

MICROBIOLOGY

MED 106-3

LABORATORY

MANUAL

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MICROBIOLOGY
MED 106-3
Course Outline

Unit #1 - Introduction, classification, history, specimen
(9 hrs) preparation, anatomy of bacteria.

LABS - preparing live mounts and staining techniques,
bacterial shapes and parts.

Unit #2 - Bacterial growth and multiplication, media types,
(15 hrs) enumeration of bacteria, ingredients and preparation,
pure cultures.

LABS - dehydrated media preparation, blood agar plates, tubed
slanted media, adjusting pH, tubed broth, controls
using known cultures.

2 LABS - Pure culture preparation from mixed cultures by: -
1) streak plate
2) transfer to new media
3) dilution
4) phenylethyl alcohol
5) selective antibiotics (S.G*. etc.)
6) bile salts
Description of bacterial colonies
Membrane filter techniques

Unit #3 - Control of microorganisms - physical conditions,
chemicals, gases, sulfonamides and antibiotics.

Unit #4 - Oral seminars by each student in the class on a
disease caused by microorganisms.

ASSIGNMENT

During the latter portion of the course, each student will give a 15 minute oral seminar, using appropriate audiovisual aids, on one of the diseases listed on the following page. • While a brief history and diagnosis of the disease as a medical problem are to be included, the seminars will concentrate on the clinical characteristics of the causative organism(s) and the laboratory techniques for detecting same.

For the oral seminars, and to be ready at least one day previous for duplication, the student will prepare a typed one page (only) summary of his findings for distribution to the others in the class for use during the seminar (sample follows)

In addition, the student will hand in a report covering his seminar of not more than five pages* This is due one week after the seminar. Seminar dates will be posted at a later date.

^ MICROBIOLOGY ASSIGNMENT

Medical Lab II

List of diseases for seminars:

Chicken Pox

Cholera

Diphtheria

Gonorrhoea

Gastroenteritis (food poisoning)

Malaria

Measles

Meningitis

Mumps

Pneumonia

Poliomyelitis

Rabies

Rheumatic Fever

Scarlet Fever

Smallpox

Syphilis

Tetanus

Tuberculosis

Typhoid Fever

Yellow Fever

WHOOPING COUGHHistory

- bacteria discovered in 1906 by Bordet and Gengou
- named Bordetella pertussis
- Group - Hemoglobinophilic Family- Parvobacteriaceae

Morphology

- bacillus - width = 0.2 - 0.3 microns length = 0.5 microns
- single, pairs or clumps - gram negative, nonmotile, nonsporing

Bacteriological Diagnosis

1. Cough-plate method—agar plate is exposed to cough (bacteria in
2. Nasopharyngeal swabs—smear across culture plate (sputum)
3. Blood sample—15-30 thousand lymphocytes per cubic millimeter

Physiological Characteristics

- optimum temperature is 37 C growth range 5 C to 40* C
- aerobic, hemolytic
- killed by drying, exposure to sunlight, treatment with disinfectants

Cultural Characteristics

1. Bordet-Gengou medium—3% solution of agar contains 1% peptone, 1% glycerol, 25% potato extract, plus an equal quantity of blood.
 - sterilised and solidified in test tubes - melted and poured into petri dishes when needed
 - culture plates inoculated with bacterial cells
 - 48-72 hours incubation -colonies appear gray and raised
2. -other media—blood agar—colonies have clear zone around them due to destruction of red blood cells
3. -addition of penicillin to Bordet-Gengou medium—stops growth of other respiratory bacteria but does not affect Bordetella pertussis

Immunity

- natural immunity after an attack of whooping cough
- vaccine—made from Bordetella pertussis organisms which are killed and preserved and possess required antigens
- vaccination—combine pertussis vaccine with diphtheria and tetanus alum toxoids to get vaccine known as DPT
- three doses of 0.5 ml given at 2,3, and 4 months of age
- two boosters at 15 months and 3 years old
- pure pertussis vaccine is given to young children exposed to whooping cough
- a person with whooping cough can be treated with convalescent serum to lessen severity and length of disease

STUDENT EVALUATION

Term Tests:

There will be four term tests, at the end of each unit, and one based on the material presented in the seminars. Each will be worth 50 marks. The student must pass each test.

