicator which turns a bright pink at basic pH.

Inoculate separate urea slants by streaking the surface with samples of *S. enteritidis* and *P. mirabilis* from the H-E plates. Incubate 24 to 48 hours at 37°C and record which of the organisms produces urease at the next lab session.

E. Differentiation between: Glucose +/-, Lactose +/-, H$_2$S +/-, and Gas-producing/non-producing Gram-negative bacilli using Kligler's Iron Agar

This is an essential early test medium in studying Gram-negative bacilli to characterize their abilities to utilize glucose and lactose, to produce acid and perhaps gas, and to produce H$_2$S from sulfur-containing amino acids. As such, it is an all important initial step to choosing further procedures in identifying such bacteria. Demonstrations of bacterial fermentative properties depend on the proportions of lactose (10 parts) to glucose (1 part). Phenol red is an indicator of acid pH and ferrous citrate will indicate H$_2$S production by the formation of a black precipitate. The initial appearance of the medium is an amber-rose translucent tubed slant. A more complete explanation for the use of the Kligler Iron Agar Slant can be found in the appendices of this manual.

Inoculate separate Kligler slants with *E. coli* and *S. enteritidis* from MacConkey plates. To inoculate, streak bacteria in the slant and then stab to the bottom. This establishes growth in both an aerobic and an anaerobic environment. Incubate at 37°C and examine at the next lab.
F. Differentiation between *E. coli* and *E. aerogenes* by IMViC.

Identification of the various Enterobacteriaceae is based for the most part on biochemical tests of which the IMViC series is one of the oldest. This test series was commonly used to determine whether Gram-negative organisms, which may be present in a water source, were *E. coli* (a sign of fecal contamination) or *Enterobacter* (which may be a soil saprophyte). In other words, this is a common test for fecal contamination of water supplies.

The acronym IMViC is derived from the following tests:

I) **Indole production from tryptophan.** Certain organisms hydrolyze tryptophan to indole and serine. This is detected by testing a culture of the growing organism for the presence of indole using Kovac's (Ehrlich's) reagent (paradimethylamino-benzaldehyde dissolved in alcohol and HCl). In this lab you will inoculate a motility-indole-ornithine agar tube (MIO) and later test for indole production.

II) **Methyl Red.** A pH indicator which turns red at pH 4.5. It identifies bacteria which produce predominantly acid end products such as formic, acetic, and lactic acids from sugar fermentations.

III) **Voges Proskauer.** The Voges Proskauer reagent identifies bacteria which carry out a butylene-glycol type fermentation. Such organisms convert pyruvate to a mixture of products such as acetoin and 2,3-butane diol. The VP reagent (reagent A) contains creatinine in a basic solution. In the presence of KOH (reagent B), acetoin is converted to diacetal which forms a pink-colored complex with creatinine.

IV) **Citrate Utilization.** Some organisms can use citrate as a sole carbon source. Growth of such organisms on citrate slants results in a blue color change in the normally green colored slant. Carry out separate IMViC series on pure *E. coli* and *E. aerogenes* cultures from MacConkey plates.

1. Stab MIO agar (indole test).
2. Inoculate glucose (MRVP) broth with a loop (methyl red test & Voges Proskauer test).
3. Streak surfaces of citrate slants (citrate test).
Note: The IMViC cultures must be grown 24 to 48 hours before reading. Many of the tests will require addition of reagents before reading. This will be detailed in the next lab. Record your results in the next laboratory session. Leave all slants' caps loose.

3. Other Procedures

A. API Strips

API strips are commercially available test kits for determining several different biochemical characteristics of Gram- isolates. Your lab instructor will demonstrate how they are used.

B. The Acid-Fast Stain.

This is a very special procedure applied only to mycobacteria and a few other related genera. They are difficult to stain by conventional procedures such as the Gram stain because they are coated by complex lipids. Once stained by vigorous procedures they are very resistant to decolorization. The original acid fast staining procedure uses heat to force the stain into the cell. Here, we use a variation of that technique. Instead of heat, the Kinyoun technique forces the stain through the lipid coatings of pertinent bacteria by the use of the surfactant Tergitol.

Acid Fast Stain Procedure

1. Mark off 2 sections of a glass slide with a grease pencil.
2. In one section emulsify a small sample of the *M. smegmatis* in a drop of water and in the other section do the same thing with a sample of *E. coli*; allow to air dry.
3. Heat fix as in the Gram stain.
4. Flood the slide for 5 min. with the carbol fuchs in reagent containing Tergitol No. 7.
5. Decolorize with acid-alcohol (3% HCL in EtOH) until color no longer flows from the smear area on the slide.
6. Rinse with water, drain and dry.
7. Flood the slide with brilliant green.
8. Rinse with water, drain and dry.
9. Examine under the oil-immersion objective.

Acid fast bacteria stain red – Acid fast negative stains greenish blue.
LAB REPORTS

Each individual in the group must complete their own report. Lab reports for M & I Labs II, III and IV are handed in as a single paper. Each of these labs concludes with a blank table to record the results of that lab session. These tables along with a short introduction and short discussion constitute the expected laboratory report.
REPORT – M & I Laboratory III

1. Examination of mixed cultures.

2. Optochin sensitivity testing

3. Bacitracin sensitivity testing

4. Growth characteristics of enterics on H-E agar and MacConkey Agar.

5. Acid-fast stain results.
LABORATORY IV
BACTERIAL IDENTIFICATION (continued)

INTRODUCTION

This laboratory session will be used in part to tabulate the results of the biochemical tests conducted in the previous laboratory. Several additional tests, commonly used in bacterial identification, will also be demonstrated.

PROCEDURES

Materials

Cultures

1. Blood agar cultures of the following:
   
   A. *Staphylococcus aureus*
   B. *S.epidermidis*
   C. *Streptococcus pyogenes*
   D. *Pseudomonas aeruginosa*
   E. *Salmonella enteritidis*

Reagents

1. Kovacs reagent
2. Methyl-Red reagent
3. Voges-Proskauer reagent (A & B)
4. Plasma for coagulase test
5. Oxidase reagent
6. $\text{H}_2\text{O}_2$
7. Polyvalent anti-*Salmonella* antisera

Methods

1. Coagulase Test

The enzyme coagulase induces clot formation in plasma. This test is used to differentiate between *S.aureus* and *S.epidermidis*.

Place a drop of plasma and a drop of water onto a glass slide. Using a loop, mix a small quantity of bacteria into each drop. Don't let drops dry out. Look for the formation of small clots. In the past, coagulase positive were
regarded as the pathogenic staphylococci (S. aureus) while the coagulase negative strains were regarded as non-pathogenic (S. epidermidis). This test identifies the more pathogenic S. aureus from its coagulase-negative relatives. However, it is important to remember that coagulase-negative staphylococci can also cause infections, particular opportunistic infections.

