KOMAR UNIVERSITY OF SCIENCE AND TECHNOLOGY
COLLEGE OF SCIENCE - DEPARTMANT OF MEDICAL LABORATORY SCIENCE

MEDICAL BACTERIOLOGY LABORATORY MANUAL

MLS3430C

Prepared by:

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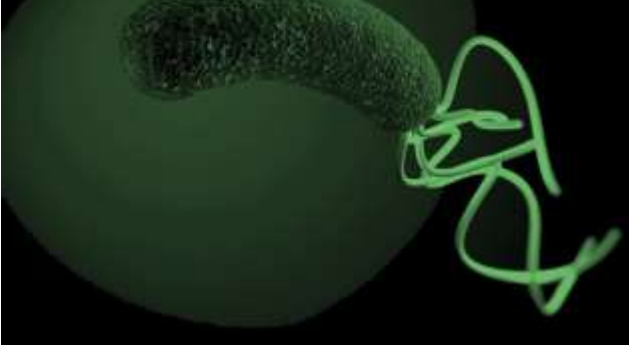

Fall 2016

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Preface

This lab manual is prepared to direct MLS students to acquire an in-depth knowledge of diagnostic strategies in Medical Bacteriology. This field is the culmination of several years of study. It is an exciting time for students, and offers unique experiences in the medical laboratory setting. Students will achieve from this experience benefits comparable to the effort they put forth.

The lab manual preparation process was done in light of Harley & Prescott: **Laboratory Exercises in Microbiology**, Fifth Edition, 2002, Morello *et al.*, **Laboratory Manual and Workbook in Microbiology**, Seventh Edition, 2003, Kayser *et al.*, **Medical Microbiology**, 2005 and Gillespie−Bamford **Medical Microbiology and Infection at a Glance**, Fourth Edition, 2012 books.

The first three labs of the manual are devoted to essential microbiology where aseptic techniques, staining and culture media preparations are covered. Then metabolic and enzymatic principles of bacterial diagnosis are explained followed by grouping of medically important bacteria with regard to their sources of sample collection.

Procedures listed from Week 2 to Week 14 are referring to four main staining techniques and different biochemical and metabolic activity experiments 1 to17.

The last lab session is to familiarize students with lab automation along with antimicrobial susceptibility tests.

Dr Sirwan M Muhammed & Alan Ahmed

Spring 2016

Week 1: General Guidelines about Medical Bacteriology Lab

Lab Safety

1. All materials and clothes other than those needed for the laboratory are to be kept away from the work area.
2. A lab coat or other protective clothing must be worn during lab. The lab clothing is not to be worn outside of the laboratory.
3. Clean the lab table before and after lab with the disinfectant solution provided.
4. Wash hands before leaving lab.
5. Any item contaminated with bacteria or body fluids must be disposed of properly. Disposable items are to be placed in the BIOHAZARD container. Reusable items are to be placed in the designated area for autoclaving prior to cleaning. Sharps are to be disposed of in the appropriate container.
6. Reusable items should have all tape and marks removed by the student before being autoclaved.
7. Because organisms used in this class are potentially pathogenic, aseptic technique must be observed at all times. NO eating, drinking, application of cosmetics or smoking is allowed. Mouth pipetting is not allowed.
8. Cuts and scratches must be covered with Band-Aids. Disposable gloves will be provided on request.
9. Long hair should be tied back while in lab.
10. All accidents, cuts, and any damaged glassware or equipment should be reported to the lab instructor immediately.
11. Sterilization techniques will involve the use of Bacticinerators that are fire and burn hazards.
12. Bacticinerators reach an internal temperature of 850^{o}C or 1500^{o}F. Keep all combustibles (capable of catching fire) away from the Bacticinerators. Do not leave inoculating loops or needles propped in the Bacticinerator.
13. Microscopes and other instruments are to be cared for as directed by the instructor.
14. It is the responsibility of the student to know the location and use of all safety equipment in the lab (eyewash, fire extinguisher, etc).
15. Cultures may not be removed from the lab. Visitors are not allowed in the lab.
16. Doors and windows are to be kept closed at all times.
17. For the best lab experience, read labs before coming to class. Make notes as necessary. Wait for a laboratory Background by the instructor before starting work.

Every student must sign and return a copy of these Laboratory Safety Rules to the laboratory instructor at the end of class.

I have read and understand the above rules and agree to follow them.

Signed……Date……. Name ……

Professionalism

The student is expected to conduct himself/herself in a professional manner at all times. The ability to **communicate** in a **respectful** manner under all circumstances is an expectation of a professional. The student must remember that all patient information is **privileged** and as such strict **confidentially** must be maintained. The student should realize that in some ways his/her education is just beginning, and to remain current during the work years ahead, it is important to participate in **continuing education activities** on a routine basis.

A Professional Medical Laboratory Science student will:

1. Demonstrate the ability to ask pertinent questions or for assistance if needed.
2. Demonstrate the ability to work independently within student guidelines.
3. Communicate courteously, effectively and professionally with instructors, laboratory staff, other healthcare personnel, patients, and visitors.
4. Demonstrate interest and enthusiasm for the medical laboratory science profession.
5. Accept evaluation of performance as constructive when offered by instructor laboratory personnel, and follow through with suggestions made.
6. Adhere to laboratory safety regulations in each clinical area.
7. Maintain a clean, organized work area.
8. Utilize reagents and supplies judiciously.
9. Replenish supplies required in the laboratory work area.
10. Demonstrate self-confidence in the operation of equipment and in the performance of laboratory procedures.
11. Report patient laboratory results only to authorized personnel.
12. Maintain the confidentiality of all privileged information.
13. Cooperate with other laboratory personnel to creat**e** a pleasant and efficient work environment.
14. Demonstrate the ability to concentrate on the laboratory test procedure being performed and the need to avoid distractions.
15. Demonstrate organizational skills through ability to coordinate the quantity of work needed to be done with the time available for its completion.
16. Practice acceptable quality assurance as established for each clinical area.
17. Defend the policy of running quality control samples according to laboratory protocol.
18. Coordinate theory with laboratory analysis to appropriately judge patient data.
19. Recognize technical problems and plan possible corrective action.
20. Maintain composure and work quality under stressful conditions.

21. Demonstrate concern for professional self-image and that of the medical laboratory science profession by practicing ethical behaviour, participating in professional activities and attending professional seminars to maintain knowledge base.

Sample Handling

The first step in the accurate diagnosis of infectious diseases is the proper collection and handling of specimens. These pre-analytical, analytical, and post-analytical factors are essential for quality assessment in the laboratory.

Specimen handling involves the following steps:

1. Correct identification of patient
2. Correct specimen collection
3. Correct use of appropriate specimen containers
4. Correct labelling of forms and containers
5. Timeliness of transport
6. Correct identification of special procedures based on suspected pathogens
7. Correct handling in the laboratory with respect to selection of growth media, stains, incubation
8. Times and temperatures
9. Correct reporting of results
10. Correct time for specimen collection – blood cultures, etc.
11. Correct identification of special handling – ice, prechilled tubes, spin immediately, etc.

Principles of Sterilization and Disinfection

Sterilization is defined as the killing or removal of all microorganisms and viruses from an object or product. Disinfection means rendering an object, the hands or skin free of pathogens. The term asepsis covers all measures aiming to prevent contamination of objects or wounds. Disinfection and sterilization makes use of both physical and chemical agents.

Aseptic Techniques

Objectives

To implement aseptic techniques so that bacteriological examinations are performed accurately and examiners are safe from being infected by pathogenic Bacteria. In other words, to prevent microbial contamination of materials or wounds.

Background

The Medical Bacteriology laboratory, whether in a classroom or a working diagnostic laboratory, is a place where cultures of microorganisms are handled and examined. This type of activity must be carried out with good aseptic technique in a thoroughly clean, well-organized workplace. In **aseptic technique**, all materials that are used have been sterilized to kill any microorganisms contained in or on them, and extreme care is taken not to introduce new organisms from the environment. Even if the microorganisms you are studying are not usually considered pathogenic (disease producing), *any* culture of *any* organism should be handled as if it were a potential pathogen. With current medical practices and procedures, many patients with lowered immune defences survive longer than they did before. As a result, almost any microorganism can cause disease in them under the appropriate circumstances. Each student must quickly learn and continuously practice aseptic laboratory technique. It is important to prevent contamination of your hands, hair, and clothing with culture material and also to protect your neighbours from such contamination. In addition, you must not contaminate your work with microorganisms from the environment. The importance of asepsis and proper disinfection is stressed throughout this manual and demonstrated by the experiments. Once these techniques are learned in the laboratory, they apply to almost every phase of patient care, especially to the collection and handling of specimens that are critical if the laboratory is to make a diagnosis of infectious disease.

Principles of Diagnosis

Identification of the organism causing an infectious process is frequently essential for effective antimicrobial and supportive therapy. Initial treatment may be empiric, based upon the microbiologic epidemiology of the infection and the patient's symptoms. However, definitive microbiologic diagnosis of an infectious disease usually involves one or more of the following five basic laboratory techniques, which guide the physician along a narrowing path of possible causative organisms:

1. Direct microscopic visualization of the organism;
2. Cultivation and identification of the organism;
3. Detection of microbial antigens [immunohematology];
4. Detection of an inflammatory or host immune response to the microorganism [immunohematology];
5. Detection of microbial DNA or RNA [molecular biology] and

All laboratory studies must first be directed by the patient's clinical information (that is, their history and physical examination), and then evaluated, taking into consideration the sensitivity and specificity of the test.

DIAGNOSIS OF BACTERIAL INFECTIONS

Microscopy and Stains

Microscopic examination of stained or unstained specimens is a relatively simple and inexpensive, but much less sensitive method than culture for detection of small numbers of bacteria. A specimen must contain at least 105 organisms per milliliter before it is likely that organisms will be seen on a smear.

Gram staining is a very useful procedure in diagnostic microbiology. Most specimens submitted when bacterial infection is suspected should be smeared on glass slides, Gram-stained, and examined microscopically. On microscopic examination, the Gram reaction (purpleblue indicates gram-positive organisms; red, gram-negative) and morphology (shape: cocci, rods, fusiform, or other; of bacteria should be noted.

Specimens submitted for examination for mycobacteria should be stained for acid-fast organisms: The most sensitive fluorescent stains for mycobacteria detection, such as auramine- rhodamine, should be used. Confirmation of a positive fluorescent stain is usually performed using one of the nonfluorescent acid-fast stains, either Ziehl-Neelsen stain or Kinyoun stain.

Culture Systems

For diagnostic bacteriology, it is necessary to use several types of media for routine culture, particularly when the possible organisms include aerobic, facultatively anaerobic, and obligately anaerobic bacteria. The standard medium for specimens is blood agar, usually made with 5% sheep blood. Most aerobic and facultatively anaerobic organisms will grow on blood agar. Chocolate agar, a medium containing heated blood with or without supplements, is a second necessary medium; some organisms that do not grow on blood agar, including pathogenic Neisseria and Haemophilus, will grow on chocolate agar. A selective medium for enteric gram negative rods (either MacConkey agar or eosin–methylene blue [EMB] agar) is a third type of medium used routinely. Specimens to be cultured for obligate anaerobes must be plated on at least two additional types of media, including a highly supplemented agar such as brucella agar with hemin and vitamin K and a selective medium containing substances that inhibit the growth of enteric gram-negative rods and facultatively anaerobic or anaerobic gram-positive cocci.

Broth cultures in highly enriched media are important for back-up cultures of biopsy tissues and body fluids such as cerebrospinal fluid. Broth cultures may give positive results when there is no growth on solid media because of the small number of bacteria present in the inoculums.

Week 2 & 3: Culture Media Inoculation & Colony Isolation

Background:

Given that many clinical specimens contain a mixed flora of microorganisms. When these specimens are set up for culture, if only one isolation plate were inoculated, a great deal of time would be spent in subculturing and sorting through the bacterial species that grow out. Instead, the microbiologist uses several types of primary media at once (i.e., a battery) to culture the specimen initially. In general, the primary battery has three basic purposes:

1. To culture all bacterial species present and see which, if any, predominates
2. To differentiate species by certain characteristic responses to ingredients of the culture medium
3. To selectively encourage growth of those species of interest while suppressing the normal flora

The basic medium on which a majority of bacteria present in a clinical specimen will grow contains agar enriched with **blood** and other nutrients required by pathogens. The blood, which provides excellent enrichment, is obtained from animal sources, most often from sheep. The use of human blood (usually obtained from outdated collections in blood banks) in culture media is not recommended because it may contain substances such as antimicrobial agents, antibodies, and anticoagulants that are either inhibitory to the growth of fastidious microorganisms or interfere with characteristic reactions.

In addition to basic nutrients, **differential media** contain one or more components, such as a particular carbohydrate, that can be used by some microorganisms but not by others. If the microorganism uses the component during the incubation period, a change occurs in an indicator that is also included in the medium.

Selective media contain one or more components that suppress the growth of some microorganisms without seriously affecting the ability of others to grow. Such media may also contain ingredients for differentiating among the species that do survive.

Objectives:

- **After completing this lab, students are expected to attain proficiency in:**
- Collecting sample aseptically
- Culturing collected samples on different culture media
- Staining and microscopy techniques
- Following sample labelling

- Obtaining single colony

Materials

- Nutrient agar plates
- Blood agar plates
- MacConkey or (EMB) agar plates
- Mannitol salt agar plates (MSA)
- Gram stain
- Simulated faecal suspension, containing *Escherichia coli*, *Pseudomonas aeruginosa*, and *Staphylococcus epidermidis*

Procedure

1. Inoculate the simulated faecal specimen on nutrient agar, blood agar, EMB, and MSA plates. Streak each plate for isolation of colonies. Incubate at 35°C.
2. Make a Gram stain (see page 61) of the faecal suspension and examine it.

For Culturing techniques, Microscopy, and Staining;

Refer to Introduction to Microbiology Lab Manual (prepared by Dr Belal, Fall 2015).

Culture Characterization of Bacteria:

When a single bacterial culture is grown using different forms of media (broth, slants, deeps, and plates), it displays a collective pattern of growth that is unique to its species.

This unique pattern of growth is referred to as its Culture Characteristics.

An organism's culture characteristics can help distinguish it from other organisms, since each bacterial species typically has a unique pattern of growth.

Bacterial and fungal colonies often have distinctive characteristics— Characteristics of bacterial colonies including Shape, margin, elevation (side view), size, texture, appearance, pigmentation (color), and optical properties are described by a variety of terms. These will help in identifying the microbial species that formed the colony, as follows:

Appearance: Shiny or dull

Optical property: Opaque, translucent, transparent

Pigmentation: Pigmented (purple, red, yellow)

Nonpigmented (cream, tan, white)

Texture: Rough or smooth

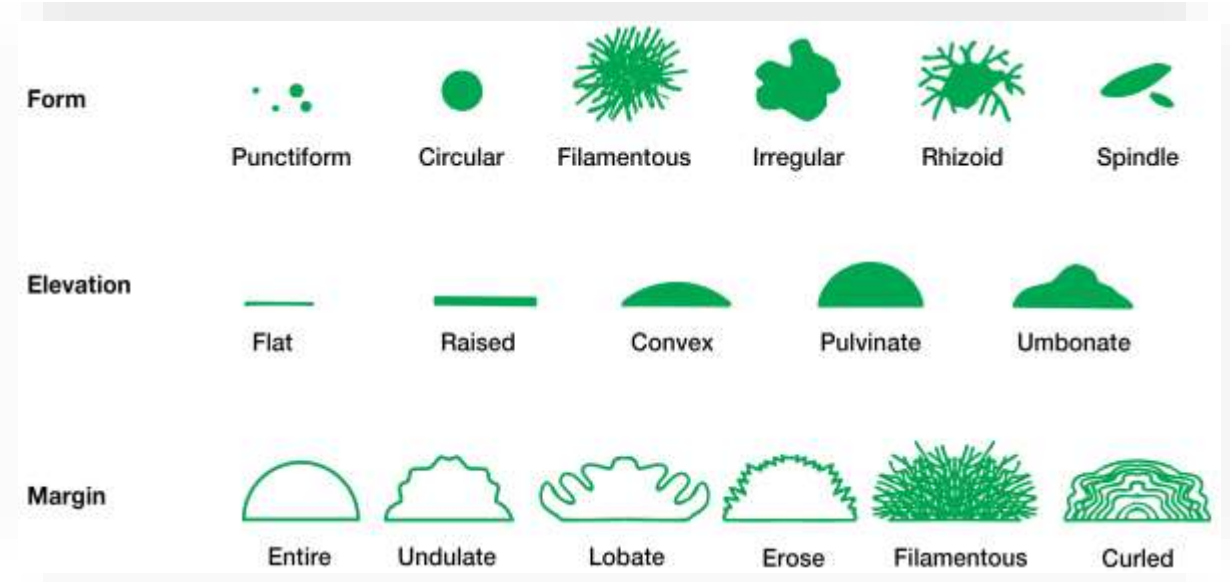


Figure 1 Culture Charesteristics

Results

After overnight incubation features of simulated faecal specimen cultures to be written as bellow:

Medium	Gross Morphology of Each Type	Gram-Stain Reaction and Microscopic Morphology Colony of Each Colony Type	Presumptive Identification*

Figure 2 Different Bacterial Colonial Morphologies

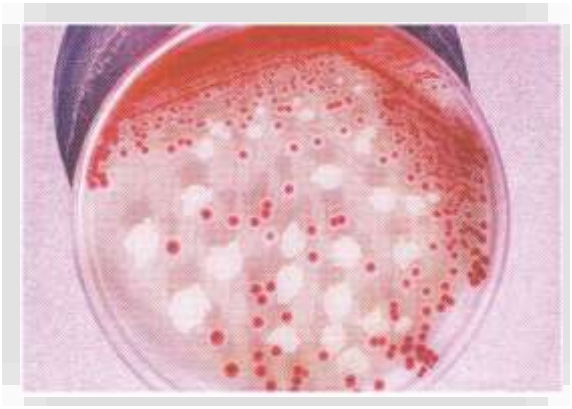


Figure 3 Streak Plate Showing Isolation of Two Different Bacterial Types

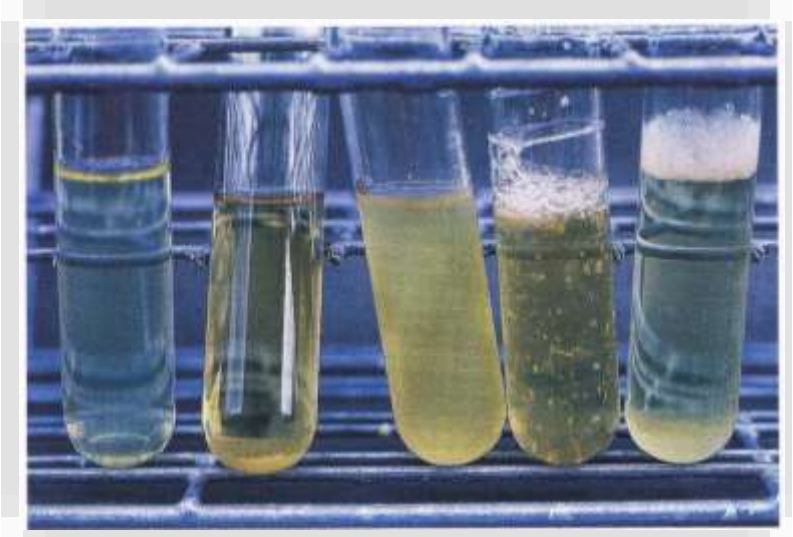


Figure 4 Bacterial growth characteristics in broth. From LEFT to RIGHT: uninoculated tube, precipitation reaction, turbidity, flocculation, pellicle formation.

Review Questions

- Why is MacConkey agar selective as well as differential?
- Why is blood agar useful as a primary isolation medium?
- Define a differential medium and discuss its purpose.

Week 4: Spore-Forming Gram-Positive Bacilli (Bacillus & Clostridium Species)

BACILLUS SPECIES

The genus *Bacillus* includes large aerobic, gram-positive rods occurring in chains. Most members of this genus are saprophytic organisms prevalent in soil, water, and air and on vegetation, such as *Bacillus cereus* and *Bacillus subtilis*.

Morphology and identification

Typical Organisms

The typical cells, measuring 1 x 3-4 µm, have square ends and are arranged in long chains; spores are located in the center of the nonmotile bacilli.

Culture

Colonies of *B anthracis* are round and have a "cut glass" appearance in transmitted light. Hemolysis is uncommon with *B anthracis* but common with *B cereus* and the saprophytic bacilli. Gelatin is liquefied, and growth in gelatin stabs resembles an inverted fir tree.

Growth Characteristics

The saprophytic bacilli use simple sources of nitrogen and carbon for energy and growth. The spores are resistant to environmental changes, withstand dry heat and certain chemical disinfectants for moderate periods, and persist for years in dry earth. Animal products contaminated with anthrax spores (eg, hides, bristles, hair, wool, bone) can be sterilized by autoclaving.

CLOSTRIDIUM SPECIES

The clostridia are large anaerobic, gram-positive, motile rods. Many decompose proteins or form toxins, and some do both. Their natural habitat is the soil or the intestinal tract of animals and humans, where they live as saprophytes. Among the pathogens are the organisms causing botulism, tetanus, gas gangrene, and pseudomembranous colitis.

Morphology and Identification

Typical Organisms

Spores of clostridia are usually wider than the diameter of the rods in which they are formed. In the various species, the spore is placed centrally, subterminally, or terminally. Most species of clostridia are motile and possess peritrichous flagella.

Culture

Clostridia are anaerobes and grow under anaerobic conditions; a few species are aerotolerant and also grow in ambient air. Anaerobic culture conditions are discussed in Chapter 21. In general, the clostridia grow well on the blood-enriched media or other media used to grow anaerobes.

Colony Forms

Some clostridia produce large raised colonies (eg, *C perfringens*); others produce smaller colonies (eg, *C tetani*). Some clostridia form colonies that spread on the agar surface. Many clostridia produce a zone of β-hemolysis on blood agar. *C perfringens* characteristically produces a double zone of β-hemolysis around colonies.

Growth Characteristics

Clostridia can ferment a variety of sugars; many can digest proteins. These metabolic characteristics are used to divide the Clostridia into groups, saccharolytic or proteolytic. Milk is turned acid by some and digested by others and undergoes “stormy fermentation” (ie, clot torn by gas) with a third group (eg, *C perfringens*). Various enzymes are produced by different species.

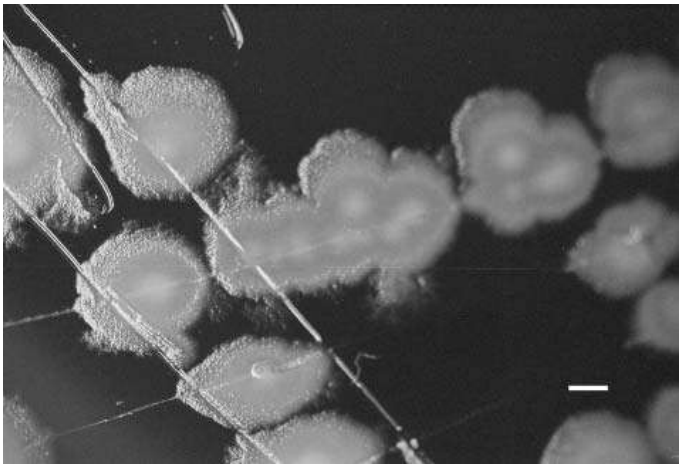


Figure 5 Bacillus anthracis Colonies

Figure 6 Bacillus cereus Colonies

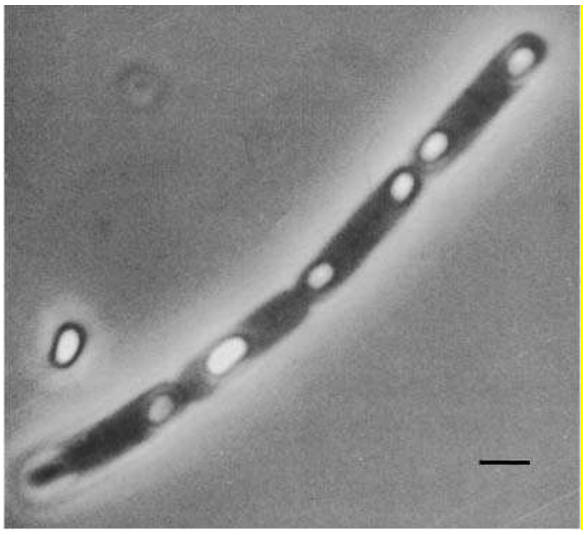


Figure 7 Endospores

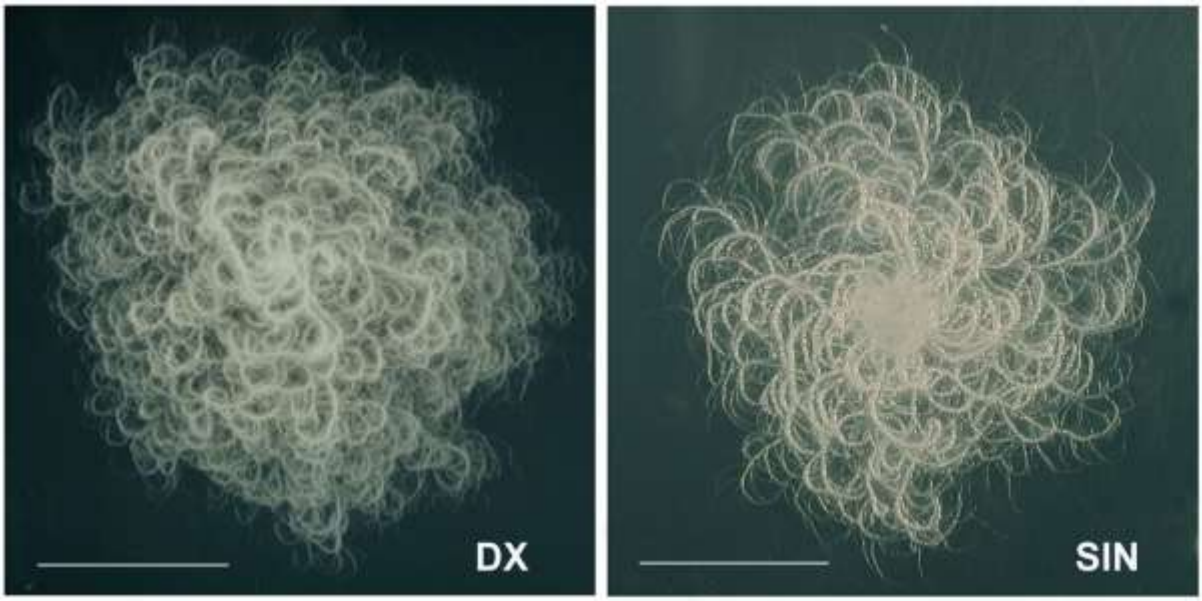


Figure 8 Bacillus mycoides Colonies

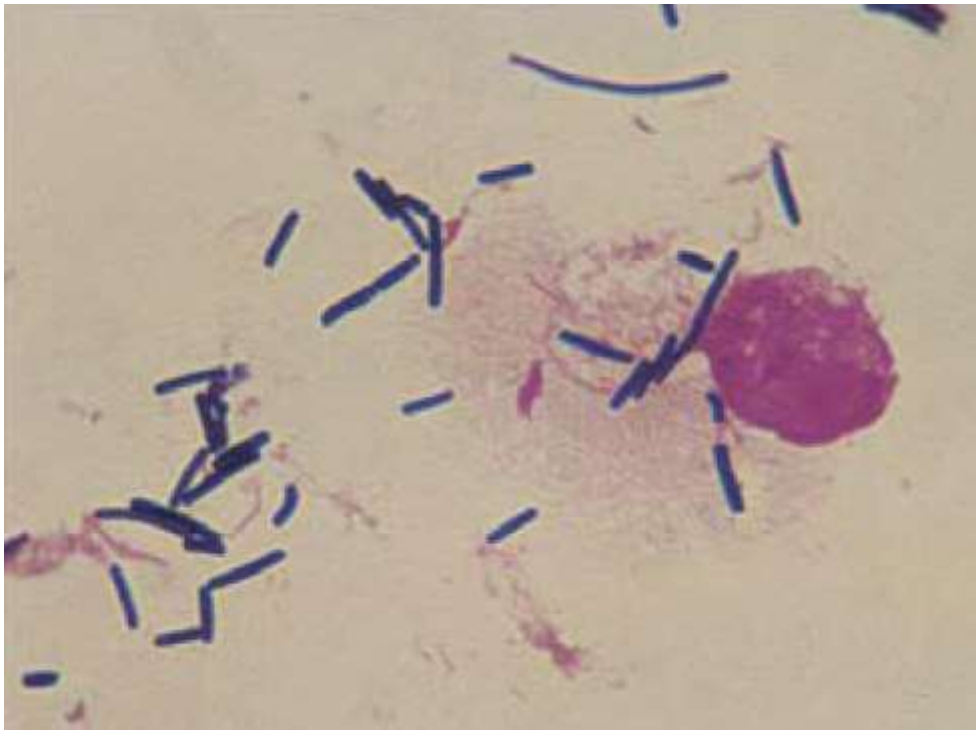


Figure 9 Clostridium perfringens

Laboratory diagnostic techniques

Bacillus anthracis:

- Gram stain and culture of blood, respiratory secretions or lesions
- Serology
- PCR

Bacillus cereus:

- Clinical grounds
- Culture and Gram stain of implicated food

Clostridia:

Primarily a clinical diagnosis; organism is rarely isolated.

Materials

- Gram Stain
- Endospore Stain
- Starch, Blood & Nutrient Agar

Procedure

1. Inoculate your samples on blood and nutrient agar
2. Stain obtained single colonies with Gram (see page 61) and Endospore stains (see page 62)
3. Do Starch Hydrolysis Test (see page 69)

Results

Record your result as follows:

Bacteria Sample	Colony Characteristics on:		Cell Characteristics with	
	Blood Agar	Nutrient Agar	Gram Stain	Spore Staing
1				
2				

Review questions

- What is the benefit of gram stain in this lab?
- Why don't you need to use MacConkey Agar Media for cultivation?
- Why is iodine used to detect starch hydrolysis?
- Did you see spores of ***Bacillus mycoides***?
    - If yes why?
    - If not why?

Week 5: Non-Spore-Forming Gram-Positive Bacilli: Corynebacterium, Propionibacterium Corynebacterium diphtheria

CORYNEBACTERIUM DIPHTHERIAE

Morphology and Identification

Characteristically, they possess irregular swellings at one end that give them the "club-shaped" appearance. Irregularly distributed within the rod (often near the poles) are granules staining deeply with aniline dyes (metachromatic granules) that give the rod a beaded appearance.

On **blood agar**, the C diphtheriae colonies are small, granular, and gray with irregular edges and may have small zones of hemolysis. On agar containing **potassium tellurite**, the colonies are brown to black with a brown-black halo because the tellurite is reduced ntracellularly. *C diphtheriae* and other corynebacteria grow aerobically on most ordinary laboratory media. On Loeffler serum medium, corynebacteria grow much more readily than other respiratory organisms, and the morphology of organisms is typical in smears made from these colonies.

Gram-Positive Anaerobes / Gram-Positive Bacilli:

Propionibacterium

Propionibacterium species are members of the normal microbiota of the skin, oral cavity, large intestine, conjunctiva, and external ear canal. Their metabolic products include propionic acid, from which the genus name derives. On Gram stain, they are highly pleomorphic, showing curved, clubbed, or pointed ends; long forms with beaded uneven staining; and occasionally coccoid or spherical forms. ***Propionibacterium acnes***, often considered an opportunistic pathogen, causes the disease **acne vulgaris** and is associated with a variety of inflammatory conditions.