Lab Work:

The laboratory work will be worth 100 marks based on assignments to be handed in (60 marks) and the student's performance, neatness and cleanliness in the laboratory. (40 marks)

Assignment (seminar):

The assignment covering the laboratory techniques for detecting microorganism - caused diseases will be marked out of 100, based on the oral presentation (50 marks) and written material handed in (50 marks).

The total course marks will be 400. Students will receive grades based on their course average and consistency of performance. Each student must complete all course requirements satisfactorily. The pass mark is 50% for each.

TEXT: Pelczar, M.J., Jr. and R.D. Reid. 1972. Microbiology
New York, McGraw-Hill. 948p.

REFERENCE TEXTS:

Bodily, H.L., and E.L. Updyke. 1970. Diagnostic procedures
for bacterial, mycotic and parasitic infections. 4th ed.
New York, Am. Publ. Health Assoc.

Cruickshank, R., J.P. Duguid and R.H.A. Swain. 1974.
Medical microbiology. Edinburgh, E. & S. Livingstone.

Frobisher, M., R.D. Hinsdill, K.T. Crabtree and C.R. Goodheart
1974. Fundamentals of microbiology, 9th ed. Philadelphia, W.B
Saunders. 850p.

Lennette, E.H., E.H. Spaulding and J.P. Truant. 1974.
Manual of clinical microbiology. 2nd ed. Washington, Amer
Assoc. Microbiol.

Raphael, S.S. 1976. Lynch's Medical laboratory technology,
3rd ed. Toronto, W.B. Saunders.

Wistreich, G. A., and M. D. Lechtman. 1976. Laboratory
exercises in Microbiology. 3rd ed. Beverley Hills,
Glencoe Press. 310 p.

NOTE TO THE STUDENT

LABORATORY REGULATIONS

1. To prevent contamination of street clothes, always wear a laboratory coat or apron during the class period. Remove this protective apparel before leaving the laboratory.
2. It is your responsibility to keep all equipment in good working condition.
3. Never remove equipment, media, or microbial cultures from the laboratory.
4. Place items such as textbooks, coats, and other personal belongings in the area specified by your instructor. Never let them clutter the operating space on the laboratory bench, where they might become contaminated through exposure to microbial cultures.
5. Never eat, drink, or smoke in the laboratory.
6. Wash your hands thoroughly with detergent and water before leaving the laboratory. Carry out this procedure even in the case of a coffee break.

LABORATORY OPERATING PROCEDURES

1. The laboratory class period begins promptly. At the beginning of the period the instructor will give a lecture demonstration. He will give general directions designed to make your work easier and more efficient.
2. Before and after each class period, wipe desk tops with the disinfectant (.1% javex) provided for this purpose. Your instructor will show you how to discard contaminated items.
3. In the event of a laboratory accident, such as the spilling or dropping of a live culture, remain calm. Carry out the following procedure:
 - (a) Report the accident to your instructor- as soon as possible.
 - (b) Place paper towels over the spilled material.
 - (c) Pour disinfectant (1% javex) liberally over the towels.
 - (d) After 15 minutes remove and dispose of the towels in the receptacle designed for disposal of contaminated materials.
4. In the event of a fire or a personal injury such as a cut or burn, report the emergency to your instructor immediately. Know the locations of the nearest fire extinguisher and first aid kit.
5. Leave all laboratory facilities and equipment in good order at the close of each period. Waste paper and contaminated glassware should be placed in receptacles provided. Bring to the attention of the instructor any defective equipment.
6. You are expected to read each laboratory exercise and carry out suggested reading assignments before you come to class. The instructor will designate which parts of each exercise will be undertaken during each class period.
7. As you perform your experiments, record data in ink. Make sketches and labels in pencil. Always perform exercises in the sequence given.
8. Unless your instructor specifies otherwise, each experiment is to be completed within one week. Your lab notebook is a valuable record. Keep it up to date, using procedures outlined by your instructor.

9. The student is to keep the incubator LOCKED at all times outside the regular lab time. Replace the key to its proper location.

MATERIALS TO BE PROVIDED BY THE STUDENT

1. One clean laboratory coat or apron
2. Textbook and laboratory manual
3. One 4H drawing pencil
4. Colored pencils -- red, blue, and green
5. Two packages of matches
6. Slide box (optional)
7. One wax marking pencil or a washable-ink felt pen

OBLIGATIONS OF THE STUDENT

1. The student shall make a neat clear and concise record of the results of each of his experiments.
2. The above to be handed in for each laboratory one week after the laboratory session for marking by the instructor.
3. Laboratory reports to be accumulated in a looseleaf folder after marking for the duration of the semester.
4. Students will be assigned a grade on his neatness, cleanliness and efficiency in the laboratory.

