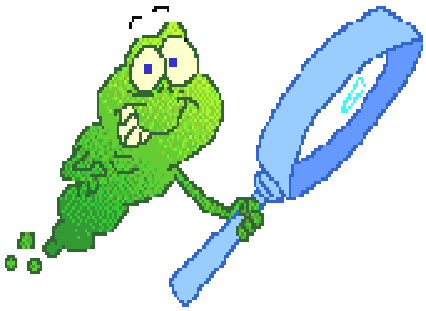
Diagnostic Medical Microbiology

Laboratory Manual





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INTRODUCTION

This diagnostic microbiology manual is designed to be used in conjunction with lecture textbook and other resources. Microbiological techniques are different in many ways when compared with other laboratory disciplines. Although results are not obtained in a short time, the time required to perform the test is very short. Most of the techniques are simple, yet requires a great deal of theoretical background to be correctly interpreted. For this reason, each laboratory exercise was supplemented with the theory behind it.

As a guide for the student, each experiment was started by the title OBJECTIVE, which means what is required from the student to learn upon completion of the exercise. It is in the form of questions (name, define, discuss, etc.). Students should try answering all the questions when reading and working each exercise.

Before performing any of the exercises in this manual, one should read the safety precaution and measures as well as the exercise (the materials needed, the procedures, and the expected results). This will ensure the safety of the student and also will ensure good results.

General View on the Parameters Used in the Process of Microorganism Identification

Before one can proceed to identify a microorganism, the characteristics of that organism have to be determined in details. The major characteristics which are observed include the following:

A. Cultural Characteristics

In clinical terms, it is the shape, size, color, elevation and other characteristics of the colony formed on the culture plate. In taxonomy, it includes the nutrient requirements for the growth of the organism and the physical factors such as temperature, pH and the incubation period. These factors are used to identify certain pathogenic species but less commonly used in routine procedures.

The cultural characteristics of a microorganism usually vary depending on the media used and many other factors. Some experienced microbiologists could have a good guess about the identity of a microorganism just by its cultural characteristics, but this was proven to be a bad technique. Students as well as microbiologists are advised to follow strict procedures for the identification of isolates from clinical specimens.

B. Morphology and Staining

This includes the microscopic appearance of a stained preparation of the organism. Useful information to be taken into account, are the size of the individual cells, cell shape and arrangement and staining reaction if differential staining procedures is used.

EXAMPLE: A gram stained film prepared from a pure culture of certain microorganism shows the following:

-Small spherical cells "Cocci"
-Arranged in clusters
-Gram-positive = violet in color

Some laboratories which have a little facility could give the report of a microbiological examination of a clinical specimen just by stating their morphological characteristics and the sensitivity testing results.

C. BIOCHEMICAL CHARACTERISTICS

Frequently, the identity of a species requires detailed knowledge of its biochemical activities, since other characteristics are not sufficiently distinctive or differential. For example, the bacterium *Escherichia coli*, a normal inhabitant of our intestinal tract, is indistinguishable microscopically from Salmonella typhi, the bacterium that causes typhoid fever. However, if these two bacteria are examined for their metabolic (or biochemical) characteristics, they are found to be very different and distinguishable on this basis.

Numerous laboratory techniques are available for the characterization of microorganisms. In general, the microorganism is grown in the presence of a specific substrate, after which the culture is examined to determine what chemical changes have taken place. This subject will be discussed in details in other parts of the handout.

D. SEROLOGICAL CHRACTERISTICS

Sometimes, to identify a species as *E. coli* is insufficient, for the reason that some strains of this organism are non-pathogenic and others are highly associated with diseases. Serological testing in such case will identify the exact strain number based on testing against prepared specific antisera.

In-Vivo serological tests (skin tests) are of great value in the diagnosis of many bacterial, fungal and viral infections.

E. OTHER CHARACTERISTICS

To identify some strains of bacteria, one may need to look for other characteristics than those mentioned above. Phage typing and animal inoculation are examples of uncommon techniques used in the identification process.

General Information

The microbiology laboratory is considered to be vital and the take the great amount of the general work load of the laboratory. Receiving and recording specimens, culturing, staining, isolation and identification of pathogens and doing sensitivity tests for the isolated pathogens are the major tasks.

Who Can Request Laboratory Services

1. All licensed physicians, dentists and optometrists.
2. All public health nurses and physicians assistants.
3. Local Health Departments.
4. Communicable Disease Specialists.

Reports shall be given only to the submitter. Private individuals will not receive reports.

Information For Microbiology Laboratory Staff

General Requirements for Collecting and Submitting Specimens

Proper collection and adequate amounts of specimen are required. The following criteria should be used as guidelines:
Medical Group employees who handle laboratory specimens have relatively high rates of work-related hepatitis and other transmittable diseases. Loosely capped containers and soiled requisitions sent to the laboratory are a significant risk to all who come in direct contact with these contaminated materials or areas contaminated by such materials. Therefore, laboratory staff will not accept soiled laboratory requisitions/leaking specimen containers.

When needed, a written test request must include the following information:

Patient details

- Hospital No.
- Name: First name and family name
- Sex
- Date of birth/Age
- Address
- Social security no. (insurance)
- For females: whether pregnant or lactating
- Details of illness
- Presenting signs/symptoms
- Duration/date of onset
- Recent travel history
- Immunizations

Identification of Specimens

- Type of specimen (Exact source and nature of specimen)
- Collection date and time
- Laboratory number
- Laboratory findings
- Tests requested
- Ordering physician

Also, **ALL** specimens must be properly labeled with the patient's full name, date and time of collection, and specimen source.

Swab Specimens: Separate specimens must be submitted for each specific request, i.e., one for bacterial culture, one for fungal culture, etc.

Special Culture / Specimen Requirements:

Anaerobic Specimens: Submit in anaerobic transport containers or in a sealed syringe with no bubbles.

SPECIMEN COLLECTION

Proper specimen collection, container labeling, and culture requests are the responsibility of the ordering physician. Technologists in the Clinical Microbiology Laboratory will be familiar with specimens of choice and proper collection techniques.

The technologist in the laboratory will directly handle specimens of clinical and environmental source which are received from the Postal Service or hand carried to the laboratory.

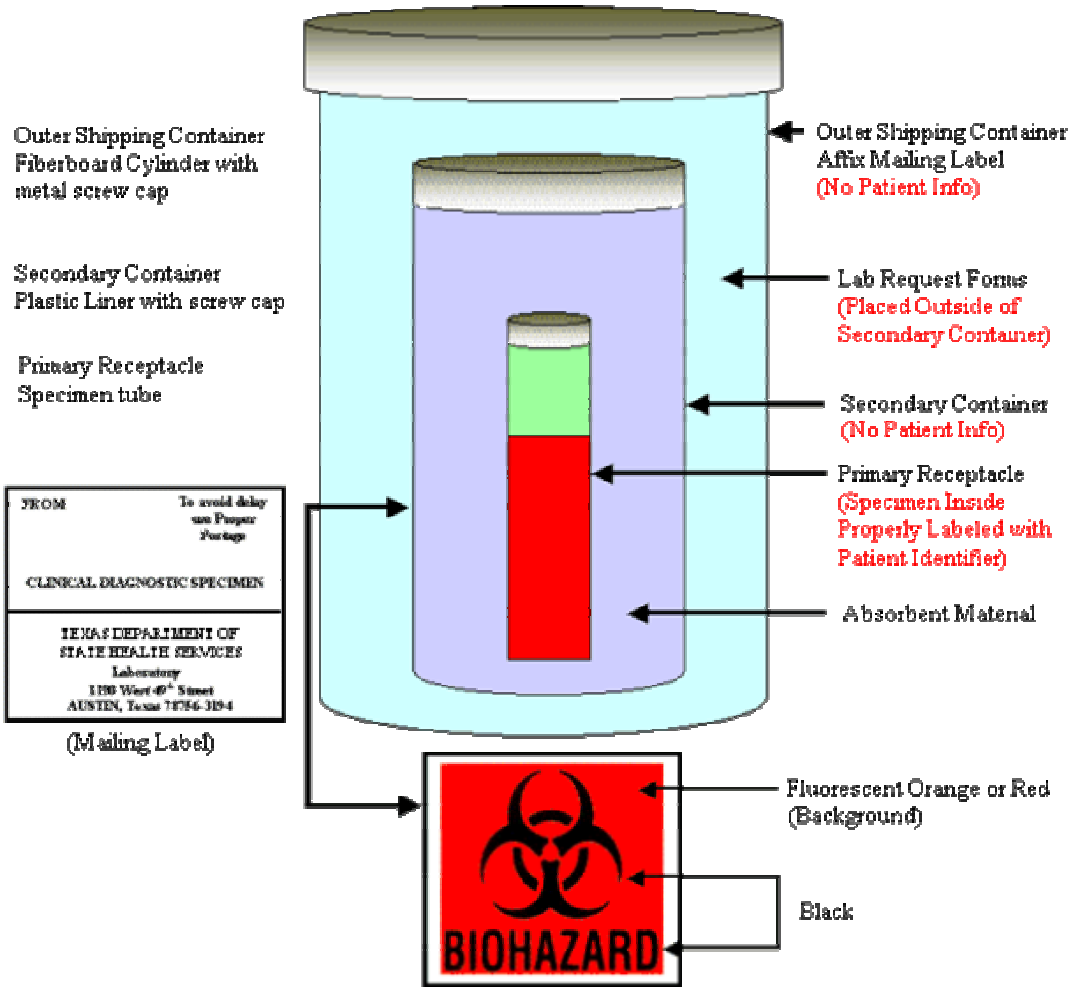
The technologists will handle the clinical specimens completely by the following procedure.

SPECIMEN HANDLING AND STORAGE

Specimen containers and requisitions will be delivered to the **Clinical Microbiology Central Processing Area (CPA)** within the specified period (depending on the specimen source and type) of collection. Upon receipt, the CPA staff will check requisitions for completeness. Specimens will be stored properly until they are picked-up by the microbiology staff. The CPA staff will assign numbers for the specimens and indicate them on the original requisitions. When **STAT** requests are received, the CPA staff will immediately notify the microbiology supervisor and arrange for the immediate delivery of the specimen.

SPECIMEN DELIVERY

The Clinical microbiology Laboratory recommends that a specimen should be transported to the laboratory as soon as possible (a maximum delay is indicated for each type of specimen; see next sections for individual instructions). If more than one specimen is received for one type of analysis, the Processing Area staff will note on the requisition "**Duplicate Specimen**" in red ink.



Actively growing cultures of organisms for identification should be submitted on tubed media appropriate for the organism being submitted. Seal the tube with water proof tape. All specimen containers should be closed tightly or sealed in order to prevent leakage and contamination. Media in Petri dishes or liquid cultures are not an acceptable transport media.

LABELING, LOGBOOK

Upon receiving the specimen and requisition with complete data., record it in the microbiology log book in numeral order. The number assigned to the specimen is written on the specimen container and the requisition form, culture media containers, and culture media plates. In addition, date and time of processing and the name of the patient should be written clearly on all

culture plates, tubes, slides or whatever used in the processing of the specimen.

SPECIMEN REJECTION CRITERIA.

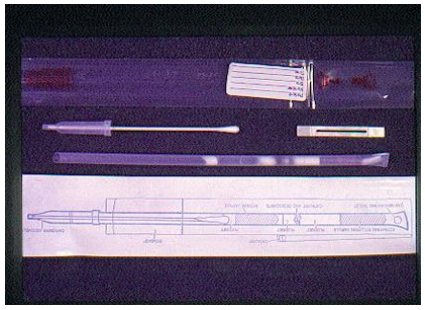
General Issues

In general, specimens for the microbiology laboratory are unacceptable if any of the following conditions apply:

1. The information on the label doesn't match the information on the request form.
2. The specimen was transported in an improper container or at wrong temperature .
3. The quantity of the specimen is insufficient to carry out all the required examination.
4. Leaking specimen

BACTERIOLOGY

- Blood received in blood culture bottles is unsuitable for fungal isolation.
- Saliva is unacceptable for culture. Submit "deep cough or induced sputum."
- Multiple urine, stool, sputum, or routine throat specimens sent on the same day from the same source from the same patient



Other specimens unsatisfactory for cultures are:

- Specimens in fixative (Formalin).
- Dried out swabs.
- Foley Catheter tips.
- 24 hour urine/sputum for routine bacteria, or fungi.
- Urine held two hours or more at room temperature.
- Fluids received in culturette tubes.
- Swab material for anaerobic culture not in the proper anaerobic transport.
- Gram stains for *Neisseria gonorrhea* on vaginal or anal crypt specimens are not diagnostic and will not be performed.
- Stool specimens for culture from a patient who has been an inpatient greater than 5 days will not be performed.
- Anaerobic cultures on vaginal, cervical, urine (unless suprapubic tap), sputum or fecal specimen.

Every effort should be made to contact the physician or unit if a specimen is rejected. *The physician will be informed about the reason/s for specimen rejection.*

REPORTING OF MICROBIOLOGY RESULTS
(Pathogenic Organisms versus Normal Flora)
Reporting of Bacteriology Results.

CSF, Body Fluids, Blood, and Wounds: Positive gram stains, upon preliminary examination, will be reported to the physician. Identification of an organism isolated will be performed on all aerobic organisms as appropriate.

Ear: Potential pathogens, i.e., ***S. aureus*, gram-negative organisms** will be identified and antimicrobial sensitivities performed.

Eye: Report identification on any organisms isolated. Sensitivities as appropriate

Gastrointestinal: Routine screen for **Salmonella**, **Shigella**, and special cultures for **Campylobacter**, **Vibrio**, and ***E. coli* 0157:H7**. The lab will report Pseudomonas and Staphylococcus aureus. Proteus will be reported on the pediatric patients. Negative cultures will be reported as "**No Enteric Pathogens Isolated**."

Lower Respiratory (Aspirates): Report any pathogenic organisms isolated

Nasal/Nasopharyngeal: Report any gram negative rod, ***S. aureus, S. pneumoniae, H. Influenzae, N. meningitidis*, Group A Streptococcus.**

Skin: Predominant organism will be identified. No sensitivity will be performed on **coagulase negative Staph**.

Sputum: Specimens evaluated as adequate for culture are screened for potential pathogens. **Legionella** must be specifically requested.

Throat Cultures: Routinely screened for **Group A Strep**. Reported as Positive for **Beta-hemolytic Strep. Group A**, or Negative for Group A Strep. Physician must specify if a culture is to be screened for other than group A Strep. A positive Strep list is provided daily to Primary Care and Emergency Room for immediate follow-up.

Mouth Cultures: Must specify organism of interest to be screened, i.e., ***C. albicans, C. diptheriae,*** etc., or Cystic Fibrosis patient for potential pathogens. Tooth sockets from dental clinic will be screened for any predominating organism.

Urines: Report identification and antimicrobial sensitivities on colony counts greater than 10,000. Female Urine will be screened for ***S. saprophyticus***. Susceptibility testing will not be performed on **Streptococci (other than Enterococci**), ***Corynebacterium species**, or **Lactobacillus***. Plates with three or more organisms will be reported as "three or more organisms, please repeat." Mixed flora of less than 10,000 colonies each will be reported as "**Normal Skin Flora**."

Vaginal/Cervical: Report predominant organism. Mixed cultures of **Lactobacillus**, **diphtheroids**, **staphylococcus**, **alpha streptococcus, Acinetobacter**, members of **Enterobacteriaceae and yeast** will be considered **Normal Vaginal Flora**.

Reporting of Susceptibility Testing Results.

After initial identification and susceptibility testing, susceptibility testing will be performed every 4-5 days on biotypically identical organisms isolated from the same patient from the same site. Cultures will be held 48 hours should the clinician feel that repeat susceptibility testing is indicated.

Reporting of TB/MYCOLOGY Results.

All acid-fast and fungal organisms isolated will be reported.

Urine Culture

Aim of the test
An etiological diagnosis of bacterial urinary tract infection by quantitative cultivation of the urine with identification and susceptibility test of the isolated bacteria(s).

Types of specimen
Urine (Midstream urine), suprapubic aspiration, catheterized urine.

Criteria of specimen rejection
Un-refrigerated specimen older than 2 hours may be subject to overgrowth and may not yield valid results; unlabeled specimen; mislabeled specimen; specimen in expired transport container; 24 hours urine specimens.

Pathogens and commensals

Urine specimen	
Common pathogens	**commensal flora**
Neisseria gonorrhoeae	the urine is sterile except for the urethral mucosa which support the growth of microflora as:
E. coli and other Enterobacteriaceae	
Enterococcus spp	Diphtheroid bacilli
Staphylococcus aureus	*Lactobacillus* spp
Staph saprophyticus	Coagulase negative *Staphylococc*i
Corynebacterium jeikeium	α Haemolytic *Streptococci*
Acinetobacter spp	Bacillus spp
Pseudomonas spp	Non pathogenic *Neisseria spp.*
Gardnerella vaginalis	Anaerobic cocci
ß-haemolytic streptococci	Commensal *Mycobacterium*
Salmonella spp (early stage of infection)	Commensal *Mycoplasma spp.*
Parasites	
Schistosoma haemetobium	
Trichomonas vaginalis	

Pre specimen processing

Patient preparing
Instruct the procedures for the patient

Specimen collection

- Collection of midstream urine for bacterial investigation:
- Patient not needing assistance:
- Give the patient a suitable container.
- Instruct the patient before the collection, preferably with illustration.
- Tell the patient not to touch the inside or rim of the container.

Male:

1. If not circumcised, draw back the foreskin.

2. Begin to urinate, but pass the first portion into the toilet.

3. Collect the mid-portion of urine into the container, and pass the excess into the toilet.

Female:

1. Squat over the toilet and separate the labia with one hand.

2. Void the first portion of urine into the toilet.

3. Collect the mid-portion of urine into the container and pass the excess into the toilet.

Infants:

- Have ready: Clean, preferably sterile container of appropriate size or a plastic bag, cotton wool or gauze pads, handwarm soapy water.

1. Clean the external genitals.

2. Give the child as much liquid as possible just prior to the collection.

3. Seat the child on the lap of the mother, nurse or ward attendant.

4. Collect as much urine as possible in the container or plastic bag when the child urinates.

Note: First morning specimens yield highest bacterial counts from overnight incubation in the bladder, and are the best specimens. Colony count interpretation standards are based on controlled studies from first early morning collections. Forced fluids or random specimens dilute the urine and may cause reduced colony counts. Hair from perineum will contaminate the specimen. The stream from a male may be contaminated by bacteria from beneath the prepuce. Bacteria from vaginal secretions, vulva or distal urethra may contaminate transport. Organisms from hands or clothing might contaminate. Receptacle must be sterile. Read Patient Preparation.

Who will collect the specimen
Midstream urine is collected by the patient. If disabled, nursing staff will assist in collection. For catheterized specimen, nursing staff will collect the specimen. Suprapubic aspiration is performed by the physician.

Quantity of specimen
To fill line on transport tube (~20 mL)

Time relapse before processing the sample
The maximum time allowed for processing a urine sample is 2 hours from the time of collection

Storage
At room temperature unless delay is inevitable; it must be refrigerated



Specimen processing

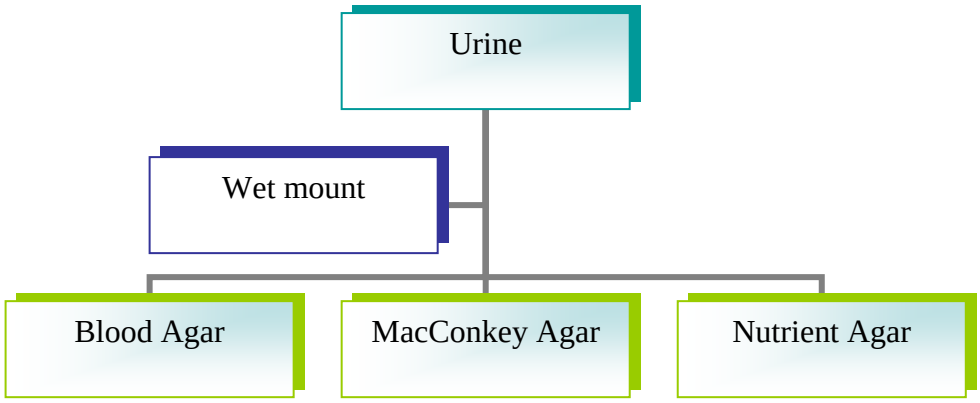
Media

1. Blood Agar,
2. MacConkey Agar
3. Nutrient Agar

Culturing procedure

- Mix the urine sample to re-suspend microorganism present.
- Dip a 1 µl calibrated loop in vertical position in the urine and remove the loop and use the collected fluid to inoculate a nutrient agar plate that will be used for urine plate count.
- Take another loop to streak Blood agar and another loop to streak MacConkey agar plates.
- Streak the Nutrient agar plate to cover all surface area.

A plate count of 10,000 CFU/ml of pure culture should be considered positive and isolated organism should be identified and sensitivity test will be performed.



Post specimen processing

Interfering factors:
Patient on antibiotic therapy.
Improper sample collection.

Result reporting:
Report wet mount as an initial report.
Report the isolated pathogen and its sensitivity pattern as a final report.

Turn around time:
Wet mount results should be available 1 hour after specimen receipt.
Isolation of a possible pathogen can be expected after 2-3 days. Negative culture will be reported out 1-2 days after the receipt of the specimen.

Additional information
A single culture is about 80% accurate in the female; two containing the same organism with count of 10^5 or more represents 95% chance of true bacteriuria; three such specimens mean virtual certainty of true bacteriuria. Urinary tract infection is significantly higher in women who use diaphragm-spermicide

contraception, perhaps secondary to increased vaginal pH and a higher frequency of vaginal colonization with *E. coli*. A single clean voided specimen from an adult male may be considered diagnostic with proper preparation and care in specimen collection. If the patient is receiving antimicrobial therapy at the time the specimen is collected, any level of bacteriuria may be significant. When more than two organisms are recovered, the likelihood of contamination is high; thus, the significance of definitive identification of the organisms and susceptibility testing in this situation is severely limited. A repeat culture with proper specimen collection including patient preparation is often indicated. Periodic evaluation of diabetics and pregnant women for asymptomatic bacteriuria has been recommended.[2] Institutionalized patients, especially elderly individuals, are prone to urinary tract infections, which can be severe. Cultures of specimens from Foley catheters yielding multiple organisms with high colony counts usually represents colonization of the catheter and not true significant bacteriuria. Most laboratories limit the number of organisms which will be identified when recovered from urine to two. Similarly, most do not routinely perform susceptibility tests on isolates from presumably contaminated specimens. Failure to recover aerobic organisms from patients with pyuria or positive Gram's stains of urinary sediment may indicate the presence of mycobacteria or anaerobes. As the number of patients who are chronically catheterized increases, so does the controversy on what constitutes a diagnostic specimen. Few clinical studies have been performed to support the identification of more then two organisms or implicate usual site flora (eg, diptheroids, alpha or gamma streptococci, and coagulase-negative staphylococci other than *S. saprophyticus*).

Blood Culture

Aim of the test
An etiological diagnosis of bacteremia by aerobic and anaerobic cultivation of the blood, with identification and susceptibility test of the isolated organism(s). Blood culture should be made for cases with suspected septicemia, endocarditis, and bacteremia secondary to localized infections (pneumonia, intraabdominal abscesses, pyelonephritis, epiglottitis, meningitis). In this case the blood culture may provide an etiological diagnosis of the localized infection.

Types of specimen
Whole blood

Criteria of specimen rejection
Blood collected in tubes or bottles other than aerobic and anaerobic blood culture bottles. If the information on the label does not match that of the request form. Specimens for anaerobic blood culture received in aerobic bottles or vice versa.

Pathogens

Blood is a sterile body fluid and normally contains commensals

Common pathogens	
Streptococcus spp	*Bacteroides fragilis* and other anaerobic bacteria
Staphylococcus aureus	Coagulase negative staphylococci
Listeria monocytogenes	Enteric gram negative bacilli
Corynebacterium jeikeium	*Neisseria meningitides*
Haemophilus influenza	Non fermenter gram negative bacilli
Salmonella typhi	
Pseudomonas aeruginosa	
Fungi	
Candida albicans	*Cryptococcus neoformans*
Other *candida* spp	*Coccidoides immitis*
Histoplasma capsulatum	

Pre specimen processing

Patient preparing

The major difficulty in interpretation of blood cultures is potential contamination by skin flora. This difficulty can be markedly reduced by careful attention to the details of skin preparation and antisepsis prior to collection of the specimen.

Skin preparation: First cleanse the vein puncture site with isopropanol. Then use tincture of iodine or povidone iodine to disinfect the site using progressively larger concentric circles. Iodine should remain in contact with skin for about 1 minute or until dry to ensure disinfection. The vein puncture site must not be palpated after preparation. Blood is then drawn. Following vein puncture, alcohol is used to remove the iodine from the site.

Specimen collection
Blood cultures should be drawn prior to initiation of antimicrobial therapy. If more than one culture is ordered, the specimens should be drawn separately at no less than 30 minutes apart to rule out the possibility of transient bacteremia by self-manipulation by the patient of mucous membranes in the mouth caused by brushing teeth, etc or by local irritations caused by scratching of the skin.

The time of collection must be indicated. Strict aseptic technique is essential. If present remove the plastic cap from the blood culture bottles, swab the stoppers with tincture of iodine or povidone iodine and allow to dry. Collect 20 mL blood in a sterile plastic syringe and inoculate at least 10 mL blood (as indicated on bottle) into each bottle or use Vacutainer® and butterfly collection set and monitor the fill using the graduations on the side of the bottle. For more information about the amount of blood, please refer to the blood bottles manufacturer's user guide.

Quantity of specimen
Volume inoculated in sets of culture bottles for aerobic and anaerobic cultivation

Children below 2 years	I mL of venous blood in 2 bottles
Children *2-5* years	2 mL of venous blood in 4 bottles
Children 6-10 years	3 mL of venous blood in 4 bottles
Children 11-15 years	*5* mL of venous blood in 4 bottles
Children above 15 years and adults	5 mL venous blood in three sets of bottles (6 bottles).

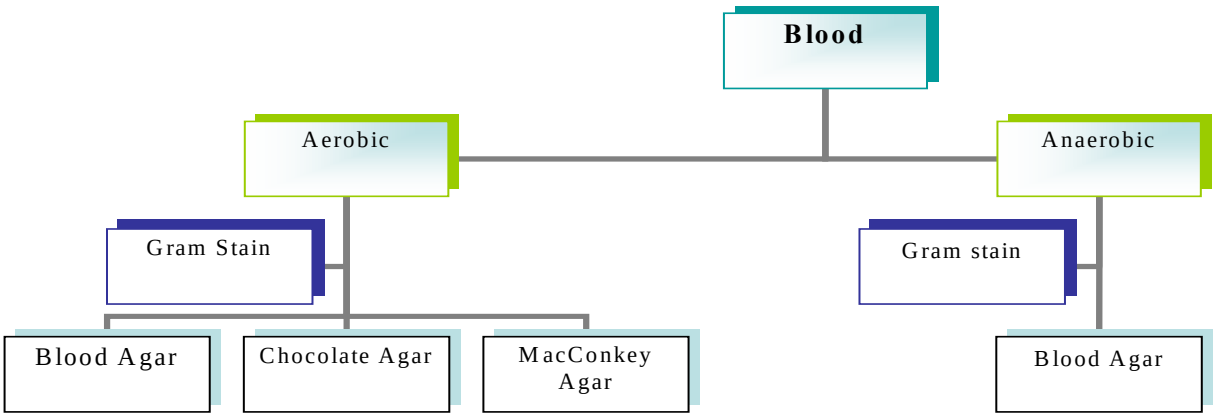
Storage
Pre-incubate or maintain specimen at room temperature. Do not refrigerate

Container
One aerobic and one anaerobic blood culture bottle. Do **not** vent.

Specimen processing

Media
Aerobic Blood culture bottle
Anaerobic Blood culture bottle
MacConkey Agar
Blood Agar
Chocolate Agar

Method
Blood is injected to both aerobic and anaerobic bottles and incubated for up to 10 days at 37 ^{o}C. Discard as negative after the 10 days incubation period is expired. During the incubation period, a gram stain and subculture onto appropriate media should be done. See diagram below:



Post specimen processing

Interfering factors

Patient on antibiotic therapy

Result reporting:
Any isolated organism will be reported. Antibiotic sensitivity will also be included with the report.

Turn around time
Initial blood culture results will be reported as soon as it shows growth. Final results with sensitivity will be issued after 24-48 hours of the initial report. Negative results will be issued after 10 days of culture submission.

Interpretation of Positive Blood Cultures

- Virtually any organism, including normal flora, can cause bacteremia
- A negative culture result does not necessarily rule out bacteremia; false-negative results occur when pathogens fail to grow
- A positive culture result does not necessarily indicate bacteremia; false-positive results occur when contaminants grow.
- Gram-negative bacilli, anaerobes, and fungi should be considered pathogens until proven otherwise.
- The most difficult interpretation problem is to determine whether an organism that is usually considered normal skin flora is a true pathogen.

Limitations

Three negative sets of blood cultures in the absence of antimicrobial therapy are usually sufficient to exclude the presence of bacteremia. One set is seldom ever sufficient.[1] Prior antibiotic therapy may cause negative blood cultures or delayed growth. Blood cultures from patients suspected of having *Brucella* or *Leptospira* must be requested as special cultures. Consultation with the laboratory for special culture procedures for the recovery of these organisms prior to collecting the specimen is recommended. Yeast often are isolated from routine blood cultures. However, if yeast or other fungi are specifically suspected, a separate fungal blood culture should be drawn along with each of the routine blood culture specimens. See separate listing for proper collection of Blood Fungus Culture. *Mycobacterium avium* complex (MAC) is frequently recovered from blood of immunocompromised patients, particularly those with acquired immunodeficiency syndrome, AIDS. Special procedures are required for the recovery of these organisms; Contact lab.





Cerebrospinal fluid (CSF)

Aim of the test
Diagnosis of the bacteria or fungal meningitis by microscopic examination and culture with identification and susceptibility test of the isolated organism.

Types of specimen
CSF
Criteria of specimen rejection
Non sterile container and the general causes for rejection stated in the introduction

Pathogen and commensals

Infection of C.S.F
CSF is a sterile fluid and does not contain any commensals, however, care should be taken not to contaminate the specimen with skin normal flora during collection.
Common bacterial pathogen
Haemophilus influenzae
Neisseria meningitis
Streptococcus pneumoniae
Group A & B *streptococci*
Gram negative bacilli
Listeria monocytogenes
Treponema pallidum (rare)
Brucella (rare)
Salmonella (rare)
Toxoplsma (rare)
Microbes that cause chronic meningitis
M. tuberculosis
Cryptococcus neoformans
Coccidoides immitis
Histoplasma capsulatum
Blastomyces dermatitides
Candida spp.
Nocardia
Actinomyces

Pre specimen processing

Specimen collection

Who will collect the specimen
Only physicians

Quantity of specimen
3 ml of CSF is sufficient for culture

Time relapse before processing the sample
CSF is an emergency specimen and should be processed immediately

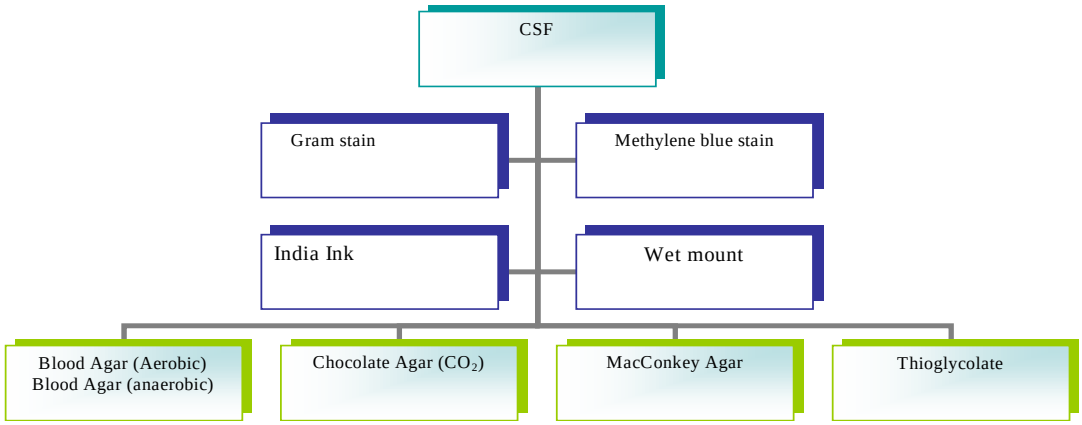
Storage
Room Temperature

Specimen processing

Media

- 2 Blood Agar
- Chocolate Agar
- MacConkey Agar
- Fluid Thioglycollate

Culturing procedure
As a general rule in CSF and body fluid specimens for culture, centrifuge clear specimen and inoculate plates and do staining from sediments. While turbid specimens may not be centrifuged.



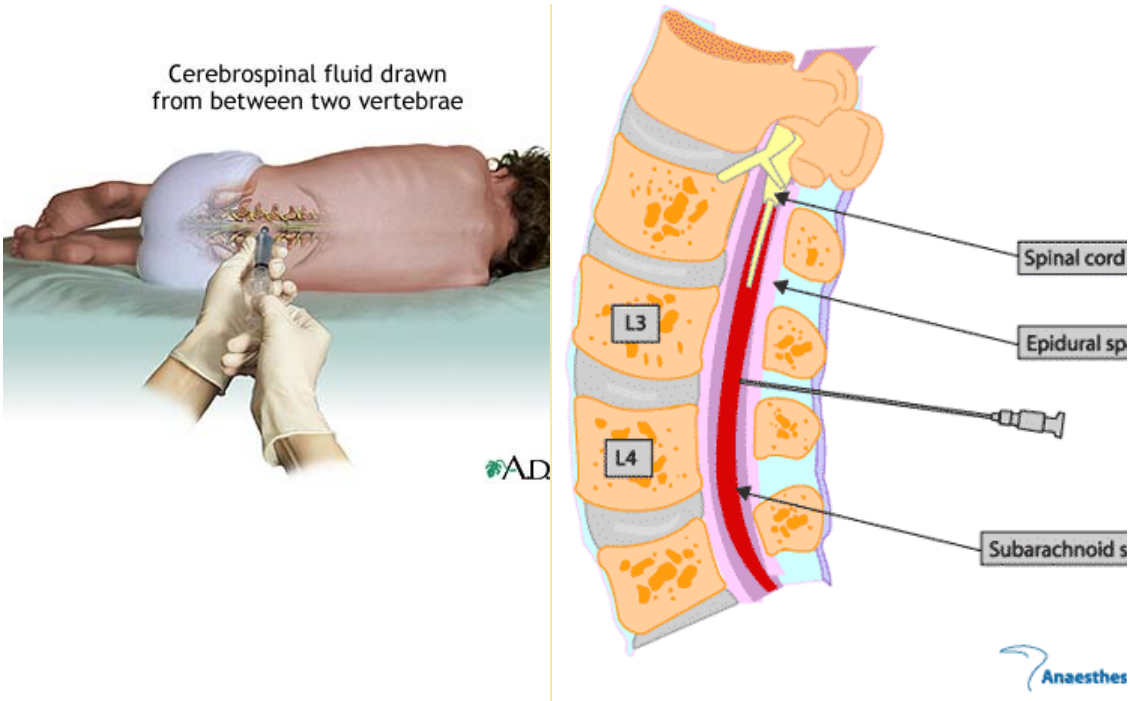
Post specimen processing

Interfering factors
Patient on antibiotic therapy.
Improper sample collection.

Result reporting
Results of the microscopy and all positive cultures of CSF are reported immediately to the treating physician. Negative bacterial results are sent out 72 hours after the CSF is received.

Turn around time
Gram stain result is reported within 30 minutes of specimen receipt
Positive Culture results = 3- 5 days
Negative Culture results = 2-3 days

Additional information
Several antigen detection methods are available for the direct detection of the polysaccharide capsular antigen of *H. influenzae*, *N. meningitidis*, *S. pneumoniae* and Group B streptococci in CSF which showed specificity and sensitivity of about 90-97%. Direct detection of Cryptococcus antigen in CSF is also available which replaced India ink in many laboratories.
The routine culture for CSF does not include all organisms mentioned in the above table.



Body Fluid Culture, Sterile

Synonyms
Culture, Body Fluid, Sterile, Routine; Sterile Body Fluid Culture, peritoneal, pericardial, plural, ascitic, synovial, etc.

Aim of the test
Isolate and identify pathogenic organisms from normally sterile body fluids and perform sensitivity test

Types of specimen
Aseptically aspirated body fluid (e.g., , synovial, peritoneal fluid).