Figure 10 Corynebacterium diphtheriae Stained Cells LEFT & Colonies RIGHT

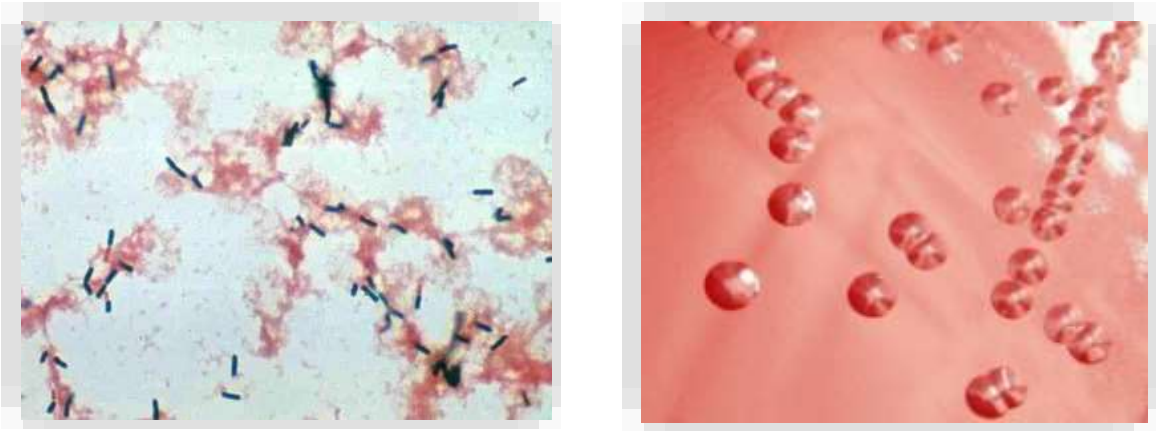


Figure 11 Listeria monocytogenes Stained Cells LEFT & Colonies RIGHT

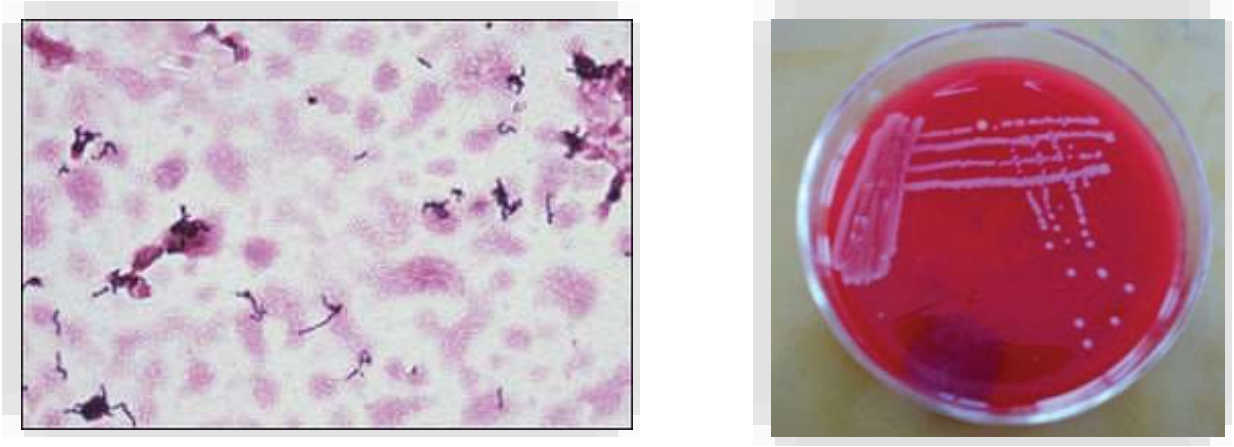


Figure 12 P. acnes Stained Cells LEFT & Colonies RIGHT

Laboratory diagnostic techniques

Corynebacterium diptheriae:

- Elek test to document toxin production (ELISA for toxin is now the frontline)
- Toxin produced by toxin-producing strains diffuses away from growth.
- Antitoxin diffuses away from the strip of filter paper.
- Precipitin lines form at zone of equivalence.

Listeria monocytogenes:

- Blood or CSF culture
- CSF wet mount [motility] or Gram stain

Materials

- Gram Stain
- Blood & Nutrient Agar
- H_2O_2 for catalase test

Procedure

1. Inoculate swab samples on Nutrient & Blood Agar Plates and incubate them at 37°C for 24 Hours.
2. After overnight incubation, observe morphology of colonies and Gram (see page 61) stained bacterial cells
3. Then do catalase test for the suspected bacilli forms (see page 91)

Results

Bacteria Sample	Colony Characteristics on:		Bacterial Cell Response with	
	Blood Agar	Nutrient Agar	Gram Stain	Catalase H_2O_2
1				
2				

Review Questions

- What is the substrate of the catalase reaction? Why are bubbles produced in a positive catalase test?
- Why will a false-positive catalase test result if the organisms are tested on a medium containing blood?

Week 6: Staphylococci & Streptococci

Staphylococci

Morphology and identification

Typical Organisms

Staphylococci are spherical cells about 1 μm in diameter arranged in irregular clusters. Single cocci, pairs, tetrads, and chains are also seen in liquid cultures. Young cocci stain strongly gram positive; on aging, many cells become gram negative. Staphylococci are nonmotile and do not form spores.

Culture

Staphylococci grow readily on most bacteriologic media under aerobic or microaerophilic conditions. They grow most rapidly at 37° C but form pigment best at room temperature (20 – 25° C). Colonies on solid media are round, smooth, raised, and glistening. ***S aureus*** usually forms gray to deep golden yellow colonies. ***S epidermidis*** colonies usually are gray to white on primary isolation; many colonies develop pigment only upon prolonged incubation. No pigment is produced anaerobically or in broth. Various degrees of hemolysis are produced by ***S aureus*** and occasionally by other species. The genus ***Staphylococcus*** contains two species, ***S saccharolyticus*** and ***S aureus*** subsp. ***anaerobius***, which initially grow only under anaerobic conditions but become more aerotolerant on subcultures.

Growth Characteristics

The staphylococci produce **catalase**, which differentiates them from the streptococci. Staphylococci slowly ferment many carbohydrates, producing lactic acid but not gas. Proteolytic activity varies greatly from one strain to another. Staphylococci are relatively resistant to drying, heat (they withstand 50° C for 30 minutes), and 9% sodium chloride. ***S aureus*** produces **coagulase**, an enzyme-like protein that clots oxalated or citrated plasma. **Clumping factor** is responsible for adherence of the organisms to fibrinogen and fibrin.

The Streptococci

The streptococci are gram-positive spherical bacteria that characteristically form pairs or chains during growth. They are widely distributed in nature. Some are members of the normal human microbiota; others are associated with important human diseases attributable to the direct effects of infection by streptococci or in other cases to an immunologic response to them.

STREPTOCOCCI OF PARTICULAR MEDICAL INTEREST:

Streptococcus pyogenes

Morphology and Identification

Typical Organisms

Individual cocci are spherical or ovoid and are arranged in chains. The cocci divide in a plane perpendicular to the long axis of the chain. The members of the chain often have a striking diplococcal appearance, and rodlike forms are occasionally seen. Streptococci are gram positive; however, as a culture ages and the bacteria die, they lose their gram positivity and can appear to be gram negative; for some streptococci, this can occur after overnight incubation.

Culture

Most streptococci grow in solid media as discoid colonies, usually 1 – 2 mm in diameter. ***S pyogenes*** is β -hemolytic; other species have variable hemolytic characteristics.

Growth Characteristics

Energy is obtained principally from the utilization of glucose with lactic acid as the end product. Growth of streptococci tends to be poor on solid media or in broth unless enriched with blood or tissue fluids. Nutritive requirements vary widely among different species. The human pathogens are most exacting, requiring a variety of growth factors. Growth and hemolysis are aided by incubation in 10% CO2. Most pathogenic hemolytic streptococci grow

best at 37°C. Most streptococci are facultative anaerobes and grow under aerobic and anaerobic conditions.

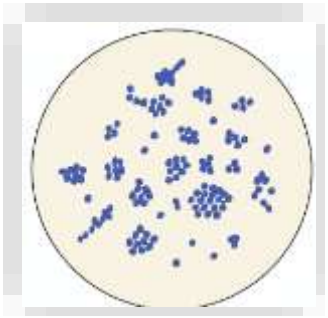


Figure 13 Staphylococci Cell Morphology

Figure 14 Staphylococci Colonies in Nutreint & Blood Agar

Figure 15 Manitol Fermentation RIGHT & Non-fermenting Staphylococci

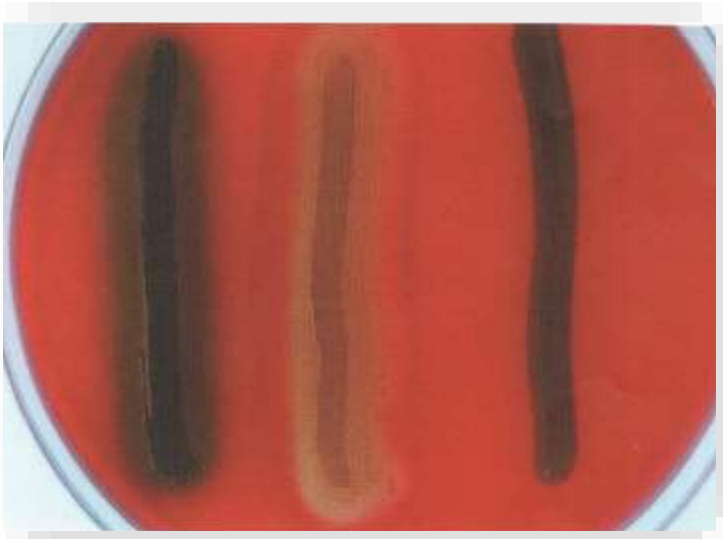


Figure 16 LEFT to RIGHT Alpha, Beta & Gamma Hemolysis

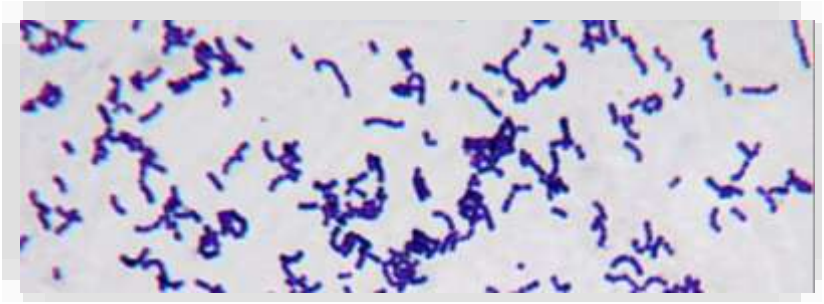


Figure 17 Stained Streptococci Cells

Classification:

- The streptococci are a large and heterogeneous group of bacteria and no one system suffices to classify them. Yet, understanding the classification is key to understanding their medical importance.
- The streptococci of greatest medical significance are *S. pyogenes, S. agalactiae,* and *S. pneumoniae.* Of lesser importance are *S. faecalis, S. faecium,* and *S. bovis.*

Streptococci species are classified based on:

a. **hemolytic capacity** (**α**, **β**, **γ** hemolysis)

b. **The antigenicity** of a carbohydrate occurring in their cell walls (Lancefield antigen).

Hemolytic Capacity

Streptococci can be broadly classified according to the hemolytic reaction on blood agar:

- ***α-hemolytic:*** Is the partial destruction of RBCs and produces a greenish discoloration of the agar around the colonies. ***S. pneumoniae.***
- ***β-hemolytic:*** Colonies on blood agar are completely hemolyses the red cells around their colonies. ***S. pyogenes and S. agalactiae***
- ***γ-hemolytic:*** is the absence of macroscopically visible hemolytic zones. ***Streptococcus bovis***

Lancefield Groups

- Many streptococci and enterococci have a polymeric carbohydrate (C substance) in their cell walls called the Lancefield antigen.
- They are classified in Lancefield groups A-V based on variations in the antigenicity of this antigen.
- These antigenes were labeled Group A, Group B, Group C, and so on. Currently three Lancefield Groups are of medical importance:
- Group A, Group B, and Group D. Of the organisms used in this lab the following correlations apply:
- ***Group A Strep****--Streptococcus pyogenes*
- ***Group B Strep****--Streptococcus agalactiae*
- ***Group D Strep****--Streptococcus bovis*

Table 1 Classification of Streptococci

Hemolysis	Lancefield group	Species
β	A	*Streptococcus pyogenes*
	B	*Streptococcus agalactiae*
	C	*Streptococcus dysgalactiae*
	D	*Enterococcus* spp.
α or γ	D	*Enterococcus* spp.
	D	*Streptococcus bovis complex* (reclassified into at least four new species as described in text)
	None	Viridans group*
α	None	*Streptococcus pneumoniae*

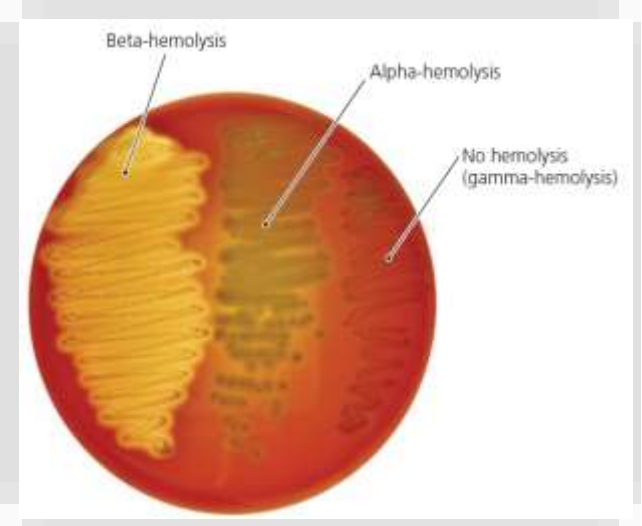


Figure 18 β, α and γ Hemolysis of Streptococci

The CAMP Test for Group B Streptococci

- The CAMP test is used to differentiate Group B ***Streptococcus agalactiae*** (**+**) from other *Streptococcus* species (**-**).
- Group B streptococci can be distinguished from other beta-hemolytic streptococci by their production of a substance called the CAMP factor. This term is an acronym for the names of the investigators who first described the factor: *C*hristie, *A*tkins, and *M*unch-*P*etersen. The substance is a peptide that acts together with the beta-hemolysin produced by some strains of *Staphylococcus aureus,* enhancing the effect of the latter on a sheep blood agar plate. This effect is sometimes referred to as *synergistic* hemolysis
- When streaked perpendicularly to an *S. aureus* subsp. *aureus* streak on blood, an **arrow head shaped zone** of hemolysis forms and is a positive result

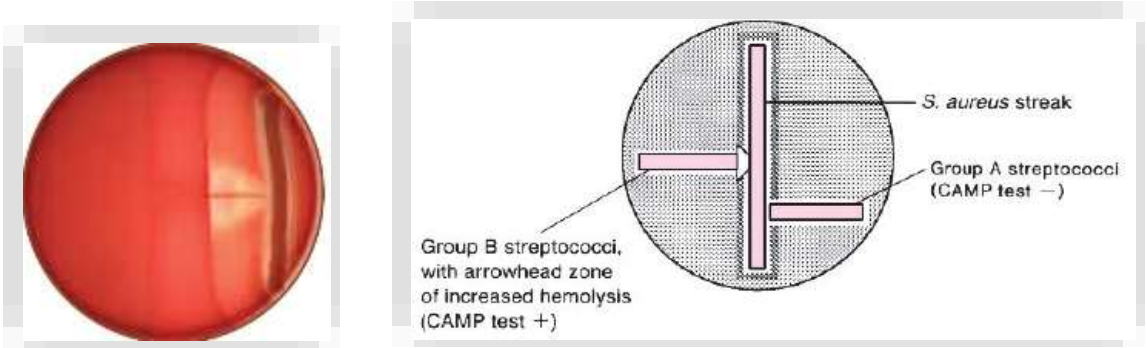


Figure 19 CAMP Test

CAMP Test Method

1. With an inoculating loop, streak a strain of ***S. aureus*** down the center of a blood agar plate.
2. On one side of the plate, inoculate a strain of group **B *Streptococcus*** by making a streak at a 90° angle, starting 5 mm away from the ***S. aureus*** and extending outward to the edge of the agar.
3. On the other side of the plate, inoculate a strain of group **A *Streptococcus,*** again at a 90° angle from the ***S. aureus,*** as in step This streak should not be directly opposite the group B inoculum.
4. Incubate the plate aerobically at 35°C for 18 to 24 hours.

Interpretation of Results

Observe the area of hemolysis surrounding the *S. aureus* streak. At the point adjacent to the streak of group B streptococci, you should see an arrowhead-shaped area of increased hemolysis indicating production of the CAMP factor. There should be no change in the hemolytic zone adjacent to the streak of group A streptococci, most strains of which do not produce the CAMP factor.

Laboratory diagnostic techniques

Staphylococci:

- Gm + cocci in grapes, **Catalase** differentiates from Strep.

Staphylococcus aureus:

- Betahemolysis, Coagulase +ve, Yellow (Au) pigment

Staphylococcus epidermidis:

- Coagulase -ve
- Novobiocin sensitive

Staphylococcus saprophyticus:

- Coagulase -ve
- Novobiocin resistant

Streptococci:

Streptococcus pyogenes [Group A Streptococcus]:

- The rapid strep test (ELISA-based) misses approximately 25% of infections.
- Culture all negatives.
- Antibodies to streptolysin 0 (ASO) titer of > 200 is significant for rheumatic fever.

Streptococcus pneumoniae:

- Gram stain of CSF
- PCR of CSF
- Quellung reaction: positive (swelling of the capsule with the addition of type-specific antiserum)
- Latex particle agglutination: test for capsular antigen in CSF

Enterococcus faecalis/faecium:

- Culture on blood agar
- Antibiotic sensitivities

Materials

- Gram Stain
- Blood & Manitol Salt Agar MSA
- H_2O_2 for catalase test
- Blood Plasma for coagulase test
- Novobiocin discs

Procedure

1. Inoculation on Blood & MSA Plates
2. Gram Stain (see page 61)
3. Catalase & Coagulase Test (see page 91 & 94)
4. Novobiosin sensitivity test (see page 109)

Results

Bacteria Sample	Colony Characteristics on:		Bacterial Cell Response with		
	Blood Agar	MSA Agar	Gram Stain	TEST	
				Catalase H_2O_2	Coagulase
1					
2					

Novobiocin Sensitivity Result for Coagulase Negative Sample: [----------]

Review Questions

- What is coagulase?
- What is Novobiocin?
- How Manitol is fermented?