Laboratory #1
SIMPLE MOUNTS

After completing this exercise you should:

1. be able to prepare and examine microscopically, hanging drop and temporary wet mount specimens;
2. be aware of size differences and shapes of bacteria;
3. be able to distinguish motility (vital movement) of bacteria.

Direct examination of live microorganisms can be extremely useful in determining size and shape relationships, motility, and reactions to various chemicals or immune sera. Two methods are in general use for studies of this type: hanging drop and wet mount techniques. Both methods maintain the natural shape of organisms and reduce the distorted effects which can occur when specimens are dried and fixed. Because the majority of microorganisms are not too different in either color or refractive index from the fluid in which they are suspended, a light source diminished in intensity is advisable for viewing purposes.

"A" - TEMPORARY WET MOUNTS (TWM)

Perform a wet mount on each culture provided as follows:

1. Obtain a very small quantity of bacterial culture on your sterile transfer needle.
2. Mix with a drop of distilled water on a cleaned glass slide.
3. Place a clean cover slip over the mixture. Petroleum jelly may be used to reduce evaporation from the sides of the cover slip.
4. Observe under the microscope and record bacterial shape for each of the cultures provided.

Organisms: Bacillus subtilis, Sarcina lutea, Rhodospirillum rubrum

"B" - HANGING-DROP MOUNT

Perform as follows:

1. Obtain a small quantity of a culture of a mobile bacteria (Azotobacter chroococcum)
2. Make a suspension with distilled water on a cleaned slide.
3. Apply petroleum jelly to the edges on one surface of a clean glass coverslip.
4. Transfer a small drop to the centre of the coverslip.
5. Invert a glass slide with the concave portion over the liquid drop and press down slightly. Lift the preparation and turn right side up so the liquid remains suspended from the coverslip.
6. Observe. Record bacterial shape and behaviour.
7. Repeat using a culture of Euglena, a representative motile algae.

Organisms: A. chroococcum, Euglena sp.

Reference: Pages 72 - 73, 79 - 85, textbook, colour atlas

WASH ALL GLASSWARE etc. and sterilize in drying oven.

Laboratory #2
SIMPLE STAINS

After completing this exercise you should;

1. be able to carry out the Gram stain procedure correctly;
2. be able to differentiate between Gram - positive and Gram - negative reactions;
3. recognize the value of the Gram stain procedure;
4. be proficient in carrying out the acid-fast procedure;
5. be able to distinguish between acid-fast and non-acid-fast reactions;
6. be able to carry out a negative staining technique;
7. be able to identify bacterial capsules.

"A" - GRAM STAIN

The Gram differentiation is based upon the color reactions exhibited by bacteria when they are treated with crystal violet dye followed by an iodine-potassium-iodide solution. Certain organisms lose the violet color rapidly when ethyl alcohol is applied, while others lose their color more slowly. After the decolorization step, a counterstain -- usually safranin -- is used. Bacteria resistant to decolorization will retain a blue or purple color and will not take the counterstain. Such organisms are referred to as Gram-positive. Those microorganisms unable to retain the crystal violet stain will take the counterstain and consequently exhibit a pink or red color. The term Gram-negative is used to describe these organisms. It is important to note that this differential is based on the rate at which the dye leaves the cell, and is not due to any absolute characteristic of bacteria. For this reason, the procedure must be performed with great care.

Since the original work of Gram, many investigators have attempted to determine the mechanism of the Gram stain reaction. Most theorists have been able either to explain much of the data that has accumulated through the years of

investigation, or offer an adequate mechanism for the Gram stain reaction. Recently, a theory has been proposed based upon permeability differences to the decolorizing solvent. In 1964, Bartholomew and Salton each reported the current data which strongly favor the permeability concept.