Criteria of specimen rejection
Inappropriate specimen transport device; mislabeled specimen; unlabeled specimen; specimen received after prolonged delay (usually more than two hour); specimen received in expired transport media

Pathogen and commensals

infection of sterile body fluid	
all body fluid are sterile	
common pathogenic of precarditis and myocarditis	***Pleural fluid***
Mycoplasma pneumoniae	*Staphylococcus aureus*
Chlamydia trachomatis	*Streptococcus pneumoniae*
Mycobacterium tuberculosis	*Haemophilus influenzae*
Staphylococcus aureus	*Enterobacteriacae*
Streptococcus pneumoniae	*Pseudomonas spp.*
Enterobacteriacae and other gram negative bacilli	*Anaerobic bacteria*
	Mycobacterium tuberculosis
Bones and joints	*Actinomyces spp.*
Staphylococcus aureus	
Streptococcus pyogenes	**Peritoneal fluid**
Haemophilus influenzae	*Streptococcus pneumoniae*
Enterobacteriacae	Group A streptococci
Mycobacterium spp	Enterobacteriacae
Neisseria gonorrheae	Other gram negative bacilli
Streptococcus pneumoniae	Staphylococci
	Neisseria gonorrheae
	Chlamydia trachomatis

Pre specimen processing

Patient preparing
Swab skin over the site of puncture with 2% tincture of iodine in concentric circles.

Note: Iodine should remain in contact with skin for at least 1 minute prior to puncture to ensure complete antisepsis. Following puncture, 70% alcohol is used to remove iodine from skin.

Specimen collection
Contamination with normal flora from skin, rectum, vaginal tract, or other body surfaces should be avoided. Indicate the specific source and pertinent clinical history on the request form.

Who will collect the specimen
Physician

Quantity of specimen
1-5 mL is adequate.

Time relapse before processing the sample
Body fluids should be treated as CSF specimens and should processed immediately.

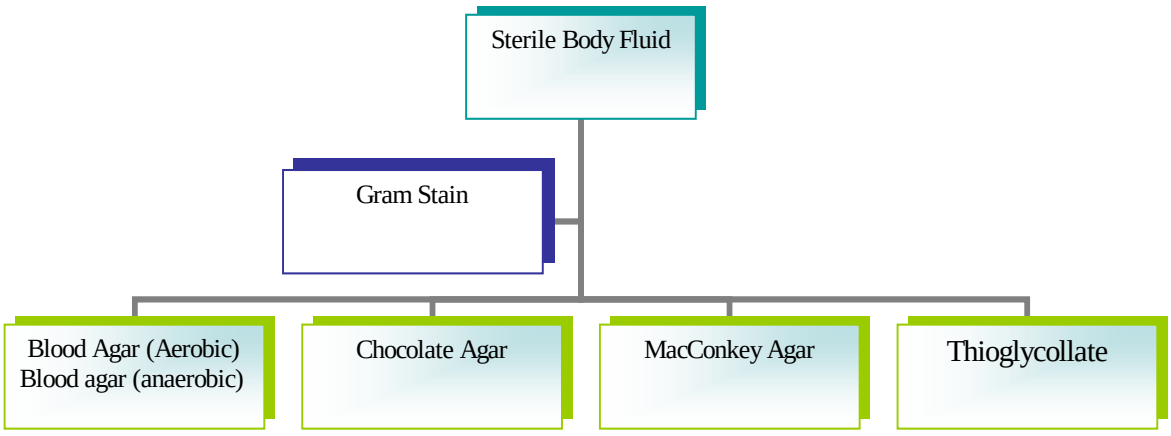
Storage
Maintain specimen at room temperature. Do not refrigerate.

Specimen processing

Media

1. Blood Agar (2 plates)
2. Chocolate Agar,
3. MacConkey Agar
4. Thioglycollate broth

Culturing procedure

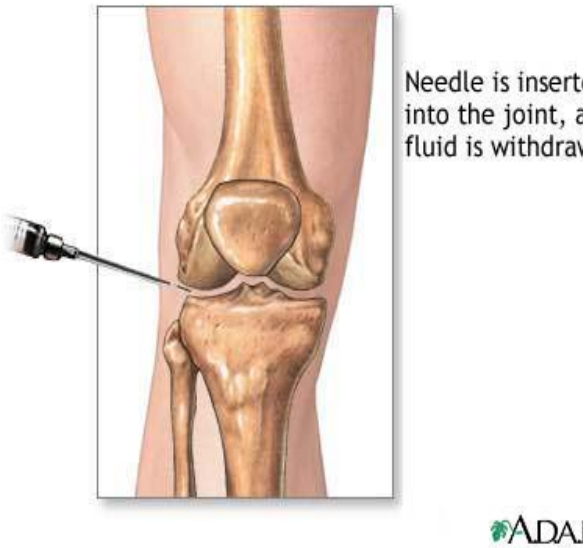


Post specimen processing

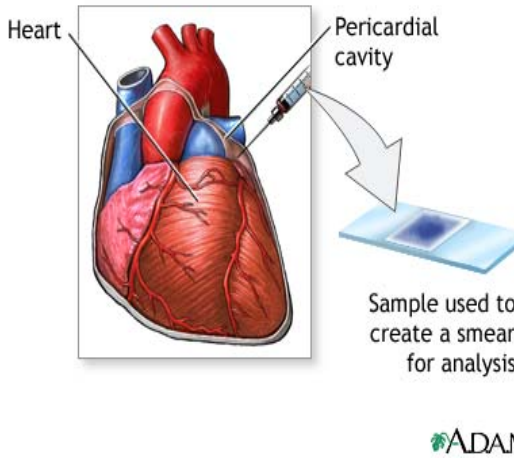
Interfering factors:
Patient on antibiotic therapy.
Improper sample collection.

Result reporting:
Report Gram stain finding as an initial report.
Report the isolated pathogen and its sensitivity pattern as a final report.

Turn around time:
Gram stain and wet mount results should be available 1 hour after specimen receipt.
Isolation of a possible pathogen can be expected after 2-3 days. Negative culture will be reported out 1-2 days after the receipt of the specimen.



Synovial fluid collection



Pericardial fluid

Conjunctival Discharge

Aim of the test
An etiological diagnosis of bacterial conjunctivitis by aerobic cultivation with identification and susceptibility test of the isolated bacteria .

Types of specimen
Discharge from the eye(s).

Criteria of specimen rejection
Inappropriate specimen transport device; mislabeled specimen; unlabeled specimen; specimen received after prolonged delay (usually more than two hour); specimen received in expired transport media

Pathogen and commensals

Infection of the EYE	
Common pathogen	**Commensals Bacteria**
Streptococcus pyogenes	*Staphylococcus epidermidis*
Pseudomonas aeruginosa	*Lactobacillus spp*
Chlamydia trachomatis	*Propionibacterium spp*
Streptococcus pneumoniae	*Staphylococcus aureus*
Haemophilus influenzae	Various Enterobacteriaceae
Haemophilus aegyptius	Various *streptococcus spp*
Staphylococcus aureus	Occasion *pseudomonas aeruginosa*
Neisseria gonorrheae	
Acremonium curvularia	

Pre specimen processing

Specimen collection

1. Pull down the lower eyelid so that the lower conjunctival fornix is exposed.
2. Swab the fornix without touching the rim of the eyelid with the sterile cotton swab.
3. Place the swab immediately in a bacterial transport medium or, if the specimen is brought to the laboratory immediately, in a sterile test tube with *0.5* mL of buffered saline (pH 7).

Quantity of specimen
Sufficient amount on swab

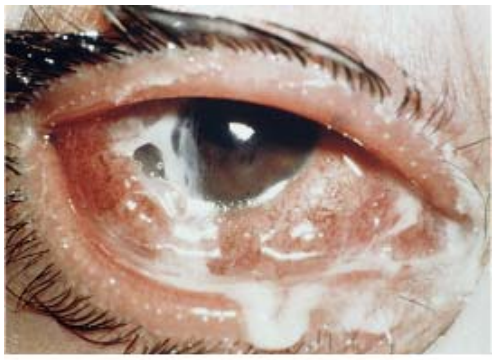
Time relapse before processing the sample
Eye specimen should be processed immediately because tears contains lysosomes which may kill the organism

Storage
Refrigerated (2-8 ^{0}C).

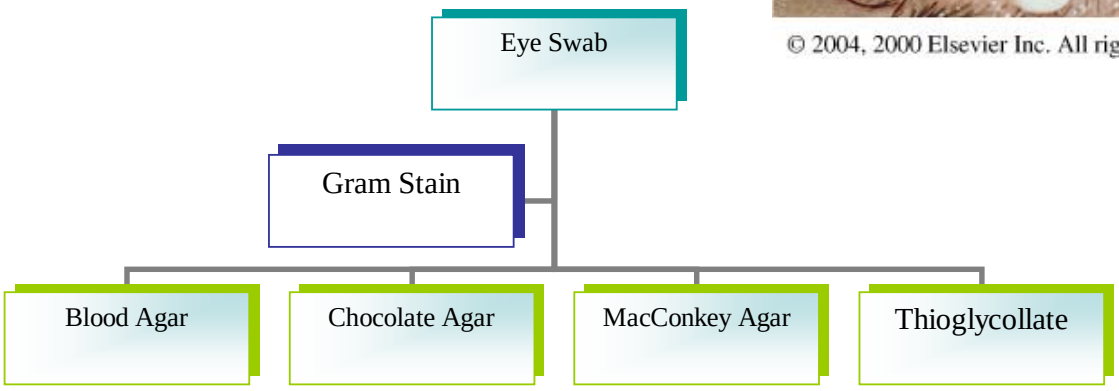
Specimen processing

Media

- Blood Agar
- Chocolate Agar
- MacConkey Agar
- Fluid Thioglycollate


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Culturing procedure



Post specimen processing

Result reporting:
Report Gram stain finding as an initial report.
Report the isolated pathogen and its sensitivity pattern as a final report.

Turn around time:
Gram stain results should be available 1 hour after specimen receipt.
Isolation of a possible pathogen can be expected after 2-3 days. Negative culture will be reported out 1-2 days after the receipt of the specimen.

Additional information
All bacteria isolated in fair amounts and not resembling contaminants will be identified and tested for antibiotic susceptibility, including susceptibility to chloramphenicol.

- If trachoma is suspected, conjunctival scraping should be smeared onto a microscopic slide, air-dried and fixed in absolute methanol. Chlamydia antigen detection systems are available for this purpose.

Ear Discharge

Aim of the test
Aetiological diagnosis of external or media otitis by aerobic and anaerobic culture with identification and susceptibility test of the isolated organism(s).

Types of specimen
Pus from the external or middle ear.

Criteria of specimen rejection
Inappropriate specimen transport device; mislabeled specimen; unlabeled specimen; specimen received after prolonged delay (usually more than two hour); specimen received in expired transport media

Who will perform the test
Medical technologist, Microbiologist

Who is authorized to order the test
Physician

Pathogen and commensals

Infection of Ear	
common pathogens	**Commensal flora** are present in the external ear canal
Staphylococcus aureus	*Staphylococcus epidermidis*
Streptococcus pyogenes	*Lactobacillus spp.*
Pseudomonas aeruginosa	*Propionibacterium spp.*
Other Gram negative bacilli	*Staphylococcus aureus*
Streptococcus pneumoniae	Various Enterobacteriaceae
Haemophilus influenzae	Various *Streptococcus* spp
Anaerobic bacteria	*Candida spp*. other than albicans
Proteus spp.	Occasion *Pseudomonas aeruginosa*

Pre specimen processing

Patient preparing
Instruct the patient with the procedures

Specimen collection

1. Collect a specimen of the discharge on a thin, sterile cotton wool or dacron swab.
2. Place the swab in a container with the transport medium, breaking off the swab stick to allow the stopper to be replaced tightly.
3. Label the specimen and send it to the laboratory.

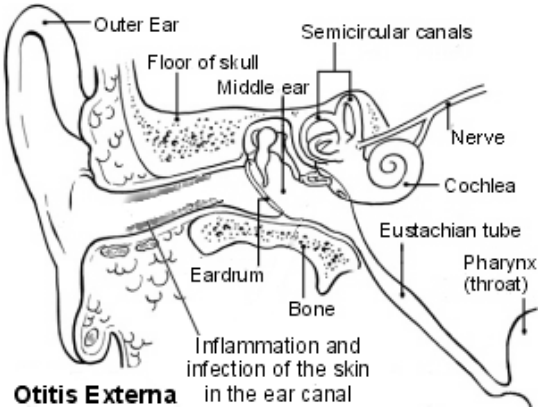
Time relapse before processing the sample
Not more than 2 hours

Storage
Refrigerated (2-8 ^{0}C).

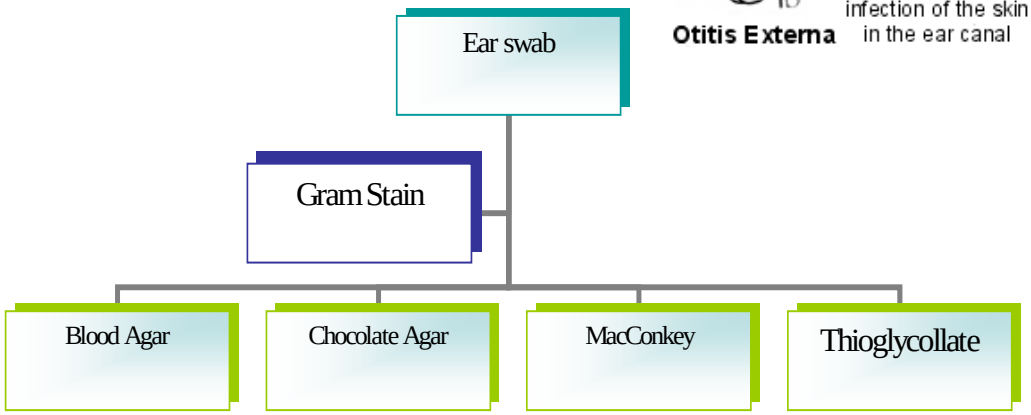
Specimen processing

Media

1. Blood Agar,
2. Chocolate Agar,
3. MacConkey Agar
4. Fluid thioglycollate



Culturing procedure



Post specimen processing

Interfering factors:
Patient on antibiotic therapy.
Improper sample collection.

Result reporting:
Report Gram stain finding as an initial report.
Report the isolated pathogen and its sensitivity pattern as a final report.

Additional information

For external ear infections only *Staphylococcus aureus, Streptococcus pyogenes, Pseudomonas aeruginosa, Vibrio* spp. and *Aspergillus* will be looked for and reported. For middle ear infections only pneumococcus, *Streptococcus pyogenes, Haemophilus influenzae* and *Staphylococcus aureus* will be reported with a susceptibility test. For the chronic discharging ear, *Bacteroides* species and fungi will also be reported in addition to the organisms reported for middle ear infections

Genital Culture and sensitivity

Aim of the test
Isolate and identify potentially aerobic pathogenic organisms including *Gardnerella vaginalis* and group B *Streptococcus*; establish the diagnosis of gonorrhea, medical/legal cases.

Types of specimen
Swab of vagina, cervix, discharge, aspirated endocervical, endometrial, prostatic fluid, or urethral discharge. Use swab to inoculate Jembec for transport to the laboratory and recovery of *Neisseria gonorrhoeae*; swab should also be sent in transport device.

Criteria of specimen rejection
Inappropriate specimen transport device; mislabeled specimen; unlabeled specimen; specimen received after prolonged delay (usually more than 72 hours); specimen received in expired transport media or container.

Pathogen and commensals

Genital Tract Infection	
Pathogenic bacteria	**Commensals bacteria**
Neisseria gonorrheae	Coagulase negative *Staphylococci*
Group B *Streptococci*	*Corynebacterium spp*.
Gardnerella vaginalis	*E.coli* and other coliform
Enterococcus spp.	Many species of anaerobic
Certain anaerobes including *Actinomyces spp.*	
Haemophilus ducreyi	
Treponema pallidum	
Mycoplasma spp.	
Enterobacteriaceae	
Chlamydia trachomatis	
Fungi	
Candida albicans	
Parasite	
Trichomonas vaginalis	
Viruses:	
Herpes simplex virus	
Human papilloma virus	

Pre specimen processing

Specimen collection
Females: Do **not** use lubricant on speculum. Cervical mucous should be removed first before inserting swab into endocervical canal, move swab from

side to side allowing several seconds for absorption of organisms by the swab. Return swab to the transport tube and label.
Males: Using small wire swab, gently scrape the anterior urethral mucosa or, use a swab to collect specimen of urethral discharge.

To overcome some of the problems involved in obtaining adequate amount for the recovery of organisms in suspected cases of STD, kits containing swabs, slides suitable for collecting such specimens are now available and could be used in physicians offices.

Quantity of specimen
Sufficient amount on swab

Time relapse before processing the sample
30 min.

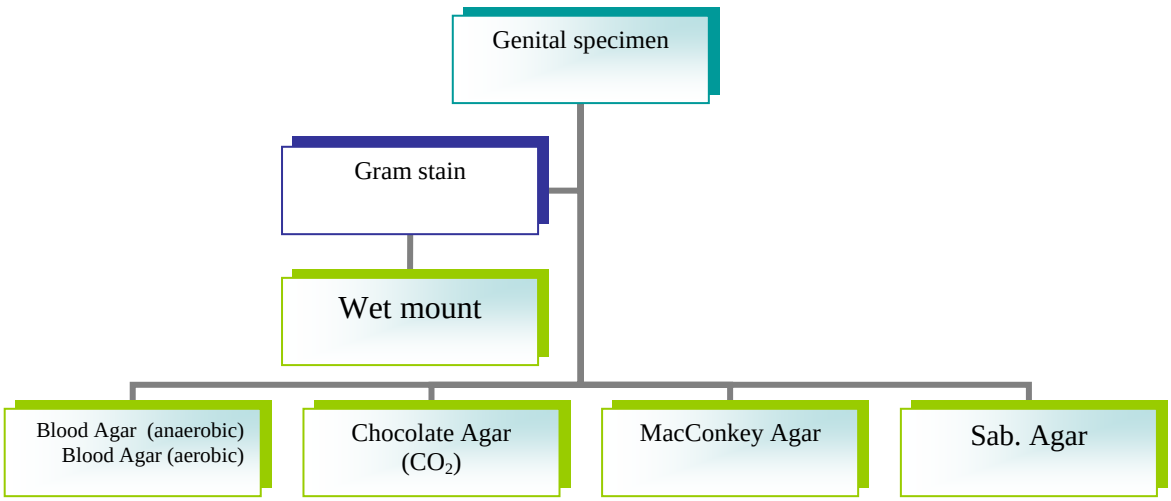
Storage
Maintain specimen swab at room temperature. Do not refrigerate.

Specimen processing

Media

- Blood Agar (2 plates)
- Chocolate Agar,
- MacConkey Agar
- Sab. Agar

Culturing procedure
Streak two blood agar plates, one chocolate, MacConkey and Sab agar plate. Do wet mount t examine for Clue cell and Trichomonas, and gram stain to check the predominant organisms.

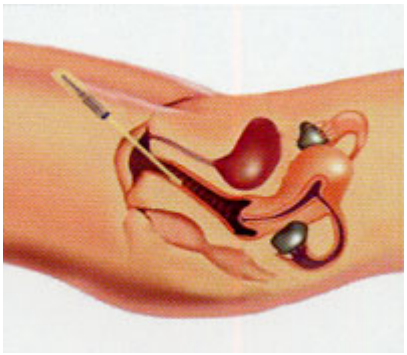


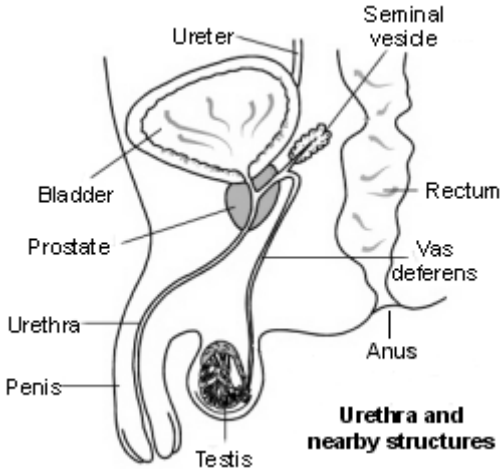
Post specimen processing

Interfering factors:
Patient on antibiotic therapy.
Improper sample collection.

Result reporting:
Report wet mount Gram stain finding as an initial report.
Report the isolated pathogen and its sensitivity pattern as a final report.

Turn around time:
Gram stain and wet mount results should be available 1 hour after specimen receipt.
Isolation of a possible pathogen can be expected after 2-3 days. Negative culture will be reported out 1-2 days after the receipt of the specimen.





Pus(wound, Abscesses, Burns and sinuses) culture & sensitivity

Aim of the test
Isolate and identify aerobic and anaerobic pathogenic organisms pus specimen.

Types of specimen
Swabs from the infected area or aspiration from deep wounds. Swabs in anaerobic transport media for the isolation of anaerobes.

Criteria of specimen rejection
Inappropriate specimen transport device; mislabeled specimen; unlabelled specimen; dried samples and specimen received after prolonged delay (usually more than 72 hours); specimen received in expired transport media

Pathogen and commensals

Pus Infection	
Pathogenic bacteria	**Commensals bacteria**
Pseudomonas aeruginosa	Alpha haemolytic streptococci
Proteus spp	*Corynebacterium spp*.
E. coli	Coagulase negative Staph.
Klebsiella spp	*Propionobacterium spp.*
Morganella	*Bacillus spp.*
Providencia	
Streptococcus pyogenes	
Staphylococcus aureus	
Enterococcus spp.	
Clostridium perfringens	
Fusobactrium spp	
Peptostreptococcus spp	
Mycobacterium tuberculosis	
Nocardia spp.	
Actinomyces israelii	

Pre specimen processing

Specimen collection
Pus from abscess is best to collected at the time, the abscess is incised and drained. Using sterile technique, aspirate or collect from drainage tube up to 5 ml of pus, transfer to sterile container. If pus is not being discharged use sterile cotton wool swab to sample from the infected site ,extend the swab deeply into the depth of the lesion. Immerse the swab in container of transport medium , label it and send to the laboratory as soon as possible

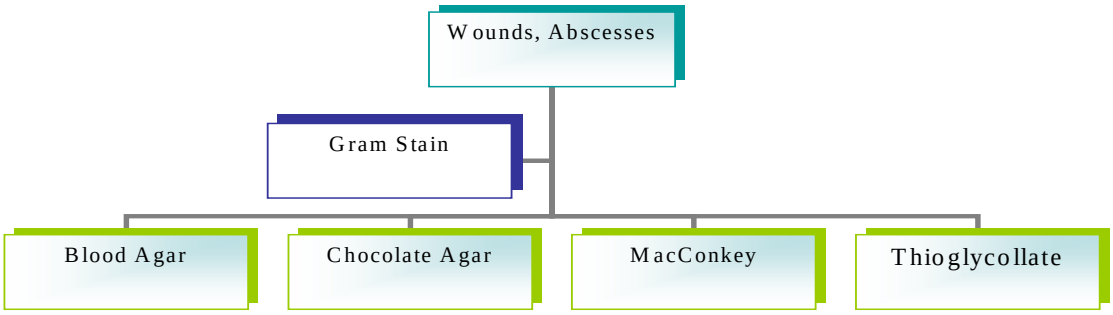
Quantity of specimen
Sufficient amount on swab, or aspiration in transport media or syringe.
Time relapse before processing the sample
30 min.
Storage
Maintain specimen swab at room temperature. Do not refrigerate.

Specimen processing

Media

- Blood Agar,
- Chocolate Agar,
- MacConkey Agar
- Thioglycollate broth

Culturing procedure
Streak one blood agar plates, one chocolate, MacConkey and inoculate thioglycollate broth tube. Gram stain to check the presence or absence and if present the type or types and the predominant organisms.



Post specimen processing

Interfering factors:
Patient on antibiotic therapy.
Improper sample collection.
Result reporting:
Report Gram stain finding as an initial report.
Report the isolated pathogen/s and its sensitivity pattern as a final report.
Turn around time:
Gram stain results should be available 1 hour after specimen receipt.
Isolation of a possible pathogen can be expected after 2-3 days. Negative culture will be reported out 1-2 days after the receipt of the specimen.

Additional information
Contamination of the specimen with normal flora is one of the major obstacles in obtaining good results. Care should be taken to avoid contaminating the specimen with normal flora. This could e accomplished by swabbing superficial infected wounds with 70% alcohol.

Sputum Culture and Sensitivity

Aim of the test
An etiological diagnosis of lower respiratory tract infection by microscopic examination and culture with identification and susceptibility test of the isolated organism.

Types of specimen
Sputum, Transtracheal aspirates, translaryngeal aspiration, bronchoalveolar lavage .

Criteria of specimen rejection
Saliva (report as "***Improper specimen, only saliva, please resubmit***"

Pathogen and commensals

Infection of lower respiratory tract
The lower respiratory tract consist of the following part : Left and right pleural cavity, bronchioles, pleural space, diaphragm, mediastinum
The common pathogens
Streptococcus pneumoniae
Haemophilus influenzae
Staphylococcus aureus
Klebsiella pneumoniae and other Enterobacteriaceae
Moraxella catarrhalis
Mycobacterium spp.
Fusobacterium spp.
Bordetella spp
Chlamydia pneumoniae
Legionella spp.

Pre specimen processing

Patient preparing
Patient is asked to wash oral cavity by gargling with water 3-4 times.

Specimen collection

Deep cough and collect sputum in a wide mouth sterile container. All expectorated sputum is contaminated to some degree with secretion of the Oropharyngeal cavity, which contains a wide variety of commensal bacteria, some of which are potential pathogens of the lower respiratory tract (*S. pneumonia, Haemophilus influenzae*). Since the sputum reflect the infection in the bronchi and the lung. Contamination Oropharyngeal secretion should be kept to a minimum.

Early morning sputa is preferred because they contain pooled overnight secretion in which, pathogenic bacteria are more likely to be concentrated.

The specimen should be collected in a sterile, wide-mouth container with tightly fitted screw-cap lid.

Who will collect the specimen
The patient

Quantity of specimen
3 ml

Time relapse before processing the sample
30 min.

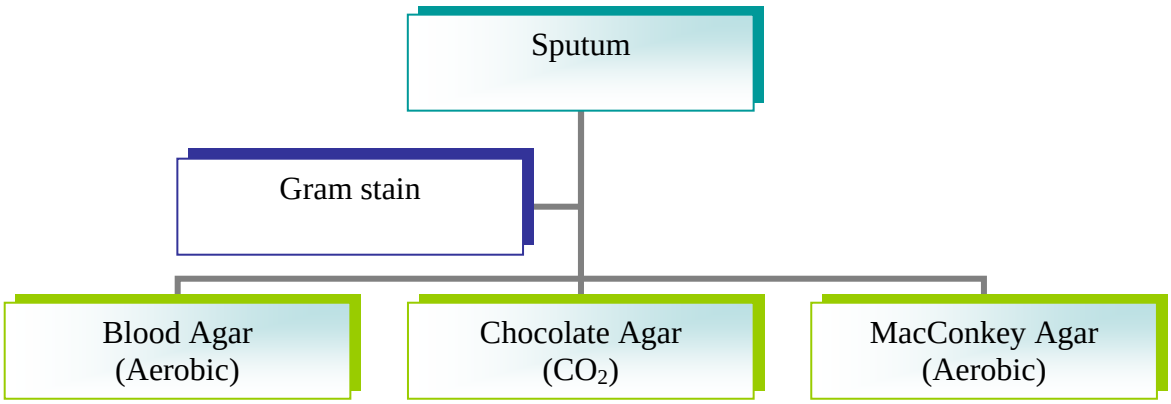
Storage
4 ^{o}C for not more than 2 hours

Specimen processing

Media

- Blood Agar,
- Chocolate Agar,
- MacConkey Agar

Culturing procedure
Inspect the sample and select bloody purulent portion and inoculate blood agar, chocolate agar, and MacConkey Agar and perform a gram stain from the specimen. Incubate the plates as indicated by the chart. Identify according to the attached charts in appendix V.



Post specimen processing

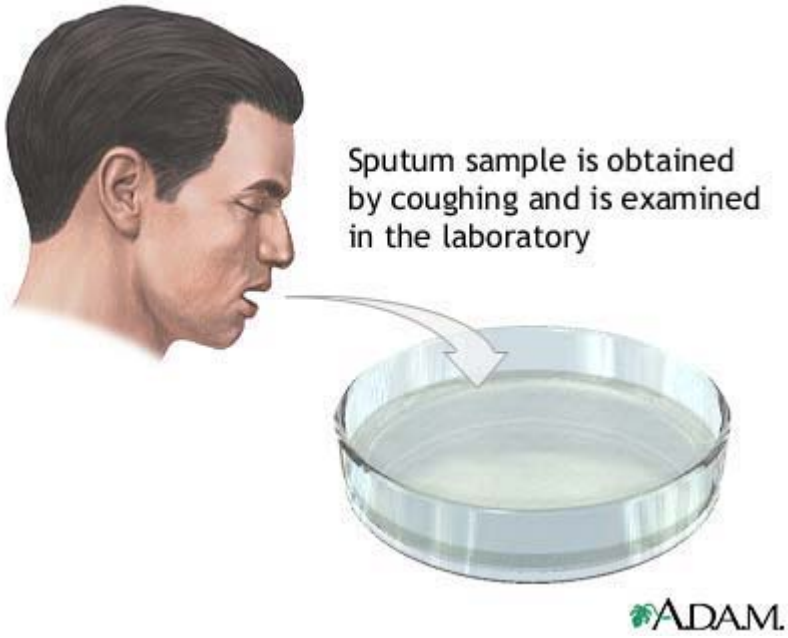
Interfering factors:
Patient on antibiotic therapy.
Improper sample collection.

Result reporting:
Report Gram stain finding as an initial report.
Report the isolated pathogen and its sensitivity pattern as a final report.

Turn around time:
Gram stain results should be available 1 hour after specimen receipt. Isolation of a possible pathogen can be expected after 2-3 days. Negative culture will be reported out 1-2 days after the receipt of the specimen.

Additional information
During the collection of sputum, it is usually contaminated with saliva, therefore, careful collection and interpretation of results is required.
Translaryngeal (transtracheal) aspiration could be requested if the patient:

1. Is debilitated and can not spontaneously expectorate a sputum sample.
2. Routine sputum specimens have failed to recover a causative organism in cases of bacterial pneumonia.



Stool Culture,

Routine, Salmonella & Shigella

Aim of the test
Detect bacterial pathogenic organisms in the stool; diagnose typhoid fever, enteric fever, bacillary dysentery, *Salmonella* infection

Types of specimen
Stool or rectal swab or stool (fresh random) in fecal transport system

Criteria of specimen rejection
Formed stool, specimen contaminated with urine, residual soap, or disinfectants. Specimens received in grossly leaking transport containers; diapers; dry specimens; specimens submitted in fixative or additives;

Pathogen and commensals

Gastrointestinal tract	
Common pathogens	**Commensal flora**
Helicobacter pylori	Enterobacteriaceae other than the common pathogens
Salmonella spp.	*Bacteroides spp*
E. coli O157:H7	*Streptococcus spp*
Staphylococcus aureus	*Lactobacilli*
Campylobacter spp.	*Pseudomonas spp.*
Vibrio cholerae	Coagulase negative *staphylococci*
Yersinia enterocolitica	*Bacteroides*
Clostridium difficile	*Clostridium*
Shigella spp.	*Peptostreptococcus*

Pre specimen processing

Patient preparing
Instruct the patient on how the specimen should be collected and transferred to the container; provide him/her with sticks and containers.

Specimen collection
A single stool specimen cannot be used to rule out bacteria as a cause of diarrhea. More than two specimens should only be submitted from patients for whom there is a high degree of suspicion.

The stool should be collected on collected in sterile bedpan. A sample is transferred with the sticks to the container. The specimen should contain at least 5 g of faeces and, if present, those parts that contain blood and/or mucus should be selected. The specimen should not be contaminated with urine. Close the lid.

Rectal swab: Pass swab beyond anal sphincter, carefully rotate, and withdraw. Swabbing of lesions of rectal wall or sigmoid colon during proctoscopy or sigmoidoscopy is preferred.

Duodenal or sigmoid aspirate: Specimen should be collected by a physician trained in this procedure

Who will collect the specimen
The patient. If stool is unobtainable, nursing staff or physician will collect fecal swab.

Quantity of specimen
The specimen should contain at least 5 g of faeces

Time relapse before processing the sample
Stool samples should be examined and cultured as soon as possible after collection. As the stool specimen cools, the drop in pH will inhibit the growth of most *Shigella* spp. and some *Salmonella* spp.

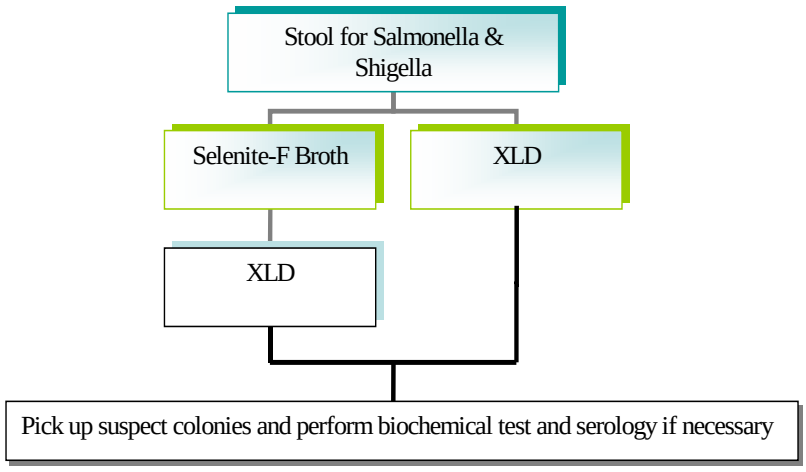
Storage
Refrigerated (2-8 °C)

Specimen processing

Media
- SSA or XLD
- Selenite-F broth or tetrathionate

Reagents
- API 20 E Kit
- Salmonella and Shigella antiserum (polyvalent and monovalent)

Culturing procedure
1 gram of stool is transferred to a tube of Selenite-F broth and a loop is streaked on XLD or SSA. Incubate at 37 ^{o}C. After an overnight incubation do subculture from Selenite-F broth onto a fresh plate of XLD or SSA.



Post specimen processing

Interfering factors

Patient on antibiotic therapy.
Improper sample collection.

Result reporting

A positive report will be issued only in case salmonella or shigella were isolated, otherwise, a negative report will be issued**.**

Turn around time

Negative results are sent out 48 hours after receipt of the specimen.
Results of positive cultures can be expected in 3-4days.

Additional Information

Indications for stool culture include:

- Bloody diarrhea
- Fever
- Tenesmus (is the constant feeling of the need to empty the bowel, accompanied by pain, and cramping)
- Severe or persistent symptoms
- Recent travel to a third world country
- Known exposure to a bacterial agent
- Presence of fecal leukocytes

Notes

In enteric fever caused by *Salmonella typhi*, *S. choleraesuis*, or *S. enteritidis*, blood culture may be positive before stool cultures, and blood cultures are indicated early; urine cultures may also be helpful.

Stool samples should be examined and cultured as soon as possible after collection. As the stool specimen cools, the drop in pH will inhibit the growth of most *Shigella* spp. and some *Salmonella* spp.

Diarrhea is common in patients with the acquired immunodeficiency syndrome (AIDS). It is frequently caused by the classic bacterial pathogens as well as unusual opportunistic bacterial pathogens and parasitic infestation. (*Giardia*, *Cryptosporidium*, and *Entamoeba histolytica* frequently reported.) *Cryptosporidium* and *Pneumocystis* can occur with AIDS. Rectal swabs are useful for the diagnosis of *Neisseria gonorrhoeae* and *Chlamydia* infections. AIDS patients are also subject to cytomegalovirus, *Salmonella*, *Campylobacter*, *Shigella*, *C. difficile*, herpes, and *Treponema pallidum* gastrointestinal tract involvement.

Syndrome (anatomic site)	Features	Characteristic Etiologies
Gastroenteritis (stomach)	Vomiting	Rotavirus
		Norwalk virus
		Staphylococcal food poisoning
		Bacillus cereus food poisoning
Enteritis (small bowel)	Watery diarrhea Large-volume stools, few in number	Enterotoxigenic *Escherichia coli*
		Vibrio cholerae
		Any enteric microbe
		Inflammatory bowel disease
Dysentery, colitis (colon)	Small-volume stools containing blood and/or mucus and many leukocytes	*Shigella*
		Campylobacter
		Salmonella
		Invasive *E. coli*
		Plesiomonas shigelloides
		Aeromonas hydrophila
		Vibrio parahaemolyticus
		Clostridium difficile
		Entamoeba histolytica
		Inflammatory bowel disease

Diarrhea Syndromes Classified by Predominant Features

In acute or subacute diarrhea, three common syndromes are recognized: gastroenteritis, enteritis, and colitis (dysenteric syndrome). With colitis, patients have fecal urgency and tenesmus. Stool are frequently small in volume and contain blood, mucus, and leukocytes. External hemorrhoids are common and painful. Diarrhea of small bowel origin is indicated by the passage of few large volume stools. This is due to accumulation of fluid in the large bowel before passage. Leukocytes indicate colonic inflammation rather than a specific pathogen. Bacterial diarrhea may be present in the absence of fecal leukocytes and fecal leukocytes may be present in the absence of bacterial or parasitic agents (ie, idiopathic inflammatory bowel disease). See table. Although most bacterial diarrhea is transient (1-30 days) cases of persistent symptoms (10 months) have been reported. The etiologic agent in the reported case was *Shigella flexneri* diagnosed by culture of rectal swab. In infants younger than 1 year of age, a history of blood in the stool, more than

10 stools in 24 hours, and temperature greater than 39°C have a high probability of having bacterial diarrhea. Diarrhea is also a common side effect of long term antibiotic treatment. Although often associated with *Clostridium difficile*, other bacteria and yeasts have been implicated.