2. Catalase Test

The ability to produce this enzyme which decomposes $2\text{H}_2\text{O}_2$ to $2\text{H}_2\text{O}$ and $\text{O}_2$ is important in characterizing various groups of bacteria, notably Gram positive cocci and mycobacteria.

Carefully remove some bacteria from a colony, place on a microscope slide and add a drop or two of $\text{H}_2\text{O}_2$. A positive reaction is an immediate effervescence (bubbling). Note that when working with blood agar plate cultures it is important that no tiny bits of medium be carried along with the bacterial mass because erythrocytes are rich in catalase.

Staphylococci are catalase positive while streptococci are catalase negative. This test is often the first step in differentiating between these two important groups of bacteria.

3. Oxidase Test

All of the members of the genus Neisseria and some other bacterial species possess a respiratory enzyme which is capable of oxidizing the organic dye aniline. This is a test which uses various organic chemicals as surrogate substrates to detect bacterial production of indophenol (cytochrome) oxidase. Many bacteria produce this enzyme, but tests for it are particularly useful in studying species of Gram-negative cocci and non-fermentative Gram-negative rods.

To do this test, a few drops of oxidase reagent are added to bacteria transferred to a filter paper. A positive reaction is a progressive color change of the tested bacterial growth from pink to red to purple to black. A negative reaction is no change. Note the color change that occurs when a few drops of the oxidase reagent are placed on Pseudomonas aeruginosa bacteria transferred to a filter paper. You should include a negative control such as E. coli to assure yourself of the test’s validity.

4. Serologic Identification of Salmonella

Antibodies to surface antigens can cause bacteria carrying these antigens to
agglutinate or clump. Serologic typing is a frequently used epidemiological tool which is important in identifying sources of infection.

To demonstrate what this type of reaction looks like, emulsify small samples of the *Salmonella* culture provided into separate drops of polyvalent anti-*Salmonella* antisera and water. You should include a negative control such as *E. coli* or a *Proteus* species to assure yourself of the test's validity.

5. Methods for Scoring IMViC Test Results

Following incubation of cultures, perform the following steps to obtain results:

a. To test for **indole** and **motility**: Add approximately 5 to 7 drops of Kovac's reagent to the top of the agar. Red color is scored positive (i.e., indole present). No change in color is scored negative. Observe culture for motility which will be seen as growth of cells down the sides of the tube away from the initial stab.

b. **Methyl Red** and **Voges-Proskauer**: Divide the broth into 2 aliquots (you simply need 1 additional tube into which half of the volume will be placed).

**Tube 1**: Test for methyl red by adding approximately 10 drops of methyl red. If the broth turns orange-red (indicating acid pH), the results are scored positive. No color change indicates a negative result.

**Tube 2**: Add 5 drops of "reagent B" (KOH which increases pH), then add 10 drops of "reagent A" (α-naphthol). Wait 5 min. A pink color is scored as a positive VP result. No change in color is scored negative for VP.

c. **Citrate**: Blue color in agar is scored positive. No color change is negative.

6. Scoring of Urease Results

Following incubation, bright pink is positive, orange-yellow is negative.
7. Scoring Kligler's Iron Agar Results

Look for color change to yellow in the butt of the tube to indicate acid production following fermentation. A black precipitate indicates H₂S production. Bubbles or a breaking up of the agar indicates gas production as a byproduct of fermentation. Examine your tubes inoculated in the last lab session. Use the table in the appendices of this manual to help interpret your results.

LAB REPORTS

Each individual in the group must complete their own report. Lab reports for M & I Labs II, III and IV are handed in as a single paper. Each of these labs concludes with a blank table to record the results of that lab session. These tables along with a short introduction and short discussion constitute the expected laboratory report.
REPORT – M & I Laboratory IV

Coagulase Test.

Catalase Test.

Oxidase Test.

Serologic Test.

IMViC Test

<table>
<thead>
<tr>
<th>species</th>
<th>I</th>
<th>M</th>
<th>Vi</th>
<th>C</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>E. coli</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>E. aerogenes</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Urease Production

Kliger Iron Agar Slant
INTRODUCTION

In the diagnosis of infectious disease, preliminary evaluation of patient specimens can provide much information while awaiting laboratory results. For example, respiratory tract infections may be attributed to bacterial, fungal, viral or Mycoplasma etiologies. In many cases, the definitive etiology is of obvious importance to physicians in order for them to institute appropriate antimicrobial therapy as rapidly as possible. For an accurate diagnosis, many parameters are evaluated including the patient's clinical history, microbiological work-up, etc.

PROCEDURES

The purpose of these exercises is to simulate how this information may be gathered, interpreted and confirmed. You will be provided with case histories of patients with active infections, bacterial in most cases. At the first session, you will be given an appropriate sample containing the causative agent of disease which accounts for the symptoms noted in the case history. There may be other organisms present in your sample as well. Your objective is to diagnose the etiology of each disease, taking into account both the patient’s history and the results you obtain in your lab work-up. Keep an account of your observations and justify your selection of culture media and diagnostic tests performed to work-up the sample. Use information learned in Labs II, III and IV in selecting appropriate methods for identification of organisms in your sample. You will work during this and the following two sessions to complete your identification. These observations will be included in your group lab report.

i. **Note:** 1. All samples should be Gram-stained as well as cultured. The suspected etiologic agent should be taken into account in selecting the appropriate culture media and incubation conditions.

You will also test for the antibiotic resistance/sensitivity of your isolate, once you have a pure culture, as a means of suggesting appropriate therapy.
The Basic Concepts of Testing for Antibiotic Resistance

Today a major concern in the treatment of infectious diseases is the constantly increasing resistance of bacteria to once effective antibiotics. Consequently, in many bacterial infections, it is necessary to determine the antibiotic resistance profile of each isolate in order to insure effective treatment. The simplest, most expedient and most commonly used procedure is the Kirby-Bauer disk diffusion method. This involves completely covering an agar plate with the isolated pure culture of bacteria and then placing on the agar surface paper disks impregnated with known concentrations of various antibiotics. After incubation, zones of no growth will surround possibly effective antibiotic disks while the rest of the plate surface will show solid growth. The diameters of growth inhibition must be correlated with charts of each antibiotic since these compounds vary greatly in the rate they diffuse through the agar gel matrix due to molecular size, charge, etc.

You will carry out the Kirby-Bauer procedure for your diagnostic unknown isolate as a means to evaluate possibly effective antibiotics. Your overall analysis will include a qualitative set of observations regarding potentially effective antibiotics.

CASE PRESENTATIONS & LAB REPORTS

All students must attend the case presentation session and be prepared to present your case to your peers. These are group presentations. Lab reports for these sessions are also to be done as a group effort. Please make sure to divide the work evenly. Presentations and write-ups should include some or all of the following and anything else your group deems important:

1. Case history summary-What are the important facts?
2. Differential diagnosis-What are possible etiologic agents? Are there some more likely candidates than others? Why?
3. Lab findings-What tests did you do to identify the agents? Why? Were there tests you would have like to do, but were not able to do?
4. What are the properties of the etiologic agent (culture characteristics, virulence factors...)
5. Prognosis/Treatment.