The general procedure is quite simple – perhaps deceptively so. It involves the application of the primary dye, then the iodine solution, and finally alcohol. It is customary to wash off excess reagent after each step. But excessive washing can remove the dye or dye-iodine complexes within the cells and consequently greatly affect the overall staining reaction. As the decolorization step is probably the most critical, precautions should be taken to guard against misleading results. The most commonly employed control is the use of a mixed smear of a Gram-positive coccus and a slender Gram-negative rod on a portion of the same slide used for the unknown culture. The final step in the procedure, the application of the counterstain, must be performed very carefully. Given too much exposure time, the counterstain will replace the primary dye in Gram-positive organisms.

Perform the gram stain as follows:

1. Place a drop of the two suspensions to be tested on separate cleaned slides.
2. Smear the suspensions with the edge of a coverslip to form a thin film over the slide.
3. Cover the film with crystal violet (0.5% sol'n. in distilled water) and stain for h minute.
4. Pour off the excess stain, wash with distilled water, then wash off with Lugol's iodine and leave to act for h minute.
5. Decolorize with ethanol until no more stain comes out of the preparation.
6. Wash with distilled water.
7. Apply safranin (0.5% aqueous sol'n.) as a counterstain.
8. Pour off counterstain, wash in distilled water and dry by passing slide back and forth through low bunsen burner flame.
9. Record colour of bacteria from each suspension.

Organisms: E. coli, B. subtilis

"B" - ZIEHL - NEELSEN STAIN

The acid-fast staining procedure was developed by Paul Ehrlich in 1882. He found that tubercle bacilli retained a dye reagent composed of crystal violet and aniline in water even after a wash treatment with an acidified ethanol solution. Subsequent to this initial technique, changes in methodology resulted in the formulation of the Ziehl-Neelsen procedure, which will be used in the exercise. One of the advantages of the Ziehl-Neelsen procedure involves use of reagents with better preserving quality than those used by Ehrlich. Basic fuchsin in aqueous 5% phenol is the primary staining reagent. Methylene blue is the counter-stain, replacing Bismarck brown, as used by Ehrlich.

As in the case of the Gram stain reaction, the integrity of the cell wall (or more specifically structurally intact cells) is required for the demonstration of the acid-fast nature of microorganisms. Because of this property, the mechanism of the acid-fast reaction may again be due to permeability factors.

In this staining procedure, the primary dye, acid fuchsin, is formulated with phenol to allow permeation through the waxlike cell walls of the mycobacteria. The slide is usually heated in order to facilitate permeation. Ethyl alcohol is employed as the decolorizer. However, the reagent is prepared with hydrochloric acid in order to aid in the decolorization of non-acid-fast cells.

Proceed as follows:

1. Prepare film smear as previously.
2. Flood film with strong carbolfuchsin and heat until steam rises. Allow to stain for 5 minutes keeping the stain hot and adding more if needed.
3. Wash well with distilled water.
4. Decolourize with 20% sulfuric acid. (3% hydrochloric acid in 95% ethanol is used for pathogenic forms).
5. Wash in distilled water.
6. Counterstain with Loeffler's methylene blue for 10-15 seconds.
7. Wash and blot dry, using fresh paper for each slide to avoid transferring cells from one film to another.
8. Repeat using alternate decolorizer.

9. Record carefully colour of cells from each culture.

Organisms: M. smegmatis, M. tuberculosis (from lab and pathogenic)

"C" - NEGATIVE STAINING

The bacterial capsule, although external to the cell, is synthesized partially in the cytoplasm. Probably the synthesis continues in the area of the cell wall before the capsule is deposited. Indistinct in many bacteria, the capsule is well developed in some. These include Streptococcus (Diplococcus) pneumoniae. Clostridium perfringens and Klebsiella pneumoniae. Capsules appear to increase the virulence of organisms by protecting them from the defense mechanisms of their host. In addition, they impart specific immunologic properties to some microorganisms. Pneumococci, for example, are differentiated on the basis of the antigenic characteristics of their capsules, which are polysaccharide in nature. Demonstration of the presence of pneumococcus capsules is accomplished by means of a serologic test known as the Quellung reaction, first described by Neufeld in 1902. The reaction is characterized by the occurrence of capsular swelling when pneumococci of a specific type are mixed with homologous antisera. In our exercise, the presence of capsules will be demonstrated by a simpler, nonspecific method called the negative stain.