Limitations

Yersinia sp, *Vibrio, E. coli O157:H7, and Campylobacter spp.* will not be isolated **unless specifically requested**; These organisms are fastidious and have very specific requirements for growth.

Stool Culture, E. coli O157:H7

Aim of the test
Detect *E. coli* O157:H7 from stool specimen or rectal swab and perform sensitivity test. The Latex test will demonstrate by slide agglutination, *E. coli* strains possessing the somatic O157 antigen and Flagellar H7 antigen.

Types of specimen
Stool or rectal swab or stool (fresh random) in fecal transport system

Criteria of specimen rejection
Formed stool, specimen contaminated with urine, residual soap, or disinfectants. Specimens received in grossly leaking transport containers; diapers; dry specimens; specimens submitted in fixative or additives;

Pre specimen processing

Patient preparing
Instruct the patient on how the specimen should be collected and transferred to the container; provide him/her with sticks and containers.

Specimen collection
See specimen collection under stool culture, routine.
Quantity of specimen
The specimen should contain at least 5 g of feces

Time relapse before processing the sample
Stool samples should be examined and cultured as soon as possible after collection.

Storage
Refrigerated (2-8 °C)

Specimen processing

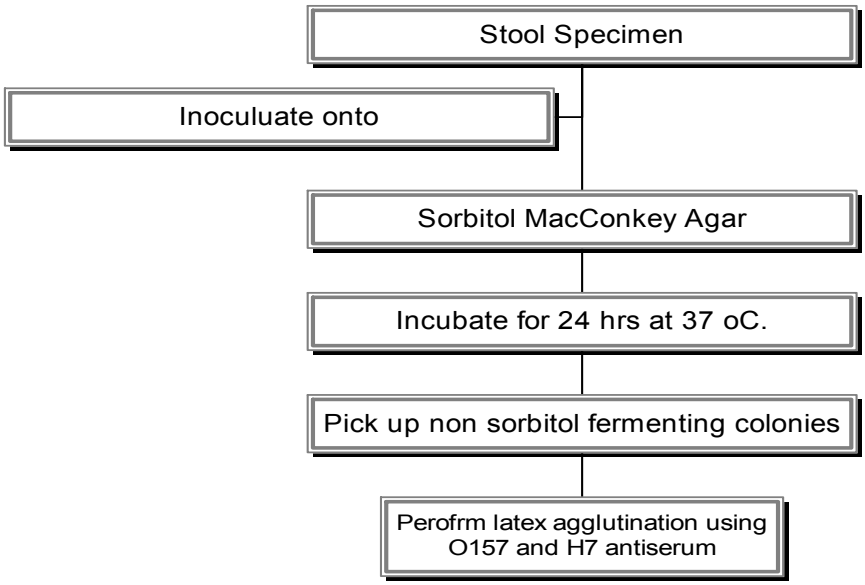
Media
Sorbitol MacConkey Agar (SMAC)

Culturing procedure
A loopful of stool is streaked on Sorbitol MacConkey. Incubate at 37 ^{o}C. Under aerobic conditions. Examine plates for non-sorbitol fermenting colonies (NSF).
NSF colonies may be taken from SMAC plates or alternatively NSF isolates may be inoculated onto non-selective agar media for testing.
It is necessary to test up to 10 separate NSF colonies to ensure a high probability of detection from mixed cultures.

LATEX PROCEDURES

1) Bring the latex reagents to room temperature. Make sure the latex suspensions are mixed by vigorous shaking. Expel any latex from the dropper pipette for complete mixing.
2) Dispense 1 drop of the Test latex onto a circle of the black slide. Place it close to the edge of the circle..
3) Add some loopfuls or a Pasteur pipette drop of saline to the circle. Ensure that the latex and saline do not mix at this stage.
4) Using a loop, pick off a portion of the colony to be tested and carefully emulsify in the saline drop.
5) Mix the Test latex and suspension together and spread to cover most of the reaction area using the loop. Flame the loop.
6) Rock the slide in a circular motion, observing for agglutination. Do not rock the card for more than 1 minute and do not use a magnifying glass.
7) If no agglutination occurs, then proceed to test other NSF colonies if these are present.
8) If agglutination with the test reagent does occur, then it is necessary to test a further portion of the colony with the control reagent to ensure that the isolate is not an autoagglutinating strain.
9) When finished, dispose of the reaction slide into disinfectant.

Stool culture of E. coli O157:H7



Interpretation

a) Positive result - Agglutination of the Test latex occurs within 1 minute. No agglutination of the Control latex. Perform biochemical tests to confirm that the organism is an *E. coli* strain.
b) Negative result - no agglutination of the Test latex.
c) Non-interpretable result - clumping of the Control latex.

Post specimen processing

Interfering factors

Patient on antibiotic therapy.
Improper sample collection.

Result reporting

A positive report will be issued only in case *E. coli* O157:H7 were isolated, otherwise, a negative report will be issued**.**

Turn around time
Negative results are sent out 72 hours after receipt of the specimen. Results of positive cultures can be expected in 4-5days.

Stool Culture, *Vibrio* spp.

Aim of the test
Isolate *Vibrio cholera* from stool specimen and perform antibiotic sensitivity testing.

Types of specimen
Fresh random stool, rectal swab if stool is unavailable.

Criteria of specimen rejection
Specimens received in grossly leaking transport containers; diapers; dry specimens; specimens submitted in fixative or additives; specimens received in expired transport media or incorrect transport device; inappropriate specimen transport conditions (not in a C&S vial or in an overfilled C&S vial); specimens received after prolonged delay in transport (usually more than 72 hours); specimens stored or transported frozen; wooden shaft swab in transport device; unlabeled specimen or name discrepancy between the specimen label and the request

Pathogen and commensals
See stool culture, routine

Pre specimen processing

Patient preparing
Instruct the patient on how the specimen should be collected and transferred to the container; provide him/her with sticks and containers.

Specimen collection
The stool should be collected in sterile bedpan. A sample is transferred with the stick to the container. The specimen should contain at least 5 g of feces and, if present, those parts that contain blood and/or mucus should be selected. The specimen should not be contaminated with urine. Close the lid.

Rectal swab: Pass swab beyond anal sphincter, carefully rotate, and withdraw. Swabbing of lesions of rectal wall or sigmoid colon during proctoscopy or sigmoidoscopy is preferred.

Duodenal or sigmoid aspirate: Specimen should be collected by a physician trained in this procedure

Who will collect the specimen
The patient. If stool is unobtainable. Nursing staff of physician will collect fecal swab.

Quantity of specimen
The specimen should contain at least 5 g of feces

Time relapse before processing the sample
Stool samples should be examined and cultured as soon as possible after collection.

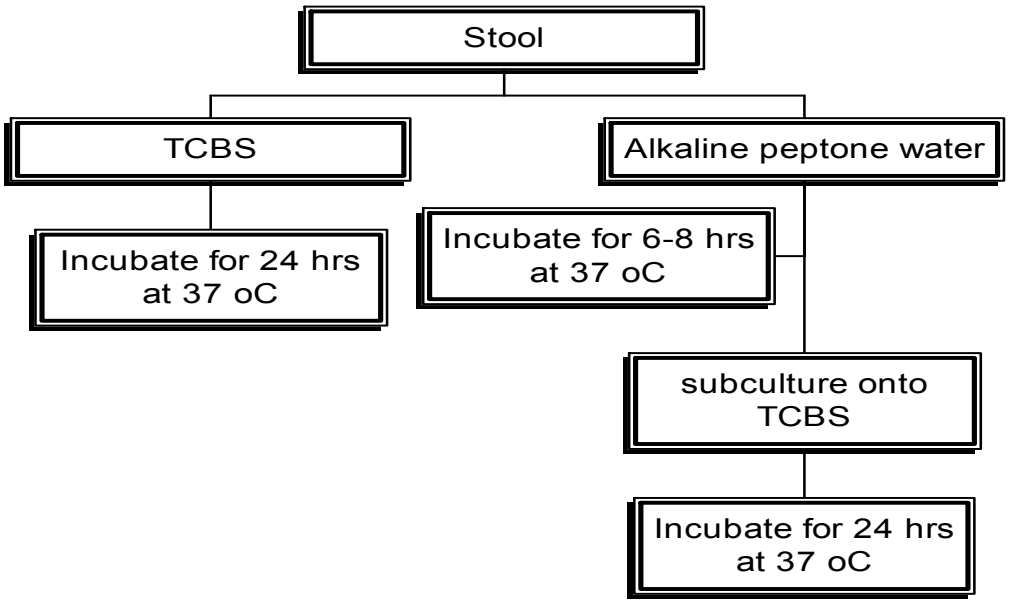
Storage
Maintain specimen at room temperature until shipment.

Specimen processing

Media
Alkaline peptone water
TCBS (Thiosulfate Citrate Bile salt Sucrose Agar)

Culturing procedure
A loopful of stool is streaked onto the surface of a TCBS plate and about one gram is inoculated into a tube containing alkaline peptone water, incubate at 37 ^{o}C. After 6-8 hours make a subculture from the alkaline peptone water onto the surface of a new plate of TCBS. Incubate at 37 ^{o}C for 24 hours. See schematic diagram

Isolation of Vibrio cholera from Stool



Post specimen processing

Interfering factors
Patient on antibiotic therapy.
Improper sample collection.

Result reporting

A positive report will be issued only in case *Vibrio cholera* were isolated, otherwise, a negative report will be issued**.**

Turn around time
Negative results are sent out 72 hours after receipt of the specimen. Results of positive cultures can be expected in 4-5days.

Throat Swab for

Beta-Haemolytic *Streptococcus* Culture, Group A Only

Aim of the test
Isolate and identify group A beta-hemolytic streptococci; establish the diagnosis of strep throat infection.

Types of specimen
Material from posterior pharynx, tonsils, or other inflamed area.

Criteria of specimen rejection
Inappropriate specimen transport device; mislabeled specimen; unlabeled specimen; specimen received after prolonged delay (usually more than 72 hours); specimen received in expired transport

Pathogen and commensals

infection of the upper respiratory tract	
Common pathogenic bacteria	**commensal flora**
Beta-Haemolytic streptococci group A	the upper respiratory tract consist of the following parts nasal cavity, pharynx, nasal pharynx, oropharynx, laryngopharenx, larynx-trachea-left and right primary bronchi
Streptococcus pneumoniae	α haemolytic streptococci
Staphylococcus aureus	Neisseria species other than N. gonorrhea
Klebsiella spp and other Enterobacteriaceae	Coagulase negative staphylococci
Bacteroides spp. and other anaerobes	*Staph. aureus* (occasionally)
Corynebacterium diphtheria	*Haemophilus haemolyticus*
Neisseria gonorrheae	Enterobacteriaceae
Bordetella pertussis	*Candida albicans*
	Occasionally β-haemolytic *streptococcus* other than group A
	Diphtheroides

Pre specimen processing

Patient preparing
Instruct the procedures for the patient

Specimen collection
Both tonsillar pillars and the oropharynx should be swabbed. Do not allow the swab to touch the tongue.

1. The patient is instructed to tilt his/her head back and breath deeply. The tongue is gently depressed with a tongue blade to visualize the tonsillar fossa and posterior pharynx.

2. The swab is extended between the tonsillar pillars and behind the vulva, care should b taken not to touch the lateral walls of the buccal cavity or the tongue to minimize contamination with commensal bacteria.
3. The posterior pharynx should be firmly rubbed with the swab.
4. After collection, the swab should be placed immediately into sterile tube or other suitable container for transport to the laboratory.

Who will collect the specimen
Physician, Medical technologist, Microbiologist, experienced nurse.

Quantity of specimen
One or two swabs

Time relapse before processing the sample
30 min.

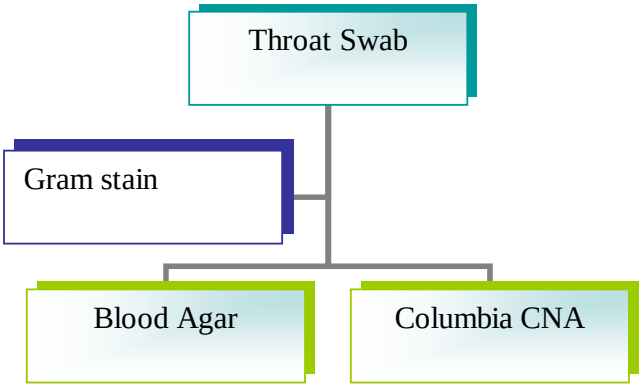
Storage
Maintain specimen at room temperature

Specimen processing

Media

- Blood Agar,
- Columbia CNA

Culturing procedure
Streak the swab across blood agar plate and Columbia CNA to make a line that divide the plate into two halves, and using a sterile loop, streak by crossing the line to produce isolated colonies. Make few stabs in the agar. Do a gram stain from the swab noting the predominant organism.



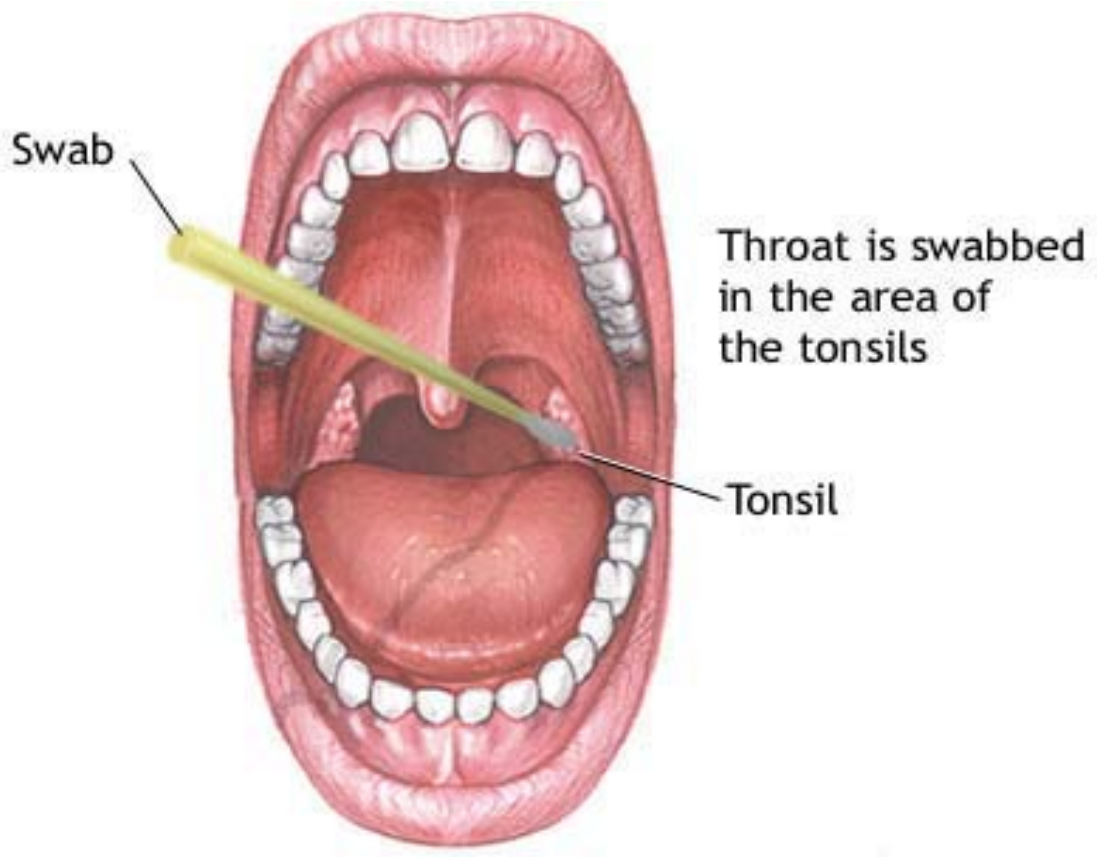
Post specimen processing

Interfering factors:
Patient on antibiotic therapy.
Improper sample collection.

Result reporting:
Report wet mount Gram stain finding as an initial report.
Report the isolated pathogen and its sensitivity pattern as a final report.

Turn around time:
Gram stain results should be available 1 hour after specimen receipt.
Isolation of a possible pathogen can be expected after 2-3 days. Negative culture will be reported out 1-2 days after the receipt of the specimen.

Additional information
Rheumatic fever remains a concern all over the world and serious complications including sepsis, soft tissue invasion, and toxic shock-like syndrome have been reported to be increasing in frequency; therefore, timely diagnosis and early institution of appropriate therapy remains important. Timely therapy may reduce the acute symptoms and overall duration of streptococcal pharyngitis. The sequelae of poststreptococcal glomerulonephritis and rheumatic fever are diminished by early therapy.



Isolation and Identification of Enterobacteriaceae and *Pseudomonas*

Part 1

OBJECTIVES

After completing this Exercise, the student will be able to perform the following objectives:

A. **ENTEROBACTERIACEAE: FERMENTATIVE, GRAM-NEGATIVE, ENTERIC BACILLI**
   1. Name the bacterial family to which the most commonly encountered organisms isolated from clinical specimens belong.
   2. List five characteristics used to place bacteria into the family Enterobacteriaceae.
   3. State what infections are caused by *Salmonella* and by *Shigella* and how they are transmitted to humans.
   4. Name four strains of *Escherichia coli* that may infect the gastrointestinal tract.
   5. Name five genera of Enterobacteriaceae considered as common opportunistic pathogens, state their normal habitat, and list four common types opportunistic infections that they all may cause.
   6. Name several predisposing factors that make one more susceptible to urinary tract infections.
   7. In terms of CFUs, state the laboratory culture standard for a urinary tract infection.
   8. Define nosocomial infection.
   9. State the significance of endotoxins in infections caused by many of the Enterobacteriaceae.
   10. Discuss the significance of R plasmids in our attempts to treat infections caused by the Enterobacteriaceae.

B. ***PSEUDOMONAS* AND OTHER NONFERMENTATIVE, GRAM-NEGATIVE BACILLI**
   1. Name the most common nonfermentative gram-negative rod that infect humans and list five types of opportunistic infections it may cause.

C. **ISOLATION OF ENTEROBACTERIACEAE AND *PSEUDOMONAS***
   1. 1. State the usefulness of XLD agar and Pseudosel agar for the isolation of Enterobacteriaceae and *Pseudomonas*.

D. **DIFFERENTIATING BETWEEN THE ENTEROBACTERIACEAE AND *PSEUDOMONAS***
   1. State how to differentiate *Pseudomonas aeruginosa* from the Enterobacteriaceae using the following tests:
      a. Oxidase test
      b. Fermentation of glucose
      c. Production of pigment and fluorescent products
      d. Odor

E. **IDENTIFYING THE ENTEROBACTERIACEAE USING RAPID METHODS**

1. Briefly describe the Enterotube®II.
2. Briefly describe the API20E system.
3. Be acquainted with the computerized system of identification.

DISCUSSION

A. ENTEROBACTERIACEAE: THE FERMENTATIVE, GRAM-NEGATIVE, ENTERIC BACILLI

Bacteria belonging to the family Enterobacteriaceae are the most commonly encountered organisms isolated from clinical specimens. The **Enterobacteriaceae** is a large diverse family of bacteria commonly referred to as the **fermentative, gram-negative, enteric bacilli**, indicating that they are gram-negative rods which can **ferment sugars**. Many are normal flora of the intestinal tract of humans and animals. Some infect the intestinal tract. Members of this family have the following five characteristics in common:

1. They are **gram-negative rods**
2. If motile, they possess a **peritrichous arrangement of flagella**
3. They are **facultative anaerobes**
4. They are **oxidase negative**
5. All species **ferment the sugar glucose** but otherwise vary widely in their biochemical characteristics.

Twenty-six genera and over 100 species of Enterobacteriaceae have been recognized. Some of the more common clinically important genera of the family Enterobacteriaceae include:

Salmonella	*Citrobacter*	*Morganella*
Shigella	*Enterobacter*	*Yersinia*
Proteus	*Serratia*	*Edwardsiella*
Escherichia	*Klebsiella*	*Providencia*

Several genera of Enterobacteriaceae are associated with **gastroenteritis** and food-borne disease. These include *Salmonella, Shigella*, certain strains of *Escherichia coli*, and certain species of *Yersinia*. All intestinal tract infections are **transmitted by the fecal-oral route**.

Any infection caused by ***Salmonella*** is called a **salmonellosis**. The majority of *Salmonella* cause **diarrhea**, but one species, *S. typhi,* may disseminate into the blood and cause a severe form of salmonellosis called typhoid fever.

Any ***Shigella*** infection is called a **shigellosis.** *Shigella* may produce cytotoxins that cause abscesses and ulcers to appear in the large intestines resulting in **dysentery** (diarrhea with blood, mucous, and white blood cells in the stool).

While *Escherichia coli* is one of the dominant normal flora in the intestinal tract of humans and animals, **some strains** can cause infections of the intestines. **Enterotoxic *E. coli* (ETEC)** produce enterotoxins that cause the loss of sodium ions and water from the intestines resulting in a **watery diarrhea**. Over half of all travelers' diarrhea is due to ETEC. **Enteropathogenic *E. coli* (EPEC)** also cause a **watery diarrhea**, probably by adhering to intestinal mucosal cells and interfering with their function. **Enteroinvasive *E. coli* (EIEC)** invade intestinal epithelial cells causing a **dysentery**-type syndrome. Finally, **Verotoxin-producing *E. coli* (VTEC)**, such as *E. coli* 0157:H7, produce a verotoxin (also called shiga-like toxin) that kills intestinal epithelial cells causing a **bloody diarrhea**. In rare cases, the verotoxin enters the blood and is carried to the kidneys where it damages vascular cells and causes **hemolytic uremic syndrome**.

Several species of ***Yersinia***, such as *Y. enterocolitica* and *Y. pseudotuberculosis* also causes of **diarrheal disease**.

Many other genera of the family Enterobacteriaceae are **normal flora of the intestinal tract** and are considered **opportunistic pathogens**. The most common genera of Enterobacteriaceae causing opportunistic infections in humans are ***Escherichia coli, Proteus, Enterobacter, Klebsiella, Citrobacter***, and ***Serratia***. They act as opportunistic pathogens when they are introduced into body locations where they are not normally found, especially if the host is debilitated or immunosuppressed. They all cause the same types of opportunistic infections, namely, **urinary tract infections, wound infections, pneumonia, and septicemia** and represent, along with *Staphylococcus aureus* wound infections, the leading cause of hospital-acquired or **nosocomial infections**.

The most common infection caused by these opportunistic Enterobacteriaceae is a **urinary tract infection (UTI)**. Among the nonhospitalized and nondebilitated population, UTIs are more common in females, but anyone can become susceptible to urinary infections in the presence of predisposing factors that cause functional and structural abnormalities of the urinary tract. These abnormalities increase the volume of residual urine and interfere with the normal clearance of bacteria by urination. Such factors include paraplegia, spina bifida, scar tissue formation, and catheterization. The laboratory culture standard for a UTI is the presence of **more than 100,000 CFUs** (colony-forming units) **per ml of midstream urine or any CFUs from a catheter-obtained urine sample**.

Wound infections are due to fecal contamination of external wounds or a result of wounds that cause trauma to the intestinal tract (surgical wounds, gunshot wounds, etc.).

Although they sometimes cause **pneumonia**, gram-negative bacilli account for less than 5% of the bacterial pneumonias requiring hospitalization.

Gram-negative septicemia is a result of these opportunistic bacteria getting into the blood. They are usually introduced into the blood from some other infection site, such as an infected kidney, wound, or lung.

Since all of these organisms are gram-negative, the lipid A moiety of the outer membrane may act as an **endotoxin**. Endotoxin, especially when in the blood, can lead to inflammation, high fever, hypotension, capillary damage, intravascular coagulation, tissue degradation, and irreversible shock. Many of the Enterobacteriaceae also possess **R (resistance) plasmids**. These plasmids are small pieces of circular nonchromosomal DNA that may code for **multiple antibiotic resistance** In addition, the plasmid may code for a **sex pilus**, enabling the bacterium to pass R plasmids to other bacteria by **conjugation**.

B. *PSEUDOMONAS* AND OTHER NONFERMENTATIVE GRAM-NEGATIVE BACILLI

Nonfermentative gram-negative bacilli refer to gram-negative rods or coccobacilli that **cannot ferment sugars**. The nonfermentative gram-negative bacilli are often normal inhabitants of soil and water. They may cause human infections when they colonize immunosuppressed individuals or gain access to the body through trauma. However, less than one-fifth of the gram-negative bacilli isolated from clinical specimens are nonfermentative bacilli. By far, the **most common** gram-negative, nonfermentative rod that causes human infections is *Pseudomonas aeruginosa*.

Pseudomonas aeruginosa is also an **opportunistic pathogen**. It is a common cause of nosocomial infections and can be found growing in a large variety of environmental locations. In the hospital environment, for example, it has been isolated from drains, sinks, faucets, water from cut flowers, cleaning solutions, medicines, and even disinfectant soap solutions. It is especially dangerous to the debilitated or compromised patient. Like the opportunistic Enterobacteriaceae, it is a gram-negative rod, it is frequently found in small amounts in the feces, and it causes similar opportunistic infections: **urinary tract infections, wound infections, pneumonia, and septicemia**. In addition, it is a significant cause of **burn infections**, colonizes the respiratory tract of people with cystic fibrosis, can cause a destructive eye infection, and causes folliculitis (infection of hair follicles). Like other opportunistic gram-negative bacilli, *Pseudomonas aeruginosa* also releases endotoxin and

frequently possesses R plasmids. A number of other species of *Pseudomonas* have also been found to cause human infections.

Other nonfermentative gram-negative bacilli that are sometimes opportunistic pathogens in humans include *Acinetobacter, Aeromonas, Alcaligenes, Eikenella, Flavobacterium,* and *Moraxella*.

C. ISOLATION OF ENTEROBACTERIACEAE AND *PSEUDOMONAS*

To isolate Enterobacteriaceae and *Pseudomonas*, specimens from the infected site are plated out on any one of a large number of **selective and differential media** such as EMB agar, Endo agar, Deoxycholate agar, MacConkey agar, Hektoen Enteric agar, and XLD agar.

XLD agar is **selective for gram-negative bacteria**. In addition, different gram-negative bacilli, due to their biochemical reactions, **produce different appearing colonies**. Typical reactions for some of the Enterobacteriaceae and *Pseudomonas* are shown below:

1. ***Escherichia coli*:** flat yellow colonies; some strains may be inhibited.
2. ***Enterobacter* and *Klebsiella***: mucoid yellow colonies.
3. ***Proteus*:** red to yellow colonies; may have black centers.
4. ***Salmonella*:** usually red colonies with black centers.
5. ***Shigella* and *Pseudomonas***: red colonies without black centers.

The biochemical reasons for these color reactions will be discussed in exercise2. Some species and subspecies, however, may not show typical reactions.

Pseudosel agar is **selective for *Pseudomonas aeruginosa*** and also stimulates *P. aeruginosa* to produce its characteristic **pigment** as well as **fluorescent products**. *Pseudomonas aeruginosa* will typically produce a green to blue water-soluble pigment on this agar and will also fluoresce when the plate is placed under a short wavelength ultraviolet light.

D. DIFFERENTIATING BETWEEN THE ENTEROBACTERIACEAE AND *PSEUDOMONAS*

Once the gram-negative rod is isolated, a number of tests can be performed to determine if it is one of the Enterobacteriaceae or if it is *Pseudomonas*. Several of these tests are listed below:

1. Production of the enzyme **oxidase**. The oxidase test is based on the bacterial production of an oxidase enzyme. Cytochrome oxidase, in the presence of oxygen, oxidizes the **para-amino dimetheylanaline** oxidase test reagent in a Taxo-N® disc to form a rose-colored compound **indophenol**. The

Enterobacteriaceae are oxidase-negative; *Pseudomonas aeruginosa* and most other nonfermentative gram-negative rods are oxidase-positive. The procedure for the oxidase test is described later in this Exercise.

2. **Fermentation of glucose.** All of the Enterobacteriaceae ferment the sugar glucose; *Pseudomonas aeruginosa* and other nonfermentative gram-negative rods will not.
3. **Pigment production**. None of the Enterobacteriaceae produces pigment at 37 ^{o}C; *Pseudomonas aeruginosa* produces a green to blue, water soluble pigment called pyocyanin. It also produces a product called fluorescein that will fluoresce under short wavelength (254nm) ultraviolet light. Pseudosel agar can be used to stimulate the production of pigment and fluorescent products.
4. **Odor**. Most of the Enterobacteriaceae have a rather foul smell; *Pseudomonas aeruginosa* produces a characteristic fruity or grape juice-like aroma due to production of an aromatic compound called **aminoacetophenone**.

Some common biotypes of *Pseudomonas* as well as all members of the Enterobacteriaceae can also be identified by means of biochemical tests found in commercially produced systems such as the API-20E® System or the Enterotube®II (discussed below).

E. IDENTIFYING THE ENTEROBACTERIACEAE USING THE ENTEROTUBE®II AN API 20E SYSTEMS

A number of techniques can be used for the identification of specific species and subspecies of Enterobacteriaceae. Speciation is important because it provides data regarding patterns of susceptibility to antimicrobial agents and changes that occur over a period of time. It is also essential for epidemiological studies such as determination of nosocomial infections and their spread.

In an effort to simplify the speciation of the Enterobacteriaceae and reduce the amount of prepared media and incubation space needed by the clinical lab, a number of self-contained **multi-test systems** have been commercially marketed. Some of these multi-test systems have been combined with a computer-prepared manual to provide identification based on the overall **probability of occurrence** for each of the biochemical reactions. In this way, a large number of biochemical tests can economically be performed in a short period of time, and the results can be accurately interpreted with relative ease and assurance.

The Enterotube®II is a self-contained, compartmented plastic tube containing 12 different agars (enabling the performance of a total of 15 standard biochemical tests) and an enclosed inoculating wire. After inoculation and incubation, the resulting combination of reactions, together with a Computer Coding and Identification System (CCIS), allows for easy identification. The various biochemical reactions of the Enterotube®II and their correct interpretation is discussed in

exercise 2. Although it is designed to identify members of the bacterial family Enterobacteriaceae, it will sometimes also identify common biotypes of *Pseudomonas* and other nonfermentative gram-negative bacilli.

The API 20E is a strip containing 20 miniaturized tests that is used in the identification of Enterobacteriacae and gram negative non-fermenters (pseudomonas and similar organisms). After inoculation, the strips are incubated at 37 oC for 18-24 hours. The results are read and recorded in a special form supplied with the strip. A 7 digit number is then calculated from the results and then entered into special software for final identification.

ORGANISMS (Trypticase Soy agar plate cultures)

Possible unknowns include:



1. *Escherichia coli*
2. *Enterobacter aerogenes*
3. *Enterobacter cloacae*
4. *Proteus mirabilis*
5. *Proteus vulgaris*
6. *Salmonella enteritidis*
7. *Klebsiella pneumoniae*
8. *Citrobacter freundii*
9. *Pseudomonas aeruginosa*

CAUTION: TREAT EACH UNKNOWN AS A PATHOGEN! Inform your instructor of any spills or accidents. **WASH YOUR HANDS WELL** before you leave the lab.

MATERIALS

Taxo N® disk, alcohol, dropper bottle of distilled water, platinum inoculating loop, and **either** a plate of XLD agar and an Enterotube®II **or** a plate of Pseudosel agar and an Enterotube®II and API 20 E Kit.

PROCEDURE (to be done in pairs)

Each pair will be given one of the above unknowns. You will determine its identity doing the tests below.

1. Using the Trypticase Soy agar culture of your unknown, first perform an **oxidase test** as follows:
    a. Using alcohol-flamed forceps remove a Taxo-N® disc and moisten it with a drop of sterile distilled water.
    b. Place the moistened disc on the colonies of the **Trypticase Soy agar plate culture** of your unknown.
    c. Using a platinum loop, scrape off some of the colonies and spread them on the Taxo-N® disc.

In the **immediate test**, oxidase-positive reactions will turn a **rose color within 30 seconds**. Oxidase-negative will not turn a rose color. This reaction only lasts a couple of minutes. In the **delayed test**, oxidase-positive colonies within 10 mm of the Taxo-N® disc will **turn black within 20 minutes** and will remain black. If the bacterium is oxidase-negative, the growth around the disc will not turn black.

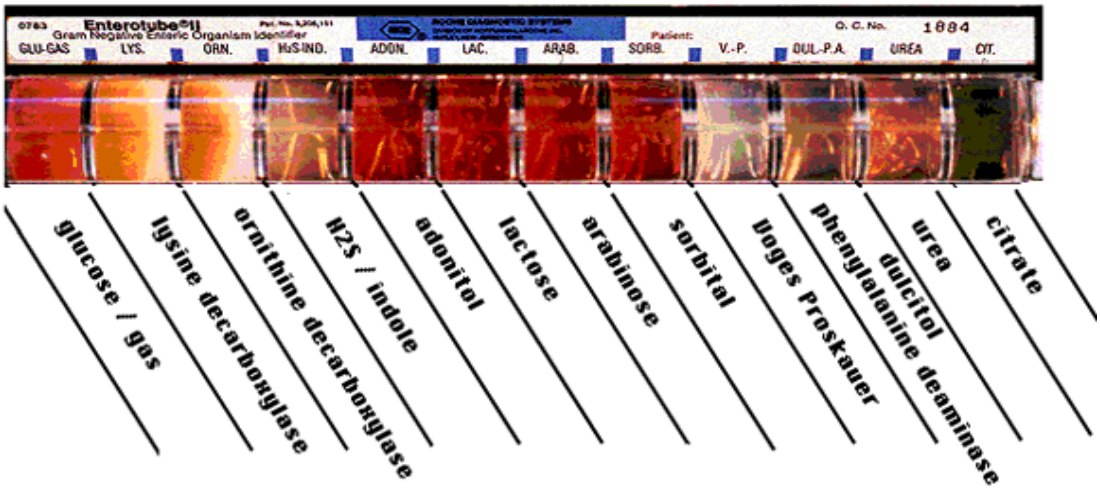
***Pseudomonas aeruginosa* and most other nonfermentative, gram-negative bacilli are oxidase-positive; all of the Enterobacteriaceae are oxidase-negative.**

Record your oxidase test results in the Results section of Exercise 2.

2. Perform a **gram stain** on your unknown. All of the Enterobacteriaceae as well as *Pseudomonas* are gram-negative bacilli. **Record the results of your gram stain in the Results section of Lab 2**.
3. If your unknown is **oxidase-negative**, do the following inoculations:
    a. Streak your unknown for isolation on a plate of **XLD agar**. Incubate at **37 ^{o}C**.
    b. Inoculate an **Enterotube®II** as follows:
        1. Remove both caps of the Enterotube®II and with the **straight end of the inoculating wire**; pick off the equivalent of a colony from your unknown plate. **A visible inoculum should be seen on the tip and side of the wire**.
        2. **Inoculate** the Enterotube®II by grasping the **bent-end of the inoculating wire**, twisting it, and withdrawing the wire through all 12 compartments using a turning motion.
        3. **Reinsert the wire** into the tube (use a turning motion) **through all 12 compartments** until the notch on the wire is aligned with the opening of the tube. (The tip of the wire should be seen in the citrate compartment.) **Break the wire at the notch** by bending. Do not discard the wire yet.
        4. Using the broken off part of the wire, **punch holes through the cellophane which covers the air inlets located on the rounded side of the last 8 compartments**. Your instructor will show you their correct location. Discard the broken off wire in the disinfectant container.
        5. **Replace both caps** and incubate the Enterotube®II **on its flat surface at 37 ^{o}C**.
    c. If your unknown is **oxidase-positive**, do the following inoculations:
        1. Streak your unknown for isolation on a plate of **Pseudosel agar**. Incubate at **37 ^{o}C**.
        2. Inoculate an **Enterotube®II** as described above in step 3b.

Enterotube Reactions

CONTROL



d. If you are asked to perform API 20 E

Inoculation of the strip: - stock kept in cold room. Supplied in aluminium pouches which once opened can be resealed and then may be used for ten months or until expiry date on pack.