32
CASE 1

A 5-year old boy presented with the chief complaint of low grade fever of 2 weeks duration associated with loss of appetite and malaise. Three days prior to admission, he developed a non-throbbing generalized headache, neck stiffness and progressive drowsiness. He became lethargic and had several episodes of vomiting not related to food intake. The past medical history was essentially negative. There was no history of head trauma. The patient lived with his grandmother who gave a history of chronic cough.

On physical examination, temperature was 38.9°C, pulse 90/minute, BP 110/75 mm Hg, respiratory rate 28/minute, weight 18 kg. Examination revealed an ill appearing, apathetic and drowsy young boy with a stiff neck. Fundi were normal. The patient had positive Kernig’s and Brudzenski’s signs with increased deep tendon reflexes in both lower extremities. Babinski’s sign was negative. There were no localizing neurological deficits. There was no generalized lymphadenopathy. The remainder of the physical examination was within normal limits.

LABORATORY DATA:
CBC: Hgb 12 gm%, Hct 37%, WBC 12,000/ cu mm, PMN 70%, lymphocytes 28%, monocytes 2%. Urinalysis: normal. Blood chemistry: normal BUN, sugar, calcium and electrolytes. Lumbar puncture: opening pressure 300 mm; CSF color clear; cells 70 WBC, 60% lymphocytes; sugar 28 mg% (blood sugar 95 mg%), protein 110 mg%.

A sample of cerebral spinal fluid is presented for your analysis.
CASE 2

A 22-year old male student was admitted to the Mount Sinai Hospital on September 14 with a chief complaint of shortness of breath, fever and blood-flecked sputum. One week prior to admission, the patient experienced symptoms which he described as a mild cold, with sore throat, cough, headache and general malaise. Three days prior to admission while riding the subway home from school, the patient experienced a severe shaking chill, which persisted for about 10 minutes. That evening, his cough became worse and productive of sputum for the first time.

On physical examination, the patient appeared acutely ill and toxic, temperature of 104°F and respiration was accompanied by grunting.

A sputum sample from this patient is provided.
CASE 3

Earlier this week, 17 cases of acute gastrointestinal disease occurred among 33 students and instructors at a class banquet.

Symptoms included nausea and vomiting (100%), diarrhea (90%), and abdominal cramps and pain (55%). All affected sought medical aid. Ten were hospitalized, and the remaining 7 were treated in the hospital emergency room. Incubation periods of the illness ranged from 1.3 to 5.5 hours with a median of 3.0 hours.

Food histories obtained from 23 of the guests implicated potato salad as the vehicle of transmission. The attack rate among those who ate potato salad was 79% (11/14), while 0% (0/9) of those not eating potato salad became ill.

A sample of the potato salad is provided for your examination.
CASE 4

A 68-year-old female presents to the emergency room in June because of pain in her left leg, chills and fever. She was in satisfactory health until March when she underwent coronary artery bypass surgery. Three months later she had a temperature of 38.8°C and chills that resolved spontaneously in a few days without therapy. One week later she again experienced fever and shaking chills. On the second day of fever she received oral doxycycline hyclate. The next day erythema and tenderness were noted in her left leg and she was admitted to the hospital.

On examination her temperature is 103°F, BP 100/70 mm Hg, respiratory rate 22/minute, pulse 80/minute. There were soft, tender nodes palpated in the left inguinal area. There was diffuse erythema, increased heat and swelling. Small abrasions and blisters were present over the anterior tibial region. A phlebogram performed the day after admission was normal. Plain films of the left leg were normal.

LABORATORY DATA:

WBC 16.0/ cu mm with 89% segmented forms, 7% bands and 2% lymphocytes.
SGOT 30 U/L
LDH 96 U/L
CPK 100 U/l

An aspirate of the blister from the abraded area has been provided for your examination.
CASE 5

A 29-year-old female is seen in the emergency room for nausea, fever, dysuria, oliguria and back pain of 24 hours duration. She has also experienced vaginal itching and discharge of 3 days duration. She is presently taking Ovral 21. The last day of her previous menstrual cycle was three weeks ago.

LABORATORY DATA:
Macroscopic urinalysis - glucose - trace; ketones - +; protein - 100 mg/dl; blood - +; bilirubin - 0; urobilinogen - <1; nitrite - +; specific gravity - > 1,035; color - amber and cloudy; pH 7.5.

Microscopic urinalysis - WBC - > 100/high power field (hpf); RBC - 10/hpf; many budding yeast; many mucous threads; many bacteria.

You have been provided with a fresh clean-voided urine for microbiological workup.
CASE 6

A 36-year-old male was admitted to the hospital because of fever and leukopenia. Evaluation revealed acute leukemia and he was begun on prednisone, 6-mercaptopurine and allopurinol. Anti-bacterial therapy included gentamicin and carbenicillin. Two weeks after the first round of chemotherapy, the patient developed painful dysphagia and severe retrosternal pain and hiccups. His temperature spikcd to 102°F, pulse - 100/minute, respirations - 20/minute and BP - 160/90 mm Hg. Examination of the mouth and throat revealed erythema and white patches. An upper GI series was normal. Fiberoptic esophagagogastroduodenoscopy was done. The esophagus showed areas of superficial ulceration covered by yellowish-white membrane. The stomach, duodenal bulb and postbulbar areas were found to be normal.

LABORATORY DATA:

<table>
<thead>
<tr>
<th>Test</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>WBC</td>
<td>1000/cu mm</td>
</tr>
<tr>
<td>platelets</td>
<td>100,000/ cu mm</td>
</tr>
<tr>
<td>HCT</td>
<td>16.2%</td>
</tr>
<tr>
<td>HGB</td>
<td>5.5 g/dl</td>
</tr>
<tr>
<td>ESR</td>
<td>&gt; 150 mm/hr</td>
</tr>
<tr>
<td>SGOT</td>
<td>15 U/L</td>
</tr>
<tr>
<td>LDH</td>
<td>69 U/L</td>
</tr>
<tr>
<td>CPK</td>
<td>15 U/L</td>
</tr>
</tbody>
</table>

A throat swab has been provided for your examination.
CASE 7

A 64-year-old man was hospitalized because of symptoms of lower urinary tract obstruction. On the third hospital day, he underwent cytoscopy, which confirmed suspected prostatic hypertrophy, bladder dilatation and hypertrophy, and mild hydronephrosis. Twelve hours later he developed fever, hypotension and tachypnea.

Physical examination revealed an acutely ill man with cold and clammy extremities. Blood pressure was 80/40 mm Hg, pulse rate 120/min., respiratory rate 30/min. and temperature 102°F. Examination of the chest and heart, EKG and chest roentgenogram, was normal. Blood and urine are provided for your examination.
CASE 8

A 53-year-old female with a history of chronic alcoholism was seen in the emergency room this morning complaining of left pleuritic chest pain of four days duration. While in the emergency room she experienced a profuse sweat and severe shaking chills. The patient gave a history of several such shaking episodes in the previous four days.