Week 7 & 8: Enteric Gram-Negative Rodes (Enterobacteriaceae)

Morphology and Identification

Typical Organisms

The Enterobacteriaceae are short gram-negative rods. Typical morphology is seen in growth on solid media in vitro, but morphology is highly variable in clinical specimens.

Culture

E coli and most of the other enteric bacteria form circular, convex, smooth colonies with distinct edges. Enterobacter colonies are similar but somewhat more mucoid. Klebsiella colonies are large and very mucoid and tend to coalesce with prolonged incubation. The salmonellae and shigellae produce colonies similar to *E coli* but do not ferment lactose. Some strains of *E coli* produce hemolysis on blood agar.

Growth Characteristics

Carbohydrate fermentation patterns and the activity of amino acid decarboxylases and other enzymes are used in biochemical differentiation. Some tests, such as the production of indole from tryptophan, are commonly used in rapid identification systems, but others, such as the Voges-Proskauer reaction (production of acetylmethylcarbinol from dextrose), are used less often. Culture on “differential” media that contain special dyes and carbohydrates (eg, eosin-methylene blue [EMB], MacConkey, or deoxycholate medium) distinguishes lactose-fermenting (colored) from non–lactose-fermenting colonies (nonpigmented) and may allow rapid presumptive identification of enteric bacteria. Many complex media have been devised to help in identification of the enteric bacteria. One such medium is triple sugar iron (TSI) agar, which is often used to help differentiate salmonellae and shigellae from other enteric gram-negative rods in stool cultures.

Escherichia

E coli typically produce positive test results for indole, lysine decarboxylase, and mannitol fermentation and produces gas from glucose (TSI is A/A +Ve gas). An isolate from urine can

be quickly identified as E coli by its hemolysis on blood agar, typical colonial morphology with an iridescent “sheen” on differential media such as EMB agar, and a positive spot indole test result.

Shigella

Shigellae are nonmotile and usually do not ferment lactose but do ferment other carbohydrates, producing acid but not gas (TSI is K/A, -Ve gas). They do not produce H2S. The four Shigella species are closely related to E coli. Many share common antigens with one another and with other enteric bacteria.

Salmonella

Salmonellae are motile rods that characteristically ferment glucose and mannose without producing gas but do not ferment lactose or sucrose. Most salmonellae produce H_2S. They are often pathogenic for humans or animals when ingested.

- Methyl red test positive
- Voges-Proskauer test negative
- Citrate positive (growth on Simmon's citrate agar)
- Lysine decarboxylase positive
- Urease negative
- Indole production negative

Laboratory diagnostic techniques

Escherichia coli:

- Gram-negative rod
- Oxidase negative
- E. coli is a lactose fermenter: colonies with iridescent green sheen on EMB

Salmonella enterica Subsp. typhi

- Organisms can be isolated from blood, bone marrow, urine, and tissue biopsy from the rose spots if present.

Salmonella enterica Subspecies Other Than typhi (S. enteritidis, S. typhimurium):

- Culture on Hektoen agar, H2S production

Shigella Species:

- Isolation from stool during illness and culture on selective media

Materials

- Gram Stain
- MacConkey & Kligler Iron Agar
- Reagents for [Indole, Methyl Red, Voges-Proskauer, Citrate] tests

Procedure

1. Inoculate simulated fecal sample on MacConkey & Kligler Iron Agar (see page 86) then Gram Stain (see page 61)
2. Do IMViC [Indole, Methyl Red, Voges-Proskauer, Citrate] test (see page 73, 75, 77 & 79)

Results

Comparison Between →	*E. coli*		*Salmonella*	
Media / Reaction	**Expected**	**Result**	**Expected**	**Result**
Indole	+		-	
Methyl Red	+		+	
Voges-Proskauer	-		-	
Citrate Utilization	-		+	
Colony Color on MacConkey Agar	Pink		Colorless	
Ferment Lactose	+		-	

Review questions

How could you differentiate between *E. coli* & *Salmonella*? State the principle behind the fact.

Week 9: Vibrios, Campylobacters, Helicobacter & Associated Bacteria

VIBRIO CHOLERAE

Morphology and Identification

Typical Organisms

Vibrios are among the most common bacteria in surface waters worldwide. They are curved aerobic rods and are motile, possessing a polar flagellum

Culture

V cholerae produces convex, smooth, round colonies that are opaque and granular in transmitted light. *V cholera* and most other vibrios grow well at 37°C on many kinds of media, including defined media containing mineral salts and asparagine as sources of carbon and nitrogen. *V cholera* grows well on thiosulfate-citrate-bile-sucrose (TCBS) agar, a media selective for vibrios, on which it produces yellow colonies (sucrose fermented). Vibrios are oxidase positive, which differentiates them from enteric gram-negative bacteria. Characteristically, vibrios grow at a very high pH (8.5–9.5) and are rapidly killed by acid. Cultures containing fermentable carbohydrates therefore quickly become sterile. *V. cholerae* on Kligler's iron agar (KIA), which contains glucose and lactose, is similar to those of nonlactose-fermenting Enterobacteriaceae (K/A, no gas, no H_2S)

CAMPYLOBACTER

Morphology and Identification

Typical Organisms

C jejuni and the other campylobacters are gram-negative rods with comma, S, or "gull wing" shapes. They are motile, with a single polar flagellum, and do not form spores.

Growth Characteristics

C jejuni and the other campylobacters pathogenic for humans are positive for both oxidase and catalase. Campylobacters do not oxidize or ferment carbohydrates. Gram-stained smears

show typical morphology. Nitrate reduction, hydrogen sulfide production, hippurate tests, and antimicrobial susceptibilities can be used for further identification of species.

HELICOBACTER PYLORI

Morphology and Identification

Typical Organisms

H pylori has many characteristics in common with campylobacters. It has multiple flagella at one pole and is actively motile.

Culture

H pylori grows in 3–6 days when incubated at 37°C in a microaerophilic environment, as for *C jejuni*. The media for primary isolation include Skirrow's medium with vancomycin, polymyxin B, and trimethoprim, chocolate medium, and other selective media with antibiotics (eg, vancomycin, nalidixic acid, amphotericin). The colonies are translucent and 1–2 mm in diameter.

Growth Characteristics

H pylori is oxidase positive and catalase positive, has a characteristic morphology, is motile, and is a strong producer of urease.

Laboratory diagnostic techniques

Vibrio cholera:

- Culture stool on TCBS
- Oxidase positive

Campylobacter jejuni:

- Culture on Campylobacter or Skirrow agar at 42°C

Helicobacter pylori:

- Biopsy with culture; histology with Giemsa or silver stain
- Breath test: ^{13}C-urea swallowed; ammonia+^{13}C-$C0_2$ exhaled
- Serology

Materials

- MacConkey Agar
- Kligler Iron Agar
- Thiosulfate-citrate-bile-sucrose (TCBS) Agar
- Gram Stain
- Reagents for Indole, Methyl Red, Voges-Proskauer, Citrate, Catalase, Urease & Oxidase tests

Procedure

1. Inoculate simulated fecal sample on MacConkey thiosulfate-citrate-bile-sucrose (TCBS) & Kligler Iron Agar (see page 86) then Gram Stain (see page 61)
2. Do IMViC [Indole, Methyl Red, Voges-Proskauer, Citrate] test (see page 73, 75, 77 & 79)
3. Do Catalase, Oxidase & Urease test (see page 91, 97 & 99)

Results

Serological test results if applicable: [---------]

Result of *Vibrio* culture on TCBS: [Describe the Colonies]

Review questions

Differentiate between *Vibrio cholera* & *Helicobacter pylori* on the basis of morphology and biochemical reactions.

Week 10: Pseudomonads & Anaerobic Bacteria

THE PSEUDOMONAD GROUP *Pseudomonas aeruginosa*

Morphology and Identification

Typical Organisms

P aeruginosa is motile and rod shaped. It is gram negative and occurs as single bacteria, in pairs, and occasionally in short chains.

Culture

P aeruginosa is an obligate aerobe that grows readily on many types of culture media, sometimes producing a sweet or grapelike or corn taco–like odor. Some strains hemolyze blood. *P aeruginosa* forms smooth round colonies with a fluorescent greenish color. It often produces the nonfluorescent bluish pigment pyocyanin, which diff uses into the agar. Other Pseudomonas species do not produce pyocyanin.

Biochemical reactions

- Catalase-positive
- Oxidase-positive
- Nitrate reduction-positive
- Indole test-negative
- Methyl red test-negative
- Vp test-negative
- Citrate test-positive
- Urease test-negative

Laboratory diagnostic techniques

Pseudomonas aeruginosa:

- Gram stain and culture; it produces pyocyanin, pyoverdin

Materials

- MacConkey Agar
- Gram Stain
- Reagents for Indole, Methyl Red, Voges-Proskauer, Citrate, Catalase, Urease & Oxidase tests

Procedure

1. Inoculate samples on MacConkey Agar, then Gram Stain (see page 61)
2. Do IMViC [Indole, Methyl Red, Voges-Proskauer, Citrate] test (see page 73, 75, 77 & 79)
3. Do Catalase, Oxidase & Urease test (see page 91, 97 & 99)

Results

The characteristics of colonies will be described completely: [----------]

Review questions

What is the characteristic color of colonies of *Pseudomonas aeruginosa*? And what is the source of this color?

Week 11: Haemophilus, Bordetelia & Legioneliae Haemophilus genus

THE HAEMOPHILUS SPECIES

Morphology and Identification

This is a group of small, gram-negative, pleomorphic bacteria that require enriched media, usually containing blood or its derivatives, for isolation. *Haemophilus influenzae* type b is an important human pathogen.

Haemophilus influenza

Culture

On chocolate agar, flat, grayish brown colonies are present after 24 hours of incubation. IsoVitaleX in media enhances growth. *H influenzae* does not grow on sheep blood agar except around colonies of staphylococci ("satellite phenomenon").

THE BORDETELLAE

Morphology and Identification

Typical Organisms

The organisms are minute gram-negative coccobacilli. With toluidine blue stain, bipolar metachromatic granules can be demonstrated. A capsule is present.

Culture

Primary isolation of *B pertussis* requires enriched media. Bordet-Gengou medium (potato-blood-glycerol agar).

Growth Characteristics

The organism is a strict aerobe and it is oxidase and catalase positive but nitrate, citrate, and urea negative, the results of which are useful for differentiating among the other species of bordetellae

LEGIONELLA PNEUMOPHILA

Morphology and Identification

Typical Organisms

Legionellae are fastidious, aerobic gram-negative bacteria. They often stain poorly by Gram's method and are not seen in stains of clinical specimens.

Culture

Legionellae can be grown on complex media such as buffered charcoal yeast extract agar with α-ketoglutarate and iron (BCYE) at a pH of 6.9, temperature of 35°C, and 90% humidity. Antibiotics can be added to make the medium selective for Legionella species. Legionellae grow slowly; visible colonies are usually present after 3 days of incubation. Colonies that appear after overnight incubation are not Legionella species. Colonies are round or fl at with entire edges. They vary in color from colorless to iridescent pink or blue and are translucent or speckled.

Growth Characteristics

The legionellae are catalase positive. *L pneumophila* is oxidase positive; the other legionellae are variable in oxidase activity. *L pneumophila* hydrolyzes hippurate; the other legionellae do not. Most legionellae produce gelatinase and β-lactamase; *L micdadei* produces neither gelatinase nor β-lactamase.

Laboratory diagnostic techniques

Haemophilus influenza:

- Blood or CSF culture on chocolate agar
- PCR
- Antigen detection of capsule (latex particle agglutination)

Bordetella pertussis:

- Fastidious/delicate: Regan-Lowe or Bordet-Gengou media; either direct cough plates or nasopharyngeal cultures

- Difficult to culture from the middle of paroxysmal stage on
- Direct immunotluorescence (DFA) on nasopharyngeal smear
- PCR and serologic tests available

Legionella pneumophila:

- Diagnosis: DFA (direct fluorescent antibody) on biopsy, (+) by Dieterle silver stain
- Antigen urine test for one serogroup only
- Fourfold increase in antibody

Materials

- Blood, Chocolate & MacConkey Agar
- Gram & capsule Stain
- Reagents for Citrate, Gelatinase, Catalase, Urease & Oxidase tests

Procedure

1. Inoculate samples on Blood, Chocolate & MacConkey Agar, then Gram (see page 61) & capsule Stain (see page 63)
2. Do Citrate, Gelatinase, Catalase, Oxidase & Urease test (see page 79, 91, 97, 99 & 101)

Results

Media / Stain /Reaction	Bacterial Response
Blood Agar	
Chocolate Agar	
MacConkey Agar	
Gram Stain	
Capsule Stain	
Citrate	
Gelatinase	
Catalase	
Urease	
Oxidase	

Review questions

Can you culture *Bordetella pertussis* on Blood Agar?

- If yes, how?
- If no, why?

Week 12: Brucelia, Yersinia, Franciselia & Pasteurelia Brucella genus

THE BRUCELLAE

Morphology and Identification

The brucellae are obligate parasites of animals and humans and are characteristically located intracellularly. They are relatively inactive metabolically. *Brucella melitensis* typically infects goats; *Brucella suis*, swine; *Brucella abortus*, cattle; and *Brucella canis*, dogs. Other species are found only in animals.

Typical Organisms

The appearance in young cultures varies from cocci to rods 1.2 µm in length, with short coccobacillary forms predominating. They are gram negative but often stain irregularly, and they are aerobic, nonmotile, and nonspore forming.

Culture

Small, convex, smooth colonies appear on enriched media in 2–5 days.

Growth Characteristics

Fresh specimens from animal or human sources are usually inoculated on trypticase-soy agar or blood culture media. Whereas *B abortus* requires 5–10% CO_2 for growth, the other three species grow in air. Brucellae use carbohydrates but produce neither acid nor gas in amounts sufficient for classification. Catalase and oxidase are produced by the four species that infect humans. Hydrogen sulfide is produced by many strains, and nitrates are reduced to nitrites.

FRANCISELLA TULARENSIS

Morphology and Identification

Typical Organisms

F tularensis is a small, gram-negative coccobacillus. It is rarely seen in smears of tissue.

Specimens

Blood is taken for serologic tests. The organism may be recovered in culture from lymph node aspirates, bone marrow, peripheral blood, deep tissue, and ulcer biopsies.

Culture

Growth requires enriched media containing cysteine. In the past, glucose-cysteine blood agar was preferred, but *F tularensis* grows on commercially available hemin containing media such as chocolate agar, modified Thayer-Martin agar, and buffered charcoal yeast extract (BCYE) agar used to grow Legionella species. Media should be incubated in CO_2 at 35–37°C for 2–5 days.

Biochemical/Test Reactions .

- Oxidase: Negative .
- Catalase: Weak positive .
- Urease: Negative

YERSINIA PESTIS

Morphology and Identification

Morphology and Identification

Y pestis is a gram-negative rod that exhibits striking bipolar staining with special stains such as Wright, Giemsa, Wayson, or methylene blue. It is nonmotile. It grows as a facultative anaerobe on many bacteriologic media. Growth is more rapid in media containing blood or tissue fluids and fastest at 30°C. In cultures on blood agar at 37°C, colonies may be very small at 24 hours. A virulent inoculum, derived from infected tissue, produces gray and viscous colonies, but after passage in the laboratory, the colonies become irregular and rough. The organism has little biochemical activity, and this is somewhat variable.

Y pestis produces nonlactose-fermenting colonies on MacConkey agar, and it grows better at 25°C than at 37°C. The organism is catalase positive; indole, oxidase, urease negative; and nonmotile. The last two reactions are useful in differentiating *Y pestis* from other pathogenic yersiniae.

PASTEURELLA

Morphology and Identification

Pasteurella species are primarily animal pathogens, but they can produce a range of human diseases. Pasteurellae are nonmotile gram-negative coccobacilli with a bipolar appearance on stained smears. They are aerobes or facultative anaerobes that grow readily on ordinary bacteriologic media at 37°C. They are all oxidase positive and catalase positive but diverge in other biochemical reactions.

Laboratory diagnostic techniques

Brucella:

- Culture is hazardous.
- Serum agglutination test, fourfold increase in titer
    - Antibodies against Brucella > 1 : 160 considered positive

Francisella tularensis:

- Serodiagnosis; culture is hazardous.
- DFA

Yersinia pestis:

- Clinical specimens and cultures are hazardous.
- Serodiagnosis or direct immunotluorescence
- "Safety pin'' staining

Pasteurella:

- Rarely cultured because routine prophylaxis is common

Materials

- BloodAgar
- Chocolate Agar
- MacConkey Agar
- Gram Stain
- Reagents for Indole, Methyl Red, Voges-Proskauer, Citrate Catalase, Urease & Oxidase tests

Procedure

1. Inoculate samples on Blood, Chocolate & MacConkey Agar, then Gram Stain (see page 61)
2. Do IMViC [Indole, Methyl Red, Voges-Proskauer, Citrate] test (see page 73, 75, 77 & 79)
3. Do Catalase, Oxidase & Urease test (see page 91, 97 & 99)

Results

Serological test results if applicable: [---------]

Review questions

Why cultures of *Brucella, Francisella tularensis* & *Yersinia pestis* are considered hazardous?