1. Take an incubation tray and lid and add about 5 ml of water to the tray before adding a strip to prevent the it from drying out.
2. Record the isolates reference number on the elongated tab and on a blood agar plate (isolation medium).
3. Perform oxidase test on an identical colony. SOP 001.
4. Take a bijou of sterilized water and make a light suspension of the organism to be identified.
5. Use this to inoculate the strip and plate out one drop on the labelled plate for a purity check.
6. Fill both tube and cupule in CIT, VP, and GEL tests.
7. Fill only tubes and not cupules of other tests.
8. Create anaerobiosis in the tests ADH, LDC, ODC, H_2S and URE by filling cupule with liquid paraffin.
9. Incubate strip and plate overnight in the hot room.

Place the pipettes into a discard pot and the strips into a tin for autoclaving prior to disposal.

Reading the strip:-

1. After 18-24 hours incubation read the strip by referring to the reading table.
2. Record all results that do not need the addition of reagents on the result sheet.

3. The following tests require the addition of reagents before reading.

TDA test: add one drop of TDA reagent. A dark brown color indicates a positive.
IND test: add one drop of JAMES reagent. The reaction takes place immediately: a pink coloration in the whole cupule indicates a positive reaction.
NO_2 test: add drop of each of NIT 1 and NIT 2 reagents to the GLU tube. Wait 2 to 3 minutes. A red color indicates a positive result.
VP test: add one drop of VP1 and VP2 reagents. Wait ten minutes. A bright pink or red color indicates a positive result.



Isolation and Identification of Enterobacteriaceae and *Pseudomonas*

Part 2

OBJECTIVES

After completing this Exercise, the student will be able to perform the following objectives:

ISOLATION OF ENTEROBACTERIACEAE AND *PSEUDOMONAS*

1. Interpret the results of XLD agar and Pseudosel agar.

IDENTIFICATION OF *PSEUDOMONAS*

1. Interpret the results of the following tests:
    a. Oxidase test
    b. Pigment production on Pseudosel agar
    c. Fluorescence under ultraviolet light on Pseudosel agar
    d. Odor
2. Recognize an organism as *Pseudomonas aeruginosa*

IDENTIFICATION OF ENTEROBACTERIACEAE USING AN ENTEROTUBE®II

1. Interpret the results of an Enterotube®II and the result of API 20 E
2. Identification software will be demonstrated.

In Exercise 1, you learned how to isolate members of the Enterobacteriaceae, differentiate them from *Pseudomonas*, and identify them by biochemical testing. Today, you will learn how to **interpret the results of the various media you inoculated in Exercise 1**.

A. **THE OXIDASE TEST**

In Exercise 1 you performed on your unknown, an oxidase test using a Taxo N® disc. Record the results of your oxidase test in the Results section of today's Exercise if you did not do so last time.

B. **THE GRAM STAIN**

In Exercise 1 you also did a gram stain on your unknown. Record your gram stain results in the Results section of today's Exercise if you did not do so last time.

C. **ISOLATION OF ENTEROBACTERIACEAE ON XLD AGAR**

In EXERCISE 1, if the **oxidase test done on your unknown was negative**, you inoculated a plate of XLD agar. Xylose Lysine Desoxycholate (XLD) agar contains sodium desoxycholate, which **inhibits the growth of gram-positive bacteria** but permits the growth of gram-negatives. It also contains the sugars lactose and sucrose, the amino acid L-lysine, sodium thiosulfate, and the pH indicator phenol red.

If the gram-negative bacterium **ferments lactose and/or sucrose, acid end products** will be produced and cause the colonies and the phenol

red in the agar around the colonies to **turn yellow**. If lactose and sucrose are not fermented but the **amino acid lysine is decarboxylated, ammonia, an alkaline end product** will cause the phenol red in the agar around the colonies to **turn a deeper red**. Sometimes the **sugars are fermented** producing acid end products and **lysine is broken down** producing alkaline end products. in this case **some of the colonies and part of the agar turns yellow and some of the colonies and part of the agar turns a deeper red**. If **hydrogen sulfide** is produced from thiosulfate reduction, part or the entire **colony will appear black**. Well isolated colonies are usually needed for good results. Typical colony morphology on XLD agar is as follows:

1. *Escherichia coli*: flat yellow colonies; some strains may be inhibited.
2. *Enterobacter* and *Klebsiella*: mucoid yellow colonies.
3. *Proteus*: red to yellow colonies; may have black centers.
4. *Salmonella*: usually red colonies with black centers
5. *Shigella* and *Pseudomonas*: red colonies without black centers

Keep in mind, however, that some species and subspecies do not show typical reactions. Typical reactions for our strains are as follows:

1. *Escherichia coli*
2. *Proteus vulgaris*
3. *Enterobacter aerogenes*
4. *Salmonella enteritidis*
5. *Enterobacter cloacae*
6. *Klebsiella pneumonia*
7. *Proteus mirabilis*

Record the results of your XLD agar in the Results section of today's Exercise.

D. ISOLATION AND IDENTIFICATION OF PSEUDOMONAS ON PSEUDOSEL AGAR

As was mentioned in Exercise 1, a number of tests can be performed to determine if your unknown is *Pseudomonas*. These tests include:

1. **Oxidase**. The oxidase test is based on the bacterial production of an oxidase enzyme. Cytochrome oxidase, in the presence of oxygen, oxidizes the para-amino dimetheylanaline oxidase test reagent in a Taxo-N® disc. In the **immediate test**, oxidase-positive reactions will turn a **rose color within 30 seconds**. Oxidase-negative will not turn a rose color. This reaction only lasts a couple of minutes. In the **delayed test**, oxidase-positive colonies within 10 mm of the Taxo-N® disc will **turn black within 20 minutes** and will remain black. If the bacterium is oxidase-negative, the growth around the disc will not turn black.

   ***Pseudomonas aeruginosa* and most other nonfermentative, gram-negative bacilli are oxidase-positive; all of the Enterobacteriaceae are oxidase-negative.**

2. **Fermentation of glucose.** All of the Enterobacteriaceae ferment the sugar glucose; *Pseudomonas aeruginosa* and other nonfermentative gram-negative rods will not. (Note: You can use TSIA to detect glucose fermentation)
3. **Pigment production**. None of the Enterobacteriaceae produces pigment at 37C; *Pseudomonas aeruginosa* produces a green to blue, water soluble pigment called pyocyanin. It also produces a product called fluorescein that will fluoresce under short wavelength (254nm) ultraviolet light.
4. **Odor**. Most of the Enterobacteriaceae have a rather foul smell; *Pseudomonas aeruginosa* produces a characteristic fruity or grape juice-like aroma due to production of an aromatic compound called **aminoacetophenone**.

(Some common biotypes of *Pseudomonas* as well as all members of the Enterobacteriaceae can also be identified by means of biochemical tests found in commercially produced systems such as the Enterotube®II, as will be discussed below.)

We will now identify one of the unknowns as *Pseudomonas aeruginosa* by means of the above mentioned tests.

In Exercise 1, if your **oxidase test was positive**, you inoculated a plate of Pseudosel agar with your unknown. Pseudosel agar contains **cetrimide** which inhibits most bacteria other than *Pseudomonas aeruginosa*. It also stimulates *P. aeruginosa* to produce the pigment pyocyanin as well as fluorescein, a fluorescent product. *Pseudomonas aeruginosa* will typically produce a **green to blue, water-soluble pigment** and will also **fluoresce** when the plate is placed under a short wavelength (254nm) ultraviolet light.

5. Look for the production of the **green to blue, water soluble pigment** on the Pseudosel agar plate and record the results in the Results section of today's Exercise.
6. Look for the production of **fluorescent products** by placing the Pseudosel agar plate under a short wavelength ultraviolet lamp and looking for fluorescence. Record the results in the Results section.
7. To detect **odor**, lift the lid of the pseudosel agar plate and, using your hand, fan towards your nose. Record the results in the Results section.
8. The **oxidase test** was done last time. It is already recorded.
9. **Glucose fermentation** results are part of the Enterotube®II described below.
10. Since some common biotypes of *Pseudomonas* can be identified with the Enterotube®II, we will also see how those results come out. Keep in mind, however, that the Enterotube®II is designed for identifying members of the bacterial family Enterobacteriaceae and not necessarily nonfermentative gram-negative bacilli such as *Pseudomonas*. Record the Enterotube®II

results (as determined after following the instructions below in section E) in the Results section.

E. **IDENTIFYING MEMBERS OF THE ENTEROBACTERIACEAE WITH THE ENTEROTUBE®II**

The Enterotube®II contains 12 different agars that can be used to carry out 15 standard biochemical tests. Interpret the results of your Enterotube®II using the instructions below. For more detail on the 15 biochemical tests in the Enterotube®II, see **Table 2 A**.

Table 2A: Interpretation of the Enterotube II.

Compartment	Reaction	Negative	Positive
1	glucose fermentation	red/orange	yellow
1	gas production	wax not lifted	wax lifted

Remarks: Glucose - Any degree of yellow is positive. Acid end products from glucose fermentation turn the pH indicator from red (alkaline) to yellow (acid). Remarks: Gas - Positive is a definite and complete separation of the white wax overlay from the surface of the glucose medium. Detects gas from glucose fermentation.

Compartment	Reaction	Negative	Positive
2	Lysine decarboxylase activity	Yellow	Purple

Remarks: Any degree of purple is positive. Alkaline end products from the decarboxylation of lysine changes the pH indicator from pale yellow (acid) to purple (alkaline).

Compartment	Reaction	Negative	Positive
3	Ornithine decarboxylase activity	Yellow	Purple

Remarks: Any degree of purple is positive. Alkaline end products from the decarboxylation of ornithine changes the pH indicator from pale yellow (acid) to purple (alkaline).

Compartment	Reaction	Negative	Positive
4	Hydrogen sulfide production	Beige	Black
4	Indole production **(done last)**	Colorless	Red

Remarks: Hydrogen sulfide - Only a true black is positive. Reduction of thiosulfate produces hydrogen sulfide which reacts with iron salts to produce black ferric sulfide.
Remarks: Indole - **This test is not interpreted until all other compartments have been read**. Kovac's Reagent must be added before reading. Indole, produced from the breakdown of tryptophan, reacts with Kovac's reagent turning it red.

Compartment	Reaction	Negative	Positive
5	Adonitol fermentation	Red/orange	Yellow

Remarks: Any degree of yellow is positive. Acid end products from adonitol fermentation turn the pH indicator from red (alkaline) to yellow (acid).

Compartment	Reaction	Negative	Positive
6	Lactose fermentation	red/orange	yellow

Remarks: Any degree of yellow is positive. Acid end products from adonitol fermentation turn the pH indicator from red (alkaline) to yellow (acid).

Compartment	Reaction	Negative	Positive
7	Arabinose fermentation	Red /orange	Yellow

Remarks: Any degree of yellow is positive. Acid end products from adonitol fermentation turn the pH indicator from red (alkaline) to yellow (acid).

compartment	Reaction	Negative	Positive
8	Sorbitol fermentation	Red /orange	Yellow

Remarks: Any degree of yellow is positive. Acid end products from adonitol fermentation turn the pH indicator from red (alkaline) to yellow (acid).

Compartment	Reaction	Negative	Positive
9 **(not normally used)**	Voges-Proskauer	Colorless	Red

Remarks: This test is not used unless required later as a confirmatory test. Acetoin produced during the production of butylene glycol from glucose fermentation reacts with the added reagents KOH and alpha naphthol and turns red.

Compartment	Reaction	Negative	Positive

10	Dulcitol ferm.	Not yellow	Yellow
10	PA	Not black/smoky gray	Black/smoky gray

Remarks: Dulcitol - Yellow or pale yellow is positive. Any other color is negative. Acid from dulcitol fermentation turns the pH indicator from green (alkaline) to yellow (acid).
Remarks: PA - Pyruvic acid produced from deamination of phenylalanine reacts with ferric salts in the medium turning it black.

Compartment	Reaction	Negative	Positive
11	Urea hydrolysis	Beige	Red/purple

Remarks: Hydrolysis of urea forms ammonia which causes the pH indicator to turn from yellow (acid) to red/purple (alkaline).

Compartment	Reaction	Negative	Positive
12	Citrate utilization	Green	Blue

Remarks: Any degree of blue is positive. Utilization of citrate produces alkaline products turning the pH indicator from green (acid) to blue (alkaline).

1. Interpret the results of **glucose** fermentation in **compartment 1**.
   - any yellow = +; red or orange = -
   - If positive, circle the number 2 under glucose on your Results page.
2. Interpret the results of **gas** production also in **compartment 1**.
   - wax lifted from agar = +; wax not lifted from agar = -
   - If positive, circle the number 1 under gas on your Results page.
3. Interpret the results of **lysine** decarboxylase in **compartment 2**.
   - any purple = +; yellow = -
   - If positive, circle the number 4 under lysine on your Results page.
4. Interpret the results of **ornithine** decarboxylase in **compartment 3**.
   - any purple = +; yellow = -
   - If positive, circle the number 2 under ornithine on your Results page.
5. Interpret the results of **H2S** production in **compartment 4**.
   - true black = +; beige = -
   - If positive, circle the number 1 under H2S on your Results page.

6. **Indole** production also in **compartment 4**. Do not interpret the indole test at this time. Add Kovac's reagent only after all other tests have been read (see **step 16** below).
7. Interpret the results of **adonitol** fermentation in **compartment 5**.
    - any yellow = +; red or orange = -
    - If positive, circle the number 2 under adonitol on your Results page.
8. Interpret the results of **lactose** fermentation in **compartment 6**.
    - any yellow = +; red or orange = -
    - If positive, circle the number 1 under lactose on your Results page.
9. Interpret the results of **arabinose** fermentation in **compartment 7**.
    - any yellow = +; red or orange = -
    - If positive, circle the number 4 under arabinose on your Results page.
10. Interpret the results of **sorbitol** fermentation in **compartment 8**.
    - any yellow = +; red or orange = -
    - If positive, circle the number 2 under sorbitol on your Results page.
11. **Voges-Praskauer** test in **compartment 9**. This test is not used unless a final VP confirming test is later called for. **Skip this compartment**.
12. Interpret the results of **dulcitol** fermentation in **compartment 10**.
    - yellow = +; any other color = -
    - If positive, circle the number 1 under dulcitol on your Results page.
13. Interpret the results of **PA** deaminase also in **compartment 10**.
    - black or smoky gray = +; any other color = -
    - If positive, circle the number 4 under PA on your Results page.
14. Interpret the results of **urea** hydrolysis in **compartment 11**.
    - red or purple = +; beige = -
    - If positive, circle the number 2 under urea on your Results page.
15. Interpret the results of **citrate** utilization in **compartment 12**.
    - any blue = +; green = -
    - If positive, circle the number 1 under citrate on your Results page.
16. Using a hot inoculating loop, burn a small hole in the top of **compartment 4 (H2S/Indole)**. Using a sterile Pasteur pipette, add 2-3 drops of **Kovac's reagent** to the indole test by dropping it through the hole.
    - pink or red = +; colorless = -
    - If positive, circle the number 4 under indole on your Results page.
17. **Add all the circled numbers in each bracketed section** and enter the sum in the space provided below the arrow on your Results page.

18. Locate the 5 digit number in the **Computer Coding and Identification System (CCIS)** booklet and find the best identification in the column entitled "ID Value." (Should more than one organism be listed, the confirmatory tests indicated in the CCIS would normally then have to be performed. In addition, an identification of *Salmonella* or *Shigella* would usually be confirmed by direct serologic testing.
19. If there are any problems, consult your instructor.
20. **Every student should do a complete set of results for three different unknowns today: *Pseudomonas aeruginosa* and 2 different Enterobacteriaceae**.

F. API 20 E Interpretation

Follow instruction and read according to the following table

TEST	REAGENT	RESULTS	
		Negative	**Positive**
ONPG	Nil	Colorless	Yellow
ADH	Nil	Yellow	Orange
LDC	Nil	Yellow	Orange
ODC	Nil	Yellow	Orange
CIT	Nil	Pale green/yellow	Blue green/blue
H_2S	Nil	Colorless/greyish	Black deposit
URE	Nil	Yellow	Red/orange
TDA	TDA / Immediate	Yellow	Dark brown
IND	JAMES / Immediate	Colorless	Pink
VP	VP 1+2 / 10 mins	Colorless	Pink/red
GEL	Nil	No diffusion black	Diffusion black
GLU	Nil	Blue/blue green	Yellow
MAN	Nil	Blue/blue green	Yellow
INO	Nil	Blue/blue green	Yellow
SOR	Nil	Blue/blue green	Yellow
RHA	Nil	Blue/blue green	Yellow
SAC	Nil	Blue/blue green	Yellow
MEL	Nil	Blue/blue green	Yellow
AMY	Nil	Blue/blue green	Yellow
ARA	Nil	Blue/blue green	Yellow
NO_2	NIT 1 + NIT 2 / 2-3 mins.	Yellow	Red
OX			

RESULTS

A. **If the oxidase test performed on the unknown on Exercise 1 was positive, record the results here.**

1. **Oxidase test**

Results (+ or -)	
Description of immediate test	
Description of delayed test	

2. **Gram stain**

Gram reaction (purple = gram +; pink = gram -)	
Shape	

3. **Growth on Pseudosel agar**

Growth (+ or -)	
Conclusion	

4. **Pigment on Pseudosel**

Pigment (+ or -)	
Color and solubility of pigment	

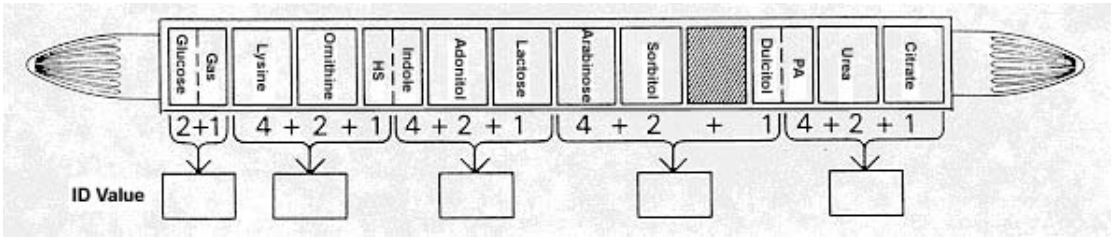
5. **Fluorescence on Pseudosel agar under ultraviolet light**

Fluorescence	

6. **odor on Pseudosel agar**

Description of odor	

7. **Enterotube®II**



IDENTITY OF ORGANISM

B. **If the <u>oxidase test</u> performed on the unknown on Exercise 1 was <u>negative</u>, record the results here.**
   1. **Oxidase**

Results (+ or -)	
Description of immediate test	
Description of delayed test	

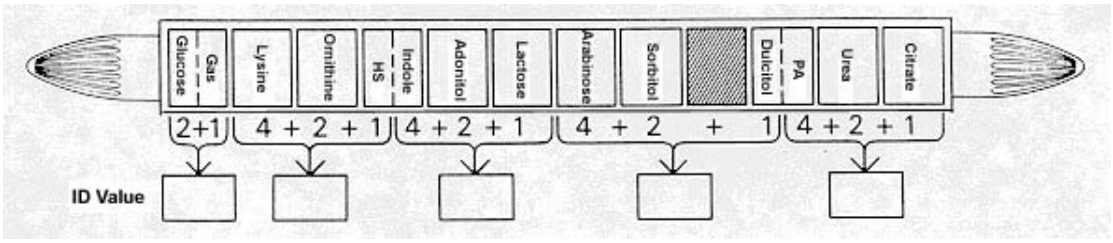
2. **Gram stain**

Gram reaction (purple = gram +; pink = gram -)	
Shape	

3. **XLD agar**

Growth (+ or -)	
Conclusion (G+ or G-)	
Description of colonies	
Probable organism(s)	

4. **Enterotube®II**



IDENTITY OF ORGANISM

_____

C. **If the <u>oxidase test</u> performed on the unknown on Exercise 1 was <u>negative</u>, record the results here.**

1. **Oxidase test**

Results (+ or -)	
Description of immediate test	
Description of delayed test	

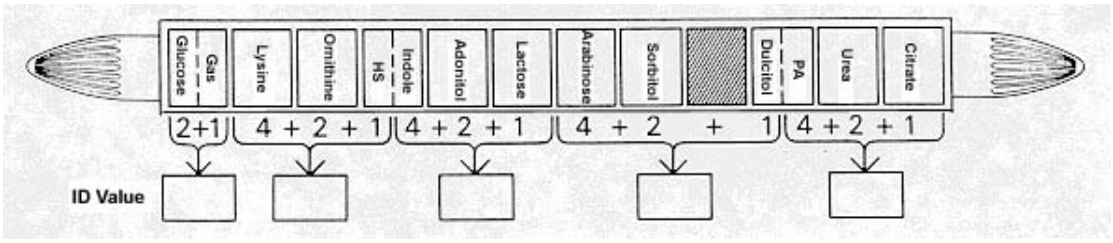
2. **Gram stain**

gram reaction (purple = gram +; pink = gram -)	
shape	

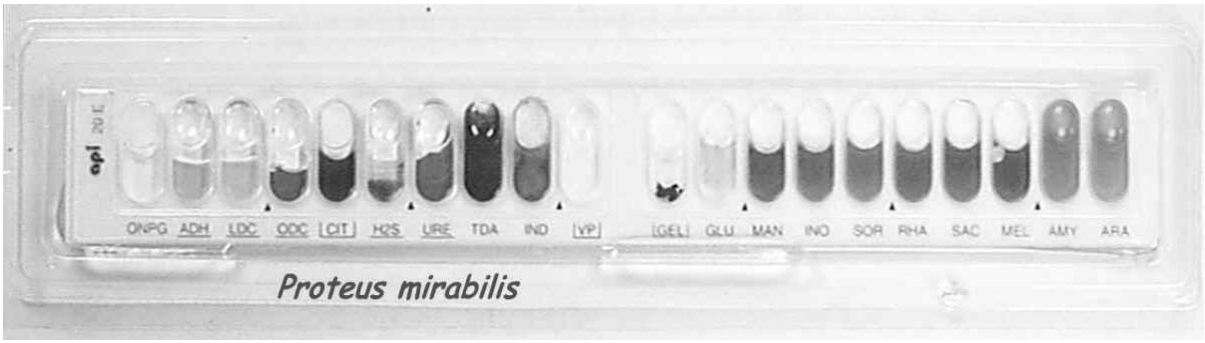
3. **XLD agar**

Growth (+ or -)	
Conclusion (G+ or G-)	
Description of colonies	
Probable organism(s)	

4. **Enterotube®II**



IDENTITY OF ORGANISM



Isolation and Identification of

Streptococci and Enterococci

OBJECTIVES

After completing this Exercise, the student will be able to perform the following objectives:

GENERAL DISCUSSION

1. State the gram reaction and morphology of the streptococci.
2. State two ways the streptococci are classified.
3. Describe alpha hemolysis, beta hemolysis, and gamma reaction on Blood agar plates.
4. State what is meant by the Lancefield system.
5. State the Lancefield group of streptococcus that is the most common cause of acute streptococcal infections in humans and name five other Lancefield groups that frequently cause human infections.

A. **THE BETA STREPTOCOCCI**
   - **DISCUSSION**
     1. State what the term "group A beta" means when referring to streptococci.
     2. State the genus and species of the group A beta streptococci.
     3. State the most common infection caused by *Streptococcus pyogenes* and name six other infections it may cause.
     4. Name two autoimmune diseases associated with the group A beta streptococci.
     5. State the genus and species of the group B streptococci.
     6. State the normal habitat of the group B streptococci, name three infections they may cause in newborns, and describe how the infants become colonized.
     7. Name three infections the group B streptococci may cause in adults.
   - **ISOLATION AND IDENTIFICATION OF GROUP A BETA STREPTOCOCCI**
     1. Describe the appearance of group A beta streptococci on Blood agar.
     2. State why Blood agar is usually stabbed during streaking when isolating beta streptococci.
     3. Describe the reaction of group A beta streptococci to a Taxo A® disc containing bacitracin.

- **RESULTS OF GROUP A BETA STREPTOCOCCI**
    1. Identify an organism as a group A beta streptococcus (or *Streptococcus pyogenes*) and state the reasons why when it is seen growing on a Blood agar plate with a Taxo A® disc containing bacitracin.
    2. Recognize beta hemolysis on Blood agar.

B. **THE GROUP D STREPTOCOCCI: THE FECAL STREPTOCOCCI**

- **DISCUSSION**
    1. Name two fecal streptococci that commonly infect humans.
    2. State the Lancefield group of the enterococci.
    3. Name an infection commonly caused by *Streptococcus bovis*.
    4. Name four infections commonly caused by *Enterococcus faecalis*.
- **ISOLATION AND IDENTIFICATION OF ENTEROCOCCI**
    1. Describe the reactions of enterococci in SF broth and on Bile Esculin agar.
       State the gram reaction and morphology of the enterococci.
- **RESULTS OF THE ENTEROCOCCI**
    1. Identify an organism as an *Enterococcus* and state the reasons why when it is seen growing in SF broth (*Streptococcus faecalis* broth) and on Bile Esculin agar.

C. **THE PNEUMOCOCCUS**

- **DISCUSSION**
    1. State the genus and species of the pneumococcus.
    2. State the gram reaction and morphology of *Streptococcus pneumoniae*.
    3. State the natural habitat of *Streptococcus pneumoniae* and name four infections it may cause in humans.
- **ISOLATION AND IDENTIFICATION OF PNEUMOCOCCI**
    1. Describe the appearance of *Streptococcus pneumoniae* on Blood agar with a Taxo P® disc containing the drug optochin.
- **RESULTS OF PNEUMOCOCCI**
    1. Identify an organism as *Streptococcus pneumoniae* and state the reasons why when it is seen growing on a Blood agar plate with a Taxo P® disc containing optochin.
    2. Recognize alpha hemolysis on Blood agar.

D. **THE VIRIDANS STREPTOCOCCI**

1. State the normal habitat of the viridans streptococci and name three infections they may cause in humans.
2. State the hemolytic reactions of the viridans streptococci on Blood agar.

GENERAL DISCUSSION

The streptococci are **gram-positive cocci** (0.5-1.0μm in diameter) which occur **in pairs and chains** of varying length. They are usually classified based on their **hemolytic properties on blood agar** and according to their **serologic groups**.

The streptococci are usually isolated on Blood agar. **Blood agar** is one of the most commonly used medium in a clinical lab. It consists of an enriched agar base (Tryptic Soy agar) to which **5% sheep red blood cells** has been added. Blood agar is commonly used to isolate not only streptococci, but also staphylococci and many other pathogens. Besides providing enrichments for the growth of fastidious pathogens, Blood agar can be used to detect hemolytic properties.

Hemolysis refers to is the lysis of the red blood cells in the agar surrounding bacterial colonies and is a result of bacterial enzymes called **hemolysins**. Although hemolysis can often be observed with the naked eye, ideally it should be examined microscopically using low power magnification, especially in cases of doubtful hemolysis. Reactions on blood agar are said to be beta, alpha, gamma, or double-zone:

1. **Beta hemolysis** refers to a **clear, colorless zone** surrounding the colony, where a **complete lysis of the red blood cells** by the hemolysins has occurred. This is best seen in subsurface colonies where the agar has been stabbed since some bacterial hemolysins, like streptolysin O, are inactivated by oxygen.
2. **Alpha hemolysis** appears as a zone of **partial hemolysis** surrounding the colony, often accompanied by a **greenish discoloration of the agar**. This is also best seen in subsurface colonies where the agar has been stabbed.
3. **Gamma reaction** refers to **no hemolysis or discoloration of the agar** surrounding the colony.
4. **Double-zone hemolysis** refers to **both a beta and an alpha zone of hemolysis** surrounding the colony.

Many of the streptococci can also be classified under the **Lancefield system**. In this case, they are divided into a number of distinct **serologic groups** on the basis of carbohydrate antigens in their cell wall. These antigenic groups are designated by the letters A through T. Lancefield serologic groups A, B, C, D, F, and G are the ones that normally infect humans, however, not all pathogenic streptococci can be identified by Lancefield typing (e.g., *Streptococcus pneumoniae*).

o DISCUSSION

Lancefield serologic groups A, B, C, D, F, and G are all streptococci that may show beta hemolysis on Blood agar. However, some group B streptococci are nonhemolytic and group D streptococci (discussed below) usually show alpha hemolysis or are nonhemolytic.

The **group A beta hemolytic streptococci (*Streptococcus pyogenes*)** are responsible for most acute human streptococcal infections. Between 5% and 20% of children are asymptomatic carriers. The most common infection is **pharyngitis** (streptococcal sore throat) with the organism usually being limited to the mucous membranes and lymphatic tissue of the upper respiratory tract. From the pharynx, however, the streptococci sometimes **spread to other areas of the respiratory tract resulting in laryngitis, bronchitis, pneumonia, and otitis media (ear infection)**. Occasionally, it may enter the **lymphatic vessels or the blood** and disseminate to other areas of the body, causing **septicemia, osteomyelitis, endocarditis, septic arthritis, and meningitis**. If it enters **injured skin**, it may cause **cellulitis.** If it produces **erythrogenic toxin** (as a result of phage conversion), it can cause **scarlet fever**. Finally, it can result in two **autoimmune diseases, rheumatic fever and acute glomerulonephritis**, where antibodies made against streptococcal antigens cross react with joint membranes and heart valve tissue in the case of rheumatic fever, or glomerular cells and basement membranes of the kidneys in the case of acute glomerulonephritis.

Virulence factors include lipoteichoic acid (which allows adherence to target host cells); M-protein (which resists phagocytic engulfment); tissue damaging toxins such as leukocidin (kills leukocytes) and erythrogenic toxin (damages endothelium); and tissue damaging enzymes such as proteinase, hyaluronidase, and Dnase (thought to be especially important in infections of the skin and soft tissues as well as the rapid spread of the organism through the lymphatics).

The **group B streptococci** (*Streptococcus agalactiae*) usually show a small zone of beta hemolysis on Blood agar, although some strains are nonhemolytic. They are often found in the **genital and intestinal tracts** of healthy adults with from 5% to 40%, depending on the population, carrying the bacteria. This reservoir, along with nosocomial transmission, provides the inoculum by which many infants are colonized at birth. Most colonized infants (and adults) remain asymptomatic; however, an estimated **0.5-1.0% of neonates colonized will develop pneumonia, septicemia, and/or meningitis** from this organism. Other infections associated with group B streptococci include **urinary tract infections, wound infections, otitis media, and infected ulcers (decubitus ulcers and ulcers associated with diabetes)**. In the bedridden patient it sometimes causes **pneumonia and meningitis**.

The **group C streptococci** (mainly *S. equisimilis* and *S. zooepidemicus*) are beta hemolytic. They sometimes cause pharyngitis and, occasionally, bacteremia, endocarditis, meningitis, pneumonia, septic arthritis, and cellulitis.

The **group F streptococci** (mainly *S. milleri*) have been isolated from abscesses of the brain, mouth, and jaw. They also sometimes cause endocarditis.

The **group G streptococci** also show beta hemolysis. They can cause serious infections of the skin and soft tissues (mainly in the compromised host) as well as endocarditis, bacteremia, and peritonitis.

All of these beta hemolytic streptococci can be identified by biochemical testing and/or by serologic testing. Today you will look at the isolation and identification of group A beta streptococci (*Streptococcus pyogenes*) by biochemical testing. Serological identification will be performed in later exercises.

- **ISOLATION AND IDENTIFICATION OF GROUP A BETA STREPTOCOCCI (*Streptococcus pyogenes*)**

Group A beta streptococci are usually isolated on **Blood agar**. They produce very **small, white to grey colonies** approximately 1mm in diameter surrounded by a zone of **beta hemolysis** around 2-3mm in diameter. There are two streptococcal hemolysins, streptolysin S and streptolysin O. Streptolysin O can be **inactivated by oxygen** so more distinct hemolysis can be seen by **stabbing the agar several times**. In this way, some of the organisms form subsurface colonies growing away from oxygen. Since both streptolysin S and streptolysin O are active in the stabbed area, a clearer zone of beta hemolysis can be seen.

To determine if the beta hemolytic organism is a group A streptococcus, direct serologic testing can be performed. Alternately, one can test for **sensitivity to the antibiotic bacitracin**. Only *Streptococcus pyogenes* among the group A beta streptococci is sensitive to bacitracin, as shown by a **zone of inhibition around a Taxo A® disc**, a paper disc containing low levels of bacitracin. Other serologic groups of streptococci are resistant to bacitracin and show no inhibition around the disc.

- **MATERIALS**(per pair)

Blood agar plates (2), 1 Taxo A (bacitracin) disc, loop, swab, tongue depressor

- **ORGANISM**

Trypticase Soy broth culture of *Streptococcus pyogenes* (a group A beta streptococcus).

HANDLE AS A PATHOGEN! WASH YOUR HANDS WELL WHEN FINISHED!

- **PROCEDURE** (to be done in pairs)

1. Take a **Blood agar plate and divide it in half** with your wax marker. **After washing your hands**, do a **throat culture** on your lab partner as follows:

a. Depress the tongue with a sterile tongue depressor (**do not lay the tongue depressor down once you pick it up**) and scrape the pharynx with a sterile swab.

b. Streak about **one-third** of your half of the plate with the **swab**. Discard the swab in the disinfectant container.

c. Spread the portion of the plate that you streaked with the swab over the remaining portion using a sterile **inoculating loop**.

d. **Stab** the agar 2-3 times in the streaked area with your loop.

e. Incubate at **37 ^{o}C** until the next lab period.

2. Streak a **second plate of Blood agar** with ***Streptococcus pyogenes*** for isolation as follows:

a. Dip a sterile **swab** in your culture of *S. pyogenes.* Squeeze the swab against the side of the tube to remove the excess inoculum.

b. Streak about **one-third** of the plate with the **swab**. Dispose of the swab in the disinfectant container.

c. Using a sterile **inoculating loop** spread the swabbed area over the remainder of the plate.

d. **Stab** the agar several times in each of the 3 growth areas with your loop. Place a **Taxo A® disc** containing bacitracin on the area of the plate that you streaked with the swab.

3. Incubate **at 37C** until the next lab period.

4. Do **gram stain** of *S. pyogenes*.

B. THE GROUP D STREPTOCOCCI: THE ENTEROCOCCI AND OTHER FECAL STREPTOCOCCI

o DISCUSSION

Streptococcus bovis and *Enterococcus faecalis* (formally *Streptococcus faecalis*) are the two most common **group D streptococci** that infect humans. Both are **normal flora of the intestinal tract**. Both organisms are among the most common organisms causing **endocarditis**. *S. bovis* endocarditis is frequently associated with patients having lesions of the large intestine, such as polyps and carcinomas. While *S. bovis* rarely causes infections other than endocarditis, *Enterococcus faecalis* is regularly isolated from **infections within the peritoneal cavity** (especially following penetrating trauma), **urinary tract infections, kidney infections, prostate infections, and infections of damaged or compromised skin** (e.g., diabetic or decubitus ulcers, and burns). Other opportunistic fecal streptococci include *E. faecium* and *E. durans*. Most group D streptococci show alpha hemolysis or no hemolysis on Blood agar, although some strains are beta hemolytic.

o ISOLATION AND IDENTIFICATION OF ENTEROCOCCI

The enterococci may be isolated and identified using various selective and differential media. Two such media are:

1. **SF broth**

SF broth contains sodium azide, which inhibits most bacteria other than enterococci. The enterococci will grow in SF broth and ferment the dextrose, turning the pH indicator **from violet to a yellow-brown color**.

2. **Bile Esculin agar**

Unlike most bacteria, the enterococci will grow in the presence of the bile salts in the medium. They hydrolyze the esculin, producing esculetin which reacts with the iron salts in the medium turning the agar **black**.

- **MATERIALS**

  1 tube of SF broth, 1 Bile Esculin agar slant, sterile swab.

- **ORGANISM**

  Trypticase Soy broth culture of *Enterococcus faecalis*

- **PROCEDURE** (to be done in pairs)
  1. Using a sterile **swab**, inoculate the **SF broth tube** and the **Bile Esculin agar slant** with *Enterococcus faecalis*.
  2. Incubate both tubes **at 37C** until the next lab period.
  3. Do a **gram stain** of the *E. faecalis*.

C. **THE PNEUMOCCUS (*Streptococcus pneumoniae*)**

- **DISCUSSION**

Streptococcus pneumoniae, or the pneumococcus, is a gram-positive lanceolate coccus usually appearing as a **diplococcus**, but occasionally appearing singularly or in short chains. Pneumococci are frequently found as normal flora of the **nasopharynx of healthy carriers**. From 10% to 40% of adults carry the bacterium in the nasopharynx. They are **the most common cause of pneumonia that requires hospitalization**, being responsible for over 90% of the cases of bacterial lobar pneumonia (which usually occurs as a secondary infection in the debilitated or immunocompromised host). The pneumococci are the major cause of **otitis media** in children and **sinusitis** in people of all ages. In addition, *S. pneumoniae* is the most common cause of **meningitis** in adults and children over 4 years of age. Septicemia and endocarditis are also sometimes caused by pneumococci. The capsule serves as the major virulence factor, enabling the pneumococcus to resist phagocytic engulfment. Pneumococci show **alpha hemolysis** on Blood agar.