Upon physical examination, the patient appeared acutely ill and toxic. A strong odor of alcohol was noted on her breath. Her oral temperature was recorded at 101°F.

A sputum sample was observed to be blood flecked and is provided for your examination. A blood sample was also taken and is provided.
CASE 9

Four days after her arrival in Acapulco, Mexico, a 37-year-old New York woman developed a mild diarrheal illness. Her illness lasted two days and she was asymptomatic upon her arrival in the United States. Five days after her original illness, she had the onset of severe diarrhea with vomiting and abdominal cramps. She was hospitalized that day. Physical examination was normal except for tachycardia.

A stool specimen was obtained on the day of admission and is provided for your examination.
CASE 10

A 19-year-old heroin addict was admitted to the hospital because of fever, shaking chills and left flank pain of one week’s duration. He had no prior history of urinary tract infection and no urinary symptoms at the time of his admission. He had hepatitis 18 months previously, for which he had been hospitalized for one month at another hospital. In all other respects, he had been in good health.

On physical examination, the patient appeared acutely ill and resisted movements because of back pain. His temperature was 103.2°F; blood pressure, 124/82 mm Hg; pulse, 88/min. and respirations, 20/min. A tender mass, 6 X 6 cm., was palpable just below the left ribcage. Blood analysis revealed WBC of 17,500 with 87% segmented neutrophils; hematocrit was 37 ml per 100 ml; and hemoglobin was 12.5 gm. per 100 ml. Routine urine analysis was normal and urine culture revealed no growth. Samples of blood were obtained for bacteriological culture and are provided for your examination.
CASE 11

A 9-month-old baby girl presented with low-grade fever, decreased appetite and irritability of 2 weeks duration. She had occasional vomiting, but no diarrhea or symptoms of upper respiratory tract infection. The mother had noticed that the patient had not been gaining weight over the past month. Patient smiled at 2 months, had no head lag at 4 months, and reached out for objects at 7 months of age. Past medical history was essentially negative.

On physical examination: temperature, 39.8°C; height, 77 cm.; weight, 7.15 kg; pulse, 100/min.; respirations 28/min. The patient was quite irritable, with non-bulging anterior fontanel, normal ear drums, no signs of upper respiratory tract infection, clear lungs, regular sinus rhythms, soft abdomen without organomegaly or masses, normal neurologic examination and no vaginal discharge. External genitalia were normal and the kidneys were palpable bilaterally.

LAB DATA
CBC: HGB - 11.0 gm%; HCT - 34 gm%; leukocytes - 9800/ cu. mm.; PMNs 78%, lymphocytes 2%, and monocytes - 2%. Urinalysis: color - turbid yellow; sp.gr. 1.005; sugar - +1; ketone - negative; RBC - 1/ hp field; WBC - 10-15/ hp field; casts - absent. Blood chemistry: normal BUN, sugar and electrolytes. Lumbar puncture: normal CSF.

Blood and urine samples are provided for your analysis.
CASE 12

A young girl, ten years old, was admitted to the hospital with a five-day history of malaise, anorexia, fever and increased sputum production. The patient’s weight was 41 pounds. This child has a unique family history: three siblings are deceased from cystic fibrosis and two other siblings are alive and show no clinical symptoms. There is no other history of family members having cystic fibrosis.

LAB DATA:

sodium - 135 meg/L; potassium - 4.8 meg/L; chloride - 100 meg/l; CO₂ - 22 meg/L; WBC - 13.2/ cu. mm.; RBC - 5.5/ cu. mm.; HGB - 13.0 g/dl; HCT - 40%.

Within 24 hours, the patient’s respiratory rate increased to 36/ min., her arterial oxygen pressure (pO₂) while receiving 40% oxygen by face mask was 45 mm Hg. The chest X-ray film showed atelectasis of the left lung. Endotracheal intubation and fiberoptic examinations were performed, which revealed purulent material in both bronchial trees.

A sputum sample from this patient is provided for your examination.
CASE 13

A 5-year old boy presented with the chief complaint of low-grade fever of 2 weeks duration associated with an earache and loss of appetite and malaise. Three days prior to admission, he developed a non-throbbing generalized headache, neck stiffness and progressive drowsiness. He became lethargic and had several episodes of vomiting not related to food intake. The past medical history was essentially negative. There was no history of head trauma.

On physical examination, temperature was 38.9°C, pulse 90/minute, BP 110/75 mm Hg, respiratory rate 28/minute, weight 18 kg. Examination revealed an ill appearing, apathetic and drowsy young boy with a stiff neck. Fundi were normal. The patient had positive Kernig’s and Brudzenski’s signs with increased deep tendon reflexes in both lower extremities. Babinski’s sign was negative. There were no localizing neurological deficits. There was no generalized lymphadenopathy. The remainder of the physical examination was within normal limits.

LABORATORY DATA:
CBC: Hgb 12 gm%, Hct 37%, WBC 12,000/ cu mm, PMN 70%, lymphocytes 28%, monocytes 2%. Urinalysis: normal. Blood chemistry: normal BUN, sugar, calcium and electrolytes. Lumbar puncture: opening pressure 300 mm; CSF color clear; cells 70 WBC, 60% lymphocytes; sugar 28 mg% (blood sugar 95 mg%), protein 110 mg%.

A sample of cerebral spinal fluid is presented for your analysis.
CASE 14

A 61-year-old insulin-dependent diabetic male presents to the hospital emergency room in October complaining of severe pain and swelling in his left forearm of one day's duration. The previous day the patient was working in a vacant lot clearing locust trees and brush. Early in the day the patient received a 1.0 cm puncture wound to the left forearm. This became contaminated with dirt as the day progressed. The patient claims that he thoroughly cleaned the wound upon arrival at home that evening.

On physical examination at the time of presentation to the ER, the patient appears critically ill with a temperature of 102°F, Pulse=80 and Respirations=18/min. The BP = 120/70 mm Hg. The head, lungs, heart and abdomen were normal. The left forearm is pale and swollen. There are splotches of erythema and a few vesicles. Crepitation is present. Pulses of the lower extremity are absent. Neurologic exam revealed absence of sensation over the left forearm in the distributions of the radial and ulnar nerves and very little sensation in the distribution of the median nerve. Serum obtained at this time appears normal in color and the urine is normal. X-ray films of the left forearm showed even distribution of gas in the soft tissues. The bones are normal.

LABORATORY DATA:
WBC 18.8/ mm$^3$ with a differential of 3% bands, 84% segmented forms, 10% lymphs, 2% monos, 1% eos. Glu=400 mg/dl; Ca=7.4 mg/dl; P 2.1 mg/dl; protein 5.6 mg/dl; BUN 15 mg/dl; bilirubin 0.6 mg/dl; SGOT 80 U/L; LDH 300 U/L; CPK 400 U/L; SGPT 10 U/L.