Week 13: The Neisseriae & Unusual Bacterial Pathogens

Neisseriae genus & Mycobacteria

The Neisseriae

Morphology and Identification

Typical Organisms

The typical Neisseria is a gram-negative, nonmotile diplococcus, approximately 0.8 µm in diameter. Individual cocci are kidney shaped; when the organisms occur in pairs, the flat or concave sides are adjacent.

Culture

In 48 hours on enriched media (eg, modified Thayer-Martin, Martin-Lewis, GC-Lect, and New York City), gonococci and meningococci form convex, glistening, elevated, mucoid colonies 1–5 mm in diameter. Colonies are transparent or opaque, nonpigmented, and nonhemolytic. *Neisseria flavescens, Neisseria cinerea, Neisseria subflava,* and *Neisseria lactamica* may have yellow pigmentation. *Neisseria sicca* produces opaque, brittle, wrinkled colonies. *Moraxella catarrhalis* produces nonpigmented or pinkish gray opaque colonies.

Growth Characteristics

The neisseriae grow best under aerobic conditions, but some grow in an anaerobic environment. They have complex growth requirements. Most neisseriae oxidize carbohydrates, producing acid but not gas, and their carbohydrate patterns are a means of distinguishing them. The neisseriae produce oxidase and give positive oxidase reactions; the oxidase test is a key test for identifying them. When bacteria are spotted on a filter paper soaked with tetramethylparaphenylenediamine hydrochloride (oxidase), the neisseriae rapidly turn dark purple.

Mycobacteria

Morphology and Identification

The mycobacteria are rod-shaped, aerobic bacteria that do not form spores. Although they do not stain readily, after being stained, they resist decolorization by acid or alcohol and are therefore called “acid-fast” bacilli.

Typical Organisms

In tissue, tubercle bacilli are thin, straight rods measuring about 0.4 × 3 μm. On artificial media, coccoid and fi lamentous forms are seen with variable morphology from one species to another. The Ziehl-Neelsen technique of staining is used for identification of acid-fast bacteria.

Culture

Semisynthetic Agar Media

These media (eg, Middlebrook 7H10 and 7H11) contain defined salts, vitamins, cofactors, oleic acid, albumin, catalase, and glycerol; the 7H11 medium also contains casein hydrolysate. The albumin neutralizes the toxic and inhibitory effects of fatty acids in the specimen or medium.

Inspissated Egg Media

These media (eg, Löwenstein- Jensen) contain defined salts, glycerol, and complex organic substances (eg, fresh eggs or egg yolks, potato flour, and other ingredients in various combinations). Malachite green is included to inhibit other bacteria. Small inocula in specimens from patients will grow on these media in 3–6 weeks.

Broth media

Broth media (eg, Middlebrook 7H9 and 7H12) support the proliferation of small inocula. Ordinarily, mycobacteria grow in clumps or masses because of the hydrophobic character of the cell surface. If tweens (water-soluble esters of fatty acids) are added, they wet the surface and thus permit dispersed growth in liquid media. Growth is often more rapid than on complex media.

Growth Characteristics

Mycobacteria are obligate aerobes and derive energy from the oxidation of many simple carbon compounds. Increased CO_2 tension enhances growth. Biochemical activities are not characteristic, and the growth rate is much slower than that of most bacteria.

Laboratory diagnostic techniques

Neisseria meningitides:

- Ferments maltose
- Presumptive diagnosis by Gram stain of petechiae or CSF
- LATEX agglutination test b/c capsular polysaccharides
- PCR

Neisseria gonorrhoeae:

- Intracellular gram-negative diplococci in PMNs from urethral smear from symptomatic male are suggestive of *N. gonorrhoeae*.
- Commonly: diagnosis by genetic probes with amplification
- Culture (when done) on Thayer-Martin medium
- Oxidase-positive colonies
- Maltose not fermented
- No capsule

Mycobacterium tuberculosis:

- Microscopy of sputum: screen with auramine-rhodamine stain (fluorescent apple-green); no antibody involved; very sensitive; if positive, confirm with acid fast stain
- PPD skin test (Mantoux): measure zone of induration at 48-72 hours; positive if:
  - 5 mm in HIV+ or anyone with recent TB exposure; AIDS patients have reduced ability to mount skin test.
  - 10 mm in high-risk population: IV drug abusers, people living in poverty, or immigrants from high TB area
  - 15 mm in low-risk population
- **Positive skin test indicates only exposure but not necessarily active disease.**
- Quantiferon-TB Gold Test: measures interferon-gamma production when leukocytes exposed to TB antigens

- Slow-growing (3-6 weeks) colonies on Lowenstein-Jensen medium (faster new systems)
- Organisms produce niacin and are catalase-negative (68°C).
- No serodiagnosis

Materials

- Blood Agar
- Chocolate Agar
- MacConkey Agar
- Gram Stain
- Acid-Fast Stain
- Reagents for Oxidase test

Procedure

1. Inoculate samples on Blood, Chocolate & MacConkey Agar, then Gram (see page 61) & Acid-Fast Stain (see page 64)
2. Do Oxidase test (see page 97)

Results

Record complete colony morphology of *Neisseria* on different Agar Plates

Record Oxidase result for your diplococci sample

Review questions

State reasons behind inability to culture *Mycobacterium tuberculosis* in routine work.

Week 14: The Analytical Profile Index (API)

API is a method commonly used to identify a wide range of microorganisms. APIs consist of a number of plastic strips, each of which has about 20 miniature compartments containing biochemical reagents. Almost all cultivatable bacterial groups and more than 550 different species can be identified using the results of these API tests. These identification systems have extensive databases of microbial biochemical reactions. The numerical clusters derived from these tests identify different strains at selected levels of overall similarity (usually >80% at the species level) on the basis of the frequency with which they share traits. (see page 106)

A / Staining Techniques

Staining Technique 1. Gram's Stain Procedure:

1. A heat-fixed smear is flooded with a basic purple dye, usually crystal violet. Because the purple stain imparts its color to all cells, it is referred to as a primary stain.
2. After 1 minute, the crystal violet is drained off and washed with distilled water. The smear is then covered with Gram's iodine, **a mordant or helper**. When the iodine is washed off, both gram-positive and gram-negative bacteria appear dark violet or purple.
3. Next, the slide is washed with alcohol (95% ethanol) or an alcohol-acetone solution. This solution is a decolorizing agent. When the procedure is carried out, the slide is held at an angle and 95% ethanol is poured until the draining solution no longer has a purple tint.
4. The alcohol is now rinsed off with distilled water and the slide is then stained with safranin, a basic red dye known as the counter stain. The staining process is allowed to be carried out for 2-3 minutes. The smear is washed again, heat dried and examined microscopically.

Staining Technique 2. Endospore Stain Procedure

1. Aseptically transfer one species of bacterium with an inoculating loop to each of the respective slides, air dry (or use a slide warmer), and heat-fix.
2. Place the slide to be stained on a hot plate or boiling water bath equipped with a staining loop or rack. Cover the smear with paper toweling that has been cut the same size as the microscope slide.
3. Soak the paper with the malachite green staining solution. Gently heat on the hot plate (**just until the stain steams**) for 5 to 6 minutes after the malachite green solution begins to steam. Replace the malachite green solution as it evaporates so that the paper remains saturated during heating.

   Do not allow the slide to become dry.
4. Remove the paper using forceps, allow the slide to cool, and rinse the slide with water for 30 seconds.
5. Counterstain with safranin for 60 to 90 seconds.
6. Rinse the slide with water for 30 seconds.
7. Blot dry with bibulous paper and examine under oil immersion. A coverslip is not necessary. The spores, both endospores and free spores, stain green; vegetative cells stain red.

Staining Technique 3. Capsule Stain Procedure:

1. With a wax pencil, label the left hand corner of a clean glass slide with the name of the bacterium that will be stained.

2. Aseptically transfer a loopful of culture with an inoculating loop to the slide. Allow the slide to air dry.

[Do not heat-fix!] Heat-fixing can cause the bacterial cells to shrink and give a false appearance to the capsule.

3.Place the slide on a staining rack. Flood the slide with crystal violet and let stand for 4 to 7 minutes.

4. Rinse the slide thoroughly with 20% copper sulfate.

5. Blot dry with bibulous paper.

6. Examine under oil immersion (a cover slip is not necessary). Capsules appear as faint halos around dark cells.

Staining Technique 4. Acid Fast Stain Procedure:

1. Prepare a smear consisting of a mixture of E. coli and M. phlei.

2. Allow the smear to air dry and then heat-fix.

3. Place the slide on a hot plate that is within a chemical hood (with the exhaust fan on), and cover the smear with a piece of paper towelling that has been cut to the same size as the microscope slide. Saturate the paper with Ziehl's carbolfuchsin. Heat for 3 to 5 minutes.

Do not allow the slide to dry out, and avoid excess flooding! Also, prevent boiling by adjusting the hot plate to a proper temperature.

A boiling water bath with a staining rack or loop held 1 to 2 inches above the water surface also works well. (Instead of using a hot plate to heatdrive the carbolfuchsin into the bacteria, an alternate procedure is to cover the heat-fixed slide with a piece of paper towel. Soak the towel with the carbolfuchsin and heat, well above a Bunsen burner flame).

4. Remove the slide, let it cool, and rinse with water for 30 seconds.

5. Decolorize by adding acid-alcohol drop by drop until the slide remains only slightly pink. This requires 10 to 30 seconds and must be done carefully.

6. Rinse with water for 5 seconds.

7. Counterstain with alkaline methylene blue for about 2 minutes.

8. Rinse with water for 30 seconds.

9. Blot dry with bibulous paper.

10. There is no need to place a coverslip on the stained smear. Examine the slide under oil immersion and record your results in the report for this exercise. Acid-fast organisms stain red; the background and other organisms stain blue or brown.

11. Examine the prepared slide of *Mycobacterium phlei*.

B / Metabolic Activities of Bacteria

Background

Microbial metabolic processes are complex, but they permit the microbiologist to distinguish among microorganisms grown in culture. Bacteria, especially, are identified by inoculating pure, isolated colonies into media that contain one or more specific biochemical. The biochemical reactions that take place in the culture can then be determined by relatively simple indicator reagents, included in the medium or added to the culture later.

Some bacteria ferment simple carbohydrates, producing acidic, alcoholic, or gaseous end products. Many different species are distinguished on the basis of the carbohydrates they do or do not utilise, as well as by the nature of end products formed during fermentation. Still others break down more complex carbohydrates, such as starch. (see page 106)

Excercise 1: Simple Carbohydrate Fermentations

1.1. Principle

The identification of some bacteria is based on that what nutrients the bacteria can utilize and the end products produced in the process. These characteristics are controlled by the enzymes which the bacteria produce. The pattern of sugars fermented may be unique to a particular species or strain as the type of enzyme(s) produced by bacteria is genetically controlled. Fermentation products are usually acid (lactic acid, acetic acid etc.), neutral (ethyl alcohol etc.), or gases (carbon dioxide, hydrogen, etc.).

Media for testing carbohydrate fermentation are often prepared as tubed broths, each tube containing a small inverted “fermentation” (or Durham) tube for trapping any gas formed when the broth is inoculated and incubated, as shown in the figure below.

Figure 20 When Durham tubes are placed inside broth tubes, gas produced by fermentation of carbohydrates in the medium can be visualized as a bubble in the inner tube (right). The organism on the middle does not produce gas when fermenting carbohydrates. Light red colour of the tube on the left indicates no fermentation.

Each broth contains essential nutrients, a specific carbohydrate, and a colour reagent to indicate a change in pH if acid is produced in the culture (the broth is adjusted to a neutral pH when prepared). Organisms that grow in the broth but do not ferment the carbohydrate produce no change in the colour of the medium, and no gas is formed. Some organisms may

produce acid products in fermenting the sugar, but no gas, whereas others may form both acid and gas. In some cases, organisms that do not ferment the carbohydrate use the protein nutrients in the broth, thereby producing alkaline end products, a result that is also evidenced by a change in indicator colour.

Objective

To distinguish bacterial species on the basis of simple carbohydrate fermentation

1.2. Materials

- Tubed phenol red glucose broth with Durham tubes
- Tubed phenol red lactose broth with Durham tubes
- Tubed phenol red sucrose broth with Durham tubes
- Slant cultures of *Escherichia coli, Serratia marcescens, Pseudomonas aeruginosa,* and *Proteus vulgaris*

1.3. Procedure

1. Inoculate growth from each of the four cultures into separate tubes of each of the three carbohydrate broths. Be certain no bubbles are inside the Durham tubes before inoculation.
2. Label each of the 12 inoculated tubes with the name of the carbohydrate it contains and the name of the bacterial culture.
3. Incubate at 35°C for 24 hours.

1.4. Results

Record your results in the following table. Use these symbols to indicate specific changes observed in the broths.

A = acid production

K = alkaline colour change

N = neutral (no change in colour)

G = gas formation

Name of Organism	Glucose	Lactose	Sucrose

1.5. Review Questions

- What is the color of phenol red at an acid pH?
- What is the function of a Durham tube?

Excercise 2: Starch Hydrolysis

2.1. Principle

Some microorganisms split apart (hydrolyse) large organic molecules and then use the component parts in further metabolic processes. Starch is a polysaccharide that is hydrolysed by some bacteria. When iodine is added to the intact starch molecule a blue-coloured complex forms. If starch is hydrolysed by bacterial enzymes, however, it is broken down to simple sugars (glucose and maltose) that do not complex with iodine, and no colour reaction is seen. The medium for this test is a nutrient agar containing starch, prepared in a petri plate. The organism to be tested is streaked on the plate. When the culture has grown, the plate is flooded with Gram's iodine solution. The medium turns blue in all areas where the starch remains intact. The areas of medium surrounding organisms that have hydrolysed the starch remain clear and colourless.

2.2. Objective

To distinguish bacterial species on the basis of starch hydrolysis

2.3. Materials

- Starch agar plates
- Slant cultures of *Escherichia coli*, *Pseudomonas aeruginosa*, and *Bacillus subtilis*
- Gram's iodine solution

2.4. Procedure

1. Take one starch plate, invert it, and with your marking pencil mark three triangular compartments on the back of the dish.
2. Inoculate one section of the agar with *E. coli*, using back-and-forth streaking; another section with *B. subtilis*; and the third with *P. aeruginosa.*
3. Label each section of the plate on the back of the dish with the name of the organism streaked in that area.
4. Incubate 24 to 48 hours at 35°C.

5. When the cultures have grown, drop Gram's iodine solution onto the plate until the entire surface is lightly covered.

2.5. Results

Read and record your results in the table.

Name of Organism Colour around Colony Positive or Negative for Starch Hydrolysis

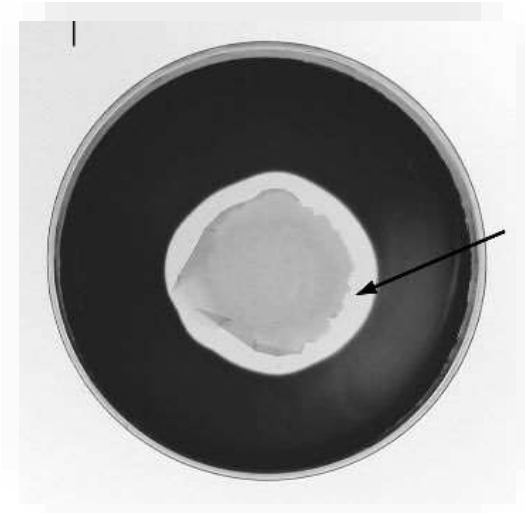


Figure 21 Clear Zone of Unstained Starch Culture Media due to Bacterial Utilization

2.6. Review Questions

- Why is iodine used to detect starch hydrolysis?

Excercise 3: Oxidation-Fermentation (OF) test

3.1. Principle

Fermentative organisms utilize the carbohydrate in both the open and sealed tubes as shown by a change in colour of the medium from green to yellow. Oxidative organisms are able to utilize the carbohydrate only in the open tube.

3.2. Objective

The test is used to differentiate those organisms that oxidize carbohydrate (aerobic utilization) from those organisms that ferment carbohydrate (anaerobic utilization).

3.3. Materials

- Slant culture of *Pseudomonas aeruginosa*
- Slant culture of *Escherichia coli*
- Tryptone or peptone agar medium containing carbohydrate (glucose or other carbohydrate) and the indicator is bromothymol blue.