- **ISOLATION AND IDENTIFICATION OF PNEUMOCOCCI (*Streptococcus pneumoniae)***

0. **Isolation on Blood agar**

Pneumococci frequently require enriched media and increased CO_2 tension for initial isolation. They are usually isolated on **Blood agar** and incubated in a

candle jar (a closed container in which a lit candle is placed to remove O_2 and increase CO_2) at 37 ^oC. On Blood agar, colonies appear small, shiny, flattened, and translucent. They are surrounded by a zone of **alpha hemolysis.** Due to autolysis with age, the colonies may show a depressed center with an elevated rim.

1. **Optochin sensitivity**

   Pneumococci are the only streptococci that are **sensitive to the drug optochin** (ethylhydrocupreine hydrochloride). This can be detected by a **zone of inhibition around a Taxo P® disc,** a paper disc containing the drug optochin, which is placed on the Blood agar plate prior to incubation.

2. **Gram stain of sputum**

   Streptococcus pneumoniae will usually appear as encapsulated, **gram-positive, lancet-shaped diplococci**.

- **PROCEDURE** (demonstration)

  Observe the demonstration of *Streptococcus pneumoniae* growing on a Blood agar plate with a Taxo P® disc containing optochin.

D. **THE VIRIDANS STREPTOCOCCI**
   - **DISCUSSION**

Several species of streptococci are known as the **viridans streptococci**. They are the **dominant normal flora in the upper respiratory tract**. Species include *S. mutans, S. sanguis, S. mitis*, and *S. salivarius*. *S. mutans* is the primary cause of **dental caries**. Viridans streptococci are responsible for between 50% and 70% of the cases of **bacterial endocarditis**, especially in people with previously damaged heart valves. They are also frequently associated with **bacteremia, deep wound infections, dental abscesses, and abscesses of internal organs**. The viridans streptococci show **alpha hemolysis or no hemolysis** on Blood agar, do not possess Lancefield group antigens, and can be differentiated from other alpha streptococci by biochemical testing.

Most of the colonies you will see on **your throat culture** you do today will be alpha and gamma viridans streptococci.

RESULTS

A. **Group A Beta Streptococci (*Streptococcus pyogenes*)**

1. **Blood agar with Taxo A® (bacitracin) disc**

Description of colony	
Type of hemolysis (alpha, beta, or gamma)	
Taxo A® disk (bacitracin) results (inhibition or no inhibition)	

2. **Gram stain**

Gram stain of *Streptococcus pyogenes*

gram reaction (purple = +; pink = -)	
shape and arrangement	

B. **Enterococci (*Enterococcus faecalis*)**

1. **SF broth**

Growth (+ or -)	
Color of broth (violet or yellow-brown)	
Fermentation of dextrose (+ or -)	

2. **Bile Esculin agar**

Growth (+ or -)	
Color of agar (black or beige)	
Hydrolysis of Esculin (+ or -)	

3. **Gram stain**

Gram stain of *Enterococcus*

Gram reaction (purple = +; pink = -)	
Shape and arrangement	

C. **Pneumococci (*Streptococcus pneumoniae*)**

1. **Blood agar with (optochin) disc**

Description of colony	
Type of hemolysis (alpha, beta, or gamma)	
(Optochin) results (inhibition or no inhibition)	

D. **Viridans streptococci**

Observe the results of your throat culture and note the type(s) of hemolysis seen.

Isolation and Identification of Staphylococci

OBJECTIVES

After completing this Exercise, the student will be able to perform the following objectives:

DISCUSSION

1. Name three common clinically important species of *Staphylococcus* and state which is most pathogenic.
2. State the sources and the portal of entry for most *Staphylococcus aureus* infections.
3. Name and describe three types of abscesses caused by *Staphylococcus aureus*.
4. Name four systemic *Staphylococcus aureus* infections.
5. State the significance of *Staphylococcus aureus* enterotoxin, the exotoxin TSST-1, and the exotoxin exfoliatin.
6. Name the infection normally caused by *Staphylococcus saprophyticus*. Name the types of infections most commonly caused by coagulase-negative staphylococci other than *Staphylococcus saprophyticus*.

ISOLATION AND IDENTIFICATION OF STAPHYLOCOCCI

1. State the gram reaction and morphology of all staphylococci.
2. Describe the typical reactions of *S. aureus, S. epidermidis,* and *S. saprophyticus* on each of the following media:
    a. Blood agar (pigment, hemolysis, novobiocin resistance)
    b. Mannitol Salt agar (for mannitol fermentation)
    c. DNase agar (for the enzyme DNase)
    d. Coagulase test with citrated rabbit plasma
    e. Staphyloslide® test for cell wall clumping factor

RESULTS

1. Recognize staphylococci in a gram stain preparation.
2. Recognize an organism as *Staphylococcus aureus* and state the reasons why after seeing the results of the following:
    a. a Blood agar plate with a novobiocin disc
    b. a Mannitol Salt agar plate
    c. a DNase agar plate
    d. a tube of citrated rabbit plasma
    e. a Staphyloslide® test

DISCUSSION

Staphylococci are often found in the human nasal cavity (and on other mucous membranes) as well as on the skin. They are **gram-positive cocci** 0.8-1.0µm in diameter and occur singly, in pairs, in short chains, and most commonly, in **irregular grape-like clusters**. The staphylococci are strongly catalase positive, reduce nitrates to nitrites, and generally tolerate relatively high concentrations of sodium chloride (7.5-10%) and tellurite. This ability is often employed in preparing media selective for staphylococci.

There are five species of staphylococci **commonly associated with clinical infections:** *Staphylococcus aureus, S. epidermidis, S. haemolyticus, S. hominis* and *S. saprophyticus*.

Staphylococcus aureus is the most pathogenic species and is implicated in a variety of infections. Approximately 30% of adults and most children are healthy nasal carriers of *S. aureus*. In the majority of *S. aureus* infections the source of the organism is either the **healthy nasal carrier** or **contact with an abscess** from an infected individual. The **portal of entry** is usually the **skin**. *S. aureus* causes pus-filled inflammatory lesions known as **abscesses**. Depending on the location and extent of tissue involvement, the abscess may be called a **pustule** (an infected hair follicle), a **furuncle** or boil (if it spreads from the hair follicle to adjacent subcutaneous tissue), or a **carbuncle** (multiple infection sites involving deeper connective tissue). It may also spread through soft tissues and cause **cellulitis**. *S. aureus* **frequently causes infections of accidental wounds and postoperative wounds**, although it can also infect healthy intact skin.

Less commonly, *S. aureus* may escape from the local lesion and spread through the blood to other body areas, causing a variety of **systemic infections** that may involve every system and organ. Such systemic infections include **septicemia, septic arthritis, endocarditis, meningitis, and osteomyelitis**, as well as **abscesses in the lungs, spleen, liver, and kidneys.** *S. aureus* **pneumonia** may also be a secondary respiratory complication of viral infections such as measles and influenza. Finally, *S. aureus* is frequently introduced into food by way of abscesses or the nasal cavity of food handlers. If it is allowed to grow and produces an **enterotoxin,** it can cause **staphylococcal food poisoning**.

Virulence factors for *S. aureus* include exotoxins such as leukocidin (kills leukocytes), alpha and delta toxins (damage tissue membranes), microcapsules (resist phagocytic engulfment and destruction), coagulase and protein A (both help resist phagocytic engulfment). Some strains also produce **TSST-1** (toxic shock syndrome toxin-1) and cause **toxic shock syndrome,** usually associated with tampon use or wounds. Some strains also produce **exfoliatin**, an exotoxin which causes **scalded skin syndrome**, an infection usually seen in infants and young children.

Since most *S. aureus* strains produce the enzyme coagulase (see the coagulase test described below), they are often referred to as **coagulase-positive staphylococci**.

Clinically common species of staphylococci other than *S. aureus* are often referred to as **coagulase-negative staphylococci**. These staphylococci are normal flora of the skin and, as such, frequently act as **opportunistic pathogens**, especially in the compromised host. *S. saprophyticus* is a relatively common cause of **urinary tract infections**, especially in healthy young women, but is seldom isolated from other sources. The great **majority of infections caused by other coagulase-negative staphylococci**, including *S. epidermidis, S. haemolyticus, and S. hominis,* are associated with **intravascular devices** (prosthetic heart valves and intra-arterial or intravenous lines) and **shunts**. Also quite common are **infections of prosthetic joints, wound infections, osteomyelitis** associated with foreign bodies, and **endocarditis**.

Although certain reactions may vary from strain to strain, a series of biochemical tests will usually differentiate the most common clinically encountered species of staphylococci. Today we will use a number of tests to determine if an unknown is *S. aureus, S. epidermidis,* or *S. saprophyticus*.

ISOLATION AND IDENTIFICATION OF STAPHYLOCOCCI

1. Blood agar with a novobiocin (NB) disc

To isolate staphylococci, clinical specimens are usually grown on Blood agar. Staphylococci produce round, raised, opaque colonies 1-2mm in diameter. The novobiocin disc is used to detect sensitivity or resistance to the antibiotic novobiocin.

Test	***Staphylococcus aureus***	***Staphylococcus epidermidis***	***Staphylococcus saprophyticus***
Hemolysis (*)	Usually beta(1)	Usually none(2)	Usually none(2)
Pigment	Often creamy gold(1)	Usually white(2)	Usually white(2)
Novobiocin test	Sensitive	Sensitive	Resistant

(*) see Exercise 3 for descriptions of hemolysis
(1) some strains do not show hemolysis and/or pigment
(2) some strains do show hemolysis and/or pigment
sensitive = zone of inhibition around disc
resistant = no zone of inhibition around disc

2. **Gram stain**

   All staphylococci appear as gram-positive cocci, usually in irregular, often grape-like clusters.

3. **Mannitol fermentation on Mannitol Salt agar (MSA)**

   Staphylococci are able to tolerate the high salt concentration found in Mannitol Salt agar and thus grow readily. If mannitol is fermented, the acid produced turns the phenol red pH indicator from red (alkaline) to yellow (acid).

Test	***Staphylococcus aureus***	***Staphylococcus epidermidis***	***Staphylococcus saprophyticus***
Mannitol fermentation	Positive	Negative	Usually positive

Positive = acid end products turn the phenol red pH indicator from red to yellow
Negative = prenol red remains red

4. **Production of deoxyribonuclease (DNase) on DNase agar**

   DNase agar contains 0.2% DNA. To detect DNase production, the plate is inoculated and incubated. After growth, the plate is flooded with 1N hydrochloric acid (HCl). DNase positive cultures show a distinct clear zone around the streak where the DNA was broken down by the DNase. DNase negative cultures appear cloudy around the growth where the acid caused the DNA in the agar to precipitate out of solution.

Test	***Staphylococcus aureus***	***Staphylococcus epidermidis***	***Staphylococcus saprophyticus***
DNase production	Positive	Negative	Negative

Positive = clear zone around growth after adding 1N HCl (no DNA remaining in the agar)
Negative = cloudy around growth after adding 1N HCl (DNA remains in the agar forming a precipitate)

5. **Production of coagulase**

   The staphylococcal enzyme coagulase will cause inoculated citrated rabbit plasma to gel or coagulate. The coagulase converts soluble fibrinogen in the plasma into insoluble fibrin.

Test	*Staphylococcus aureus*	*Staphylococcus epidermidis*	*Staphylococcus saprophyticus*
Coagulase production	Positive	Negative	Negative

Positive = plasma will gel or coagulate
Negative = plasma will not gel

6. **The Staphyloslide® test for cell wall clumping factor**

   The Staphyloslide® test detects a cell wall polypeptide clumping factor distinct from coagulase. The test uses sheep red blood cells coated with fibrinogen which are mixed with the organism. Clumping factor, if present on the bacterial cell wall, converts soluble fibrinogen to insoluble fibrin causing the sheep red blood cells to clump together.

Test	*Staphylococcus aureus*	*Staphylococcus epidermidis*	*Staphylococcus saprophyticus*
cell wall clumping factor	Positive	Negative	Negative

Positive = clumping of sheep red blood cells
Negative = no clumping of sheep red blood cells

MATERIALS (per pair)

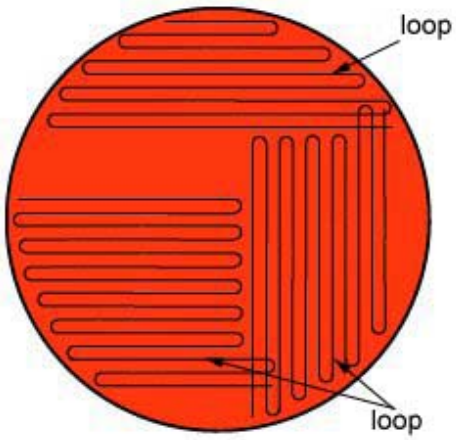
1 Blood agar plate, 2 Mannitol Salt agar plates, 1 DNase agar plate, 1 tube of citrated rabbit plasma (coagulase test), 1 novobiocin disc, inoculating loop, swab.

ORGANISMS

A Trypticase Soy broth culture of either *Staphylococcus aureus, Staphylococcus epidermidis* or *Staphylococcus saprophyticus***.**

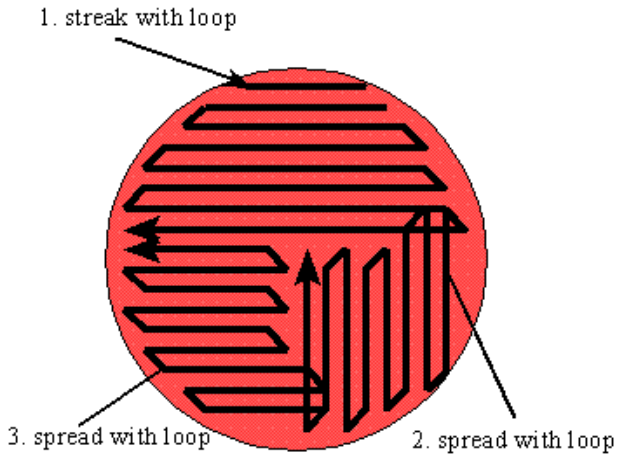
PROCEDURE (to be done in pairs)

1. Using your loop, streak your unknown for isolation on a plate of Blood agar as described below and shown in Fig. 4A.

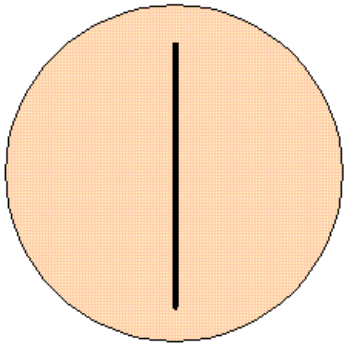


a. Streak your unknown on the plate so as to get single, isolated colonies.
b. Stab the agar several times to detect oxygen-sensitive hemolysins.
c. Place a novobiocin antibiotic disc on the plate in the area you expect to see heaviest growth.
d. ncubate at 37C until the next lab period.

2. Streak your unknown for isolation on a plate of Mannitol Salt agar (MSA) as shown in Fig. 4B. Incubate at 37C.



3. **Streak a single line of your unknown down the center of a plate of DNase agar as shown in Fig. 4C. Incubate at 37C.**



4. To determine if you are a nasal carrier of *S. aureus*, take a second plate of Mannitol Salt agar, divide it in half, and do a nasal culture on your half of the plate as follows:
    a. Stick a sterile swab up your nose.
    b. Streak about one-third of your portion of the plate with the swab. Discard the swab in the disinfectant container.
    c. Spread this out over the remainder of the plate with a sterile loop.
    d. Incubate at 37 ^{o}C until the next lab period.
5. Inoculate a tube of citrated rabbit plasma with your unknown and incubate at 37C.
6. Next lab period, observe the demonstrations of the Staphyloslide® tests performed on each of the unknowns by mixing the bacteria with fibrinogen-coated sheep red blood cells.
7. Next lab period, do a gram stain of your unknown.

RESULTS

1. Flood the surface of your DNase agar plate with 1N HCl.
2. Observe the demonstration of the Staphyloslide® test that was done on your unknown.
3. Review the expected results for each test on each unknown using the tables found earlier in this lab exercise. Interpret the results for all unknowns and fill in the following results table.

Test	**Unknown #1**	**Unknown #2**	**Unknown 3#**
Hemolysis on blood agar (alpha, beta, gamma)			
Pigment on blood agar (yellow-gold or white)			
Novobiocin (NB) disc (sensitive or resistant)			
Mannitol fermentation			

(positive or negative)			
DNase production (positive or negative)			
Coagulase production (positive or negative)			
Cell wall clumping factor (positive or negative)			

4. **Gram stain results**

   Gram stain of *Staphylococcus*

Gram reaction (purple = +; pink = -)	
Shape and arrangement	

Conclusion:

- **Unknown #1 =** _____
- **Unknown #2 =** _____
- **Unknown #3 =** _____

5. Observe the results of your nasal culture on Mannitol Salt agar. If your nasal bacteria fermented mannitol (phenol red turned yellow), run a Staphyloslide® test to confirm that the organism is *Staphylococcus aureus*.

Isolation and Identification of Neisseriae, Mycobacteria, and Anaerobes

OBJECTIVES

After completing this Exercise, the student will be able to perform the following objectives:

A. **THE NEISSERIAE**
   - **DISCUSSION**
     1. State the gram reaction and the morphology of the Neisseria.
     2. State the correct scientific names for the gonococcus and the meningococcus and indicate what disease each causes.
     3. Describe how symptoms of gonorrhea differ in the male and in the female.
     4. State the possible urogenital complications of gonorrhea in the male and in the female.
     5. Name four possible extragenital *Neisseria gonorrhoeae* infections.
     6. State how congenital gonorrhea usually appears.
     7. Give the normal habitat for *Neisseria meningitidis* and briefly describe how it reaches the meninges.
   - **ISOLATION AND IDENTIFICATION OF PATHOGENIC NEISSERIAE**
     1. Describe the appearance of a positive GC smear and indicate its significance in the diagnosis of gonorrhea.
     2. State where clinical specimens are obtained in the male and in the female for the isolation of *Neisseria gonorrhoeae*.
     3. State where clinical specimens are obtained for the isolation of *Neisseria meningitidis* when it is causing meningitis.
     4. Name a selective medium useful for the isolation of pathogenic *Neisseria* and describe how the resulting colonies will appear.
     5. State the results of *N. gonorrhoeae* and *N. meningitidis* for the oxidase test and for the fermentation of carbohydrates in CTA glucose, CTA maltose, and CTA sucrose media.
   - **RESULTS**
     1. Identify a positive GC smear and state how you can tell it is positive.
     2. Identify an organism as *N. gonorrhoeae* or *N. meningitidis* and state the reasons why when it is seen growing on MTM Chocolate agar with a Taxo N® (oxidase) disc and in CTA glucose, CTA maltose, and CTA sucrose media.

B. **THE MYCOBACTERIA**

- **DISCUSSION**
  1. Discuss one characteristic common to the genus *Mycobacterium* which allows us to distinguish this organism from most other genera of bacteria.
  2. List two pathogenic species of *Mycobacterium* and name the infection that each causes.
  3. State the significance of *Mycobacterium avium-intracellulare* complex (MAC).
- **DIAGNOSIS OF TUBERCULOSIS**
  1. State three presumptive tests for the diagnosis of tuberculosis.
  2. State how a diagnosis of active tuberculosis is confirmed.
  3. Describe the appearance of a positive acid-fast stain for tuberculosis.
- **RESULTS**
  1. Identify a positive acid-fast stain and state how you can tell it is positive.
  2. Recognize granuloma when shown a slide of a tuberculoid organ.

C. **THE OBLIGATE ANAEROBES**

- **DISCUSSION**
  1. Name the most common obligate anaerobe to cause infections in humans, state its normal habitat, and name the most common type of infections it causes.
  2. State three ways of culturing obligate anaerobes in the lab.
  3. State the normal habitat of *Clostridium perfringens* and name an infection it may cause.
- **ISOLATION AND IDENTIFICATION OF *CLOSTRIDIUM PERFRINGENS***
  1. Describe the appearance of *C. perfringens* when it is anaerobically-grown on Blood agar and in Litmus Milk.
- **RESULTS**
  1. Identify an organism as *C. perfringens* and state the reasons why when given anaerobically-grown cultures of Blood agar and Litmus Milk.

A. **THE NEISSERIAE**

DISCUSSION

The neisseriae are a group of **gram-negative cocci** usually occurring in pairs (**diplococci**). Two species of *Neisseria*, *N. gonorrhoeae* and *N. meningitidis*, are considered as true human pathogens. Both of these organisms possess pili for adherence to host cells, produce endotoxins, and resist destruction within phagocytes. *N. meningitidis* also produces a capsule to resist phagocytic engulfment.

***Neisseria gonorrhoeae* (the gonococcus)**, causes **gonorrhea**. It is estimated that there are between two and three million cases of gonorrhea per year in the U.S. Infection usually occurs following sexual contact, with the incubation period averaging 3-5 days.

In **males**, the gonococcus typically invades the anterior **urethra**, usually producing a **purulent discharge, pain upon urination, and a frequency of urination**. Up to one-third of the infected males, however, **may not show these classical symptoms** but will still be infectious. The infection may spread up the reproductive tract, infecting the **prostate, vas deferens, epididymis, and testes**, causing painful inflammation and scar tissue formation that can result in **sterility**.

In **females, 80-90%** of those initially infected are **asymptomatic or show mild symptoms**. They are, however, still infectious. Initially, the organism invades the **cervix**, the **urethra**, and frequently the **rectum**. In about 15% of the cases, the organism spreads up the reproductive tract and infects the **fallopian tubes** causing **pelvic inflammatory disease (PID)**. The resulting inflammation and scar tissue formation may result in **sterility or abnormal (ectopic or tubal) pregnancies**.

The gonococcus may also cause **extragenital infections** such as **pharyngitis** (from oral-genital sex), **ophthalmia** (from inoculation of the eyes with contaminated fingers), and **proctitis** (from anal sex). In rare cases, the organism invades the blood and disseminates, causing a **rash, septic arthritis, endocarditis, and/or meningitis**. Dissemination occurs more frequently in females.

Congenital gonorrhea is known as **ophthalmia neonatorum** and occurs as a result of the eyes of newborns becoming infected as the baby passes through the birth canal.

Neisseria meningitidis (the meningococcus) is the causative organism of meningococcal (epidemic) meningitis. *N. meningitidis* infects the **nasopharynx** of humans causing a usually mild or subclinical upper respiratory infection. However in about 15% of these individuals, the organism invades the **blood** and disseminates, causing **septicemia and/or meningitis**. A **petechial skin rash**, caused by endotoxin in the blood, appears in about 75% of the septic cases. The fulminating form of the disease, called Waterhouse-Frederichsen syndrome, can be fatal within several hours due to massive intravascular coagulation and resulting shock (probably due to endotoxin). *N. meningitidis* is especially dangerous in young children.

1. **Isolation and Identification of *Neisseria gonorrhoeae***
    a. **The GC smear**

        Identification of *N. gonorrhoeae* in **symptomatic males** is often made by performing a gram stain of the purulent discharge (called **a gonococcus or GC smear**) and

looking for **gram-negative diplococci with flattened adjacent walls and seen both inside and outside of polymorphonuclear leukocytes**. However, 50% of culture-positive females and most asymptomatic males show negative GC smears, so cultures must be performed on these.

b. **Isolation of *Neisseria gonorrhoeae***

To diagnose genital gonorrhea in males, the sample to be cultured is taken from the **urethra**. In females, cultures are taken from the **cervix and the rectum**. In nongenital gonorrhea, the infected site is cultured.

The gonococcus requires an enriched medium with increased carbon dioxide tension for growth. They are usually cultured on modified Thayer Martin (MTM) Chocolate agar. **MTM Chocolate agar is selective for pathogenic *Neisseria***. The medium contains **enrichment factors** to promote the growth of gonococci. In addition, it contains **antibiotics to inhibit normal body flora**: vancomycin to inhibit gram-positive bacteria; colistin to inhibit gram-negative bacteria; trimethoprim to suppress *Proteus*; and nystatin to inhibit yeast. The "chocolate" color is due to the hemoglobin enrichment added to the medium. Plates are then incubated under increased carbon dioxide tension such as that provided by a candle jar. (Transgrow Medium is a convenient flask containing MTM Chocolate agar and CO2.) *N. gonorrhoeae* forms small, convex, grayish-white to colorless, mucoid colonies in 48 hours at 35-37C.

c. **Identification of *Neisseria gonorrhoeae***

Once isolated, *N. gonorrhoeae* can be identified by the oxidase test, gram-staining, and carbohydrate fermentation reactions.

1. **Oxidase test**

   All *Neisseria* are **oxidase positive**. The oxidase test can be performed using a **Taxo N® disc**. A moistened Taxo N® disc can be placed on a growing culture and a **blackening of the colonies surrounding the disc indicates a positive oxidase test**. All oxidase-positive cultures would be gram stained to confirm gram-negative diplococci.

2. **Carbohydrate fermentation**

   The various species of *Neisseria* can be differentiated according to fermentation patterns using CTA glucose, CTA maltose, and CTA sucrose media. If fermentation occurs, acid end products cause the **phenol red pH indicator to turn yellow**. *N. gonorrhoeae* ferments **only glucose** whereas *N. meningitidis* ferments glucose and maltose. Nonpathogenic neisseriae usually ferment only sucrose.

2. **Isolation and Identification of *Neisseria meningitidis***

   a. **Serologic identification**

      There are 16 different serological groups of *N. meningitidis*. Group A and C usually causes the epidemic form of meningitis; Group B is common in North America. Serologic testing can be performed on cerebrospinal fluid or on organisms from skin lesions for rapid identification.. Gram stains may also be done on the cerebrospinal fluid to detect probable *Neisseria*.

   b. **Isolation of *Neisseria meningitidis***

      To isolate *N. meningitidis*, cultures are taken from the **nasopharynx, blood, cerebrospinal fluid, and skin lesions**. As with *N. gonorrhoeae,* discussed above, meningococci are cultured on an enriched, selective media such as **MTM Chocolate agar** grown under increased carbon dioxide tension. Medium to large, blue-gray, mucoid, convex, colonies form in 48 hours at 35-37C.

   c. **Identification of *Neisseria meningitidis***

      Once isolated, *N. meningitidis*, like *N. gonorrhoeae* discussed above, is identified by the oxidase test, gram staining, and carbohydrate fermentation reactions. *N. meningitidis*, like all neisseriae, is **oxidase-positive** and appears in a gram stain as **gram-negative diplococci**. In CTA fermentation tubes, *N. meningitidis* ferments **glucose and maltose**, the resulting acid end products turning the phenol red pH indicator from red to yellow.

B. THE MYCOBACTERIA

DISCUSSION

The mycobacteria are **rod-shaped** bacteria (0.4 by 3.0µ) which are said to be **acid-fast**. This means that because of their unique cell wall, when they are stained by the acid-fast procedure (Appendix C), they will resist decolorization with acid-alcohol and stain red, the color of the initial stain, carbol fuchsin. With the exception of a very few other acid-fast bacteria such as *Nocardia*, all other bacteria will be decolorized and stain blue (the color of the counterstain, methylene blue). The acid-fast stain is an important test for the genus *Mycobacterium*. Fluorescent microscopy staining may also be used to identify *Mycobacterium*.

Besides the many saprophytic forms of mycobacteria, there are two distinct pathogens in this group: ***M. tuberculosis***, the causative organism of **tuberculosis**, and ***M. leprae***, the causative agent of **leprosy**.

Mycobacterium tuberculosis **(the tubercle bacillus)** causes tuberculosis, although atypical species of *Mycobacterium* may occasionally cause tuberculosis-like infections, especially in the debilitated or immunosuppressed host. ***Mycobacterium avium-intracellulare*** **complex (MAC),** for example, frequently causes systemic infections in people with HIV/AIDS. *M. tuberculosis* is relatively resistant to many disinfectants and is also able to resist destruction within phagocytic macrophages.

Primary infection typically occurs after inhalation of the organism and subsequent generation of a peripheral lung lesion. The body responds with what is termed delayed hypersensitivity to form characteristic lesions called **granuloma** (tubercles). The formation of granuloma is actually the result of cell-mediated immune responses attempting to "wall-off" and localize infections that the body cannot effectively remove with macrophages. Although primary infection may be self-limiting, progression of the localized lesion may lead to pneumonia. The organisms may eventually die within the granuloma, or the tissue may undergo caseation, liquification, and cavitation. This can result in bronchogenic spread of the *M. tuberculosis*. In rare instances, the organism may enter the blood causing disseminated miliary tuberculosis.

Secondary infection is usually due to a relapse of either self-resolved lesions or a previously treated disease.

<u>Diagnosis of tuberculosis</u>

1. **Presumptive tests for tuberculosis**
    a. The **PPD skin test** (or other presumptive skin tests) detects **delayed hypersensitivity** to purified protein from

the cell wall of *M. tuberculosis*. A positive skin test indicates that the person has developed a cellular immunity to the organism as a result of either a **previous or a current infection**.

b. **Chest X-rays** are used to detect confluent granuloma formation in the lungs, which could be a result of past or present infection with tuberculosis or with some other pulmonary infection that may be mistaken for tuberculosis.
c. An **Acid-fast stain of the sputum** may indicate **acid-fast bacilli**, which is a presumptive test for active tuberculosis. In reporting acid-fast slide results, the slide should be observed for 10-15 minutes before considered negative. Results are reported as positive or negative for acid-fast bacilli. Sometimes the amount of acid-fast bacilli are indicated, with 3-9 per slide reported as rare, 10 or more per slide reported as few, and more than one per oil immersion field reported as numerous.

2. **Confirmation of Active Tuberculosis**

Active tuberculosis is confirmed by **culturing the organism**. Sputum is usually treated with sodium hydroxide, which is cidal for contaminants but not for *M. tuberculosis*. The liquified sputum is then neutralized, centrifuged, and the sediment is inoculated onto special enrichment media such as Lowenstein-Jensen agar slants, Middlebrook agar, or 7H 10 Oleic acid agar plates. Felsen Quadrant plates with agar containing different antimicrobial agents are also inoculated to determine drug sensitivity. Plates are incubated in a carbon dioxide atmosphere. It takes from **3-8 weeks** for colonies to form.

The above procedure represents a very simplified outline for the diagnosis of tuberculosis. Culturing of *M. tuberculosis*, atypical mycobacteria, and other clinically significant mycobacteria involves a complicated series of complex procedures carried out only in large, well-equipped labs by experienced personnel.

C. THE OBLIGATE ANAEROBES

DISCUSSION

A variety of obligate anaerobic bacteria, which are usually **normal flora** of the body, may cause human infections. Obligate anaerobes primarily cause **wound infections**, although they may participate in all varieties of infections and involve any tissue or organ. Five organisms or groups of organisms account for about two-thirds of all clinically significant anaerobic infections. These are *Bacteroides fragilis, Bacteroides melaninogenicus, Fusobacterium nucleatum, Clostridium perfringens,* and the anaerobic cocci.

Bacteroides fragilis is the **most common cause of anaerobic infections** in humans. It is also a **predominant organism of the normal human intestinal tract**. It mainly causes **wound infections**.

Bacteroides melaninogenicus is normal flora of the upper respiratory, gastrointestinal, and genitourinary tracts.

Fusobacterium species are normal flora of the upper respiratory, gastrointestinal, and genitourinary tracts.

Clostridium perfringens, as well as other clostridial species, are normal flora of the intestinal tract of various animals and may cause **gas gangrene**. *C. tetani* causes tetanus and *C. botulinum* causes botulism.

Anaerobic cocci such as *Peptostreptococcus, Peptococcus,* and *Veillonella* are also normal flora of the body.

Although anaerobic procedures are no more difficult than those used in aerobic bacteriology, strict adherence to proper technique is necessary to ensure recovery of the organism. There must be proper selection of the specimen, proper specimen collection, proper specimen transport to the lab, and provision of a proper anaerobic environment.

Common ways of **culturing obligate anaerobes in the lab** include:

1. **Brewer anaerobic jar with GasPak®**

   A GasPak® is a commercially-produced disposable hydrogen and carbon dioxide generator envelope. When water and catalyst are added, hydrogen and carbon dioxide are produced. The hydrogen then combines with oxygen to form water, thus creating an anaerobic atmosphere. The cultures are placed in a Brewer jar, water is added to the GasPak®, the lid of the jar is sealed, and the jar is placed in an incubator.

2. **Media containing reducing agents**

   Media such as **Thioglycolate medium** and Anaerobic agar contain chemicals which function as reducing agents. The reducing agents absorb oxygen and create a reduced environment required by anaerobes.

3. **Carbon dioxide incubators**

   Carbon dioxide incubators are frequently used to culture anaerobes. After the cultures are placed in the incubator, the air is evacuated and replaced by carbon dioxide gas.

Isolation and Identification of *Clostridium perfringens*

4. **Direct microscopic examination of exudates**

   Gram stains of purulent exudates from gas gangrene show **stout gram-positive bacilli** frequently surrounded by a capsule. Endospores are usually not produced on ordinary culture media or in tissues.

5. **Isolation on Blood agar**

   When inoculated onto Blood agar plates and grown anaerobically, *C. perfringens* produces smooth, glossy colonies which are usually surrounded by a **double-zone hemolysis**. The double-zone hemolysis appears as a narrow zone of beta-type hemolysis (due to theta toxin) near the colony surrounded by a wider zone of incomplete hemolysis (due to alpha toxin).

6. **Identification in Litmus Milk medium**

   In anaerobically-grown Litmus Milk cultures, enzymes of *C. perfringens* will attack the proteins and carbohydrates of the milk producing a **"stormy fermentation"** with **clotting and gas formation**.

PROCEDURE

A. ***Neisseria gonorrhoeae* and *Neisseria meningitidis***

Observe the following **demonstrations**:

1. **Positive GC smear** for *N. gonorrhoeae*. Note gram-negative diplococci inside and outside of white blood cells.
2. *N. gonorrhoeae* growing on **MTM Chocolate agar**. Note small grayish-white convex, mucoid colonies. This medium is selective for pathogenic *Neisseria*.
3. Positive **oxidase test** for *N. gonorrhoeae* using a Taxo N® (oxidase) disc. Note oxidase-positive (black) colonies around the Taxo N® disc.
4. **CTA glucose, CTA maltose, and CTA sucrose fermentation tubes** inoculated with *N. gonorrhoeae* (note positive glucose; negative maltose and sucrose) and *N. meningitidis* (note positive glucose and maltose; negative sucrose).

B. **MYCOBACTERIA**

Observe the following **demonstrations**:

1. Positive acid-fast stain of the sputum from a person with active tuberculosis. Note acid-fast (red) rods. You must **look carefully**

for the reddish acid-fast rods in the microscopic field. All other material in the sputum will pick up the blue counterstain.

2. 35 mm projection slides showing normal guinea pig organs and organs with **granuloma**.
3. Positive **chest X-ray** for the presumptive diagnosis of tuberculosis.
4. Prepare an acid-fast stain of *Mycobacterium phlei*. (Ziehl-Neelsen Method)
    a. Heat fix a smear of the sample of *Mycobacterium phlei* on a new glass slide.
    b. Cover the smear with a piece of blotting paper and flood with carbol fuchsin.
    c. Steam for 5 minutes by passing the slide through the flame of a gas burner.
    d. Allow the slide to cool and wash with water.
    e. Add the acid-alcohol decolorizing slowly dropwise until the dye no longer runs off from the smear.
    f. Rinse with water.
    g. Counterstain with methylene blue for 1 minute.
    h. Wash with water, blot dry, and observe using oil immersion microscopy.
    i. Repeat using *Staphylococcus aureus*.

    Acid-fast bacteria will appear red; non-acid-fast will appear blue.

C. **The Obligate Anaerobes**

Observe the following **demonstrations** of *Clostridium perfringens*:

1. **Endospore stain** of *C. perfringens*.
2. **Blood agar** plate of *C. perfringens* grown anaerobically. Note double-zone hemolysis.
3. **Litmus milk culture** of *C. perfringens* grown anaerobically. Note stormy fermentation of milk.

To show how special techniques such as media with reducing agents must be used to culture anaerobes, inoculate the following media with ***Clostridium sporogenes*** and incubate **at 37C**:

4. Nutrient broth
5. Thioglycolate medium

RESULTS

A. ***Neisseria gonorrhoeae* and *Neisseria meningitidis***

1. **GC smear for N. gonorrhoeae**

    Make a drawing of the GC smear, noting the gram reaction and arrangement of the bacteria and their association with the white blood cells.