An aspirate of one of the vesicles present on the forearm is presented for your examination.
CASE 15

A 9-month-old baby girl presented with low-grade fever, decreased appetite and irritability of 2 weeks duration. She had occasional vomiting, but no diarrhea or symptoms of upper respiratory tract infection. The mother had noticed that the patient had not been gaining weight over the past month. Patient smiled at 2 months, had no head lag at 4 months, and reached out for objects at 7 months of age. Past medical history was essentially negative.

On physical examination: temperature, 39.8°C; height, 77 cm.; weight, 715 kg; pulse, 100/min.; respirations 28/min. The patient was quite irritable, with non-bulging anterior fontanel, normal ear drums, no signs of upper respiratory tract infection, clear lungs, regular sinus rhythms, soft abdomen without organomegaly or masses, normal neurologic examination and no vaginal discharge. External genitalia were normal and the kidneys were palpable bilaterally.

LAB DATA
CBC: HGB - 11.0 gm%; HCT - 34 gm%; leukocytes - 9800/ cu. mm.; PMNs 78%, lymphocytes 2%, and monocytes - 2%. Urinalysis: color - turbid yellow; sp.gr. 1.005; sugar - +1; ketone - negative; RBC - 1/ hp field; WBC - 10-15/ hp field; casts - absent. Blood chemistry: normal BUN, sugar and electrolytes.

Blood and urine samples are provided for your analysis.
CASE 16

Earlier this week, 17 cases of acute gastrointestinal disease occurred among 33 students and instructors at a class banquet.

Symptoms included nausea and vomiting (60%), fever (77%), diarrhea (90%), and abdominal cramps and pain (100%). All affected sought medical aid. Twelve were hospitalized, and the remaining 5 were treated in the hospital emergency room. Incubation periods of the illness ranged from 15 to 30 hours with a median of about 19 hours.

Food histories obtained from 23 of the guests implicated potato salad as the vehicle of transmission. The attack rate among those who ate potato salad was 83%, while 0% of those not eating the salad became ill.

A sample of the potato salad is provided for your examination.
CASE 17

A 53-year-old female with a history of chronic alcoholism was seen in the emergency room this morning complaining of left pleuritic chest pain of four days duration. While in the emergency room she experienced a profuse sweat and several severe coughing episodes. The patient reported several similar symptoms occurring over the past few days and also remembered a severe shaking chill a few days before that left her feeling that she might die.

Upon physical examination, the patient appeared acutely ill and toxic. A strong odor of alcohol was noted on her breath. Her oral temperature was recorded at 101°F.

A sputum sample was observed to be blood flecked and is provided for your examination. A blood sample was also taken and is provided.
CASE 18

A 31-year-old male is rushed to the emergency room of the local hospital. The patient was well until two days ago when he developed a fever. Since then he has felt progressively worse with continued fever, muscle aches, malaise and headache. That morning he appeared delirious and fainted, at which time he was rushed to the hospital.

His wife reported that 4 days ago he had cut his arm with a carving knife. Yesterday she noticed that the area around the cut was inflamed with small spidery red lines radiating from the cut. Examination today reveals a large area of erythema and swelling surrounding the cut and a small necrotic area near the center. Several small vesicles are noted within the area of inflammation.

At the time of admission, the patient’s temperature is 104°F, BP 70/30 mm Hg, Pulse=140, Respirations=24/min. The patient's lungs are clear, the neck is supple with no signs of meningeal irritation. During examination the patient experienced several shaking chills.

You are provided with an aspirate of a vesicle from the infection on the patient’s arm and a blood sample.
An 8-week-old boy was admitted to the hospital when he was found to be cyanotic and unresponsive. Past neonatal history disclosed that the baby had been delivered vaginally after a full-term uncomplicated gestation and normal labor. There had been no symptoms of illness in the two weeks prior to the present acute episode.

In the emergency room, he had a respiratory arrest. Resuscitation was effected, and physical examination showed a lethargic infant with poor response to pain. The tympanic membranes were erythematous and bulging. The neck was supple; the anterior fontanel was open and depressed.

Examination of the CSF disclosed a protein level of 354 mg/100 ml, a white blood cell count of 30/ml, and a glucose of 1 mg/100ml.

You are provided with a blood sample and a CSF sample for microbiological analyses.
CASE 20

An 8-year-old girl sustained a puncture wound of the right big toe as a result of stepping on a rusty nail. Four days later, swelling and pain developed and persisted despite warm soaks and intramuscular administration of penicillin and tetanus toxoid. She had received tetanus toxoid immunization previously on the normal schedule for childhood vaccinations. The inguinal lymph nodes were tender. Because of the persistant pain and the development of an abscess and cellulites at the puncture side, the patient was admitted to the hospital.

X-rays showed erosion of the medial base of the proximal phalanx. Incision and drainage was performed.

You are provided with a sample of material from the infected site for microbiological workup.
INTRODUCTION

Parasites important in infectious disease range from small microorganisms to worms many feet long. Yet, these complex groupings are traditionally taught together as part of infectious disease under the general heading “Parasitology.” The purpose of this lab is to illustrate several of the types of parasites covered in the lecture portion of the course.

PROCEDURES

Each lab group will have a box of prepared slides. Please examine the slides. Parasites are often identified based solely on their distinctive appearance. It is most helpful to draw each type as a means to help you learn more about each one.

Slide #

1 & 2. Entamoeba histolytica and Giardia lamblia trophozoites:
Draw and/or describe these organisms (nucleus, etc.). How do they differ from one another? What differentiates this ameba from others?

3. Trypanosoma cruzi in heart: Typically there is a mononuclear cell infiltrate. Do you see it in your slide? Do you see organisms inside cardiac muscle cells? What stage of the lifecycle is this? What are the other stages of the T. cruzi lifecycle? Does this trypanosome differ from others?

4 & 5. Plasmodium vivax and Plasmodium falciparum smears: Some blood smears are poorly-stained and the parasites are difficult to see. Look at the edges of the smears where the blood cells are not so dense. How can you distinguish parasitized red cells from PMNs, monocytes and platelets which are sticking onto red cells? Name the different stages of the lifecycle that you see on each slide and draw them below. How do the P. vivax-infected cells look different from the P. falciparum-infected cells? Can you make a definitive diagnosis from either side? Would other malarial species look more like P. falciparum or P. vivax or something else entirely?

6-8. Toxoplasma gondii, Pneumocystis carinii and Trichinella spiralis:
Describe the organisms. Are there any similarities?
9. *Enterobius vermicularis*: This is a cross-section of a mammalian appendix showing a cross-section of the worm. There are two wing-like appendages sticking out from the cuticle on opposite sides called the "lateral alae". What is the function of these appendages? What is the common name of this worm and how do you diagnose it? Does this worm have a complex lifecycle?