3.4. Procedure

1. Inoculate the test organism to the bottom of two tubes of sterile O-F medium (always use a heavy inoculum).
2. Cover the inoculated medium of one tube with a 10 mm deep layer of sterile paraffin oil or molten wax.
3. Incubate the tubes at 35-37°C for 14 days.
4. Examine daily for carbohydrate utilization.

3.5. Results

Open tube	Sealed tube	Interpretation	Example

Yellow	Green	Oxidative organism	
Yellow	Yellow	Fermentative organism	
Green/Blue	Green	No utilization of carbohydrates	

3.6. Review Questions

- Name the indicator used in this experiment.

Excercise 4: Indole test

4.1. Principle

Certain bacteria are able to breakdown the amino acids tryptophan into indole. The test organism is cultured in a tryptophan containing medium and indole production is detected by Kovac's reagent which contains (p)-dimethylaminobenzaldehyde. This reacts with the indole to produce a red colour compound.

4.2. Objective

To determine the ability of bacteria to produce indole from utilisation of tryptophan (amino acid)

4.3. Materials

- Peptone water
- Kovac's reagent
- Slant culture of *Escherichia coli*
- Slant culture of *Enterobacter cloacae*

4.4. Procedure

1. Inoculate peptone water (or any other tryptophan containing media) with the test organism.
2. Incubate at 35-37°C overnight.
3. Add 0.5 ml of Kovac's reagent down the inner wall of the tube.
4. Shake well and examine about 1 minute later. A red colour in the reagent layer indicates the production of indole.

4.5. Results

Bright red colour ring - Indole positive

No colour change - Indole negative

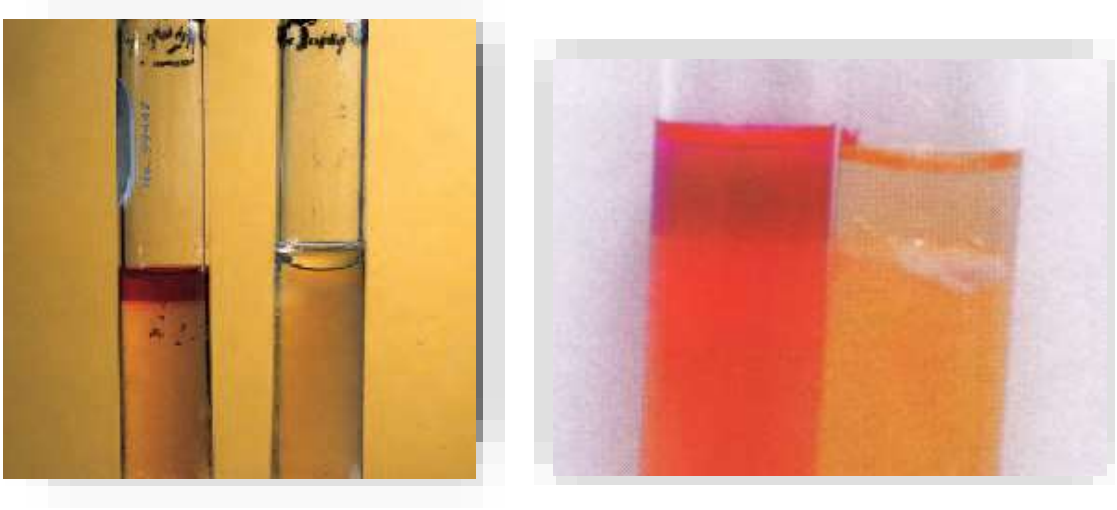


Figure 22 Indole Test; Positive [LEFT] & Negative [RIGHT]

4.6. Review Questions

- List ingredients of the culture media used in this experiment.

Excercise 5: Methyl Red (MR) test

5.1. Principle

The test detects the ability of some organisms to produce sufficient acidity from the glucose fermentation, after prolonged incubation, overcoming the pH buffering system of the medium.

5.2. Objective

Determine whether tested organism is able to produce as much acid as buffering system disturbing amount can do

5.3. Materials

- Glucose phosphate broth
- Methyl Red solution
- Slant culture of *Escherichia coli*
- Slant culture of *Enterobacter cloacae*

5.4. Procedure

1. Inoculate a colony of the test organism in 2 ml of sterile glucose phosphate broth.
2. Incubate at 35-37°C overnight.
3. Add 5 drops of Methyl Red solution.
4. Mix and read immediately.
5. Appearance of a red colour at the surface indicates a positive reaction.

5.5. Results

Bright red colour - Methyl Red positive

Orange / Yellow colour - Methyl Red negative

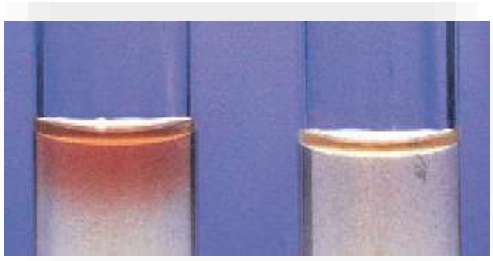


Figure 23 Methyl Red Test; Positive [LEFT] & Negative [RIGHT]

5.6. Review Questions

- Why did you shake the MR culture?

Excercise 6: Voges-Proskauer (VP) test

6.1. Principle

Some organisms produce acetoin (acetylmethylcarbinol), a neutral-reacting end product, as the main end product of the glucose fermentation. Acetoin is oxidized into diacetyl in the presence of atmospheric oxygen and alkaline condition which forms a pink compound with the creatine.

6.2. Objective

To identify organisms that mainly produce Acetoin from the fermentation of Glucose

6.3. Materials

- Glucose phosphate peptone water
- α-naphthol solution
- 40% KOH aqueous solution
- Slant culture of *Klebsiella pneumoniae*
- Slant culture of *Escherichia coli*

6.4. Procedure

1. Inoculate the test organism in to 2 ml of sterile glucose phosphate peptone water.
2. Incubate at 35-37°C for 48 hours.
3. Add 0.6 ml of α-naphthol solution followed by 0.2 ml of 40% KOH aqueous solution.
4. Shake well for maximum aeration.
5. Remove the bottle cap and leave for 1 hour at room temperature.
6. A pink – red colour indicates a positive reaction.

6.5. Results

Pink-red colour -VP positive

No pink-red colour -VP negative

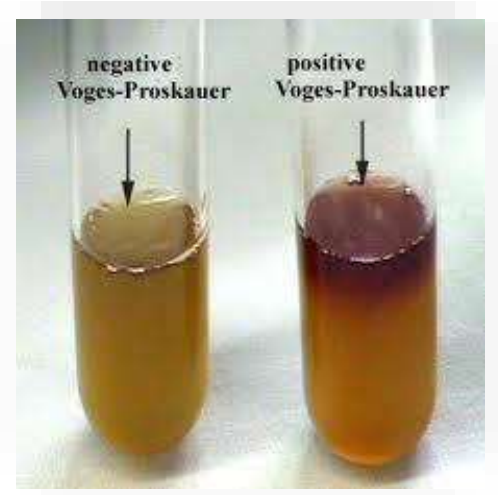


Figure 24 Voges Proskauer Test

6.6. Review Questions

- Why did you shake the VP culture?

Excercise 7: Citrate test

7.1. Principle

The test detects the ability of an organism to utilize citrate as the sole carbon and energy source and ammonia as sole source of the nitrogen.

7.2. Objective

To determine whether the tested organism can utilize citrate as sole carbon source or not

7.3. Materials

- Koser's citrate medium
- Or Simmons' citrate medium
- Slant culture of *Klebsiella pneumoniae* sub species *aerogenes*
- Slant culture of *Escherichia coli*

7.4. Procedure

1. Make a light suspension of the organism in sterile water or saline.
2. Inoculate Koser's or Simmons' citrate medium using a sterile straight wire.
3. Incubate at 35-37°C for up to 7 days, checking daily for growth.
4. In Koser's medium look for the turbidity; in Simmons' medium look for a colour change.

7.5. Results

Koser's Citrate

*Medium becomes turbid – Citrate positive

*No turbidity - Citrate negative

Simmon citrate

*Blue colour and growth on the streak line - Citrate positive

*No colour change and no growth - Citrate negative

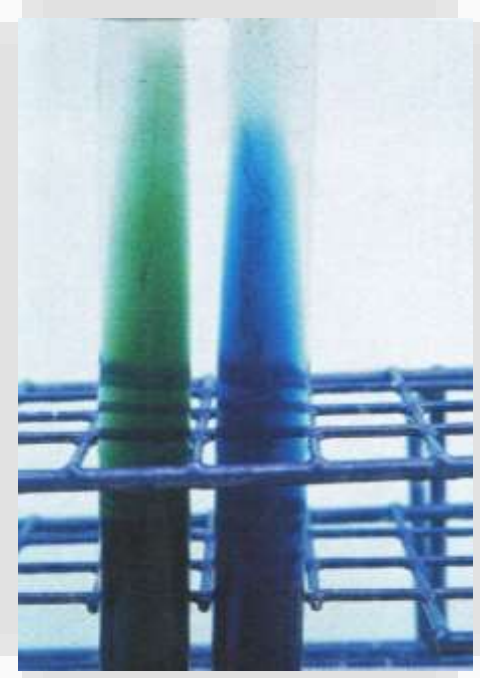


Figure 25 Positive Citrate Utilization Test [RIGHT] & Negative [LEFT]

7.6. Review Questions

- What is the culture media used for Citrate test & what is the significant ingredient?

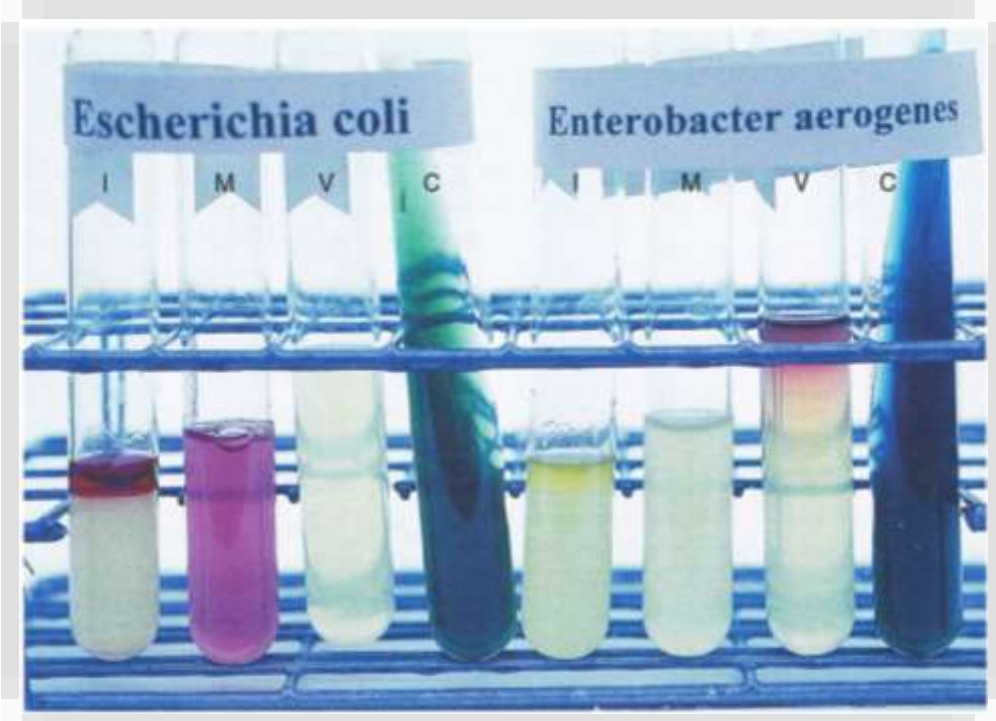


Figure 26 IMViC [Indole, Methyl Red, Voges-Proskauer, Citrate] set of reactions used to differentiate between E. coli with reaction pattern of [+, +, -, -] & Enterobacter aerogenes with [-, -, +, +]

Excercise 8: Production of Indole and Hydrogen Sulphide, and Motility

8.1. Principle

Indole is a by-product of the metabolic breakdown of the amino acid tryptophan used by some microorganisms. The presence of indole in a culture grown in a medium containing tryptophan can be readily demonstrated by adding Kovac's reagent to the culture. If indole is present, it combines with the reagent to produce a brilliant red colour. If it is not present, there will be no colour except that of the reagent itself. This test is of great value in the battery used to identify enteric bacteria.

Hydrogen sulfide is produced when amino acids containing sulfur are metabolized by microorganisms. If the medium contains metallic ions, such as lead, bismuth, or iron (in addition to an appropriate amino acid), the hydrogen sulfide formed during growth combines with the metallic ions to form a metal sulfide that blackens the medium. The most convenient medium for testing for indole and/or hydrogen sulfide production is SIM medium (SIM is an acronym for sulfide, indole, and motility). This is a tubed semisolid agar that can also be used to demonstrate bacterial motility. It is inoculated by stabbing the wire loop (or preferably a straight wire inoculating needle) straight down the middle of the agar to about one-fourth the depth of the medium and withdrawing the wire along the same path.

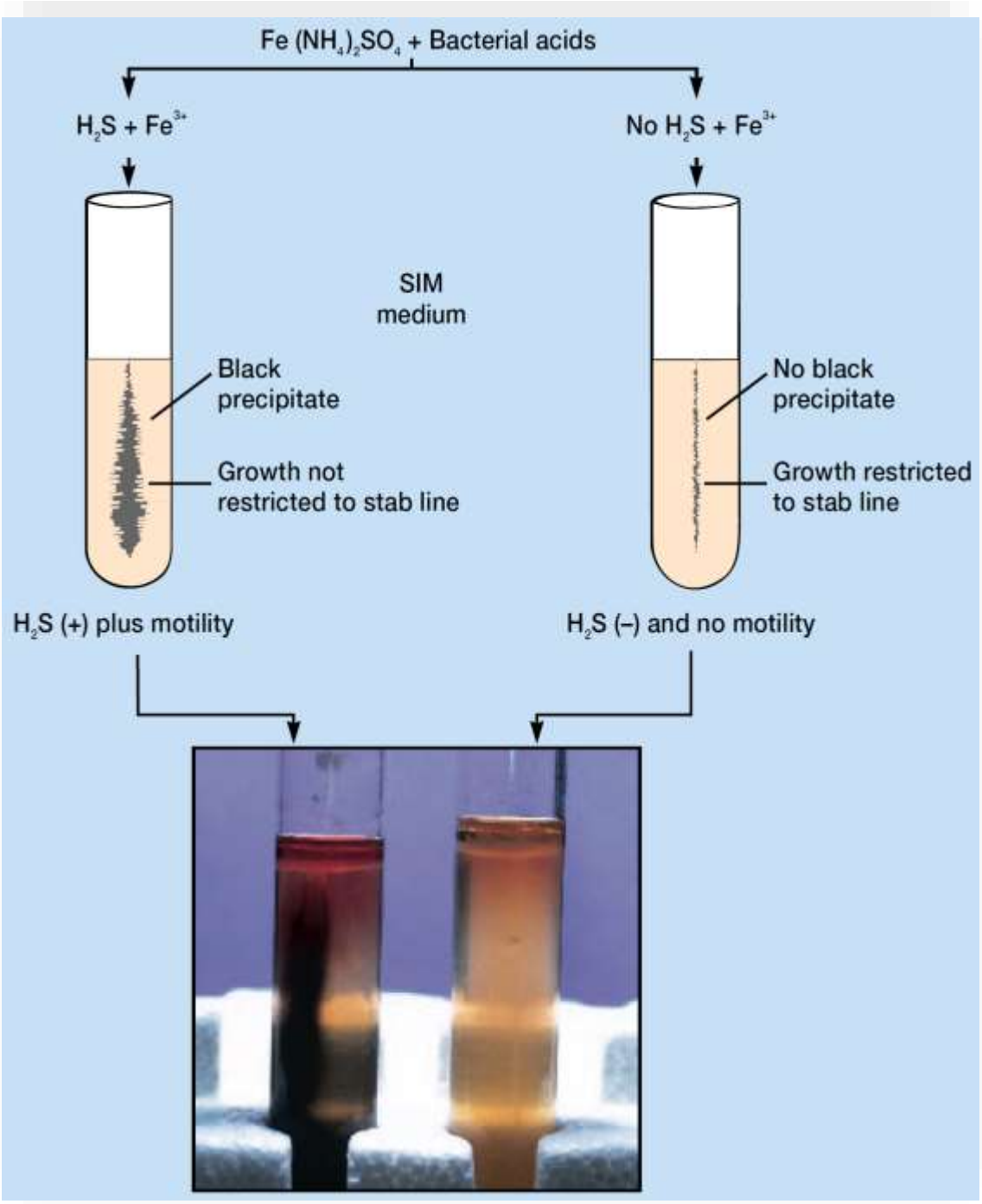


Figure 27 Hydrogen Sulfide Production and/or Motility in SIM Media

8.2. Objective

To observe how a single medium can be used to test for three distinguishing features of bacterial growth

8.3. Materials

- Tubes of SIM medium
- Xylene
- Kovac's reagent
- 5-ml pipettes
- Bulb or other pipette aspiration device
- Slant cultures of *Escherichia coli, Proteus vulgaris,* and *Klebsiella pneumoniae*
- Broth cultures of *Escherichia coli, Proteus vulgaris,* and *Klebsiella pneumoniae*

8.4. Procedure

1. Inoculate growth from each of the three slant cultures into separate tubes of SIM medium. Stab the inoculating wire straight down through the agar for a distance of about one-fourth of its depth. Quickly withdraw the wire along the same path (do not move it around in the agar).
2. Incubate the tubes at 35°C for 24 hours.
3. Examine the tubes for evidence of hydrogen sulphide production (browning or blackening of the medium). Record results.
4. Examine the tubes for evidence of motility of the organism. A motile species grows away from the line of stab into the surrounding agar. Lines of growth, or even general turbidity, can be seen throughout the tube. The growth of a nonmotile organism is restricted to the path of the stab. Record your observations.
5. Set up a hanging-drop or wet-mount preparation of each broth culture to confirm results observed in SIM medium for motility.
6. Perform the Kovac test for indole.
    a. Using a pipette bulb or other aspiration device, pipette 0.5 ml of xylene into the SIM tube (it will layer over the top surface of the agar).
    b. Pipette 0.5 ml of Kovac's reagent in the same way as you did the xylene and add it to the SIM tube.
    c. Observe the colour of the xylene layer, and record.