GC smear of *Neisseria gonorrhoeae*

Gram reaction (purple = +; pink = -)	
Shape and arrangement	

2. ***N. gonorrhoeae* on MTM Chocolate agar.**

Growth on selective MTM (positive or negative)	
Colony description	

3. ***N. gonorrhoeae* on MTM Chocolate agar with a Taxo N® disc.**

Growth on selective MTM (positive or negative)	
Colony description	
Oxidase reaction (positive or negative)	

4. **Carbohydrate fermentation in CTA medium**

Organism	**Glucose**	**Maltose**	**Sucrose**
***Neisseria gonorrhoeae* (positive or negative)**			
***Neisseria meningitidis* (positive or negative)**			

5. positive = fermentation (phenol red turns yellow) negative = no fermentation (phenol red remains red)

B. ***Mycobacterium tuberculosis***

1. **Acid-fast stain of sputum**

   Make a drawing of the acid-fast stain, noting the acid-fast reaction and the shape of the acid-fast bacteria.

   acid-fast stain of *Mycobacterium tuberculosis*

Acid-fast reaction (red = +; blue = -)	
Shape	

2. Describe the granuloma seen in the guinea pig organs.

C. **The Obligate Anaerobes**

1. ***Clostridium perfringens***
   a. **Blood agar** (anaerobically grown)

Colony description	
Type of hemolysis	

   b. **L**
   itmus Milk (anaerobically grown)
   c.
   d.
   e.
   f.
   g.

Color pink or lavender	
Stormy fermentation (positive or negative)	

   Pink = acid (fermentation)
   blue = alkaline (no fermentation)
   stormy fermentation = clotting of protein and gas production

2. Observe the tubes of Nutrient broth and Thioglycolate medium inoculated with *Clostridium sporogenes*.

nutrient broth (growth or no growth)	
thioglycolate medium (growth or no growth)	

Serology, Part 1

Direct Serologic Testing

OBJECTIVES :

After completing this Exercise, the student will be able to perform the following objectives:

A. **INTRODUCTION TO SEROLOGICAL TESTING**
   1. Define serology.
   2. Define antigen and state what may act as an antigen.
   3. Define antibody and state where they are primarily found in the body.
   4. Define direct serologic testing and indirect serologic testing.

B. **USING ANTIGEN-ANTIBODY REACTIONS IN THE LAB TO IDENTIFY UNKNOWN ANTIGENS SUCH AS MICROORGANISMS**
   1. Define antiserum.
   2. Describe two ways of producing known antiserum.
   3. Describe the concept and general procedure for using serologic testing to identify unknown antigens (direct serologic testing).

C. **EXAMPLES OF SEROLOGIC TESTS TO IDENTIFY UNKNOWN ANTIGENS**
   - **DISCUSSION**
     1. Describe how to determine serologically whether an organism is a subgroup A, B, C, or D *Shigella*.
     2. Describe how to determine serologically whether an organism is a Lancefield group A, B, C, D, F, or G *Streptococcus*.
     3. Describe how to diagnose pregnancy serologically.
     4. Briefly describe the direct fluorescent antibody technique.
   - **RESULTS**
     1. Correctly interpret the results of the following serological tests:
        a. serological typing of *Shigella*
        b. serological typing of streptococci
        c. serological testing for pregnancy
        d. a direct fluorescent antibody test

A. INTRODUCTION TO SEROLOGIC TESTING

The immune responses refer to the **ability of the body (self) to recognize specific foreign factors (nonself)** that threaten its biological integrity. There are two major branches of the immune responses:

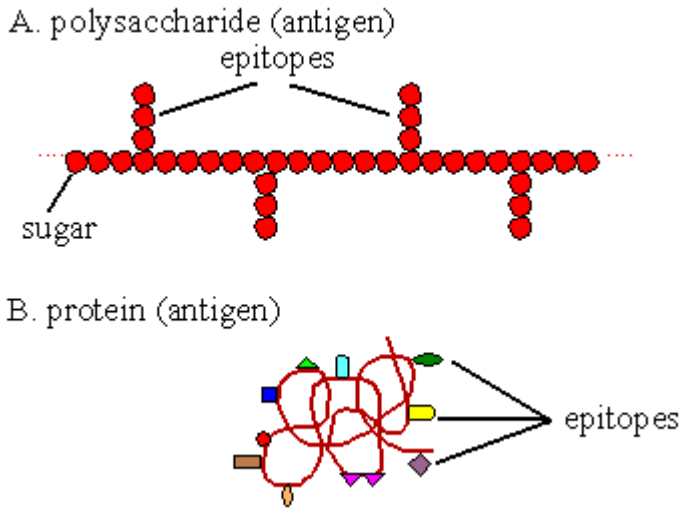
1. **humoral immunity** (the production of antibody molecules in response to an antigen; mediated by B-lymphocytes); and

2. **cell-mediated immunity** (the production of cytotoxic T-lymphocytes and cytokines in response to an antigen; mediated by T-lymphocytes).

To understand the immune responses we must first understand what is meant by the term antigen. An **antigen**, or immunogen, **is a substance the body recognizes as nonself and toward which it mounts an immune response**. Chemically, antigens are large molecular weight **proteins** (including conjugated proteins such as glycoproteins, lipoproteins, and nucleoproteins) and **polysaccharides** (including lipopolysaccharides). These protein and polysaccharide antigens are found on the surfaces of viruses and cells, including microbial cells (bacteria, fungi, protozoans) and human cells.

As mentioned above, the B-lymphocytes and T-lymphocytes are the cells that carry out the immune responses. The body recognizes an antigen as foreign when that antigen binds to the surfaces of B-lymphocytes and T-lymphocytes by way of antigen-specific receptors having a shape which corresponds to that of the antigen (similar to interlocking pieces of a puzzle). The antigen receptors on the surfaces of B-lymphocytes are antibody molecules called sIg; the receptors on the surfaces of T-lymphocytes are called T-cell receptors (TCR).

The actual portions or fragments of an antigen that react with receptors on B-lymphocytes and T-lymphocytes, as well as with free antibody molecules, are called epitopes or antigenic determinants. The size of an epitope is generally thought to be equivalent to 5-7 amino acids or 3-4 sugar residues.



In terms of infectious diseases, the following may act as antigens:

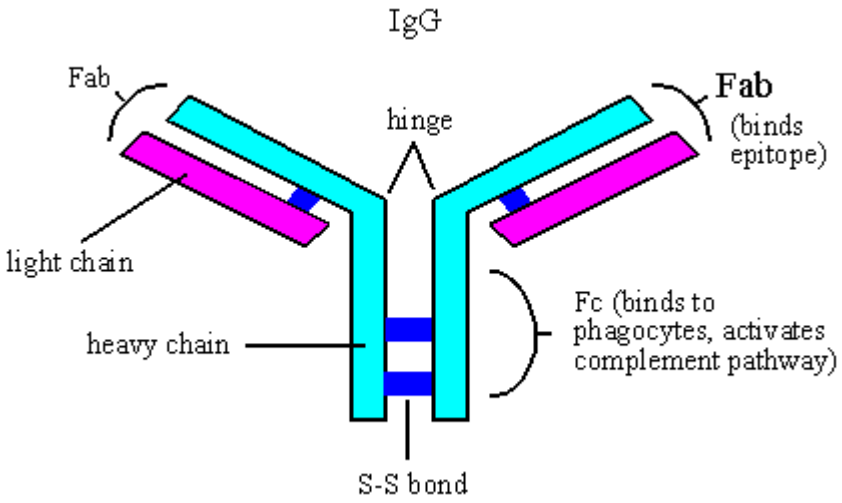
3. **microbial structures** (cell walls, capsules, flagella, pili, viral capsids, envelope-associated glycoproteins, etc.); and
4. **microbial exotoxins**

Certain **noninfectious materials** may also act as antigens if they are recognized as "nonself" by the body. These include:

5. **allergens** (dust, pollen, hair, foods, dander, bee venom, drugs, and other agents causing allergic reactions);
6. **foreign tissues and cells** (from transplants and transfusions); and
7. **the body's own cells that the body fails to recognize as "normal self"** (cancer cells, infected cells, cells involved in autoimmune diseases).

Antibodies or immunoglobulins are specific protein configurations produced by B-lymphocytes and plasma cells **in response to a specific antigen and capable of reacting with that antigen**. Antibodies are produced in the **lymphoid tissue** and once produced, are found mainly in the **plasma** portion of the blood (the liquid fraction of the blood before clotting). **Serum** is the liquid fraction of the blood after clotting.

There are 5 classes of human antibodies: IgG, IgM, IgA, IgD, and IgE. The simplest antibodies (monomers such as IgG, IgD, and IgE) are "Y"-shaped macromolecules composed of **four protein chains**: two identical heavy chains and two identical light chains. The four protein chains are connected to one another by disulfide (S-S) bonds and noncovalent bonds. The two tips of the "Y" are referred to as the **Fab portions** of the antibody. The Fab portions **provide specificity for binding an epitope on an antigen**. The bottom part of the "Y" is called the **Fc portion** and this part is responsible for the **biological activity** of the antibody (see diagram of IgG). Depending on the class of antibody, biological activities of the Fc portion of antibodies include the ability to activate the complement pathway (IgG & IgM), bind to phagocytes (IgG, IgA), or bind to mast cells and basophils (IgE). Two classes of antibodies are more complex. IgM is a pentamer, consisting of 5 "Y"-like molecules connected at their Fc portions, and secretory IgA is a dimer consisting of 2 "Y"-like molecules.



Serology refers to using antigen-antibody reactions in the laboratory for **diagnostic purposes**. Its name comes from the fact that **serum, the liquid portion of the blood where antibodies are found** is used in testing. **Serologic testing** may be used in the clinical laboratory in two distinct ways: **to identify unknown antigens** (such as microorganisms) and **to detect antibodies being made against a specific antigen in the patient's serum**.

Direct serologic testing is the use of a preparation **known antibodies**, called antiserum, to identify an **unknown antigen** such as a microorganism. This is the principle behind **Lab 6**. **Indirect serologic testing** is the procedure whereby **antibodies in a person's serum** being made by that individual against an antigen associated with a particular disease are detected using a **known antigen**. This is the principle behind **Lab 7**.

B. **USING ANTIGEN-ANTIBODY REACTIONS IN THE LABORATORY TO IDENTIFY UNKNOWN ANTIGENS SUCH AS MICROORGANISMS.**

This type of serologic testing employs **known antiserum (serum containing specific known antibodies)**. The preparation of known antibodies is prepared in one of two ways: in animals or by hybridoma cells.

1. **Preparation of known antisera in animals**.

   Preparation of known antiserum in animals involves inoculating animals with specific known antigens such as a specific strain of a bacterium. After the animals immune responses have had time to produce antibodies against that antigen, the animal is bled and the blood is allowed to clot. The resulting liquid portion of the blood is the serum and it will contain antibodies specific for the injected antigen.

However, one of the problems of using antibodies prepared in animals (by injecting the animal with a specific antigen and collecting the serum after antibodies are produced) is that up to 90% of the antibodies in the animal's serum may be antibodies the animal has made "on its own" against environmental antigens, rather than those made against the injected antigen. The development of monoclonal antibody technique has largely solved that problem.

2. **Preparation of known antibodies by monoclonal antibody technique**.

   One of the major breakthroughs in immunology occurred when monoclonal antibody technique was developed. **Monoclonal antibodies** are antibodies of a single specific type. In this technique, an animal is injected with the specific antigen for the antibody desired. After appropriate time for antibody production, the animal's spleen is removed. The spleen is rich in plasma cells and each plasma cell produces only one specific type of antibody. However, plasma cells will not grow artificially in cell culture. Therefore, a plasma cell producing the desired antibody is fused with a myeloma cell (a cancer cell from bone marrow which will grow rapidly in cell culture) to produce a **hybridoma cell**. The hybridoma cell has the characteristics of both parent cells. It will **produce the specific antibodies like the plasma cell and will also grow readily in cell culture like the myeloma cell**. The hybridoma cells are grown artificially in huge vats where they produce large quantities of the specific antibody.

   Monoclonal antibodies are now used routinely in medical research and diagnostic serology and are being used experimentally in treating certain cancers and a few other diseases.

3. **The concept and general procedure for direct serologic testing**.

   The concept and general procedure for using antigen-antibody reactions to identify unknown antigens are as follows:

   - **Concept**:

     This testing is based on the fact that **antigen- antibody reactions are very specific**. Antibodies usually react **only with the antigen that stimulated their production in the first place**, and are just as specific as an enzyme-substrate reaction. Because of this, one can use **known antiserum** (prepared by animal inoculation or monoclonal antibody technique as discussed above) to identify **unknown antigens such as a microorganisms.**

- **General Procedure:**

  A suspension of **the unknown antigen** to be identified is mixed with **known antiserum** for that antigen. One then looks for an antigen-antibody reaction.

  Examples of serologic tests used to identify unknown microorganisms include the serological typing of *Shigella* and *Salmonella*, the Lancefield typing of beta streptococci, and the serological identification of meningococci. Serological tests used to identify antigens which are not microorganisms include blood typing, tissue typing, and pregnancy testing.

4. **Detection of antigen-antibody reactions in the laboratory**

   Antigen-antibody reactions may be detected in the laboratory by a variety of techniques. Some of the commonly used techniques for observing in vitro antigen-antibody reactions are briefly described below.

   a) **Agglutination**

   Known antiserum causes bacteria or other particulate antigens to clump together or agglutinate. Molecular-sized antigens can be detected by attaching the known antibodies to larger, insoluble particles such as latex particles or red blood cells in order to make the agglutination visible to the naked eye.

   b) **Precipitation**

   Known antiserum is mixed with soluble test antigen and a cloudy precipitate forms at the zone of optimum antigen-antibody proportion.

   c) **Complement-fixation**

   Known antiserum is mixed with the test antigen and complement is added. Sheep red blood cells and hemolysins (antibodies that lyse the sheep red blood cells in the presence of free complement) are then added. If the complement is tied up in the first antigen-antibody reaction, it will not be available for the sheep red blood cell-hemolysin reaction and there will be no hemolysis. A negative test would result in hemolysis.

d) **Enzyme immunoassay (EIA)**

Test antigens from specimens are passed through a tube (or a membrane) coated with the corresponding specific known antibodies and become trapped on the walls of the tube (or on the membrane). Known antibodies to which an enzyme has been chemically attached are then passed through the tube (or membrane) where they combine with the trapped antigens. Substrate for the attached enzyme is then added and the amount of antigen-antibody complex formed is proportional to the amount of enzyme-substrate reaction as indicated by a color change.

e) **Radioactive binding techniques**

Test antigens from specimens are passed through a tube coated with the corresponding specific known antibodies and become trapped on the walls of the tube. Known antibodies to which a radioactive isotope has been chemically attached are then passed through the tube where they combine with the trapped antigens. The amount of antigen-antibody complex formed is proportional to the degree of radioactivity.

f) **Fluorescent antibody technique**

A fluorescent dye is chemically attached to the known antibodies. When the fluorescent antibody reacts with the antigen, the antigen will fluoresce when viewed with a fluorescent microscope.

C. EXAMPLES OF DIRECT SEROLOGIC TEST TO IDENTIFY UNKNOWN ANTIGENS

As stated above, this type of serologic testing uses **known antiserum (antibodies) to identify unknown antigens**. Four such tests will be looked at in lab today.

1. Serological Typing of *Shigella*

Discussion

There are **four different serological subgroups of *Shigella***, each corresponding to a different species:

- subgroup A = *Shigella dysenteriae*
- subgroup B = *Shigella flexneri*
- subgroup C = *Shigella boydii*
- subgroup D = *Shigella sonnei*

Known antiserum is available for each of the 4 subgroups of *Shigella* listed above and contains antibodies against the cell wall ("O" antigens) of *Shigella.* The suspected *Shigella* (the unknown antigen) is placed in each of 4 circles on a slide and a different known antiserum (A, B, C or D) is then added to each circle. A positive antigen-antibody reaction appears as a clumping or agglutination of the *Shigella*.

2. **Serological Typing of Streptococci**

Discussion

Many of the streptococci can be placed into serological groups called **Lancefield groups** based on carbohydrate antigens in their cell wall. Although there are 20 different Lancefield groups of streptococci, the **groups A, B, C, D, F, and G** are the ones usually associated with human infections. The **Slidex Strepto-Kit®** system is a commercial kit for typing the 6 Lancefield groups of streptococci that commonly infect humans. To make the reaction more visible, since the antigens for which one is testing are only fragments of the bacterial cell wall, the **known monoclonal antibodies** have been adsorbed to **latex particles**. In this way, when the known monoclonal antibodies react with the streptococcal cell wall antigens, agglutination of the latex particles will occur and can be easily seen with the naked eye.

3. **Serological Testing to Diagnose Pregnancy**

Discussion

The hormone **human chorionic gonadotropin (HCG)**, produced by the placenta, appears in the serum and urine of pregnant females. The HCG is composed of two subunits - alpha and beta. The **CARDS O.S.® HCG-Urine** is a one step pregnancy test that detects measurable levels of HCG as early as early as 7-10 days after conception. HCG, the unknown antigen for which one is testing, is identified in the urine by using known monoclonal antibody against human HCG.

This test uses a color immunochromatographic assay to detect the antigen-antibody reaction. Inside the plastic card is a membrane strip along which the urine flows and on which the reaction occurs. The urine is placed in the "add urine" well on the right side of the card and flows along the card from right to left. The membrane just to the left of the sample well is coated with **red latex beads to which known antibodies against the beta chain of human HCG have been attached**. If there is HCG in the urine, the beta subunit of the HCG will react with the known anti-beta HCG antibody/red latex conjugate and **this complex of HCG-antibody/red latex will become mobilized** and flow with

the urine towards the left side of the card. In the "read results" window of the card is a **vertical line to which is immobilized known antibodies against the alpha subunit of human HCG**. As the urine containing the antibody/red latex conjugate bound to the beta subunit of HCG flows past the vertical line, the alpha subunit of the HCG binds to the immobilized antibodies located on the line, trapping the complex and causing a vertical red line to appear. The vertical red line crosses the horizontal blue line preprinted in the "read results" window to form a **(+) sign**.

If the woman is not pregnant and there is no HCG in the urine, then there will be no antigen to react with the anti-beta HCG antibody/red latex conjugate to the left of the sample well and likewise, no reaction with the anti-alpha HCG antibodies immobilized along the vertical line in the "read results" window. The antibody/red latex conjugate will continue to flow to the left of the slide until it reaches the "test complete" window. Since no vertical red line forms, a **(-) sign** appears in the "read results" window.

4. **Identification of Microorganisms Using the Direct Fluorescent Antibody Technique**

   Discussion

   Certain **fluorescent dyes** can be chemically **attached to the known antibody molecules** in antiserum. The known fluorescent antibody is then mixed with the unknown antigen (such as a microorganism) fixed to a slide. After washing, to remove any fluorescent antibody not bound to the antigen, the slide is viewed with a **fluorescent microscope**.

   If the fluorescent antibody reacted with the unknown antigen, the antigen will glow or fluoresce under the fluorescent microscope. If the fluorescent antibody did not react with the antigen, the antibodies will be washed off the slide and the antigen will not fluoresce.

   Many bacteria, viruses, and fungi can be identified using this technique.

PROCEDURE

A. **Serologic Typing of *Shigella***
   1. Using a wax marker, draw **two circles** (about the size of a nickel) on each of two clean glass slides . Label the circles A, B, C, and D.
   2. Add **one drop** of the **suspected *Shigella*** (unknown antigen) to each circle. (The *Shigella* has been treated with formalin to make it noninfectious but still antigenic.)

3. Now add **one drop** of known Shigella subgroup **A** antiserum to the "A" circle, **one drop** of known *Shigella* subgroup **B** antiserum to the "B" circle, **one drop** of known *Shigella* subgroup **C** antiserum to the "C" circle, and **one drop** of known *Shigella* subgroup **D** antiserum to the "D" circle.
4. **Rotate** the slide carefully for **30-60 seconds** . **Agglutination of the bacteria, indicates a positive reaction**. **No agglutination is negative**.
5. Dispose of all pipettes and slides in the disinfectant container.

B. **Serologic Typing of Streptococci**

1. The cell wall antigens of the unknown *Streptococcus* used in this test are extracted by mixing the organism with extraction enzyme. **This step has been done for you.**
2. Place **one drop of the appropriate known streptococcal monoclonal antibody/latex conjugate** (groups A, B, C, D, F, and G) on the corresponding six circles of the slide.
3. Add **one drop of the extracted antigen** from the unknown *Streptococcus* prepared in step 1 to each circle.
4. **Spread** the antigen-antibody mixtures over the entire circles **using separate applicator sticks for each circle**.
5. . **Rock the slide back and forth** for no longer than **one minute** and look for **agglutination**.

C. **Serologic Testing to Detect Pregnancy**

1. Fill the disposable pipette to the line with urine and dispense the urine into the "add urine" well.
2. Shortly after the urine is added, a blue color will be seen moving across the "read results" window.
3. The test results can be read in the "read results" window when a distinct blue line appears in the "test complete" window (approximately 5 minutes). A (+) sign indicates a positive test; a (-) sign is a negative test.

D. **The Direct Fluorescent Antibody Technique**

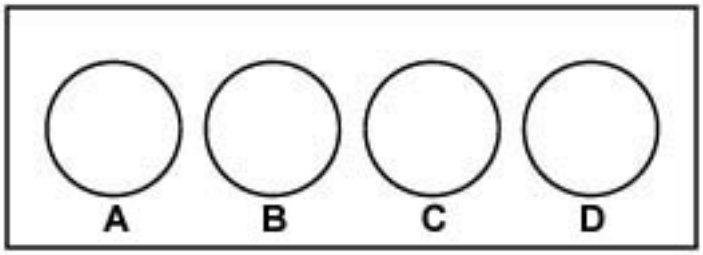
Observe the demonstration of a positive direct fluorescent antibody test.

RESULTS

A. **Serologic Typing of *Shigella***

Make a drawing of your results.

- Agglutination of bacteria is positive.
- No agglutination of bacteria is negative.



Shigella typing slide

B. **Serologic Typing of Streptococci**

Make a drawing of your results.

Streptococcus typing slide

C. **Serologic Testing to Diagnose Pregnancy**

Make a drawing of a positive pregnancy test.



positive pregnancy test

D. **The Direct Fluorescent Antibody Technique**

Make a drawing and describe a positive direct fluorescent antibody test.

positive direct fluorescent antibody test

Serology. Part 2

Indirect Serologic Testing

OBJECTIVES :

After completing this Exercise, the student will be able to perform the following objectives:

DISCUSSION

1. State the principle and the general procedure behind indirect serologic testing.
2. State the difference between a qualitative serological test and a quantitative serological test.
3. Define titer.
4. State what disease the RPR and the FTA-ABS procedures test for. Indicate which of these is a presumptive test, which is a confirming test, and why.
5. State the significance of nontreponemal antilipid (reagin) antibodies in serological testing.
6. State the significance of heterophile antibodies in serological testing.
7. State the significance of anti-deoxyribonucleoprotein antibodies in serological testing.
8. Briefly describe the indirect fluorescent antibody technique.
9. Briefly describe the EIA test for HIV antibodies and state the significance of a positive HIV antibody test.
10. State the most common reason for a false-negative HIV antibody test.

RESULTS

1. Interpret the results of the following serological tests:
    a. serologic test for infectious mononucleosis
    b. serologic test for SLE
    c. FTA-ABS test

Determine the titer of a quantitative RPR Card® test.

DISCUSSION

A. **Using antigen-antibody reactions in the laboratory to indirectly diagnose disease by detecting antibodies in a person's serum produced against a disease antigen**

   As stated in the introduction to serological testing in Exercise 6, **indirect serologic testing** is the procedure whereby **antibodies in a person's serum** being made by that individual against an antigen associated with a particular disease are detected using a **known antigen**.

1. **The concept and general procedure for indirect serologic testing**.

   The concept and general procedure for this type of serological testing are as follows:

   - **Concept**:

     This type of testing is based on the fact that **antibodies are only produced in response to a specific antigen**. In other words, a person will not be producing antibodies against a disease antigen unless that antigen is in the body stimulating antibody production.

   - **General Procedure:**

     A sample of the **patient's serum** (the liquid portion of the blood after clotting and containing antibodies against the disease antigen if the person has or has had the disease) is mixed with the **known antigen** for that suspected disease. One then looks for an antigen-antibody reaction.

   Examples of serologic tests to diagnose disease by the detection of antibodies in the patient's serum include the various serological tests for syphilis or STS (such as the RPR, the VDRL, and the FTA-ABS tests), the tests for infectious mononucleosis, the tests for the Human Immunodeficiency Virus (HIV), the tests for systemic lupus erythematosus, and tests for variety of other viral infections.

2. **Qualitative and quantitative serologic tests**.

   Indirect serologic tests may be qualitative or quantitative. A **qualitative** test only detects the presence or absence of specific antibodies in the patient's serum and is often used for screening purposes. A **quantitative** test gives the titer or amount of that antibody in the serum. **Titer** indicates how far you can dilute the patient's serum and still have it contain enough antibodies to give a detectable antigen-antibody reaction. In other words, the more antibodies being produced by the body, the more you can dilute the person's serum and still see a reaction. Quantitative serological tests are often used to follow the progress of a disease by looking for a rise and subsequent drop in antibody titer.

3. **Detection of antigen-antibody reactions in the laboratory**

   Antigen-antibody reactions may be detected in the laboratory by a variety of techniques. Some of the commonly used techniques are briefly described below.

a) **Agglutination**

Antibodies in the patient's serum cause the known particulate antigens or cells to clump or agglutinate. Molecular-sized known antigens can be attached to larger, insoluble particles such as latex particles, red blood cells, or charcoal particals in order to observe agglutination with the naked eye.

b) **Precipitation**

he patient's serum is mixed with soluble known antigen and a cloudy precipitate forms at the zone of optimum antigen-antibody proportion.

c) **Complement-fixation**

The patient's serum is mixed with the known antigen and complement is added. Sheep red blood cells and hemolysins (antibodies that lyse the sheep red blood cells in the presence of free complement) are then added. If the complement is tied up in the first antigen-antibody reaction, it will not be available for the sheep red blood cell-hemolysin reaction and there will be no hemolysis. A negative test would result in hemolysis.

d) **Enzyme immunoassay (EIA)**

The patient's serum is placed in a tube or well coated with the corresponding known antigen and becomes trapped on the walls of the tube. Enzyme-labeled anti-human gamma globulin or anti-HGG (antibodies made in another animal against the Fc portion of human antibody and to which an enzyme has been chemically attached), is then passed through the tube where it combines with the trapped antibodies from the patient's serum. Substrate for the enzyme is then added and the amount of antibody-antigen complex formed is proportional to the amount of enzyme-substrate reaction as indicated by a color change.

e) **Radioactive binding techniques**

The patient's serum is passed through a tube coated with the corresponding known antigen and becomes trapped on the walls of the tube. Radioisotope-labeled anti-human gamma globulin or anti-HGG (antibodies made in another animal against the Fc portion of human antibody and to which a radioactive isotope has been chemically attached), is then passed through the tube where it

combines with the trapped antibodies from the patient's serum. The amount of antibody-antigen complex formed is proportional to the degree of radioactivity measured.

f) **Fluorescent antibody technique**

The patient's serum is mixed with known antigen fixed to a slide. Fluorescent anti-human gamma globulin or anti-HGG (antibodies made against the Fc portion of human antibody and to which a fluorescent dye has been chemically attached) is then added. It combines with the antibodies from the patient's serum bound to the antigen on the slide causing the antigen to fluoresce when viewed with a fluorescent microscope.

B. EXAMPLES OF INDIRECT SEROLOGIC TESTS TO DETECT ANTIBODIES IN THE PATIENT'S SERUM

1. The RPR Test for Syphilis

Discussion

Syphilis is a sexually transmitted disease caused by the spirochete *Treponema pallidum*. The **RPR** (Rapid Plasma Reagin) Card® test is a **presumptive serologic screening test** for syphilis. The serum of a person with syphilis contains a **nonspecific antilipid antibody** (traditionally termed **reagin**), which is not found in normal serum. The exact nature of the antilipid (reagin) antibody is not known but it is thought that a syphilis infection instigates the breakdown of the patient's own tissue cells. Fatty substances which are released then combine with protein from *Treponema pallidum* to form an antigen which stimulates the body to produce **antibodies against both the body's tissue lipids (nonspecific or nontreponemal) as well as the *T. pallidum* protein (specific or treponemal)**. The RPR Card® test detects the nonspecific antilipid antibody and is referred to as a **nontreponemal test for syphilis**.

It must be remembered that tests for the presence of these nonspecific antilipid antibodies are meant as a presumptive screening test for syphilis. Similar reagin-like antibodies may also be present as a result of other diseases such as malaria, leprosy, infectious mononucleosis, systemic lupus erythematosus, viral pneumonia, measles, and collagen diseases and may give biologic false-positive results (BFP). Confirming tests should be made for the presence of specific antibodies against the *T. pallidum* itself. The confirming test for syphilis is the **FTA-ABS** test discussed below. Any serologic test

for syphilis is referred to commonly as an **STS** (Serological Test for Syphilis).

The **known RPR antigen** consists of **cardiolipin, lecithin, and cholesterol bound to charcoal particals** in order to make the reaction visible to the naked eye. If the patient hassyphilis, the antilipid antibodies in his or her serum will cross-react with the known RPR lipid antigens giving a visible antigen-antibody reaction (clumping).

We will do a **quantitative** RPR Card® test today in Exercise. Keep in mind that a quantitative test allows one to determine the **titer** or amount of a certain antibody in the serum. In this test, a constant amount of RPR antigen is added to dilutions of the patient's serum. **The most dilute sample of the patient's serum still containing enough antibodies to give a visible antigen-antibody reaction is reported as the titer**.

2. **Serologic Tests for Infectious Mononucleosis**

   Discussion

   During the course of infectious mononucleosis, caused by the Epstein-Barr virus (EBV), the body produces nonspecific **heterophile antibodies** which are not found in normal serum. As it turns out, these heterophile antibodies will also **cause horse or sheep erythrocytes (red blood cells) to agglutinate**.

   The infectious mononucleosis serologic test demonstrated today is a rapid **qualitative** test for infectious mononucleosis which uses specially treated **horse erythrocytes (acting as the "known antigen")** that are highly specific for mononucleosis heterophile antibodies. Agglutination of erythrocytes after adding the patient's serum indicates a positive test. Quantitative tests may be done to determine the titer of heterophile antibodies and follow the progress of the disease.

3. **Serologic Tests for Systemic Lupus Erythematosus (SLE)**

   Discussion

   Systemic lupus erythematosus or SLE is a systemic autoimmune disease. Immune complexes become deposited between the dermis and the epidermis, and in joints, blood vessels, glomeruli of the kidneys, and the central nervous system. It is four times more common in women than in men. In SLE, autoantibodies are made against components of DNA. This test is specific for the serum **anti-deoxyribonucleoprotein antibodies** associated with SLE. It is a **qualitative** test used to screen for the presence of the disease and to monitor its course.

4. **Detecting Antibody Using the Indirect Fluorescent Antibody Technique: The FTA-ABS test for syphilis**

Discussion

The **indirect** fluorescent antibody technique involves three different reagents:

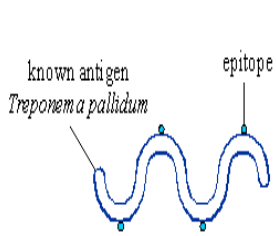
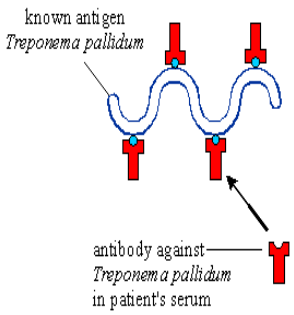
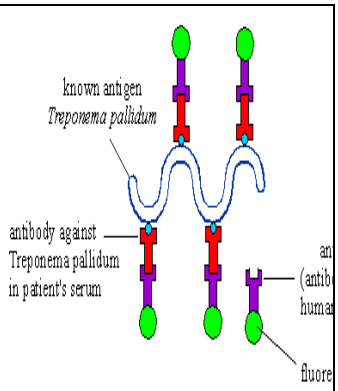
the **patient's serum** (containing antibodies against the disease antigen if the disease is present)

a) **known antigen** for the suspected disease
b) **fluorescent anti-human gamma globulin antibodies** (antibodies made in another animal against the Fc portion of human antibodies by injecting an animal with human serum. A fluorescent dye is then chemically attached to the anti-human gamma globulin (anti-HGG) antibodies.

The **FTA-ABS test** (Fluorescent Treponemal Antibody Absorption Test) **for syphilis** is an example of an indirect fluorescent antibody procedure. This is a **confirming test** for syphilis since it tests specifically for **antibodies in the patient's serum made in response to the syphilis spirochete, *Treponema pallidum***.

In this test, killed *T. pallidum,*(the known antigen), is fixed on a slide (see FTA-A) and the patient's serum is added. If the patient has syphilis, antibodies against the *T. pallidum* will react with the antigen on the slide (see FTA-B). The slide is then washed to remove any antibodies not bound to the spirochete.

To make this reaction visible, a second animal-derived antibody made against human antibodies and labelled with a fluorescent dye (fluorescent anti-human gamma globulin) is added. These fluorescent anti-HGG antibodies react with the patient's antibodies which have reacted with the *T. pallidum* on the slide (see FTA-C). The slide is washed to remove any unbound fluorescent anti-HGG antibodies and observed with a fluorescent microscope. If the spirochetes glow or fluoresce, the patient has made antibodies against *T. pallidum* and has syphilis.

		
FTA-A	FTA-B	FTA-C

5. **The EIA and Western Blot serologic tests for antibodies against the Human Immunodeficiency Virus (HIV)**

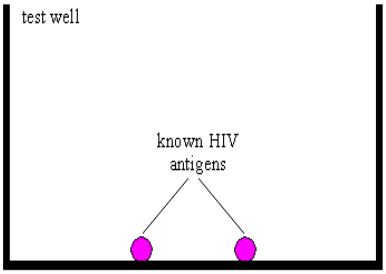
Discussion

In the case of the current HIV antibody tests, the **patient's serum** is mixed with **various HIV antigens produced by recombinant DNA technology.** If the person is seropositive (has repeated positive antigen-antibody tests), then HIV must be in that person's body stimulating antibody production. In other words, the person must be infected with HIV. The two most common tests currently used to detect antibodies against HIV are the **enzyme immunoassay or EIA** (also known as the enzyme-linked immunosorbant assay or ELISA) and the **Western blot or WB**. A person is considered to be seropositive for HIV infection only after an EIA screening test is repeatedly reactive and another test such as the WB has been performed to confirm the results.

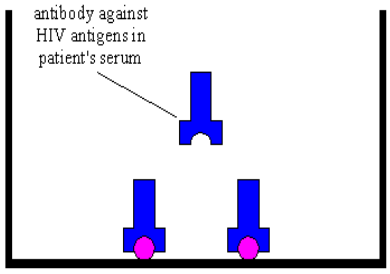
The **EIA** is less expensive, faster, and technically less complicated than the WB and is the procedure initially done as a screening test for HIV infection. The various EIA tests give a spectrophotometric reading of the amount of antibody binding to known HIV antigens.

The EIA test kit **contains plastic wells to which various HIV antigens have been adsorbed** (see EIA-A). The **patient's serum** is added to the wells and any antibodies present in the serum against HIV antigens will bind to the corresponding antigens in the wells (see EIA-B). The wells are then washed to remove all antibodies in the serum other than those bound to HIV antigens. **Enzyme-linked anti-human gamma globulin (anti HGG) antibodies** are then added to the wells. These antibodies, made in another animal against the Fc portion of human antibodies by injecting the animal with human serum,

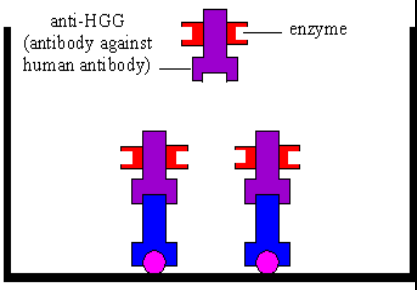
have an enzyme chemically attached. They react with the human antibodies bound to the known HIV antigens (see EIA-C). The wells are then washed to remove any anti-HGG that has not bound to serum antibodies. A **substrate specific for the enzyme** is then added and the resulting enzyme-substrate reaction causes a **color change** in the wells (see EIA-D). If there are no antibodies in the patient's serum against HIV, there will be nothing for the enzyme-linked anti-HGG to bind to and it will be washed from the wells. When the substrate is added, there will be no enzyme present in the wells to give a color change.