10 & 11. *Ancylostoma caninum* and *Taenia solium* scolex: *A. caninum* is dog hookworm that closely resembles the human worm. Does *T. solium* infect humans? What is the function of the complex structures seen in the front parts of each worm?

12. Human helminth ova: Examine the slide provided. Identify as many different ova as you can. Is there any advantage to identifying specific ova in terms of medical treatment?

**LAB REPORTS**

No lab report is required to be handed in. However, for personal knowledge, you should draw each parasite as a means to later recall it. You should also answer each of the questions listed under each slide.
LABORATORY VII
MYCOLOGY

INTRODUCTION

Fungi are eukaryotic microorganisms, which differ considerably from bacteria. Many fungi can be dimorphic, i.e. they can exist in the mold form or in the yeast form. Some fungi grow as yeast at 37°C and as molds at lower temperatures (e.g., *H. capsulatum*). Others, such as *C. neoformans* exist as yeast. *Aspergillus* sp. on the other hand, are representative of mold-like fungi. Identification and speciation of fungi relies heavily on microscopy to look for morphological differences known to exist among specific fungal species. Accurate identification in this way takes a great deal of experience. In this lab we will demonstrate some of the simplest microscope techniques and illustrate them using a few representative fungal species. You will observe several commercially-prepared slides, wet mounts that we have prepared for you and will be preparing your own India ink mounts. In all cases, associate these organisms with the diseases they cause.

**Common Laboratory Procedures Used to Identify Fungi of Medical Importance***

<table>
<thead>
<tr>
<th>Procedure</th>
<th>Advantage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wet mount of tissue or mucus-containing specimens in 100% KOH</td>
<td>Strong alkali degrades tissue and mucus and permits</td>
</tr>
<tr>
<td>visualization of fungi</td>
<td></td>
</tr>
<tr>
<td>Wet mount of portions teased from colonies and mounted in lactophenol</td>
<td>Permits observation of fungal morphology and presence of spores</td>
</tr>
<tr>
<td>cotton blue</td>
<td></td>
</tr>
<tr>
<td>Fixed slides stained with periodic-Schiff (PAS) or methenamine silver</td>
<td>Both PAS and silver stain fungal cell walls to give good contrast with</td>
</tr>
<tr>
<td>stains</td>
<td>background in tissue</td>
</tr>
<tr>
<td>sections or clinical materials</td>
<td></td>
</tr>
<tr>
<td>Sabouraud's glucose agar for culture; incubation at room temperature (RT)</td>
<td>Low pH of the medium and RT incubation favor growth of fungi over bacteria. Antibiotics may also be added to discourage</td>
</tr>
<tr>
<td>for up to 6 weeks, or at 37°C for number of days bacterial growth</td>
<td></td>
</tr>
<tr>
<td>Slide cultures, with inoculated blocks of Sabouraud's glucose agar are</td>
<td>Permits observation of relatively undisturbed fungal growth</td>
</tr>
<tr>
<td>covered with a coverslip, incubated &amp; examined in a lactophenol blue wet</td>
<td></td>
</tr>
<tr>
<td>mount</td>
<td></td>
</tr>
</tbody>
</table>

PROCEDURES

Materials

Cultures of the following fungi for observation:
- *Candida albicans*
- *Cryptococcus neoformans*
- *Aspergillus niger*
- *Penicillium chrysogenum*
- *Rhizopus tolonifer*

Fixed slide preparations of a variety of fungi:
In slide boxes A -
- *Candida albicans* - smear
- *Cryptococcus albidus* - smear
- *Mucor mucedo* - mycelium, sporangia
- Mold types
- Yeast - 3 types
In slide boxes B -
- *Aspergillus* conidiophores
- *Chaetomium* perithecia
- *Penicillium* conidia
- *Rhizopus* sporangia

Wet mounts of a variety of fungal cells:
Lacto-Phenol Cotton Blue preparations -
- *Aspergillus niger*
- *Penicillium Chrysogenum*
- *Rhizopus tolonifer*
India Ink preparations -
- *Cryptococcus neoformans*
- *Candida albicans* (for comparison)

Wet mounts and Gram stained smears of *Candida albicans* to observe special morphologies -
- *Candida albicans* from growth in plasma
- *Candida albicans* from growth on corn meal agar
Methods

A. Observations of colonial morphology
Observe the colonial morphology of each of the fungal cultures provided. DO NOT REMOVE culture lids unless told to do so. Make note of the color, size, texture and any other characteristics that you observe. Observe differences among the different cultures.

B. Microscopic examination of fungi

Fungal cells were prepared for observation by a variety of methods including simple wet mounts, wet mounts with Lacto-Phenol Cotton Blue staining, wet mounts with India Ink staining and dried smears stained with the Gram stain. The procedure for the Lacto-Phenol Cotton Blue staining and for the India Ink preparation are described below (these procedures have been completed for you).

India Ink Mounts (each group will prepare these): This is a method of demonstrating capsules in yeast such as Cryptococcus. To do this, suspend a small amount of microorganism in a drop of water on a slide. Then add a tiny amount of diluted India ink which is a colloidal suspension of minute carbon particles. Add a coverslip and examine. Where there are capsules, they will appear as clear halos holding back the tiny particles of India ink against a dark background.

Lactophenol Cotton Blue Wet Mounts (prepared for you): This is used for the microscopic examination of the filamentous fungi in a wet mount. It has three main purposes:

1. To exert an antifungal action on the organism being examined so as to reduce the hazard of infection to laboratory workers.
2. By virtue of the fluid's high viscosity to minimize the disruption of the fragile fungal structures. This is important because most fungal characterization depends on the observation of critical and often subtle aspects of microscopic morphology.
3. To provide, via the weak dye cotton blue, a background contrast making the rather transparent fungi more visible.

To make a wet mount of fungi, place a few drops of lactophenol cotton blue on a clean slide. Carefully transfer a small amount of mycelial growth into the fluid and gently tease apart. Probes are especially helpful in this operation. Add a coverslip and warm the slide gently to aid in further spreading out the growth. Examine with medium and high power objectives. Oil immersion is rarely needed.
Observe the microscopic morphology of the fungi provided for your examination, both fixed slides and wet mounts. Pay attention to the unique structures associated with each of the examples provided. Record your observations.

LAB REPORTS

No lab report is required to be handed in. However, for personal knowledge, you should draw each fungal type as a means to later recall it. Correlate each of the fungi observed to the possible infectious disease associated with it. Know how the infection is treated.
FREQUENTLY USED MICROBIOLOGICAL PROCEDURES AND MEDIA

CULTURE MEDIA

General Comments:

The choice of plating media for a given clinical specimen is governed generally by the source of the specimen and the disease suspected. The following can be used as a general guideline:

a. Specimens taken from areas of the body which are normally sterile, such as blood or spinal fluid, are cultured on rich, non-selective media (blood agar) which gives maximal opportunity for the recovery of any organisms present.

b. Specimens, such as stool samples, containing a large background of microbial species which might mask the presence of low but significant numbers of pathogens, are generally plated on media which favor the outgrowth or recognition of the pathogens. MacConkey and H-E media (see below) are two examples of such differential and selective media.