The negative stain technique incorporates material such as India ink, which is composed of particles too large to enter a cell. A small amount of culture is mixed with India ink, and the resulting smear is stained with a dye, safranin, which penetrates the bacterial cell. Upon examination of the preparation, the capsule will appear as a clear zone surrounding the cell wall. One cannot state with certainty that all of the clear zones observed are capsules, because shrinkage of cells or withdrawal and cracking of the India ink may cause anomalous results. In general, however, when a treated smear contains many uniformly shaped clear zones, it is probable that they are actual capsules and not artifacts.

Stain bacterial cells as follows:

1. Prepare 10% aqueous sol'n. of nigrosine containing 0.5% of formalin as a preservative. Filter just before use.
2. Mix a drop of liquid suspension of Klebsiella sp. on a cleaned slide with a drop of nigrosine solution.

3. Spread the mixture over a slide in a thin film.
4. Allow to dry in air and fix with gentle heat.
5. Describe the appearance of the cells.

Organisms: Klebsiella sp. (from lab & pathogenic)

Cryptococcus sp. - reference slide showing capsules.

Reference: p. 72 - 76 textbook, colour atlas

WASH ALL GLASSWARE etc. and sterilize in the drying oven.

Laboratory #3
MEDIA PREPARATION

After completing the exercise you should:

1. be able to prepare blood agar base, sterilize it and add blood to produce blood agar;
2. be able to prepare sterilized nutrient agar;
3. be able to prepare and autoclave MacConkey broth;
4. be able to adjust accurately the pH of a broth medium.

The preparation of media for the growth of bacterial colonies has been greatly simplified through the almost universal use of dehydrated prepared media available from biological supply houses. The measurement of exact quantities of numerous ingredients is no longer necessary and the extensive laboratories of these suppliers now ensure a product satisfactory for the needs of all labs.

Nevertheless, instructions furnished with the dehydrated product must be followed closely. Subsequently proper dispensation and sterilization is an absolute necessity for success. Proper storage of prepared media must be followed for later use. In addition, most laboratories feel the need to adjust the final pH of the media to that stated on the label for ensuring bacterial growth when expected.

In this laboratory you will be preparing the three major types of growth media, the standard agar plate with blood added, tubed slant agar and tubed broth. There are hundreds of variations of media used for specific purposes such as the isolation of specific groups of bacteria. You will prepare and use some of these later. This lab exercise includes the proper sterilization of the glassware as well as the prepared medium. The adjustment is also performed.

"A" - BLOOD AGAR PLATES

1. Prepare blood agar base as directed on the label of the dehydrated medium,
2. Autoclave at 15 p.s.i. (121°C).
3. Cool the base to 50°C and add 7% of defibrinated horse blood. Mix with gentle rotation and pour into petri dishes (20 ml for 15 cm dish).

4. Label and store in cold room (refrigerator) until needed.

"B" - TUBED SLANTED MEDIA

1. Prepare nutrient agar as directed on the label.
2. Pipette 10 ml amounts into each of 6 test tubes and plug with cotton batting
3. Sterilize in autoclave at 121°C for 15 minutes.
4. Label and store in cold room until needed.

"C" - TUBED BROTH MEDIA

1. Prepare MacConkey broth as directed on the label.
2. Dispense in test tubes (3 each) containing inverted vials in 10 ml quantities and plug with cotton.
3. Sterilize by autoclaving at 121°C for 15 minutes.-
4. Label and store in cold room until needed.
5. Save remainder of broth for next experiment.