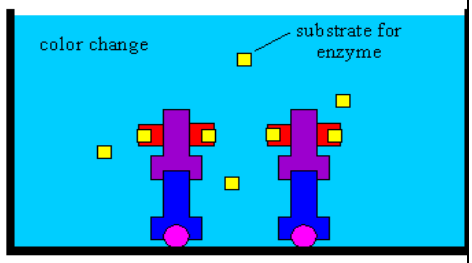
EIA-A



EIA-B



EIA-C



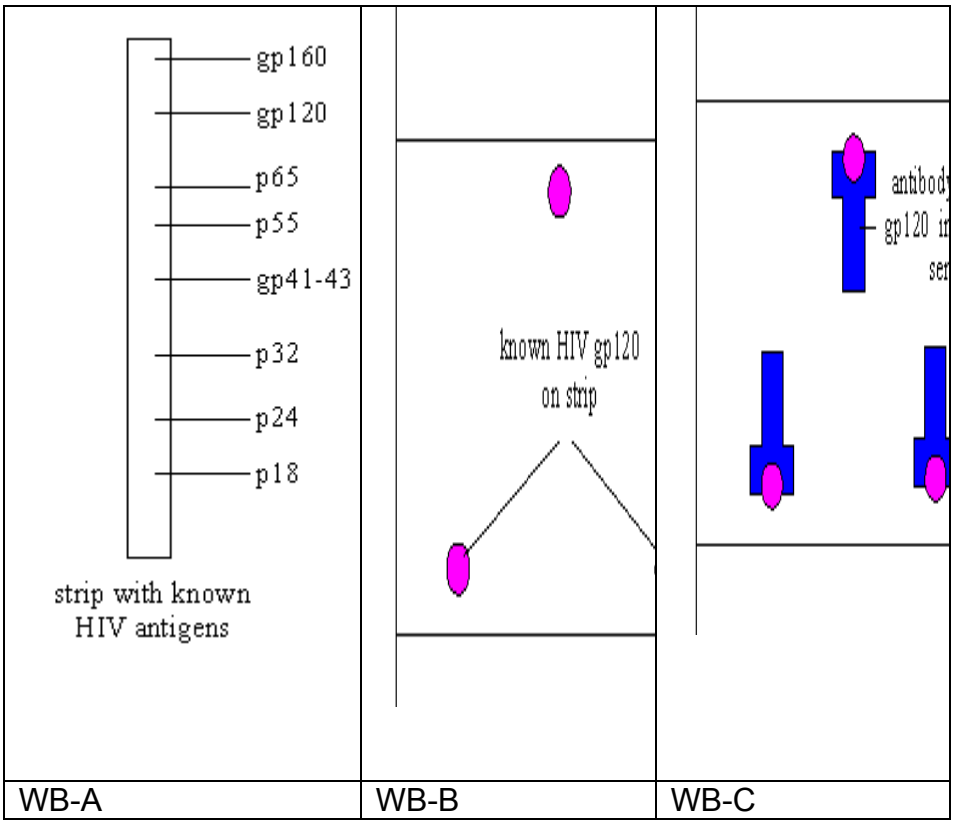
EIA-D

If the initial EIA is reactive it is **automatically repeated** to reduce the possibility that technical laboratory error caused the reactive result. If the EIA is still reactive, it is then **confirmed by the Western blot test**.

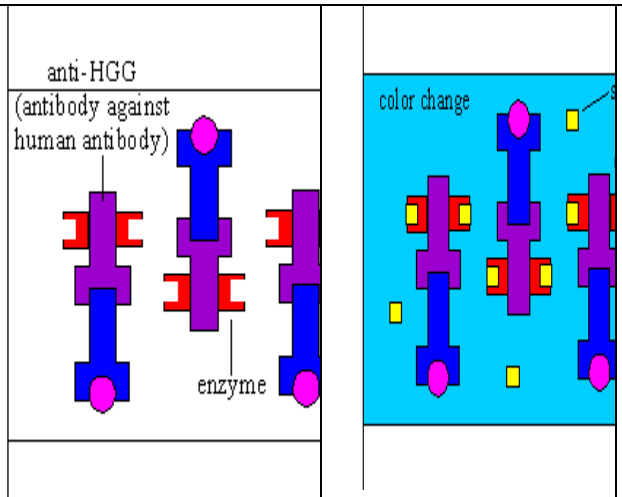
The **Western blot WB** is the test most commonly used as a confirming test if the EIA is repeatedly positive. The WB is technically more complex to perform and interpret, is more time consuming, and is more expensive than the EIAs.

With the WB, the various protein and glycoprotein antigens from HIV are separated according to their molecular weight by gel electrophoresis (a procedure that separates charged proteins in a gel by applying an electric field). Once separated, the various

HIV antigens are transferred to a nitrocellulose strip (see WB-A and WB-B). The patient's serum is then incubated with the strip and any HIV antibodies that are present will bind to the corresponding known HIV antigens on the strip (see WB-C). **Enzyme-linked anti-human gamma globulin (anti HGG) antibodies** are then added to the strip. These antibodies, made in another animal against the Fc portion of human antibodies by injecting the animal with human serum, have an enzyme chemically attached. They react with the human antibodies bound to the known HIV antigens (see WB-D). The strip is then washed to remove any anti-HGG that has not bound to serum antibodies. A **substrate specific for the enzyme** is then added and the resulting enzyme-substrate reaction causes a **color change** on the strip (see WB-E). If there are no antibodies in the patient's serum against HIV, there will be nothing for the enzyme-linked anti-HGG to bind to and it will be washed from the strip. When the substrate is added, there will be no enzyme present on the strip to give a color change.



WB-A | WB-B | WB-C



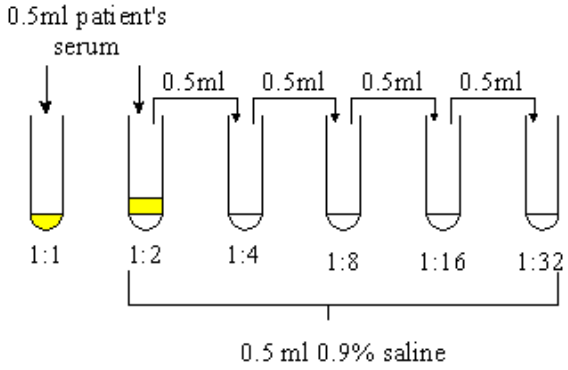
WB-D	WB-E	

It should be mentioned that all serologic tests are capable of giving occasional **false-positive and false-negative results**. The most common cause of a false-negative HIV antibody test is when a person **has been only recently infected with HIV** and **his or her body has not yet made sufficient quantities of antibodies to give a visible positive serologic test**. It generally takes between 2 weeks and 3 months after a person is initially infected with HIV to convert to a positive HIV antibody test.

PROCEDURE

A. **The RPR® Card Test for Syphilis** (demonstration)

1. Label 6 test tubes as follows: 1:1, 1:2, 1:4, 1:8, 1:16, and 1:32.
2. Using a 1.0 ml pipette, add **0.5 ml of 0.9% saline solution** into tubes **1:2, 1:4, 1:8, 1:16, and 1:32**.
3. Add **0.5 ml of the patient's serum** to the **1:1** tube (undiluted serum).
4. Add another **0.5 ml of serum** to the saline in the **1:2** tube and mix. Remove **0.5 ml from the 1:2 tube** and add it to the **1:4** tube and mix. Remove **0.5 ml from the 1:4 tube**, add to the **1:8** tube and mix. Remove **0.5 ml from the 1:8 tube**, add to the **1:16** tube and mix. Remove **0.5 ml from the 1:16 tube**, add to the **1:32** tube and mix. Remove **0.5 ml from the 1:32 tube** and **discard**. The dilution of the serum is summarized in Fig. 7A.



5. Using the capillary pipettes provided with the kit, add **a drop of each serum dilution** to separate circles of the RPR card. Spread the serum over the entire inner surface of the circle with the tip of the pipette, using a new pipette for each serum dilution.
6. Using the RPR antigen dispenser, add **a drop of known RPR antigen** to each circle. Do not let the needle of the dispenser touch the serum. Using disposable stirrers, mix the known RPR antigen with the serum in each circle.
7. Place the slide on a shaker and **rotate for a maximum of 4 minutes**.
8. Read the results as follows:
   - A definite **clumping** of the charcoal particles is reported as **reactive (R)**.
   - **No clumping** is reported as **nonreactive (N)**.

   The greatest serum dilution that produces a **reactive** result is the **titer**. For example, if the dilutions turned out as follows, the titer would be reported as 1:4 or 4 dils.

1:1	**1:2**	**1:4**	**1:8**	**1:16**	**1:32**
R	R	R	N	N	N

B. **The Serologic Tests for Infectious Mononucleosis** (demonstration)
   1. Place **one drop of each of the patient's serum** in circles on the test slide.
   2. Add **one drop of treated horse erythrocytes** (the known antigen) to each circle and mix with disposable applicator sticks.
   3. **Rock the card** gently **for 1 minute**, then **leave undisturbed for 1 minute**, and observe for agglutination of the red blood cells. Agglutination indicates the presence of **heterophile antibodies**.

C. **The Serologic Tests for Systemic Lupus Erythematosus (SLE)**
   1. Add **one drop of each of the patient's serum** to separate circles on the test slide.
   2. Add **one drop of the Latex-Deoxyribonucleoprotein reagent** (the known antigen, deoxyribonucleoprotein adsorbed to latex

particles) to each serum sample and mix with disposable applicator sticks.

3. **Rock the slide** gently **for 1 minute** and observe for agglutination. Agglutination indicates the presence of **antinuclear antibodies** associated with SLE.

D. **The FTA-ABS Test for Syphilis (Indirect Fluorescent Antibody Technique**

Observe the 35mm slide of a positive FTA-ABS test.

E. **The EIA and WB Tests for HIV Antibodies**

Observe the illustrations of the EIA and the WB tests for antibodies against HIV.

RESULTS

A. **RPR Card® Test for Syphilis (Quantitative)**

Detects nontreponemal antilipid antibodies (reagin)

Record your results:

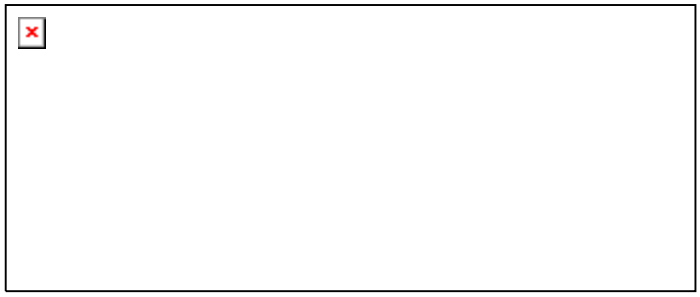
dilution	**result**
1:1	
1:2	
1:4	
1:8	
1:16	
1:32	
titer	

R = reactive (distinct clumps)
N = nonreactive (no clumps)

B. **MONO-TEST for Infectious Mononucleosis (Qualitative)**

Detects heterophile antibodies.

Draw the results of a positive and a negative test.



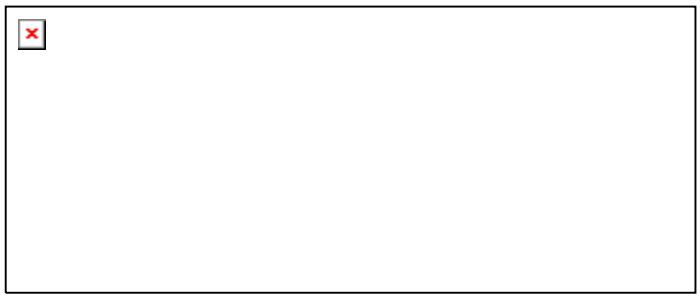
Infectious mononucleosis test slide

+ = agglutination of RBCs
- = no agglutination of RBCs

C. **Serologic test for SLE (Qualitative)**

Detects anti-deoxyribonucleoprotein antibodies.

Draw the results of a positive and a negative test.



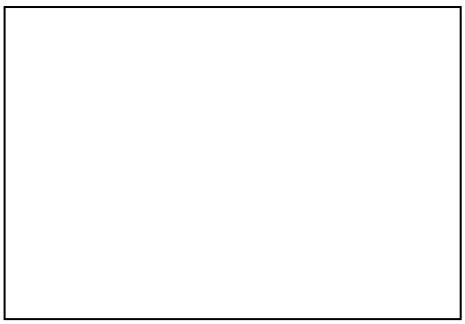
SLE test slide

+ = agglutination
- = no agglutination

D. **FTA-ABS Test for Syphilis (Confirming)**

Detects antibodies against *Treponema pallidum*

Draw the results of a positive FTA-ABS test.



positive FTA-ABS test for syphilis (fluorescent spirochetes)

Using Antimicrobial Chemotherapy to Control Microorganisms

OBJECTIVES :

After completing this Exercise, the student will be able to perform the following objectives:

A. **ANTIMICROBIAL CHEMOTHERAPEUTIC AGENTS**
   1. Define the followings: antibiotic, antimicrobial chemotherapeutic chemical, narrow spectrum antibiotic, broad spectrum antibiotic.
   2. Discuss the meaning of selective toxicity in terms of antimicrobial chemotherapy.
   3. List four genera of microorganisms that produce useful antibiotics.
   4. Describe five different major modes of action of antimicrobial chemotherapeutic chemicals and give three examples of drugs fitting each mode of action.

B. **MICROBIAL RESISTANCE TO ANTIMICROBIAL AGENTS**
   - **DISCUSSION**
     1. State five mechanisms by which microorganisms may resist antimicrobial chemotherapeutic agents.
     2. Briefly describe R plasmids and name four bacteria that commonly possess these plasmids.

C. **ANTIBIOTIC SUSCEPTIBILITY TESTING**
   - **DISCUSSION**
     1. State why antimicrobial susceptibility testing is often essential in choosing the proper chemotherapeutic agent to use in treating an infection.
     2. State what is meant by MIC.
   - **RESULTS**
     1. Interpret the results of a Bauer-Kirby antimicrobial susceptibility test when given a Mueller-Hinton agar plate, a metric ruler, and a standardized zone-size interpretation table.

DISCUSSION

A. **ANTIMICROBIAL CHEMOTHERAPEUTIC AGENTS**

Antimicrobial chemotherapy is the use of chemicals to inhibit or kill microorganisms in or on the host. Chemotherapy is based on **selective toxicity**. This means that the agent used must **inhibit or kill the microorganism** in question **without seriously harming the host**.

In order to be selectively toxic, a chemotherapeutic agent must interact with some microbial function or microbial structure which is either not present or is substantially different from that of the host. For example, in treating infections caused by prokaryotic bacteria, the agent may inhibit peptidoglycan synthesis or alter bacterial (prokaryotic) ribosomes. Human cells do not contain peptidoglycan and possess eukaryotic ribosomes. Therefore, the drug shows little if any effect on the host (selective toxicity). Eukaryotic microorganisms, on the other hand, have structures and functions more closely related to those of the host. As a result, the variety of agents selectively effective against eukaryotic microorganisms such as fungi and protozoans is small when compared to the number available against prokaryotes. Also keep in mind that viruses are not cells and, therefore, lack the structures and functions altered by antibiotics so antibiotics are not effective against viruses.

Based on their origin, there are 2 general classes of antimicrobial chemotherapeutic agents:

1. **antibiotics:** substances produced as metabolic products of one microorganism which inhibit or kill other microorganisms.
2. **antimicrobial chemotherapeutic chemicals:** chemicals synthesized in the laboratory which can be used therapeutically on microorganisms.

Today the distinction between the 2 classes is not as clear, since many antibiotics are extensively modified in the laboratory (semisynthetic) or even synthesized without the help of microorganisms.

Most of the major groups of antibiotics were discovered prior to 1955, and most antibiotic advances since then have come about by modifying the older forms. In fact, only 3 major groups of microorganisms have yielded useful antibiotics: the actinomycetes (filamentous, branching soil bacteria such as *Streptomyces*), bacteria of the genus *Bacillus*, and the saprophytic molds *Penicillium* and *Cephalosporium*.

To produce antibiotics, manufacturers inoculate large quantities of medium with carefully selected strains of the appropriate species of antibiotic-producing microorganism. After incubation, the drug is extracted from the medium and purified. Its activity is standardized and it is put into a form suitable for administration.

Some antimicrobial agents are **cidal** in action: they kill microorganisms (e.g., penicillins, cephalosporins, streptomycin, neomycin). Others are **static** in action: they inhibit microbial growth long enough for the body's own defenses to remove the organisms (e.g., tetracyclines, erythromycin, sulfonamides).

Antimicrobial agents also vary in their spectrum. Drugs which are effective against a variety of both gram-positive and gram-negative

bacteria are said to be **broad spectrum** (e.g., tetracycline, streptomycin, cephalosporins, ampicillin, sulfonamides). Those effective against just gram-positive bacteria, just gram negative bacteria, or only a few species are termed **narrow spectrum** (e.g., penicillin G, erythromycin, clindamycin, gentamicin).

If a choice is available, a narrow spectrum is preferable since it will cause less destruction to the body's normal flora. In fact, **indiscriminate use** of broad spectrum antibiotics can lead to **superinfection by opportunistic microorganisms**, such as *Candida* (yeast infections) and *Clostridium difficile* (antibiotic-associated ulcerative colitis), when the body's normal flora is destroyed. Other dangers from indiscriminate use of antimicrobial chemotherapeutic agents include **drug toxicity, allergic reactions to the drug**, and **selection for resistant strains of microorganisms**.

Below are examples of commonly used antimicrobial chemotherapeutic agents arranged according to their **mode of action:**

3. **Antimicrobial agents that inhibit peptidoglycan synthesis**. Inhibition of peptidoglycan synthesis in actively-dividing bacteria results in osmotic lysis.
    a. **Penicillins** (produced by the mold *Penicillium*)

        There are several classes of penicillins:

        1. **Natural penicillins** are highly effective against gram-positive bacteria (and a very few gram-negative bacteria) but are inactivated by the bacterial enzyme penicillinase. Examples include **penicillin G, F, X, K, O, and V**.
        2. **Semisynthetic penicillins** are effective against gram-positive bacteria but are not inactivated by penicillinase. Examples include **methicillin, dicloxacillin, and nafcillin**.
        3. **Semisynthetic broad spectrum penicillins** are effective against a variety of gram-positive and gram-negative bacteria but are inactivated by penicillinase. Examples include **ampicillin, carbenicillin, and oxacillin**. Some of the newer semisynthetic penicillins include **azlocillin, mezlocillin, and piperacillin**.
        4. **Semisynthetic broad spectrum penicillins combined with beta lactamase inhibitors such as clavulanic acid and sulbactam**. Although the clavulanic acid and sulbactam have no antimicrobial action of their own, they inhibits penicillinase thus protecting the penicillin from degradation. Examples include **amoxicillin plus**

clavulanic acid, ticarcillin plus clavulanic acid, and ampicillin plus sulbactam.

b. **Cephalosporins** (produced by the mold *Cephalosporium*)

Cephalosporins are effective against a variety of gram-positive and gram-negative bacteria and are resistant to penicillinase (although some may be inactivated by other beta-lactamase enzymes similar to penicillinase). Four "generations" of cephalosporins have been developed over the years in an attempt to counter bacterial resistance.

1. First generation cephalosporins include **cephalothin, cephapirin, and cephalexin**.
2. Second generation cephalosporins include **cefamandole, cefaclor, cefazolin, cefuroxime, and cefoxitin**.
3. Third generation cephalosporins include **cefotaxime, cefsulodin, cefetamet, cefixime, ceftriaxone, cefoperazone, ceftazidine, and moxalactam**.

c. **Carbapenems**: Carbapenems consist of a broad spectrum beta lactam antibiotic to inhibit peptidoglycan synthesis combined with cilastatin sodium, an agent which prevents degradation of the antibiotic in the kidneys. An example is **imipenem**.

d. **Monobactems**: Monobactems are broad spectrum beta lactam antibiotics resistant to beta lactamase. An example is **aztreonam**.

e. **Vancomycin** (produced by the bacterium *Streptomyces*): Vancomycin and teichoplanin are glycopeptides that are effective against gram-positive bacteria.

f. **Bacitracin** (produced by the bacterium *Bacillus*): Bacitracin is used topically against gram-positive bacteria.

4. **Antimicrobial agents that alter the cytoplasmic membrane**. Alteration of the cytoplasmic membrane of microorganisms results in leakage of cellular materials.
   a. **Polymyxin B** (produced by the bacterium *Bacillus*): Polymyxin B is used in severe *Pseudomonas* infections.
   b. **Amphotericin B** (produced by the bacterium *Streptomyces*): Amphotericin B is used for systemic fungal infections.
   c. **Nystatin**(produced by the bacterium *Streptomyces*): Nystatin is used mainly for *Candida* yeast infections.
   d. **Imidazoles** (produced by the bacterium *Streptomyces*): The imidazoles are antifungal antibiotics used for yeast infections, dermatophytic infections, and systemic fungal infections. Examples include **clotrimazole, miconazole, ketoconazole, itraconazole, and fluconazole**.
5. **Antimicrobial agents that inhibit protein synthesis**

These agents prevent bacteria from synthesizing structural proteins and enzymes.

a. Agents that **block transcription** (prevent the synthesis of mRNA off of DNA).
    1. **Rifampins** (produced by the bacterium *Streptomyces*): Rifampins are effective against some gram-positive and gram-negative bacteria and *Mycobacterium tuberculosis.* They inhibit the enzyme RNA polymerase.
    2. **Ethambutol** (a synthetic chemical): Ethambutol is used against *Mycobacterium tuberculosis*.
    3. Agents that **block translation** (alter bacterial ribosomes to prevent mRNA from being translated into proteins).
        1. Agents that bind irreversibly to the 30s ribosomal subunit and cause a misreading of the mRNA (the **aminoglycosides** produced by the bacterium *Streptomyces*). Examples include **streptomycin, kanamycin, tobramycin, and amikacin**. Most are effective against gram-positive and gram-negative bacteria.
        2. Agents that bind reversibly to the 30s ribosomal subunit and interfere with the binding of charged tRNA to the bacterial ribosome. Examples include **tetracycline, minocycline, and doxycycline**, produced by the bacterium *Streptomyces*. They are effective against a variety of gram-positive and gram-negative bacteria.
        3. Agents that bind reversibly to the 50s ribosomal subunit and block peptide bond formation during protein synthesis. Examples include **lincomycin and clindamycin**, produced by the bacterium *Streptomyces*. Most are used against gram-positive bacteria.
        4. Agents that bind reversibly to the 50s ribosomal subunit and prevent the release of uncharged tRNA from the bacterial ribosome. **Erythromycin, roxithromycin, clarithromycin, and azithromycin** are examples and are used against gram-positive bacteria and some gram-negative bacteria.
b. **Antimicrobial agents that interfere with DNA synthesis**
c. **Quinolones** (synthetic chemicals): The quinolones block bacterial DNA replication by inhibiting the DNA gyrase,

the enzyme needed by bacteria to produce their circular DNA. They are broad spectrum and examples include **norfloxacin, ciprofloxacin, enoxacin, and temafloxacin**.

d. **Co-trimoxazole** (synthetic chemicals): Co-trimoxazole is a combination of sulfamethoxazole and trimethoprim, which blocks the bacterial synthesis of folic acid needed to make DNA bases.

A list of common antimicrobial chemotherapeutic agents listed by both their generic and brand names and arranged by their mode of action can be found in at the end of this exercise

B. MICROBIAL RESISTANCE TO ANTIMICROBIAL CHEMOTHERAPEUTIC AGENTS

A common problem in antimicrobial chemotherapy is the development of resistant strains of bacteria. Most bacteria become resistant to antimicrobial agents by one or more of the following mechanisms:

1. **Producing enzymes which detoxify or inactivate the antibiotic**, e.g., penicillinase and other beta-lactamases.
2. **Altering the target site in the bacterium to reduce or block binding of the antibiotic**, e.g., producing a slightly altered ribosomal subunit that still functions but to which the drug can't bind.
3. **Preventing transport of the antimicrobial agent into the bacterium**, eg., producing an altered cytoplasmic membrane or outer membrane.
4. **Developing an alternate metabolic pathway to by-pass the metabolic step being blocked by the antimicrobial agent**, e.g., overcoming drugs that resemble substrates and tie-up bacterial enzymes.
5. **Increasing the production of a certain bacterial enzyme**, e.g., overcoming drugs that resemble substrates and tie-up bacterial enzymes.

These changes in the bacterium which enable it to resist the antimicrobial agent **occur naturally** as a result of mutation or genetic recombination of the DNA in the nucleoid, or as a result of obtaining plasmids from other bacteria. Exposure to the antimicrobial agent then **selects for these resistant strains of organism**.

As an example, many gram-negative bacteria possess **R (resistance) plasmids** which have genes coding for **multiple antibiotic resistance** through the mechanisms stated above, as well as transfer genes coding for a **sex pilus**. Such an organism can conjugate with other bacteria and transfer an R plasmid to them. *Escherichia coli, Proteus, Serratia, Salmonella, Shigella,* and *Pseudomonas* are examples of bacteria which frequently have R plasmids. Because of the problem of

antibiotic resistance, **antibiotic susceptibility testing** is usually done in the clinical laboratory to determine which antimicrobial chemotherapeutic agents will most likely be effective on a particular strain of microorganism. This is discussed in the next section.

C. ANTIBIOTIC SUSCEPTIBILITY TESTING

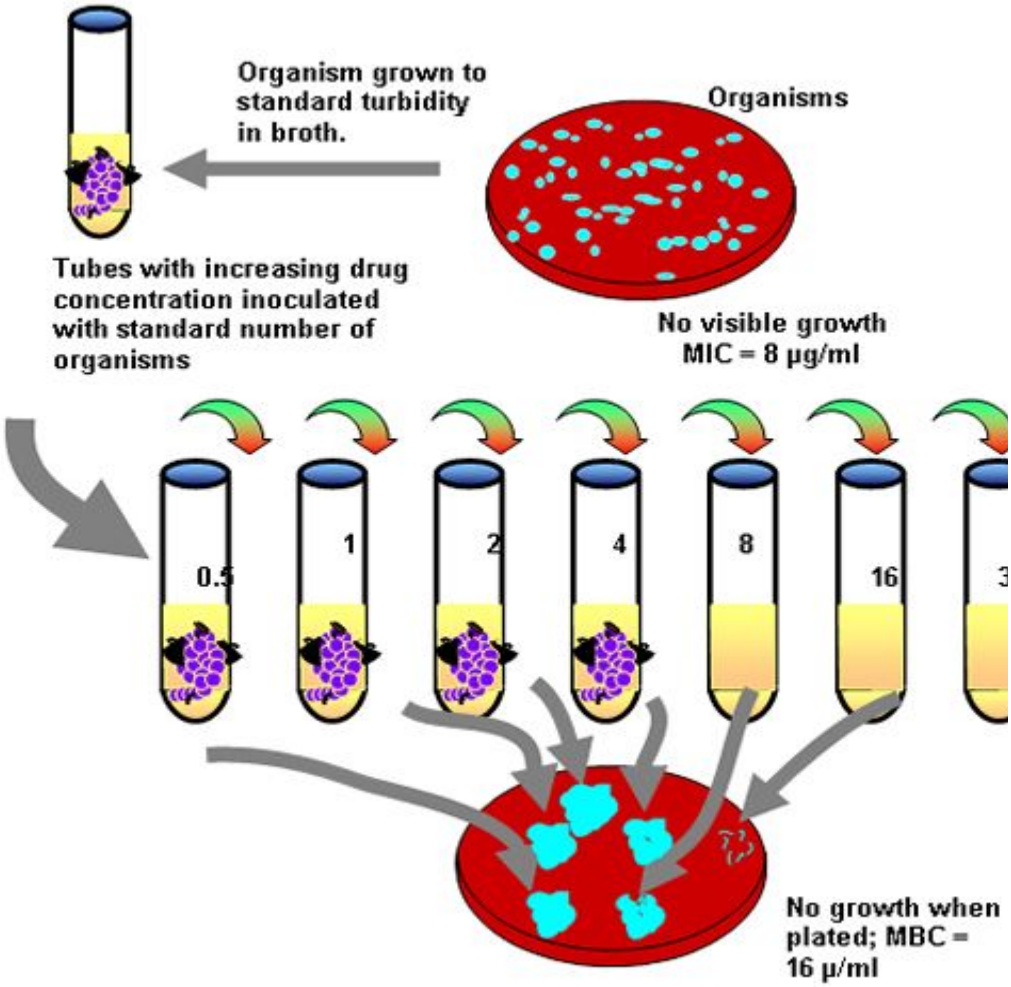
For some microorganisms, susceptibility to chemotherapeutic agents is predictable. However, for many microorganisms (*Pseudomonas, Staphylococcus aureus,* and gram-negative enteric bacilli such as *Escherichia coli, Serratia, Proteus*, etc.) there is no reliable way of predicting which antimicrobial agent will be effective in a given case. This is especially true with the emergence of many antibiotic-resistant strains of bacteria. Because of this, antibiotic susceptibility testing is often essential in order to determine which antimicrobial agent to use against a specific strain of bacterium.

Several tests may be used to tell a physician which antimicrobial agent is most likely to combat a specific pathogen:

1. Tube dilution tests

In this test, a series of culture tubes are prepared, each containing a liquid medium and a different concentration of a chemotherapeutic agent. The tubes are then inoculated with the test organism and incubated for 16-20 hours at 35C. After incubation, the tubes are examined for turbidity (growth). The lowest concentration of chemotherapeutic agent capable of preventing growth of the test organism is the **minimum inhibitory concentration (MIC)**.

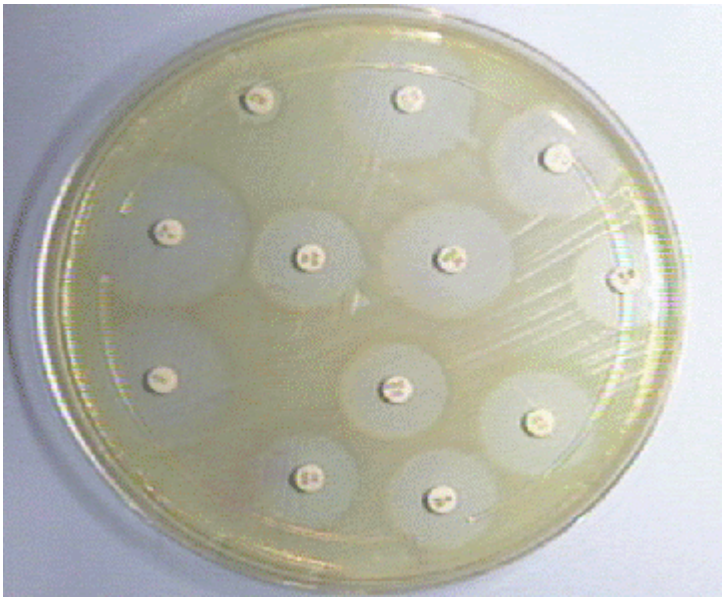
Subculturing of tubes showing no turbidity into tubes containing medium but no chemotherapeutic agent can determine the **minimum bactericidal concentration (MBC)**. MBC is the lowest concentration of the chemotherapeutic agent that results in no growth (turbidity) of the subcultures. These tests, however, are rather time consuming and expensive to perform.



2. **The agar diffusion test (Bauer-Kirby test)**

   A procedure commonly used in clinical labs to determine antimicrobial susceptibility is the Bauer-Kirby disc diffusion method. In this test, the in vitro response of bacteria to a standardized antibiotic-containing disc has been correlated with the clinical response of patients given that drug.

   In the development of this method, a single high-potency disc of each chosen chemotherapeutic agent was used. Zones of growth inhibition surrounding each type of disc were correlated with the minimum inhibitory concentrations of each antimicrobial agent (as determined by the tube dilution test). The MIC for each agent was then compared to the usually-attained blood level in the patient with adequate dosage. Categories of "Resistant," "Intermediate," and "Sensitive" were then established.



The basic steps for the Bauer-Kirby method of antimicrobial susceptibility testing are given below.

a. Prepare a **standard turbidity inoculum** of the test bacterium so that a certain density of bacteria will be put on the plate.
b. **Inoculate a 90 mm Mueller-Hinton agar plate** with the standardized inoculum so as to cover the entire agar surface with bacteria.
c. Place **standardized antibiotic-containing discs** on the plate.
d. **Incubate** the plate at 35 ^{o}C for 18-20 hours.
e. Measure the **diameter** of any resulting **zones of inhibition** in millimeters (mm).
f. Determine if the bacterium is **susceptible, moderately susceptible, intermediate, or resistant** to each antimicrobial agent using a **standardized table** (Table 8 B).

The term intermediate generally means that the result is inconclusive for that drug-organism combination. The term moderately susceptible is usually applied to those situations where a drug may be used for infections in a particular body site, e.g., cystitis because the drug becomes highly concentrated in the urine.

3. **Automated tests**

Computerized automated tests have been developed for antimicrobial susceptibility testing. These tests measure the inhibitory effect of the antimicrobial agents in a liquid medium by

using light scattering to determine growth of the test organism. Results can be obtained within a few hours. Labs performing very large numbers of susceptibility tests frequently use the automated methods but the equipment is quite expensive.

PROCEDURES

A. ANTIBIOTIC SUSCEPTIBILITY TESTING

- **MATERIALS**

  90 mm Mueller-Hinton agar plates (3)
  sterile swabs (3)
  An antibiotic disc dispenser containing discs of antibiotics commonly effective against gram-positive bacteria, and one containing discs of antibiotics commonly effective against gram-negative bacteria

- **ORGANISMS**

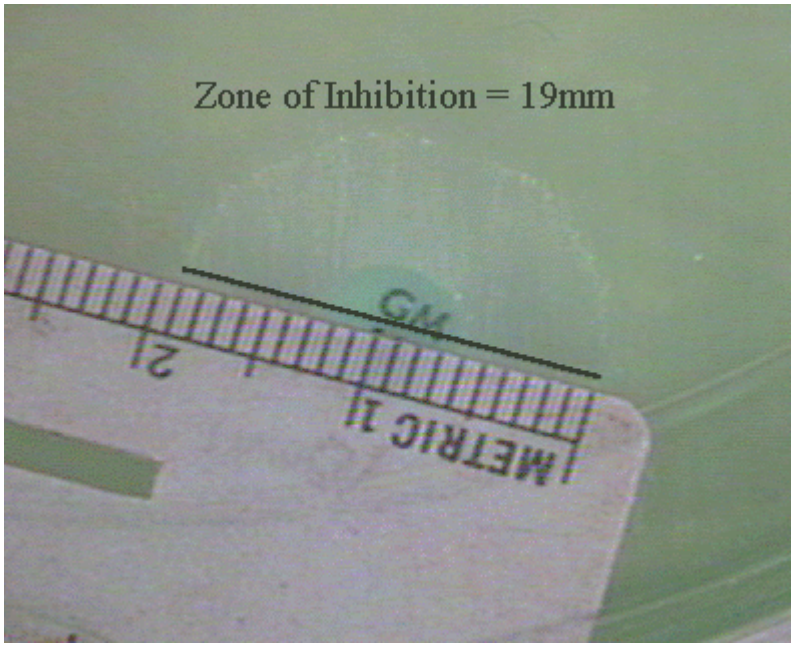
  Trypticase Soy broth cultures of *Staphylococcus aureus* (gram-positive), *Escherichia coli* (gram-negative), and *Pseudomonas aeruginosa* (gram-negative)

- **PROCEDURE**

  1. Take **3 Mueller-Hinton agar plates**. Label one *S. aureus*, one *E. coli*, and one *P. aeruginosa*.

     Using your wax marker, divide each plate into **thirds** to guide your streaking.

  2. Dip a **sterile swab** into the previously-standardized tube of *S. aureus*. **Squeeze the swab against the inner wall of the tube** to remove excess liquid.
  3. Streak the swab **perpendicular to each of the 3 lines** drawn on the plate overlapping the streaks to assure **complete coverage** of the entire agar surface with inoculum.
  4. Using the appropriate antibiotic disc dispenser, place **gram-positive antibiotic-containing discs on the plate of *S. aureus*** and **gram-negative antibiotic-containing discs on the plates of *E. coli* and *P. aeruginosa***.
  5. Make sure that one of each of the antibiotic-containing discs in the dispenser is on the plate and **touch each disc lightly with sterile forceps** to make sure it adheres to the agar surface.
  6. Incubate the 3 plates upside-down **at 37 ^{o}C** until the next lab period.
  7. Using a metric ruler, measure the **diameter of the zone of inhibition around each disc on each plate in mm** by placing the ruler on the bottom of the plate (Fig. 8B).



8. Determine whether each organism is **susceptible, moderately susceptible, intermediate, or resistant to each chemotherapeutic agent** using the standardized table (**Table 8B**) and record your results.
9.

Antimicrobial susceptibility testing by the disk diffusion method (modified Kirby-Bauer) & Antibiotic profiles

Purpose of Procedure

To test isolated bacteria for its susceptibility to antimicrobial agents

Specimen Requirements

In general a pure growth of the isolate.

Equipment

- Nichrome wire loops
- Sterile cotton swabs.