8.5. Results

Record your observations and results in the table.

Name of Organism	Sulphide	Indole	Motility	
			SIM Medium	Hanging Drop

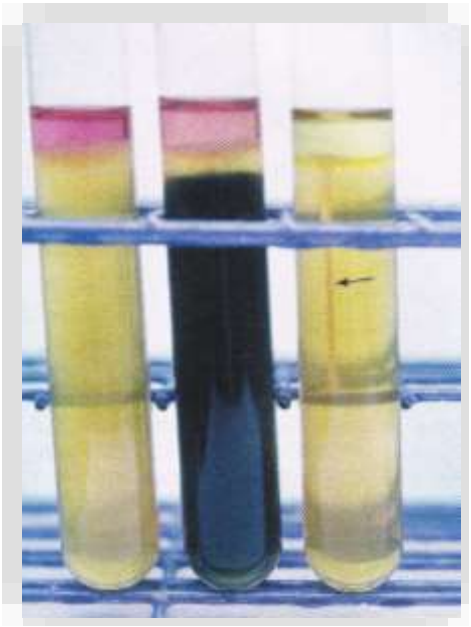


Figure 28 Reactions in Sulfide Indole Motility medium [SIM] RIGHT negative for all, MIDDLE is positive for all whilst LEFT is positive for Motility and Indole, but negative for Sulfide

8.6. Review Questions

- Name one indole-positive organism.
- How is indole produced in SIM medium? How is it detected?
- How is hydrogen sulphide demonstrated in this medium?
- Name two methods for determining bacterial motility.
- Why is it essential to have pure cultures for biochemical tests?
- Could a pH-sensitive colour indicator be used to reveal the presence of a contaminant in a fluid that should be sterile? Explain.

Excercise 9: Kligler Iron Agar (KIA)

9.1. Principle

There is an indicator (phenol red) which will change the colour of medium when acid is produced. Ratio of lactose to glucose is 10:1 in the medium [TSI Triple Sugar Iron agar is similar but sucrose is also added]. An alkalinity is produced in the slant due to the oxidative deamination of the amino acids. The organism that has the ability to ferment glucose changes the colour of the butt to yellow. But the acidity produced by glucose is not sufficient to overcome the alkalinity produced by oxidative deamination of the amino acids in the slant and change the colour of the slant. The organism that has the ability to ferment lactose changes the colour of the butt as well as the slant.

9.2. Objective

KIA is used to look for the ability of an organism to ferment lactose and glucose and to produce hydrogen sulphide. It is further used to check the production of gas while fermentation of glucose and lactose.

9.3. Materials

- KIA agar slants
- Slant Culdtures of:
    - *Alcaligenes faecalis*
    - *Escherichia coli*
    - *Proteus vulgaris*
    - *Pseudomonas aeruginosa*
    - *Shigella flexneri*

9.4. Procedure

1. Label each of the KIA agar slants with the name of the bacterium to be inoculated. Use one of the tubes as a control. Place your name and date on each tube.
2. Using aseptic technique, streak the slant with the appropriate bacterium and then

3. stab the butt. Screw the caps on the tubes but do not tighten!
4. Incubate for only 18 to 24 hours at 35°C for changes in the butt and on the slant. Tubes should be incubated and checked daily for up to seven days in order to observe blackening.
5. Then
6. Examine all slant cultures for the colour of the slant and butt, and for the presence or absence of blackening within the medium.
7. Record your results in the report
8. Using a straight wire inoculating needle, stab the butt of the tube and streak the slant; the closure should not be tight

9.5. Results

9.5.1. Different patterns in KIA

1. Un-changed slant and butt: No fermentation of glucose or lactose. This is seen with most strains of Acinetobacter.

2. Red slant with unchanged butt: Oxidative deamination of amino acids and no fermentation of glucose or lactose. This is seen with most strains of Pseudomonas.

3. Red slant and yellow butt: Oxidative deamination of amino acids, fermentation of glucose and no fermentation of lactose. This is seen with most strains of non-lactose fermenting Enterobacteriaceae.

4. Yellow slant and yellow butt: Fermentation of lactose and glucose. This occurs with Escherichia coli and other lactose fermenting enterobacteriaceae.

5. Cracks and bubbles in the medium: Gas production from glucose fermentation.

6. Blackening along the stab line or throughout the medium: Production of hydrogen sulfide. Salmonella Typhi produces small amount of blackening whereas other Salmonella species and few other species of enterobacteriaceae causes extensive blackening.

Figure 29 Kligler Iron Agar Reactions

Interpretation. (a) The tube on the left is an uninoculated control. Notice the red color. (b) The second tube from the left has a yellow slant (acid), yellow butt (acid), gas production at the bottom of the tube, and no H_2S production. This would indicate a weak lactose fermenter. (c) The third tube from the left has a red slant (alkaline), red butt (alkaline), and the black indicates H_2S production, but no gas. (d) The tube on the right has a red slant (alkaline), yellow butt (acid), H_2S production, but no gas production. This would indicate a nonlactose fermenter.

Table 2 Reporting KIA Test

	Tube a	Tube b	Tube c	Tube d
Slant	–	A	K	K
Butt	–	A	K	A
Gas	–	+	–	–
H_2S	–	–	+	+

9.6. Review Questions

1. For what bacteria would you use the TSI test?

2. Why must TSI test observations be made between 18 to 24 hours after inoculation?

3. Distinguish between an acid and alkaline slant.

4. What is the purpose of thiosulfate in the TSI agar?

5. What is meant by a saccharolytic bacterium? What reaction would it give in a TSI tube?

6. Why is there more lactose and sucrose in TSI agar than glucose?

7. What is the pH indicator in TSI agar?

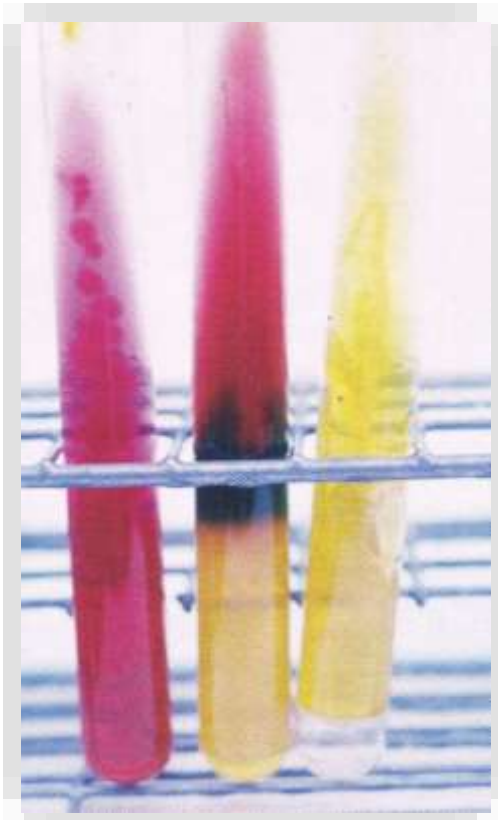


Figure 30 KIA reactions.

C / Activities of Bacterial Enzymes

Background

Enzymes are the most important chemical mediators of every living cell's activities. These organic substances catalyse, or promote, the uptake and use of raw materials needed for synthesis of cellular components or for energy. Enzymes are also involved in the breakdown of unneeded substances or of metabolic side products that must be eliminated from the cell and returned to the environment. As catalysts, enzymes promote changes only in very specific substances or substrates, as they are often called. Thus, in the previous exercise, the changes produced in simple carbohydrates and in starch substrates were brought about by different, specific enzymes.

Since enzymes appear to be limited to particular substrates, it follows that each bacterial cell must possess a large battery of different enzymes, each mediating a different metabolic process. They are identified in terms of the type of change produced in the substrate. In naming them, the suffix -ase is usually added to the name of the substrate affected. Thus, urease is an enzyme that degrades urea, gelatinase breaks down gelatin (a protein), penicillinase inactivates penicillin, and so on.

In this exercise, we shall see how many bacterial enzymes are demonstrated and how their recognition in bacterial cultures leads to identification of species.

Excercise 10: Activity of Catalase

10.1. Principle

Many bacteria produce the enzyme catalase, which breaks down hydrogen peroxide, liberating oxygen. The simple test for catalase can be very useful in distinguishing between organism groups. The hydrogen peroxide can be added directly to a slant culture or to bacteria smeared on a clean glass slide. The test should not be performed with organisms growing on a blood containing medium because catalase is found in red blood cells.

10.2. Objective

To observe bacterial catalase activity

10.3. Materials

- 3% hydrogen peroxide
- Capillary pipettes
- Pipette bulb or other aspiration device
- Nutrient agar slant cultures of *Staphylococcus epidermidis* and *Enterococcus faecalis*
- Clean glass slides
- China-marking pencil or marking pen

10.4. Procedure

1. Divide a clean glass slide into two sections with your marking pen or pencil.
2. With a sterilized and cooled inoculating loop, pick up a small amount of the Staphylococcus culture from the nutrient agar slant. Smear the culture directly onto the left-hand side of the slide. The smear should be about the size of a pea.
3. Sterilize the loop again and smear a small amount of the Enterococcus culture on the right-hand side of the slide.
4. With the capillary pipette, place one drop of hydrogen peroxide over each smear. Be careful not to run the drops together.

5. Observe the fluid over the smears for the appearance of gas bubbles. Record the results in the chart. Discard the slide in a jar of disinfectant.
6. Hold the slant culture of the Staphylococcus in an inclined position and pipette 5 to 10 drops of hydrogen peroxide onto the surface with the bacterial growth. Observe closely for the appearance of gas bubbles.
7. Repeat the procedure with the Enterococcus culture. Note whether oxygen is liberated and bubbling occurs.

10.5. Results

Record your observations and conclusions in this chart.

Organism	Slide Preparation		Tube Culture	
	Bubbling	Catalase	Bubbling	Catalase
Staphylococcus epidermidis				
Enterococcus faecalis				

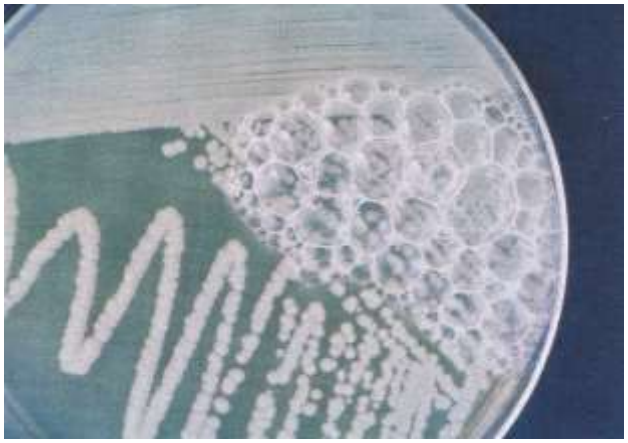


Figure 31 Catalase Test [Notice the Bubbles; Oxygen libration]

10.6. Review Questions

- What is the substrate of the catalase reaction? Why are bubbles produced in a positive catalase test?
- Why will a false-positive catalase test result if the organisms are tested on a medium containing blood?

Excercise 11: Activity of Coagulase

11.1. Principle

Coagulase is an enzyme mainly found in Staphylococcus aureus. Therefore, this test will help to distinguish Staphylococcus aureus from other commonly isolated Staphylococci.

Two forms of coagulase exist. One is bound to the cell and other one is excreted from the cell as an enzyme. Bound coagulase is known as “clumping factor” which acts directly on the fibrinogen in plasma and convert it to fibrin to form a coagulum. When the coagulase is released as an enzyme, also called “free coagulase”, reacts with the coagulase reacting factor (CRF) in plasma to form thrombin that then acts on fibrinogen in the plasma to form a fibrin clot.

Fibrinogen → Fibrin

EDTA blood is used. Citrated blood may give false positive result.

11.2. Objective

To implement coagulase activity in distinguishing between pathogenic (coagulase +ve) and non-pathogenic (coagulase -ve) staphylococci

11.3. Materials

- Plasma
- Physiological saline.
- Broth culture of *Staphylococcus aureus*
- Broth culture of *Staphylococcus epidermidis*

11.4. Procedure

11.4.1. Slide method

1. Place two separate drops of physiological saline on a clean slide.

2. Emulsify a colony and make two thick suspensions.
3. If there is no auto agglutination add a drop of plasma to one of the suspensions and mix gently.
4. Look for clumping within 10 seconds in the plasma added suspension.
5. If auto agglutination is present, this slide test cannot be read.

11.4.2. Tube test

11.4.2.1. Method 1

1. Prepare 1:10 diluted plasma in physiological saline.
2. Add 0.5 ml of diluted plasma in a tube.
3. Add 0.1 ml of an 18-24hour broth culture of the organisms.
4. Mix gently and incubate at 35-37°C.
5. Examine for formation of a coagulum after 1 hour; if no coagulum, examine at 2 and 6 hours.
6. Negative tubes should be left at room temperature overnight and re-examined.
7. Use negative and positive controls.

11.4.2.2. Method 2

1. Mix 0.5 ml undiluted plasma with an equal volume of an 18-24hour broth culture. Incubate at 37oC for 4 hours.
2. Examine after 1 and 4 hours for coagulum.
3. Negative tubes should be left at room temperature overnight and then re-examine.

11.5. Results

Formation of coagulum - Positive test

No coagulum - Negative test

11.6. Review Questions

- What is coagulase?
- How does the enzyme coagulase function?

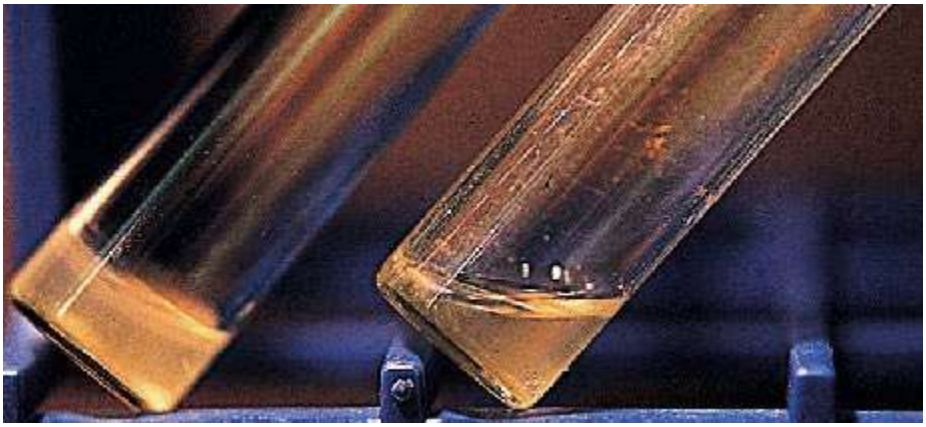
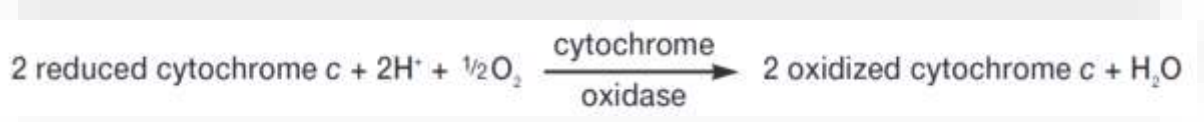


Figure 32 Coagulase Positive [LEFT] & Negative [RIGHT]

Excercise 12: Activity of Oxidase

12.1. Principle

This test indicates the presence of enzyme cytochrome oxidase. When the organism is oxidase-producing, the phenylenediamine in the reagent will be oxidized to a deep purple colour.