LABORATORY #4
MEDIA pH ADJUSTMENT

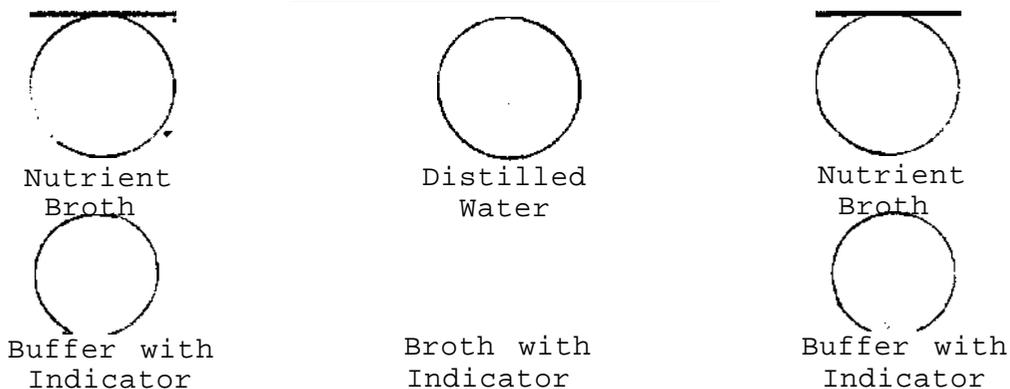
1. A set of reference buffer tubes containing phenol red at pH's from 6.8 to 8.4 have been prepared for your use.
2. Using the above series of pH indicators, determine the pH of a 100 ml sample of your MacConkey broth solution. Add 20 drops of phenol red and place the tubes in the base as shown in the diagram below and compare the colours looking horizontally through the tubes.
3. Using N/10 solution (prepared from the N/1 sol'n.) of caustic soda (NaOH), titrate a 50 ml sample of broth to adjust the pH to 7.4 using the colour comparator*
4. Calculate the required amount of N/1 NaOH required to bring the remaining 150 ml of broth to the final pH. Accurately add this amount of normal NaOH with a pipette.
5. Repeat using the pH meter. Adjust the meter to give correct readings using the reference buffers then determine the pH of your media.
6. WASH ALL glassware and STERILIZE in oven.

AUTOCLAVE STEAM PRESSURES AND CORRESPONDING TEMPERATURES

STEAM PRESSURE	TEMPERATURE	STEAM PRESSURE	TEMPERATURE	STEAM PRESSURE	TEMPERATURE
UU./OQ. In.	•F.	lb ² ./sq. In.		lb ² ./sq. In.	
0	100.0	212.0			
1	101.9	215.4	11	116.4	241.5
2	103.8	218.5	12	117.8	243.7
3	103.3	221.5	13	118.8	245.8
4	106.8	224.4	14	119.9	247.6
8	108.4	227.1	15	121.0	248.8
6	109.8	229.6	16	122.0	251.6
y	111.3	232.3	17	123.0	253.4
e	112.0	234.7	18	124.1	255.4
10	113.0	237.0	19	125.0	257.0
	116.2	239.4	20	126.0	258.8
					30
					134.5

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Colour Comparator Setup



Reference: p. 107 - 121 of your textbook.

STERILE INOCULATION METHODS

After completing this exercise you should:

1. be able to perform basic bacteriological transfer procedures;
2. recognize the characteristics of bacterial broth agar slant and plate cultures.

The proper inoculation of the three major types of media are of utmost importance in Microbiology. Unwanted organisms must be omitted and the often dangerous specimens being handled must be kept or transferred in the appropriate containers.

General special precautions are used in isolation and culturing procedures. These include flaming the lips of tubes and heating entire inoculating loop or needle to redness.

The success of your work will be clearly reflected in the results you obtain after incubation.

Bacterial species can sometimes be identified on the basis of how they appear on or in different media. The pigmentation, size, and shape of bacterial colonies as they grow on and in agar plates can provide identifying signs. Certain dangers may present themselves at the time of culture examinations. The major problems are contamination and species variation. These must be avoided if good results are to be obtained.

"A" - AGAR PLATE INOCULATION

1. Flame the entire wire length to incandescence by holding vertically in the Bunsen flame and quickly move the needle holder down through the flame so that areas adjacent to the wire are flamed slightly. Allow to cool. Always work near the burner.
2. Using little finger on right hand, remove screw cap (or cotton plug) from the tube containing S. pyogenes.

Take a thin loopful of the specimen, replace screw cap or cotton plug onto tube and place aside.

Raise cover of blood agar plate and streak the surface area back and forth in one direction. Flame loop and repeat streaks in other direction. Close plate.

Flame loop to sterilize.

Label and incubate at 42°C. Plates are inverted during incubation, but not tubes.

Repeat above using *S. coli* and a third time with cotton swab from your own throat (obtained with Q-tip).

" - TUBED SLANT INOCULATION - SURFACE

Flame loop and obtain a quantity* of *S. pyogenes* as above.

Remove cap (or cotton plug) from tube to be inoculated. Insert the loop to the base of the slant and draw it along the agar surface from bottom to top. Withdraw loop and avoid touching the sides.

Replace cap.

Flame loop to sterilize.

Label and incubate as previously.

Repeat with *E. coli* and throat swab.