Reagents Required

- Muller Hinton Agar plates
- Brain Heart Infusion Broth (5 ml amounts)
- Appropriate antibiotic discs or dispenser
- McFarland Standard (0.5)
- Pure culture of the test organism

Method

A. Preparation of Media

1. Prepare sufficient amount of MH and BHIB and sterilize by autoclaving see appendix for preparation.
2. Pour off MH medium into sterile Petri Dishes at 4 mm depth and let it stand at room temperature to solidify.

B. Preparation of the Inoculum

3. Inoculate the test organism into a BHIB tube (pick 3-5 well isolated colonies)
4. Incubate at 37 ^{o}C for 4-6 hours
5. Adjust the concentration of the inoculum by comparing the turbidity of BHIB tube to that of a 0.5 McFarland standard.

Note: If the turbidity of the inoculum is greater than that of McFarland standard, dilute with sterile BHIB. If the turbidity of the standard is greater than the inoculum, incubate further or add more of the test organism.

C. Preparation of the plates

6. Dip a sterile cotton swab into the adjusted inoculum tube and drain excess fluid by pressing the swab against the walls of the test tube.
7. Hold Muller-Hinton plate half or partially open and streak the plate using the wet cotton swab covering all the area even at the sides.
8. Place the plates aside for about 10 minutes. Allow the inoculum to dry.

D. Application of the antibiotic disks

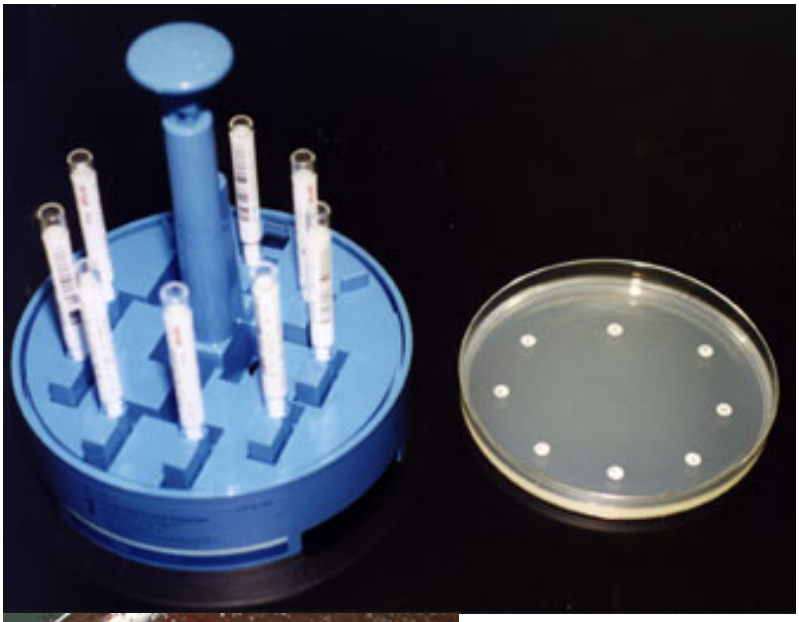
9. Using a sterile forceps or needle, apply a set of suitable antibiotic disks. Five to six disks for each plate, or 8 disks if you use the automatic dispenser.
10. Let the plates stand for 10 minutes, then incubate in inverted position at 37 ^{o}C for 18-24 hours.

E. Reading and recording the results

11. Using a ruler or caliper, measure the zone of inhibition around each antimicrobial disk and record it

F. Interpretation of the Results

12. Consult the special chart provided by the manufacturer of the antimicrobial disks and interpret results as Sensitive, Resistant, or intermediate.





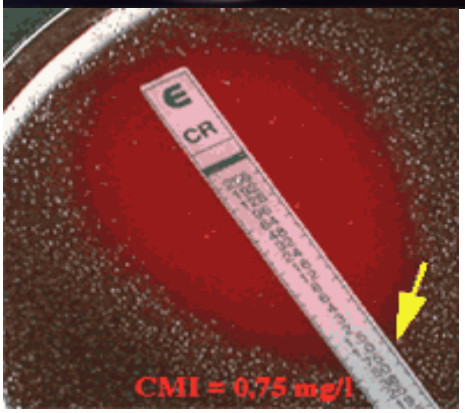

CR
CMI = 0,75 mg/l

Table 8: Zone Size Interpretive Chart for Bauer-Kirby Test

Antimicrobial agent	Disc code	R = mm or less	I = mm	MS = mm	S = mm or more
Amikacin	AN-30	15	15-16	-	16
Amoxicillin/clavulanic acid - staphylococci	AmC-30	19	-	-	20
Amoxicillin/clavulanic acid - other organisms	AmC-30	13	14-17	-	18
Ampicillin/staphylococci	AM-10	28	-	-	29
Ampicillin/ G- enterics	AM-10	11	12-13	-	14
azlocillin	AZ-75	14	15-17	-	13
Aztreonam	ATM-30	15	-	16-21	22
Carbenicillin Enterobact	CB-100	17	18-22	-	23
Carbenicillin/- *Pseudomonas*	CB-100	13	14-16	-	17
Cefamandole	MA-30	14	15-17	-	18
Cefazolin	CZ-30	14	15-17	-	18
Cefonicid	CID-30	14	15-17	-	18
Cefoperazone	CFP-75	15	-	16-20	21
Cefotaxime	CTX-30	14	-	15-22	23
Cefotetan	CTT-30	12	-	13-15	16
Cefoxitin	FOX-30	13	-	15-17	18
Ceftazidime	CAZ-30	14	15-17	-	18
Ceftizoxime/ *Pseudomonas*	ZOX-30	10	-	11	-
Ceftizoxime/other organisms	ZOX-30	14	-	15-19	20
Ceftriaxone	CRO-30	13	-	14-20	21
Cefuroxime	CXM-30	14	15-17	-	18
Cephalothin	CF-30	14	15-17	-	18
Chloramphenicol	C-30	12	13-17	-	18
Cinoxacin	CIN-100	14	15-18	-	19
Ciprofloxacin	CIP-5	15	16-20	-	21
Clindamycin	CC-2	14	15-20	-	21
Doxycycline	D-30	12	13-15	-	16
Erythromycin	E-15	13	14-22	-	23
Gentamicin	GM-10	12	13-14	-	15
Imipenem	IPM-10	13	14-5	-	16
Kanamycin	K-30	13	14-17	-	18
Methicillin /staphylococci	DP-5	9	10-13	-	14
Mezlocillin	MZ-75	12	13-15	-	16
Minocycline	MI-30	14	15-18	-	19
Moxalactam	MOX-30	14	-	15-22	23
Nafcillin/ staphylococci	NF-1	10	11-12	-	13
Nalidixic acid	NA-30	13	14-18	-	19
Netilmicin	NET-30	12	13-14	-	17
nitrofurantoin	F/M-300	14	15-16	-	17
norfloxacin	NOR-10	12	13-16	-	17
Oxacillin/ - staphylococci	OX-1	10	11-12	-	13
penicillin	P-10	28	-	-	29
streptomycin	S-10	11	12-14	-	15
sulfam+ trimethoprim	SXT	10	11-15	-	16
Tetracycline	Te-30	14	15-18	-	19
Ticarcillin	TIC-75	11	12-14	-	15
Ticarcillin/clavulanic acid	TIM-85	11	12-14	-	15
Tobramycin	NN-10	12	13-14	-	15
Trimethoprim	TMP-5	10	11-15	-	16
Vancomycin	Va-30	9	10-11	-	12

RESULTS

A. ANTIBIOTIC SUSCEPTIBILITY TESTING: BAUER-KIRBY METHOD

Interpret the results following steps 9 and 10 of the procedure and record your results in the tables below.

Staphylococcus aureus

Disc code	Antimicrobial agent	zone in mm	R	I	MS	S
	Amoxicillin/ clavulanic acid					
	Cefotaxime					
	Cefoxitin					
	Ciprofloxacin					
	Clindamycin					
	Erythromycin					
	Kanamycin					
	Methicillin					
	Penicillin					
	Streptomycin					
	sulfamethoxazole + trimethoprim					
	Tetracycline					

R = Resistant
I = Intermediate
MS = Moderately Susceptible
S = Susceptible

Escherichia coli

Disc code	Antimicrobial agent	zone in mm	R	I	MS	S
	Amikacin					
	Amoxicillin/ clavulanic acid					
	Ampicillin					
	Carbenicillin					
	Cefotaxime					
	Cefoxitin					

	Ciprofloxacin					
	Colistin					
	Gentamicin					
	Kanamycin					
	Sulfamethoxazole + trimethoprim					
	Tetracycline					

R = Resistant
I = Intermediate
MS = Moderately Susceptible
S = Susceptible

Pseudomonas aeruginosa

Disc code	**Antimicrobial agent**	**zone in mm**	**R**	**I**	**MS**	**S**
	amikacin					
	amoxicillin/ clavulanic Acid					
	ampicillin					
	carbenicillin					
	cefotaxime					
	cefoxitin					
	ciprofloxacin					
	colistin					
	gentamicin					
	kanamycin					
	sulfamethoxazole + trimethoprim					
	tetracycline					

R = Resistant
I = Intermediate
MS = Moderately Susceptible
S = Susceptible

Appendix: Common Antibacterial Antibiotics

COMMON ANTIBACTERIAL ANTIBIOTICS
Selected antibiotics and their modes of action.

1. Inhibit synthesis of peptidoglycan causing osmotic lysis.

a. **penicillins:** penicillin G, penicillin V, methicillin, ampicillin, oxacillin, amoxicillin, ticarcillin, carbenicillin, piperacillin, mezlocillin, bacampicillin.
b. **penicillins plus beta lactamase inhibitors**: amoxicillin + clavulanate (Augmentin), ticarcillin + clavulanate (Timentin), ampicillin + sulbactam, imipenem + cilastatin , piperacillin + tazobactam.
c. **cephalosporins:** cefaclor, cefadroxil, cefamandole, cefazolin (Ancef, cefixime, cefoperazone, cefepime, ceftibuten, cefprozil, cefpodoxime cefonicid, cefotaxime, cefotetan, cefoxitin, ceftazidime, ceftizoxime (Cefizox), ceftriaxone, cefuroxime, cephalexin, cephapirin, cephradine.
d. **carbapenems:** imipenem (Primaxin), metropenem (Merrem).
e. **carbacephem:** loracarbef (Lorabid).
f. **monobactems:** aztreonam (Azactam).
g. **glycopeptides:** vancomycin (Vancocin, Vancoled), teichoplanin (Targocid).

2. **Alter cytoplasmic membrane causing cellular leakage**
   polymyxins: polymyxin B

3. **Alter bacterial ribosomes causing faulty protein synthesis**
   a. **aminoglycosides:** amikacin, tobramycin, gentamicin, netilmicin, neomycin, streptomycin.
   b. **tetracyclines**: tetracycline, oxytetracycline, minocycline, doxycycline, demeclocycline.
   c. **macrolides:** erythromycin, azithromycin, clarithromycin, dirithromycin, troleandomycin.
   d. **clindamycin**
   e. **chloramphenicol**

4. **Inhibit DNA replication by blocking DNA gyrase**
   quinolones: norfloxacin, ciprofloxacin, ofloxacin, temafloxacin, enoxacin, lomefloxacin, trovafloxacin, nalidixic acid.

5. **Inhibit bacterial DNA synthesis by blocking synthesis of tetrahydrafolic acid**

a. **sulfonamides:** sulfisoxazole, sulfisoxazole+erythromycin, sulfamethoxazole, sulfamethizole, sulfasalazine.
b. **trimethoprim**
c. **trimethoprim + sulfamethoxazole**.

6. **Multiple Action: blocks aerobic energy production and synthesis of proteins, DNA, RNA, and cell walls**
   nitrofurantoin
7. **Antituberculosis drugs**
   rifampin, rifampin and isoniazid, isoniazid, ethambutol, capreomycin, cycloserine, ethionamide, pyrazinamide, streptomycin, rifabutin, rifampin + isoniazid + pyrazinamide (Rifater).

ENUMERATION OF MICROORGANISMS

OBJECTIVES:

After completing this exercise, the student will be able to perform the following objectives:

1. State the formula for determining the number of CFUs per ml of sample when using the plate count technique.
2. When given a diagram of a plate count dilution and the number of colonies on the resulting plates, choose the correct plate for counting, determine the dilution factor of that plate, and calculate the number of CFUs per ml in the original sample.
    - Plate count practice problems
        - practice problem #1
        - practice problem #2
3. State the function of a spectrophotometer.
4. State the relationship between absorbance (optical density) and the number of bacteria in a broth sample.
5. State the relationship between percent light transmitted and the number of bacteria in a broth sample.

PROCEDURE

1. Perform a serial dilution of a bacterial sample according to instructions in the lab manual and plate out samples of each dilution using the spin-plate technique.

RESULTS

1. Using a colony counter, count the number of colonies on a plate showing between 30 and 300 colonies and, by knowing the dilution of this plate, calculate the number of CFUs per ml in the original sample.

DISCUSSION

As part of daily routine, the laboratory microbiologist often has to determine the number of bacteria in a given sample as well as having to compare the amount of bacterial growth under various conditions. Enumeration of microorganisms is especially important in dairy microbiology, food microbiology, and water microbiology.

Since the enumeration of microorganisms involves the use of extremely small dilutions and extremely large numbers of cells, scientific notation is routinely used in calculations. **THE PLATE COUNT (VIABLE COUNT)**

The number of bacteria in a given sample is usually too great to be counted directly. However, if the sample is serially diluted (Fig. 9A) and then plated out on an agar surface in such a manner that **single isolated bacteria form**

visible isolated colonies, the number of colonies can be used as a measure of the number of viable (living) cells in that known dilution. However, keep in mind that if the organism normally forms multiple cell arrangements, such as chains, the colony-forming unit may consist of a chain of bacteria rather than a single bacterium. In addition, some of the bacteria may be clumped together. Therefore, when doing the plate count technique, we generally say we are determining the number of **Colony-Forming Units (CFUs)** in that known dilution. By extrapolation, this number can in turn be used to calculate the number of CFUs in the original sample.

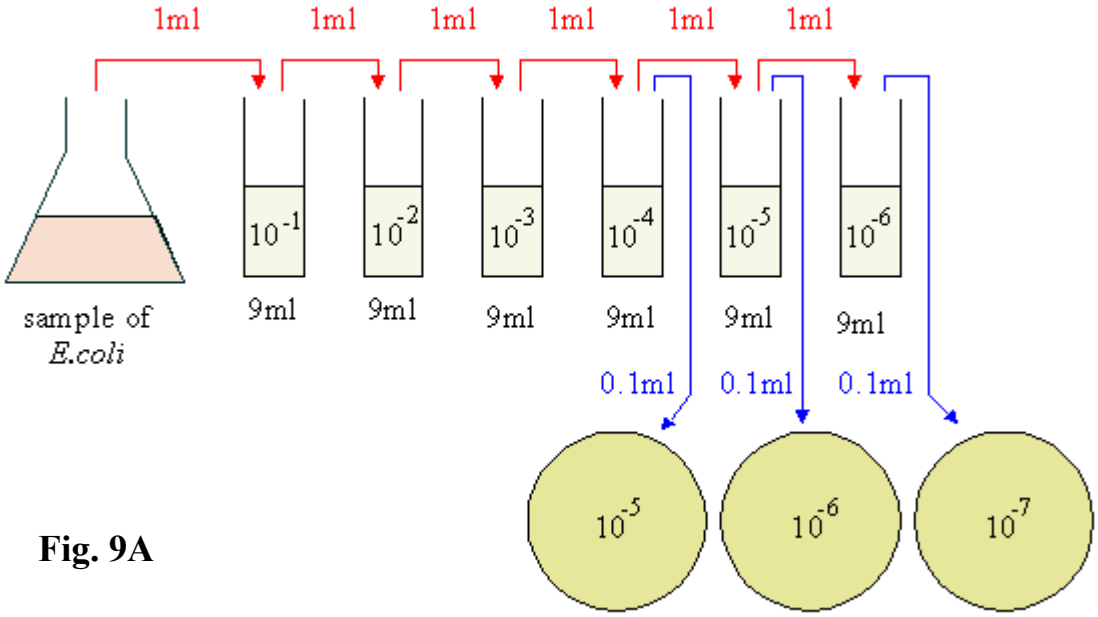


Fig. 9A

Normally, the bacterial sample is diluted by factors of 10 and plated on agar. After incubation, the number of colonies on a dilution plate showing **between 30 and 300 colonies** is determined. A plate having 30-300 colonies is chosen because this range is considered statistically significant. If there are less than 30 colonies on the plate, small errors in dilution technique or the presence of a few contaminants will have a drastic effect on the final count. Likewise, if there are more than 300 colonies on the plate, there will be poor isolation and colonies will have grown together.

Generally, one wants to determine the number of CFUs **per milliliter (ml) of sample**. To find this, the number of colonies (on a plate having 30-300 colonies) is multiplied by the number of times the original ml of bacteria was diluted (the **dilution factor** of the plate counted). For example, if a plate containing a 1/1,000,000 dilution of the original ml of sample shows 150 colonies, then 150 represents 1/1,000,000 the number of CFUs present in the original ml. Therefore the number of CFUs per ml in the original sample is found by multiplying 150 x 1,000,000 as shown in the formula below:

number of CFUs per ml of sample = number of colonies (30-300 plate) X the dilution factor of the plate counted

In the case of the example above, 150 x 1,000,000 = 150,000,000 CFUs per ml.

For a more accurate count it is advisable to plate each dilution in duplicate or triplicate and then find an average count.

A. **DIRECT MICROSCOPIC METHOD (TOTAL CELL COUNT)**

In the direct microscopic count, a counting chamber consisting of a ruled slide and a coverslip is employed. It is constructed in such a manner that a known volume is delimited by the coverslip, slide, and ruled lines. **The number of bacteria in a small known volume is directly counted microscopically and the number of bacteria in the larger original sample is determined by extrapolation.**

The Petroff-Hausser counting chamber (Fig. 9B), for example, has squares 1/20 of a millimeter (mm) by 1/20 of a mm and is 1/50 of a mm deep. The volume of one square therefore is 1/20,000 of a cubic mm or 1/20,000,000 of a cubic centimeter (cc). The normal procedure is to count the number of bacteria in **five large double-lined squares and divide by five to get the average number of bacteria per large square**. This number is then **multiplied by 20,000,000** (since the square holds a volume of 1/20,000,000 cc) to find the total number of organisms per cc in the original sample.

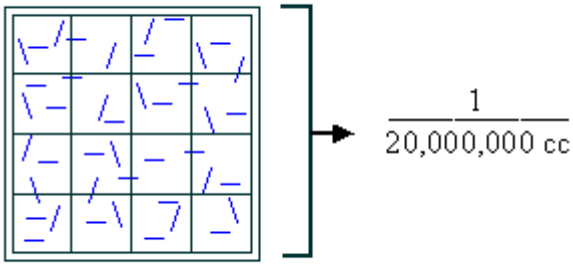


Fig. 9B

If the bacteria is diluted (such as by mixing with dye) before being placed in the counting chamber, then this dilution must also be considered in the final calculations.

The formula used for the direct microscopic count is:

number of bacteria per cc =

the average number of bacteria per large double-lined square X

the dilution factor of the large square (20,000,000) X

the dilution factor of any dilutions made prior to placing the sample in the counting chamber, e.g., mixing the bacteria with dye

B. **TURBIDITY**

When you mix the bacteria growing in a liquid medium, the culture appears **turbid**. This is because a bacterial culture acts as a colloidal suspension which blocks and reflects light passing through the culture. Within limits, the light absorbed by the bacterial suspension will be directly proportional to the concentration of cells in the culture. By measuring the amount of light absorbed by a bacterial suspension, one can estimate and compare the number of bacteria present.

The instrument used to measure turbidity is a **spectrophotometer**. It consists of a light source, a filter which allows only a single wavelength of light to pass through, the sample tube containing the bacterial suspension, and a photocell that compares the amount of light coming through the tube with the total light entering the tube.

The ability of the culture to block the light can be expressed as either percent of light transmitted through the tube or the amount of light absorbed in the tube. The **percent of light transmitted** is inversely proportional to the bacterial concentration. (The greater the percent transmittance, the lower the number of bacteria.) The **absorbance** (or optical density) is directly proportional to the cell concentration. (The greater the absorbance, the greater the number of bacteria.)

Turbidimetric measurement is often correlated with some other method of cell count, such as the direct microscopic method or the plate count. In this way, turbidity can be used as an indirect measurement of the cell count (A certain turbidity will correspond to a certain density of bacteria.)

MATERIALS

6 tubes each containing 9.0 ml sterile saline, 3 plates of Trypticase Soy agar, 2 sterile 1.0 ml pipettes, pipette filler, turntable, bent glass rod, dish of alcohol

ORGANISM

Trypticase Soy broth culture of *Escherichia coli*

PROCEDURE

A. **Plate Count** (to be done in pairs)

1. Take 6 dilution tubes, each containing 9.0 ml of sterile saline. Aseptically dilute **1.0 ml** of a sample of *E. coli* **as shown in Fig. 9B** and described below.
    a. Remove a sterile 1.0 ml pipette from the bag. **Do not touch the portion of the pipette that will go into the tubes and do not lay the pipette down**. From the tip of the pipette to the "0" line is **1 ml**; each numbered division (0.1, 0.2, etc.) represents **0.1 ml**; each division between two numbers represents **0.01 ml**.
    b. Insert the cotton-tipped end of the pipette into a **blue** 2 ml pipette filler.
    c. Flame the sample flask, insert the pipette to the bottom of the flask, and withdraw **1.0 ml** (up to the "0" line) of the sample by turning the filler knob **towards** you. Draw the sample up **slowly** so that it isn't accidentally drawn into the filler itself. Reflame and cap the sample.
    d. Flame the first dilution tube and dispense the 1.0 ml of sample into the tube by turning the filler knob **away** from you. Draw the liquid up and down in the pipette several times to rinse the pipette and help mix. Reflame and cap the tube.
    e. . **Mix the tube thoroughly** by holding the tube in one hand and vigorously tapping the bottom with the other hand. This is to assure an even distribution of the bacteria throughout the liquid.
    f. Using the same procedure, aseptically withdraw 1.0 ml from the first dilution tube and dispense into the second dilution tube. Continue doing this from tube to tube as shown in **Fig. 9B** until the dilution is completed. Discard the pipette in the disinfectant container on the benchtop.

    These pipetting and mixing techniques will be demonstrated by your instructor.

2. Using a new 1.0 ml pipette, aseptically transfer **0.1 ml from each of the last three dilution tubes** onto the surface of the corresponding plates of trypticase soy agar as shown in Figure 4B. **Note** that since only 0.1 ml of the bacterial dilution (rather than the desired 1.0 ml) is placed on the plate, the bacterial dilution **on the plate** is 1/10 the dilution of the tube from which it came.
3. Using a turntable and sterile bent glass rod, immediately spread the solution over the surface of the plates as follows:
    a. Place the plate containing the 0.1 ml of dilution on a turntable.
    b. Sterilize the glass rod by dipping the bent portion in a dish of alcohol and igniting the alcohol with the flame from your burner. Let the flame burn out.
    c. Place the bent portion of the glass rod on the agar surface and spin the turntable for about 30 seconds to

distribute the 0.1 ml of dilution evenly over the entire agar surface.
   d. Replace the lid and resterilize the glass rod with alcohol and flaming.
   e. Repeat for each plate.
   f. Discard the pipette in the disinfectant container.
4. Incubate the 3 agar plates upside down **at 37C** until the next lab period. Place the used dilution tubes in the disposal baskets in the hood.

B. **Direct Microscopic Method** (demonstration)
1. Pipette 1.0 ml of the sample of *E. coli* into a tube containing 1.0 ml of the dye methylene blue. This gives a 1/2 dilution of the sample.
2. Using a Pasteur pipette, fill the chamber of a Petroff-Hausser counting chamber with this 1/2 dilution.
3. Place a coverslip over the chamber and focus on the squares using 400X (40X objective).
4. Count the number of bacteria in 5 large double-lined squares. For those organisms on the lines, count those on the left and upper lines but not those on the right and lower lines. Divide this total number by 5 to find the average number of bacteria per large square.
5. Calculate the number of bacteria per cc as follows:

   Number of bacteria per cc =

   The average numbers of bacteria per large square X

   The dilution factor of the large square (20,000,000) X

   The dilution factor of any dilutions made prior to placing the sample in the counting chamber (2 in this case)

C. **Turbidity**

Your instructor will set up a spectrophotometer demonstration illustrating that as the number of bacteria in a broth culture increases, the absorbance increases (or the percent light transmitted decreases).

RESULTS

A. **Plate Count**
1. Choose a plate that appears to have between 30 and 300 colonies.
   - Sample 1/100,000 dilution plate.
   - Sample 1/1,000,000 dilution plate.
   - Sample 1/10,000,000 dilution plate.

   Count the exact number of colonies on that plate using the colony counter (as demonstrated by your instructor).

- Calculate the number of CFUs per ml of original sample as follows:

Number of CFUs per ml of sample = Number of colonies (30-300 plate) X The dilution factor of the plate counted

_____ = number of colonies

_____ = dilution **factor** of plate counted

_____ = number of CFUs per ml

- Record your results on the blackboard.

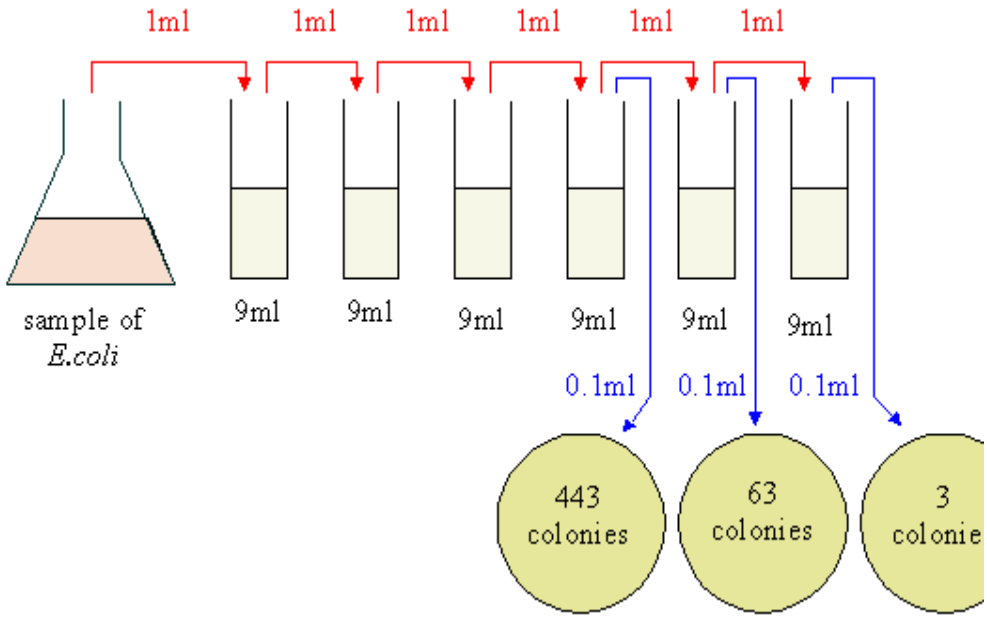
Direct Microscopic Method

Observe the demonstration of the Petroff-Hausser counting chamber.

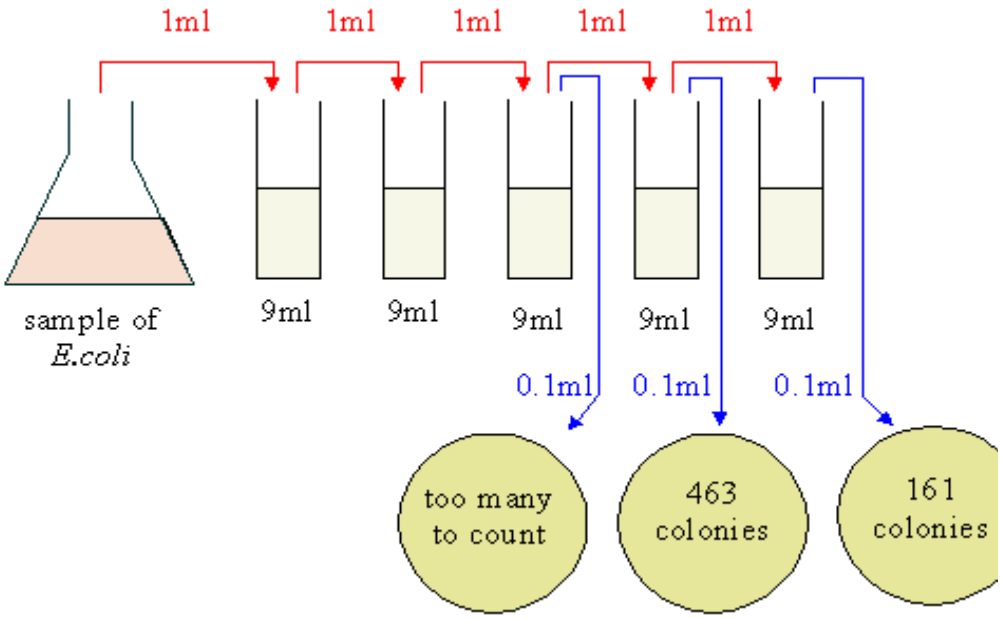
Turbidity

Observe your instructor's demonstration of the spectrophotometer.

Dilution Questions Practice



Calculate the number of bacteria in the original sample?



Calculate the number of bacteria in the original sample?

Direct Microscopic Count: Practice Problem #1

One ml of *E.coli* is mixed with 3ml of dye. A drop of this dilution is placed on a Petroff-Hausser counting chamber. Five large, double-lined squares, each containing a volume of 1/20,000,000cc, are counted giving the following results: 46, 50, 54, 58, and 52 bacteria. How many bacteria are there per cc in the original sample?

First find the average number of bacteria per square by adding the 5 numbers and dividing by 5.
46+50+54+58+52 = 260.
260 divided by 5 = 52. There is an average of 52 bacteria per square.
Multiply 52 times the dilution factor of the square which is always 20,000,000. (The square holds 1/20,000,000 of a cc. The dilution factor or inverse of 1/20,000,000 is 20,000,000/1.)
52 X 20,000,000 = 1,040,000,000

Now multiply that number by the dilution factor of the dilution made when the bacteria was mixed with dye.

One ml of bacteria was mixed with 3ml of dye. This is a 1/4 dilution. The dilution factor or inverse of 1/4 is 4/1.

1,040,000,000 X 4 = 4,160,000,000. There are 4,160,000,000 *E. coli* per cc in the original sample. (In scientific notation, the answer would be 4.16×10^9

Direct Microscopic Count: Practice Problem #2

One ml of *E. coli* is mixed with 1ml of dye. A drop of this dilution is placed on a Petroff-Hausser counting chamber. Five large, double-lined squares, each containing a volume of 1/20,000,000cc, are counted giving the following results: 61, 74, 78, 63 and 64 bacteria. How many bacteria are there per cc in the original sample?

First find the average number of bacteria per square by adding the 5 numbers and dividing by 5.

61+74+78+63+64 = 340

340 divided by 5 = 68. There is an average of 68 bacteria per square.

Multiply 68 times the dilution factor of the square which is always 20,000,000. (The square holds 1/20,000,000 of a cc. The dilution factor or inverse of 1/20,000,000 is 20,000,000/1.)

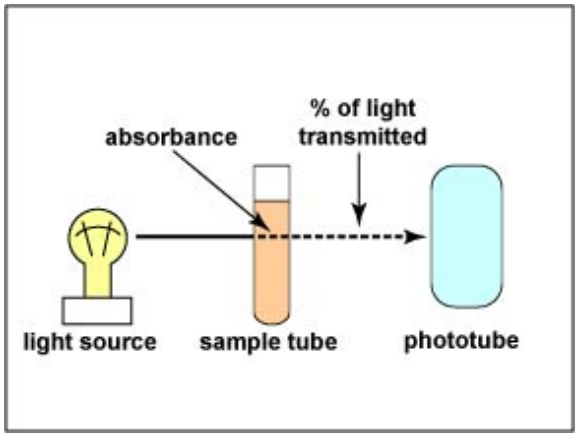
68 X 20,000,000 = 1,360,000,000

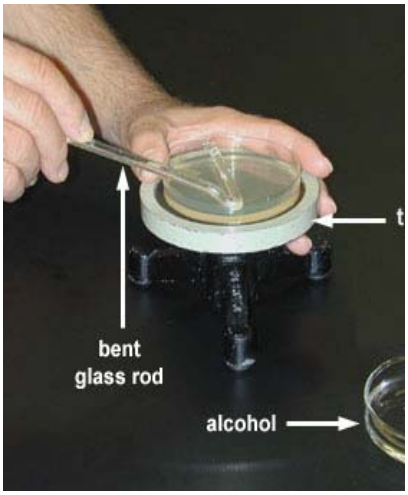
Now multiply that number by the dilution factor of the dilution made when the bacteria was mixed with dye.

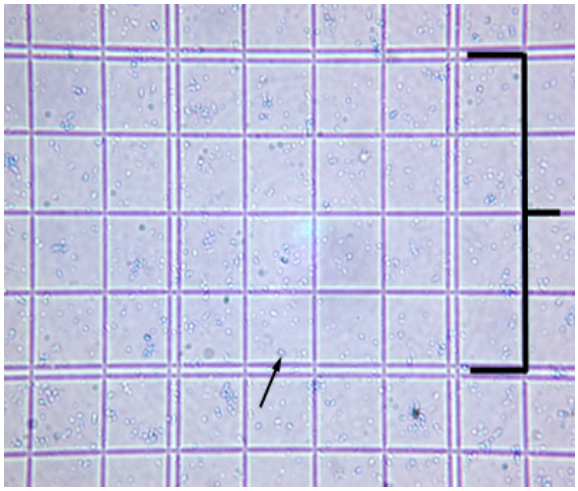
One ml of bacteria was mixed with 1ml of dye. This is a 1/2 dilution. The dilution factor or inverse of 1/2 is 2/1.

1,360,000,000 X 2 = . There are 2,720,000,000 *E. coli* per cc in the original sample. (In scientific notation, the answer would be 2.72×10^{9}






bent
glass rod
alcohol



KOH SMEAR

Aim of the test
Treatment of KOH allows rapid observation of fungal elements because it digests protein debris and clears keratinized tissue so fungi present in specimen can be seen more readily. Because of several variable factors, a KOH preparation may not reveal fungi even when they are present, the collection of the specimen by the physician, the selection of the portion of the specimen to be examined by the laboratory, and the size and number of the organism are extremely important.

The test is useful as a rapid screening a specimen for fungal elements (hyphae and spores)

Types of specimen
Superficial Mycoses.

Skin - scrapings collected with either a scalpel or the edge of a glass slide from the outer area of the lesion. Sent to the laboratory in folded black paper or any sterile container such as a sputum pot.

Hair - specimens scraped from the scalp with a scalpel or hairs plucked with forceps. Infected hairs are easily removed with their stubs.

Nail - any damaged, discolored or brittle parts of the nail are sampled by clipping the full thickness. Where a nail is grossly thickened scrapings can be taken from underneath the nail to add to the clippings.

Mucous membranes - scrapings from the mouth or vagina are better than swabs if the material is to be processed immediately. However swabs are commonly used and as yeast's quickly lose viability on drying a transport swab is preferred.

Subcutaneous mycoses.

Scrapings/crusts - can be used for microscopy and culture but bacterial contamination may be a problem. Cleaning of the site with 70 per cent alcohol prior to taking the sample may help.

Pus - should be aspirated and sent with any associated granules to the laboratory in a sterile container rather than a swab.

Biopsy specimens - transported to the laboratory in a sterile container

Pre specimen processing

Specimen collection

See types of specimens

Who will collect the specimen
Depending on the type of the specimen

Quantity of specimen
See types of specimens

Time relapse before processing the sample
As soon as possible to prevent overgrowth of contaminating bacteria or fungi

Storage
Room temperature

Specimen processing

Equipment

- Scalpel.
- Scissors.
- Nichrome wires in holders both straight and bent mycology type.
- Microscopic slides & cover slips

Reagents Required

- 20% potassium hydroxide solution. (2g potassium hydroxide in 10ml of deionized water.)
- 70% alcohol. (70 ml of industrial methylated spirit plus 30ml of deionized water.)

Method

1. Place a drop of KOH in the centre of a clean microscopic slide.
2. Place a fragment of tissue, purulent materials or scraping in KOH drop.
3. Tease the materials well enough with corner of a coverslip to give a thin preparation or break up the materials with a sterile loop.
4. Mount with coverslip.
5. Allow preparation to digest for approximately 10 minutes or longer depending on the tested materials.
6. Gently warm the slide (do not overheat)
7. Gently press on the slide to help disperse tissue materials.
8. Screen under low power objective.

9. Use high (40 X) power magnification to verify the presence of fungal elements.

Post specimen processing

Interfering factors:

Result reporting:
Report any fungal element (Description of the hyphae is important, e.g. septated or non septated)

Turn around time:
The results is expected 2 hours after specimen reception.

Additional information
Consult medical mycology text book