Media & other diagnostic procedures:

**Blood Agar**: An enriched nutrient agar containing sheep or other mammalian blood cells which act as indicators for the production of hemolysins.

**Hektoen Enteric (H-E) Agar**: A selective and differential growth medium used for the isolation of enterics. It contains bile salts which inhibit the growth of many Gram-positive organisms and selects for the growth of Gram-negative enteric organisms. The medium also contains lactose, a pH indicator, and ferric salts. Organisms which produce acid products from lactose fermentation convert the pH indicator and appear orange. This reaction is used to differentiate pathogens such as *Salmonella* and *Shigella*, which are unable to ferment lactose, from normal flora such as *E. coli* and *Klebsiella*, which are lactose fermentors. The ferric salts are present to identify organisms which produce H$_2$S which results in formation of a black precipitate (FeS). This reaction is used in differentiating *Salmonella* from *Shigella* species.

**Mueller-Hinton Agar**: A broth-based nutrient media which is used as the base medium for most antibiotic sensitivity tests.
MacConkey Agar: This is perhaps the prime medium for the detection of Gram-negative bacteria of significance in infection. It contains bile salts and crystal violet dye to inhibit Gram-positive bacteria. Therefore, virtually any colonies found on this medium will be some kind of Gram-negative bacteria - almost always of the enteric group. In addition, the medium contains lactose and the pH indicator neutral red. A major thing to know about any Gram-negative rod is whether or not it can ferment lactose to various short chain acids. Because of the components described above, colonies of lactose fermenting bacteria are colored red by the neutral red and are also often surrounded by white zones of precipitated bile salts which come out of solution at low pH's. Colonies of non-lactose fermenting bacteria are colorless and have no surrounding white zone.

Sabouraud Agar: This is the principal medium used to isolate yeasts and filamentous fungi (the "molds"). It is intended to minimize bacterial growth while favoring yeasts and fungi. It does this by a very high osmotic pressure provided by either dextrose or maltose, and by pH 5.6 at which most bacteria won't grow. The reason for this concern is that most bacteria grow far faster than yeasts and fungi and so would overwhelm the latter if measures were not taken to suppress the faster growers. As always, such attempts at suppression are not always successful, and so it is always possible that some colonies on Sabouraud agar are bacterial. Some formulations for this very important medium recommend the inclusion of such bacterial inhibitors as cycloheximide and chloramphenicol.

Chocolate Agar: This is simply super-heated blood agar (see Blood Agar). The purpose of the higher temperature is to burst the RBCs and release their contents which are otherwise unavailable to some fastidious and feeble bacteria that cannot do so for themselves. Hence this medium is used to cultivate such organisms as Neisseria, Hemophilus, etc. However, being a very rich medium, chocolate agar will grow not only these fastidious organisms but most other bacteria as well. Some specimens being tested for the presence of Neisseria such as urethral exudates and vaginal swabs are certain to contain many other bacteria which will flourish and obscure the Neisseria. Consequently, some modifications of chocolate agar have been devised to suppress the growth of most other bacteria so as to favor the growth of Neisseria. These include Thayer-Martin and Martin-Lewis agars which are basically chocolate agar to which various antibiotics have been added such as colistin, nystatin, vancomycin, etc.

Cornmeal Agar: The main ingredient of this medium literally is an aqueous extract of cornmeal and not much else. It is used to detect the formation of the characteristic chlamydospores of certain species of the yeast genus Candida. Candida: While there are numerous species of this genus, currently we are most interested in differentiating C. albicans from C. parapsilosis.
These tests help:

<table>
<thead>
<tr>
<th></th>
<th>C. albicans</th>
<th>C. parapsilosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chlamydospore</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Germ tubes</td>
<td>+</td>
<td>-</td>
</tr>
</tbody>
</table>

**Campylobacter Agar**: This consists of a very rich medium to which is added 5% sheep erythrocytes and several antimicrobials including amphotericin B, cephalothin, polymyxin B, trimethoprim and vancomycin. These are intended to inhibit the growth of most other bacteria. This medium should be incubated in a special reducing atmosphere.

**Capsule Detection**: The most common way of detecting capsules in bacteria is by observing clear halos around cells in Gram stains. There are specific capsule stains, but they are complex, lengthy, and rarely done.

**India Ink Mounts**: This is a method of demonstrating capsules in yeast such as Cryptococcus. To do this, suspend a small amount of microorganism in a drop of water on a slide. Then add a tiny amount of diluted India ink which is a colloidal suspension of minute carbon particles. Add a coverslip and examine with the oil immersion objective. Where there are capsules, they will appear as clear halos holding back the tiny particles of India ink against a dark background.

**Urea Agar**: Important ingredients of this medium are urea and the indicator phenol red. If an inoculated bacterium produces urease, that enzyme will produce ammonia from the urea and consequent alkaline pH. Phenol red which is colorless at neutral and acid pH's is red under alkaline conditions. Consequently, a change from the faint yellow color of the uninoculated slant to red indicates urease production. This medium is useful in the differentiation of Proteus species which are urease positive (often in 4-6 hours) from Salmonella and Shigella which are negative. Similarly it helps differentiate Cryptococcus (positive) from other yeasts.

**Lowenstein-Jensen (L–J) Medium**: This medium is used primarily for the cultivation of mycobacteria although it will also grow Nocardia and staphylococci. Its most important components are potato flour, homogenized eggs and the dye malachite green. There is no agar-agar. Instead, the medium is solidified in tubed slants by steaming until the eggs congeal - a sort of hardboiled medium. The purpose of the malachite green is to inhibit most other
microorganisms. This is especially important because specimens - such as sputum - are heavily contaminated by many other bacteria all growing faster and more profusely than mycobacteria. The medium is dispensed in screw-capped tubes that must be tightly sealed to prevent dehydration as such cultures have to be incubated for weeks if not months.

**Litmus Milk:** This is a simple medium that can yield a wide variety of information. It consists merely of skimmed milk and litmus indicator. Its uninoculated appearance is a purple/blue opaque liquid. After inoculation and incubation of 24 hours, any of the following results may be observed.

Litmus Test (continued):

1. A persisting purple/blue color (alkaline) indicates no lactose fermentation.
2. A pink color (acid) indicates lactose fermentation.
3. A white color indicates reduction of the litmus.
4. Solidification (usually together with reduction) indicates clotting of casein.
5. Progressive clearing and transparency indicates "peptonization" - i.e., hydrolysis of casein by proteolytic enzymes.