" - TUBED SLANT INOCULATION - STAB

Repeat B above using the straight transfer needle and stabbing the medium to *h* of its depth with a straight motion.

"D" - TUBED BROTH INOCULATION

1. Flame loop and obtain a quantity of S., pyogenes as above.
2. Remove cap (or cotton plug) from tube to be inoculated. Insert the loop down into the medium about *k* of the depth.
3. Replace cap and flame loop to sterilize.
4. Label and incubate as previously.
5. Repeat the £. coli and throat swab by rubbing loop on swab.

For "A" - "D" above record carefully the abundance and appearance of the bacterial colonies after 24 and 48 hour periods (see pages 147-150 of your text).

Organisms: Streptococcus pyogenes (pathogenic from lab, E. coli, throat swab)

Reference: colour atlas

Laboratory #5
COLONY DESCRIPTION, QUANTITATIVE DETERMINATIONS

After completing this exercise you should be able to:

1. to recognize the advantages and limitations of bacterial culture characteristics,
2. perform the plate-count technique to determine accurately the numbers of bacterial cells in a suspension.
3. perform the standard membrane filtration technique for the determination of CQliform bacteria in water samples.

The description of bacterial colonies was discussed in the previous exercise but here you will gain more experience in their description.

The plate count technique is a means whereby the numbers of viable bacterial cells found in waters, milk, foods, etc. are determined. The assumptions are that all viable cells will grow on the medium and that viable cells are not aggregated together thus forming only one instead of more colonies. Dilution is performed so a count of a reasonable (30-300) number of colonies is made.

A useful variation of the plate count technique is the membrane filter technique for water samples containing *very* few viable bacterial cells. Here-, 100 ml of water is filtered through a membrane which is in turn placed on growth inducing medium. Again, it is assumed viable cells will grow. The technique is used to determine whether water is potable or suitable for swimming. The presence of fecal coliforms suggests the possible presence of pathogenic forms.

"A" - DESCRIPTION OF BACTERIAL COLONIES

1. Observe and carefully record, according to accepted standards (pp. 147 to 150 of your text), the characteristics of the bacterial colonies from your previous experiments and others at your disposal.

Organisms: various as available.

"B" - PLATE-COUNT TECHNIQUE

1. Obtain, using sterile pipette, 1 ml of bacterial inoculum from your tubed broth suspension.
2. Transfer above to a 99 ml blank of buffered dilution water. Swirl to mix thoroughly.
3. Pipette 1 ml of this suspension (with a new sterile pipette) into a second blank and 1 ml into a sterile petri dish.
4. Repeat using 1 ml of the second suspension and add to a third blank and a second petri dish.
5. Repeat once more.
6. After addition of the inoculum to each plate, add 15-20 ml nutrient agar. Gently rotate the plate to distribute inoculum throughout the plate.
7. Allow to solidify and incubate (inverted) at 42°C for 24 hours or more.
8. Select a plate with 30 to 300 colonies and count. Draw, using wax pencil, lines to separate areas of heavy colony growth.
9. Determine the number of viable cells in the original broth suspension based on the level of dilution used.

Organisms: E. coli

¹¹C" - MEMBRANE FILTRATION TECHNIQUE

1. Place a sterile nutrient pad in a 47 mm petri dish and add approximately 2 ml of Endo agar medium.
2. Set up the sterile membrane filter apparatus as shown on page 750 of your text, rinse thoroughly with distilled water.
3. Attach to vacuum suction apparatus.
4. Place millipore filter in place with sterile forceps.
5. Filter the 100 ml of liquid to be tested from one of the water sample bottles

6. Remove the filter with sterile forceps and place over pad in petri dish
7. Incubate at 35°C for 20 hours (inverted).
8. Count the number of coliform colonies and record for each sample.

Organisms: various - as obtained in water samples.

Reference: pages 132-134, 147-149, 748-751 textbook; colour atlas.

Laboratory #6

PURE CULTURE PREPARATION

After completing this exercise you should be able:

1. to isolate individual colonies from mixed cultures by the streak plate separation;
2. to transfer a culture from a mixed plate to form a pure culture;
3. to use phenylethyl alcohol agar to separate bacterial species;
4. to demonstrate the inhibiting action of different antibiotics on bacterial growth;
5. to separate bacterial species using bile salts.