12.2. Objective

To examine whether the sample bacteria contains the respiratory enzyme cytochrome c oxidase or not

12.3. Materials

- Slant cultures of Escherichia coli & Pseudomonas aeruginosa
- Freshly prepared tetramethyl-p-phenylenediamine dihydrochloride (oxidase) reagent to make 10 ml
- Tetramethyl-p-phenylenediamine dihydrochloride 0.1 g
- Distilled water 10 ml
- Dissolve the chemical in the water. The reagent is not stable. It is therefore best prepared immediately before use.

12.4. Procedure

1. Place a piece of filter paper in a clean Petri dish and add 2-3 drops of freshly prepared oxidase reagent.
2. Using a piece of sterile stick or glass rod (not an oxidized wire loop), remove a colony of the test organism and smear it on the filter paper.
3. Look for the development of a blue-purple colour within a few seconds.

4. Same can be done using a sterile cotton bud impregnated with the oxidase solution.

12.5. Results

Record your observations within 10 seconds.

Figure 33 Oxidase Positive [RIGHT] & Negative [LEFT]

12.6. Review Questions

1. What metabolic property characterizes bacteria that possess oxidase activity?

2. What is the importance of cytochrome oxidase to bacteria that possess it?

3. Do anaerobic bacteria require oxidase? Explain your answer.

4. What is the function of the test reagent in the oxidase test?

5. The oxidase test is used to differentiate among which groups of bacteria?

6. Why should nichrome or other iron-containing inoculating devices not be used in the oxidase test?

7. Are there limitations to the oxidase test?

Excercise 13: Activity of Urease

13.1. Principle

The test detects the ability of an organism to produce enzyme urease. The test organism is cultured in a medium which contains urea and the indicator phenol red. If the organism produces urease, the enzyme will breakdown the urea to ammonia and carbon-dioxide. The medium becomes alkaline when ammonia is released which is indicated by the red-pink colour.

13.1. Objective

To observe the activity of urease and to distinguish bacteria that produce it from those that do not

13.2. Materials

- Tubes of urea broth or agar
- Slant cultures of Escherichia coli and Proteus vulgaris

13.3. Procedure

1. Inoculate a tube of urea broth or agar with E. coli, and another with the Proteus culture.
2. Incubate the tubes at 35°C for 24 hours.

13.4. Results

Record your observations.

Name of Organism	Colour of Urea Medium		Urease	
	Before Culture	After Culture	Positive	Negative

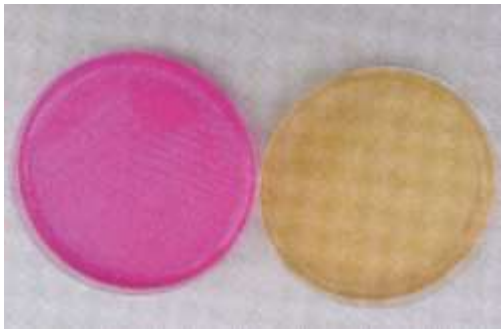


Figure 34 Urease Positive [LEFT] & Negative [RIGHT]

13.5. Review Questions

- What is a catalyst?
- Define an enzyme and a substrate. What is the value of enzyme tests in diagnostic microbiology?
- What happens to urea in the presence of urease?

Excercise 14: Activity of Gelatinase

14.1. Principle

Gelatin is a simple protein. When in solution, it liquefies at warm temperatures above 25°C. At room temperature or below it becomes solid. When bacteria that produce the enzyme gelatinase are grown in a gelatin medium, the enzyme breaks up the gelatin molecule and the medium cannot solidify even at cold temperatures. An alternative method for detecting gelatinase production is the use of X-ray film that is coated with a green gelatin emulsion. Organisms that produce gelatinase remove the emulsion from the strip.

14.2. Objective

To observe the usefulness of a gelatinase test in distinguishing between bacterial species

14.3. Materials

- Tubes of nutrient gelatin medium
- 1.25 inch strips of exposed, undeveloped X-ray film or gelatin strip
- Tubes containing 0.5 ml sterile saline
- Slant cultures of *Serratia marcescens* and *Providencia stuartii*

14.4. Procedure

1. Inoculate each of the two cultures into a separate tube of gelatin, stabbing the inoculating wire straight down through the solid column of medium.
2. Incubate the inoculated tubes and one uninoculated tube of gelatin medium at 35°C.
3. Inoculate each of the two cultures into a separate tube of 0.5 ml saline. The suspension should be very turbid.
4. Insert a strip of the X-ray or gelatin film into each saline suspension.
5. Incubate the tubes at 35°C. Observe at 1, 2, 3, 4, and 24 hours for removal of the gelatin emulsion from the strip with subsequent appearance of the transparent strip support.

6. After 24 hours, examine the nutrient gelatin tubes. The uninoculated control as well as the two inoculated cultures should be liquid. Place all three tubes in the refrigerator for 30 minutes. If at the end of this period all tubes are solidified, replace them in the incubator. If any tube is liquefied but the others are solid, record this result.
7. If tubes are reincubated, examine them every 24 hours, placing them in the refrigerator for 30 minutes each time, as in step 6.

14.5. Results

Organism	Tube Method		X-ray film method	
	Gelatinase	No. of hours if Positive	Gelatinase	No. of hours if Positive
Serratia marcescens				
Providencia stuartii				

Figure 35 Gelatinase test by tube [coloured] and strip method [Black & white]

Positive result on [RIGHT] & Negative control on [LEFT]

14.6. Review Questions

- Why is gelatin liquefied in the presence of gelatinase?
- For each enzyme, indicate one bacterial species that produces it.
    - Urease

- Catalase
- Gelatinase

Excercise 15: Phenylalaninedeaminase test / Phenylpyruvic acid (PPA) test

15.1. Principle

The test is based on the ability of organism to breakdown phenylalanine by oxidative deamination to produce phenylpyruvic acid. Phenylpyruvic acid is detected by adding FeCl3 which produces a green colour on the surface of the culture.

Phenylpyruvic Acid + Ferric Chloride → Green Complex

15.2. Objectices

Examining whether the sample bacteria is able to breakdown phenylalanine by oxidative deamination to produce phenylpyruvic acid or not.

15.3. Materials

- Phenylalanine agar Slants
- 10% aqueous solution of FeCl3
- Slant culture of *Proteus vulgaris*
- Slant culture of *Klebsiella pneumoniae* sub species pneumoniae

15.4. Procedure

1. Inoculate the slope of phenylalanine agar with the test organism.
2. Incubate at 35-37°C overnight.
3. Add 0.2 ml of freshly prepared 10% aqueous solution of FeCl3 to the culture.
4. Allow the reagent to run down the slope.
5. Watch immediately.
6. A positive reaction is indicated by a dark green colour which fades quickly.

15.5. Results

Green colour (within 5 minutes) - Positive test

No green colour - Negative test

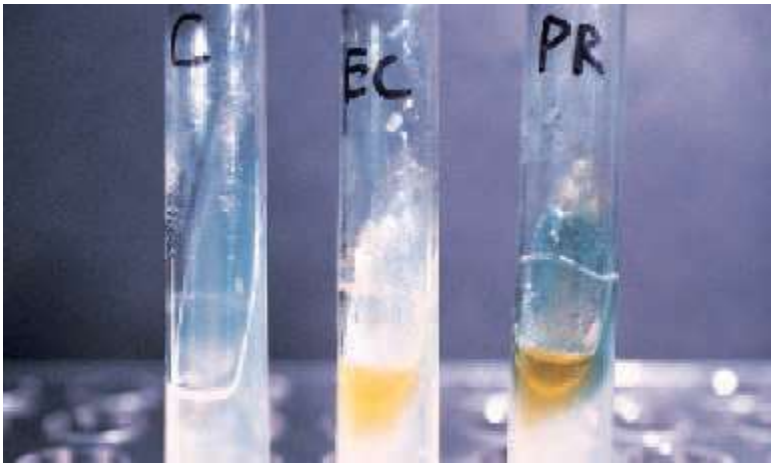


Figure 36 Phenylalanine Deamination.

(LEFT) Uninoculated control. (MIDDLE) Phenylalanine negative. (RIGHT) Phenylalanine positive.

15.6. Review Questions

1. What is the purpose of the ferric chloride in the phenylalanine deamination test?
2. When would you use the phenylalanine deamination test?
3. Name some bacteria that can deaminate phenylalanine.
4. Describe the process of deamination.

Excercise 16: API & AST tests + Lab Automation

16.1. Background

The biochemical tests performed in the preceding sections are representative of standard methods for bacterial identification. In some instances, it is possible to identify a bacterium correctly by using only a few tests, but more often an extensive biochemical "profile" is needed. Because it is expensive and time consuming to make and keep a wide variety of culture media on hand, many microbiology laboratories now use multimedia identification kits. These are commercially available and are especially useful for identifying the common enteric bacteria. The use of such kits is customarily referred to as an application of "rapid methods," even though they must be incubated overnight, as usual, before results can be read. Some of them, indeed, are rapid to inoculate, while others permit complete identification within 24 hours.

16.1. Principle

The API System (bioMérieux Inc.) is a kit for rapid identification of bacteria. This system provides, in a single strip, a series of 20 microtubules (miniature test tubes) of dehydrated media that are rehydrated with a saline suspension of the bacterium to be identified. The tests included in the strip determine whether the organism ferments glucose, mannitol, inositol, sorbitol, rhamnose, saccharose, melibiose, and amygdalin; produces indole and H_2S; splits urea; breaks down the amino acids tryptophan (same mechanism as phenylalanine), lysine, ornithine, and arginine; produces gelatinase; forms acetylmethylcarbinol from glucose (VP test); and splits the compound o-nitrophenyl-β-D-galactopyranoside (ONPG). The enzyme that acts on ONPG, called β-galactosidase, also is responsible for lactose fermentation. Some bacteria, however, are unable to transport lactose into their cells for breakdown, although they possess β-galactosidase. In lactose broth, therefore, such bacteria fail to display acid production, or do so only after a delay of days or weeks. By contrast, in ONPG medium their β-galactosidase splits the substrate in a matter of hours, producing a bright yellow end product. Thus, ONPG can be used for the rapid demonstration of an organism's ability to ferment lactose.

16.2. Objective

To observe the biochemical properties of bacteria grown in a multimedia system for rapid identification

16.3. Materials

- 20E & 20NE API System (bioMérieux Inc.) kits for rapid identification of bacteria
- Saline suspension of Escherichia coli
- Saline suspension of Proteus vulgaris

16.4. Procedure

- Inoculated two API strips with *Escherichia coli* and *Proteus vulgaris*
- incubated for 24 hours

16.5. Results

After incubation of API strips, record the results observed for each organism in the blocks provided under the following diagram.

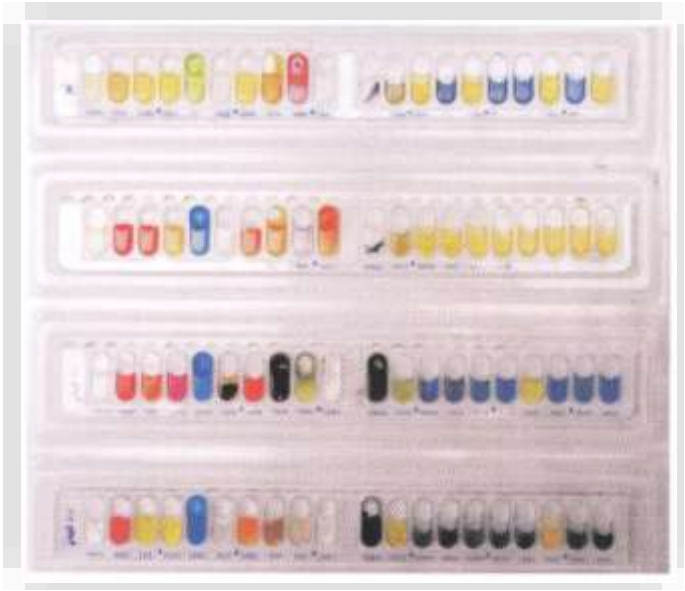


Figure 37 Reactions in API 20E.

16.6. Review Questions

- What is the significant advantage of API strips

Excercise 17: Antibiotic Susceptibility Tests for Identification

17.1. Novobiocin Susceptibility Test:

The Novobiocin Test is used to differentiate coagulase negative staphylococci.

- ***Staphylococcus saprophyticus***, which is resistant to Novobiocin **produce no clear zone**.
- ***Staphylococcus epidermidis*** *which is susceptible to Novobiocin* a large **clearing zone** around the disk will appear.

Figure 38 Novobiosin sensitive Staph. epidermidis RIGHT vs. resisting Staph. saprophyticus

17.2. Bacitracin Susceptibility Test:

This test is used to differentiate and presumptively identify β-hemolytic group A streptococci (***Streptococcus pyogenes***-susceptible) from other **β**–hemolytic streptococci (resistant).

17.3. Optochin Susceptibility Test:

This test is used to presumptively differentiate ***Streptococcus pneumoniae*** from other alpha hemolytic streptococci. ***Streptococcus pneumoniae*** is the only streptococcus susceptible to small concentrations of the antibiotic optochin.

Some Culture Media Ingredients:

Blood Agar (pH 7.3)

Infusion from beef heart ..... 500.0 g

Tryptose..... 10.0 g

Sodium chloride..... 5.0 g

Agar ..... 15.0 g

Distilled water ..... 1,000.0 ml

Note: Dissolve the above ingredients and autoclave. Cool the sterile blood agar base to 45° to 50°C and aseptically add 50 ml of sterile, defibrinated blood. Mix thoroughly and then dispense into plates while a liquid. Blood agar base for use in making blood agar also can be purchased. A combination of hemoglobin and a commercial nutrient supplement can be used in place of defibrinated blood.

Brain-Heart Infusion Agar (pH 7.4)

Calf brains, infusion from..... 200.0 g

Beef hearts, infusion from ..... 250.0 g

Proteose peptone..... 10.0 g

Dextrose..... 2.0 g

Sodium chloride..... 5.0 g

Disodium phosphate ..... 2.5 g

Agar ..... 15.0 g

Distilled water ..... 1,000.0 ml

Kligler Iron Agar (pH 7.4)

Beef extract	3.0 g
Yeast extract	3.0 g
Peptone	15.0 g
Proteose peptone	5.0 g
Lactose	10.0 g
Dextrose	1.0 g
Ferrous sulfate	0.2 g
Sodium chloride	5.0 g
Sodium thiosulfate	0.3 g
Agar	12.0 g
Phenol red	0.024 g
Distilled water	1,000.0 ml

Mannitol Salt Agar (pH 7.4)

Beef extract	1.0 g
Peptone	10.0 g
Sodium chloride	75.0 g
D–mannitol	10.0 g
Agar	15.0 g
Phenol red	0.025 g
Distilled water	1,000.0 ml

MacConkey's Agar (pH 7.1)

Bacto peptone	17.0 g
Proteose peptone	3.0 g
Lactose	10.0 g
Bile salts mixture	1.5 g
Sodium chloride	5.0 g
Agar	13.5 g
Neutral red	0.03 g
Crystal violet	0.001 g
Distilled water	1,000.0 ml

MR-VP Broth (pH 6.9)

Peptone	7.0 g
Dextrose	5.0 g
Potassium phosphate	5.0 g
Distilled water	1,000.0 ml

Mueller-Hinton Agar (pH 7.4)

Beef, infusion	300.0 g
Casamino acids	17.5 g
Starch	1.5 g
Agar	17.0 g
Distilled water	1,000.0 ml

Nutrient Agar (pH 7.0)

Peptone	5.0 g
Beef extract	3.0 g
Agar	15.0 g
Distilled water	1,000.0 ml

Note: Autoclave at 121 lb pressure for 15 minutes.

Starch Agar (pH 7.5)

Beef extract	3.0 g
Soluble starch	10.0 g
Agar	12.0 g
Distilled water	1,000.0 ml

Thioglycollate Broth (pH 7.1)

Peptone	15.0 g
Yeast extract	5.0 g
Dextrose	5.0 g
L-cystine	0.75 g
Thioglycollic acid	0.5 g
Agar	0.75 g
Sodium chloride	2.5 g
Resazurin	0.001 g
Distilled water	1,000.0 ml

Triple Sugar Iron Agar (pH 7.4)

Beef extract	3.0 g
Yeast extract	3.0 g
Peptone	15.0 g
Peptose-peptone	5.0 g
Lactose	10.0 g
Saccharose	10.0 g
Dextrose	1.0 g
Ferrous sulfate	0.2 g
Sodium chloride	5.0 g
Sodium thiosulfate	0.3 g
Phenol red	0.024 g
Agar	12.0 g
Distilled water	1,000.0 ml