Perhaps the most important use of litmus milk is in the characterization of *Clostridium perfringens* which violently ferments lactose to acid and gas as it does in tissues (hence its fame in "gas gangrene"). In litmus milk inoculated with *C. perfringens* and incubated anaerobically, what one sees is a rapid succession of acid production, reduction, solidification, peptonization, vast gas production which if incubated long enough will force the contents of the culture out of the top of the tube. This is the famous "stormy fermentation".
SPECIAL CONSIDERATIONS

Anaerobic culture:

If an anaerobic culture is requested, or if indicated by nature of specimen, appropriate agar plate is placed in the anaerobic jar, and by using "GasPak", according to directions, hydrogen and carbon dioxide gases are generated to produce an anaerobic atmosphere.

Instructions for use of "GasPak"

1. Cut off corner of envelope and place the envelope so it stands against the wall of the jar.
2. Add 10ml of tap water through the open corner of envelope.
3. Place an anaerobic indicator in the jar.
4. Promptly place lid on jar and seal.
5. Incubate jar in the regular incubator.

Agar disc diffusion susceptibility testing procedure:

Results of antimicrobial susceptibility tests guide the clinicians in initial and later treatment of the patient. They are also used for epidemiological purposes in detecting outbreaks of hospital-acquired infections and in locating resistant strains in community. Susceptibility tests results must be accurate and they must be standardized so that they can be compared with results obtained in different locations and situations.

Preparing inoculum

1. Select 4-5 similarly appearing colonies of the organism to be tested from pure culture or the primary isolation plate.
2. Dip a sterile cotton swab into the selected colonies by touching the top of each.
3. Streak the swab over the agar surface evenly.
4. Place discs on the agar with a dispenser. Press discs down gently.
5. Incubate plates immediately in the incubator.

Reading plates

1. Read the zone size around each disc. Measure zone diameters.
2. Complete inhibition of growth as judged by the naked eye.
3. Proteus mirabilis and Proteus vulgaris may swarm into areas of inhibited growth around certain antimicrobics. Zones of inhibitions are usually clearly outlined.
4. Diffusion techniques have been standardized with rapid growing pathogens, such as staphylococci and Enterobacteriaceae, and cannot be reliably applied to slow growing organisms which may show much larger zone sizes than rapid growers. Therefore, susceptibility tests on organisms which are fastidious in their nutritional requirements, require an anaerobic atmosphere (e.g. detecting sulfonamide resistance of *Neisseria meningitidis*).
ENTEROBACTERIACEAE

characteristics & identification procedures

Family characteristics

1. Gram negative rods
2. Grow well on MacConkey
3. Aerobic to facultatively anaerobic
4. All members ferment glucose with acid or acid and gas production.
5. Oxidase negative (for this test, use colonies from blood agar not MacConkey.)

Initial test used to identify Enterobacteriaceae

<table>
<thead>
<tr>
<th>Test</th>
<th>Incubation Period</th>
</tr>
</thead>
<tbody>
<tr>
<td>KIA</td>
<td>24 hr</td>
</tr>
<tr>
<td>IMVIC</td>
<td></td>
</tr>
<tr>
<td>Indole</td>
<td>24 hr</td>
</tr>
<tr>
<td>Methyl red</td>
<td>about 48 hr (3-5 days recommended)</td>
</tr>
<tr>
<td>Voges-Proskauer</td>
<td>48 hr</td>
</tr>
<tr>
<td>Citrate</td>
<td>24 hr</td>
</tr>
<tr>
<td>Motility</td>
<td>24 hr</td>
</tr>
<tr>
<td>Urease</td>
<td>24 hr</td>
</tr>
</tbody>
</table>
**Kligler’s Iron Agar (KIA)**

**Principle:**

Determination of lactose (1% concentration) glucose (0.1% conc.) fermentation, gas production, and H$_2$S production. Carbohydrate metabolism occurs both aerobically (on the slant) and anaerobically (in the butt).

**Procedure:**

Inoculate by streaking the slant, then stabbing the butt to the bottom of the tube. Incubate for 24 hours.

**Possible reactions:**

1. Fermentation of glucose only - On the slant, glucose is aerobically degraded to pyruvic acid, which initially turns the phenol red indicator yellow. Pyruvate is then further degraded to CO$_2$ and H$_2$O. If the organism can degrade only glucose, the low glucose concentration is used up, with eventual release of CO$_2$. The organism then utilizes the peptones present in the medium. This results in the release of ammonia yielding an alkaline pH, which turns the slant red.

   In the butt, glucose is degraded anaerobically. CO$_2$ cannot be released from the butt, and the acid end-products cause the butt to remain yellow.

2. Fermentation of lactose and glucose. Because the lactose concentration is 10 times that of glucose, during the 24 hr incubation period, the lactose has not yet been depleted, and an acid condition still exists. If the same KIA were read after 48 hrs. or longer, the slant would eventually turn alkaline due to depletion of lactose and the utilization of peptones.

3. Neither lactose nor glucose fermented. These results are indicative of a nonenteric Gram negative rods (not member of the family Enterobacteriaceae)

1. Hydrogen sulfite production. If H$_2$S is produced, it combines with ferric ions in the medium to yield ferrous sulfide, an insoluble black precipitate.
### Kligler Iron Agar Reactions

<table>
<thead>
<tr>
<th>KIA Reaction (slant/butt)*</th>
<th>Carbohydrate Fermented</th>
<th>Possible Organisms</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td><strong>Enterobacteriaceae</strong></td>
</tr>
<tr>
<td><strong>1.</strong> A / AG H₂S⁻</td>
<td>lactose + glucose with gas</td>
<td>E. coli&lt;br&gt;Klebsiella&lt;br&gt;Enterobacter group</td>
</tr>
<tr>
<td><strong>2.</strong> A / AG H₂S⁺</td>
<td>lactose + glucose with acid &amp; gas</td>
<td>Arizona&lt;br&gt;Citrobacter (rarely Salmonella)</td>
</tr>
<tr>
<td><strong>3.</strong> Alk / AG H₂S⁻</td>
<td>lactose – or slow + glucose with acid &amp; gas</td>
<td>Salmonella group A&lt;br&gt;Proteus – Providentia&lt;br&gt;K.E. group&lt;br&gt;E.coli</td>
</tr>
<tr>
<td><strong>4.</strong> Alk / A noG H₂S⁻</td>
<td>lactose – or slow + glucose with acid, no gas</td>
<td>Shigella&lt;br&gt;Proteus&lt;br&gt;anaerogenic Providentia&lt;br&gt;Serratia, E. coli</td>
</tr>
<tr>
<td><strong>4a.</strong> as in 4. but very slight H₂S⁺</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>5.</strong> Alk / AG H₂S⁺</td>
<td>lactose – or slow + glucose with acid &amp; gas</td>
<td>Salmonella&lt;br&gt;Proteus&lt;br&gt;Arizona&lt;br&gt;Citrobacter&lt;br&gt;Edwardsiella</td>
</tr>
<tr>
<td><strong>6.</strong> Alk / Alk no gas</td>
<td>lactose – glucose – &quot;non-fermenters&quot;</td>
<td></td>
</tr>
</tbody>
</table>

* A= acid; G= gas production; Alk = alkaline