The streak plate technique is an important means of isolating mixed colonies for further study and culture. It has become indispensable in microbiology. The wire loop containing a mixed culture is simply streaked across the agar surface to spread out the cells resulting in separate colonies.

After the colonies have been isolated, further culturing and testing is usually required for positive identification; thus the necessity of transferring single colonies to form a pure culture without contamination.

You have already encountered a basic enriched medium (blood agar). In this exercise you will use two selective media which permit the growth of one group of organisms while inhibiting the growth of other unwanted forms. Phenylethyl alcohol agar is particularly useful in inhibiting the growth of gram-negative organisms which have contaminated specimens of gram-positive species. Streptococci and other micrococci are isolated by this means.

MacConkey agar inhibits the growth of gram-positive organisms by means of a bile salts mixture. This permits the growth of gram-negative coliform organisms such as *E. coli*. This agar is used in the direct plating of water samples and sensitivity testing to antibiotics of enteric organisms.

Antibiotics are extremely useful compounds, produced from natural and artificial sources, which inhibit the growth of microorganisms. The sensitivity of various microorganisms to the numerous antibiotics varies greatly and careful selection must be made to prevent the growth of a specific specimen.

Commercially-produced filter paper discs containing an antibiotic at a specified concentration are placed on the surface of the agar which has been heavily inoculated with the microorganism to be tested. The preparation is incubated during which the antibiotic diffuses outward into the agar. At some particular distance from each disk, the minimal inhibitory concentration (MIC) is reached. Here no growth of the microorganism can occur. This is characterized by a clear zone around the disk. The diameters of such zones are measured with a ruler and compared. Remember, however, there are other considerations in selecting a specific antibiotic to control a specific bacterium.

"A" - STREAK PLATE SEPARATION

1. Flame the wire loop as described previously and allow to cool.
2. Take up a loopful of specimen from the broth suspension provided. Replace cap,
3. Raise cover of culture plate and streak about h of the surface area by passing loop back and forth in a zig-zag motion.
4. Flame loop and allow to cool.
5. Rotate plate h turn and streak again overlapping the originally streaked area.
6. Flame loop again and allow to cool.
7. Repeat 5 above. Close plate. Flame loop to sterilize.
8. Incubate at 42°C and observe after 24 hours and 48 hours.

"B" - TRANSFERRING A PURE CULTURE

1. Examine plate - select colony to be transferred.
2. Flame transfer needle - allow to cool.
3. Touch needle to selected colony.
4. Inoculate slant or another plate as previously.

5. Flame needle.

6. Incubate at 42°C for 24 hours or more. Observe.

Organisms: throat swab

"C" - PHENYLETHYL ALCOHOL SEPARATION

1. Prepare phenylethyl alcohol agar as directed on label.
2. Dispense into petri dishes and sterilize at 118 - 121°C for 15 minutes.
3. Cool and store until needed.
4. Obtain a loopful of Escherichia coli and streak half of each of phenylethyl alcohol and nutrient agar plate.
5. Sterilize transfer loop and repeat (4) above using Sarcina lutea on the other half of each plate.
6. Incubate at 38°C for 48 hours and carefully observe and record the growth on each plate.

Organisms: E. coli, S. lutea

"D" - SEPARATION WITH BILE SALTS

1. Prepare MacConkey agar as directed on the label.
2. Dispense into plates (20 ml each) and autoclave at 121°C for 15 minutes.
3. Cool and store until needed.
4. Streak separately labelled MacConkey plates with E. coli and Bacillus subtilis
Repeat on nutrient agar plates.
5. Incubate at 42°C for 24 hours.
6. Observe and record results. Repeat after 48 hours.

Organisms: E. coli, E. subtilis

Reference: 110-112, 140, 465-481 textbook, colour atlas

"E" - SEPARATION WITH ANTIBIOTICS

1. Nutrient agar plates have been prepared for your use in this lab. Each has been heavily inoculated with a different organism.
2. Using a simple numbering system acceptable to all in the class, code the eight types of antibiotic bottles and mark the plates accordingly.
3. Place the eight different disks (4 per plate) on the surface of the coded inoculated agar plates in a circle.
4. Incubate at 42°C for 24 hours.
5. Observe and record growth characteristics, measure the diameters of all MIC zones produced. Repeat after 48 hours.

Organisms: *B. subtilis*, *S. lutea*, *R. rubrum*, *A. chroococcum*, or other growing in nutrient agar.