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# LABORATORY HANDBOOK

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VETERINARY BACTERIOLOGY & MYCOLOGY  
VPM 201, 2011



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ATLANTIC VETERINARY COLLEGE

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**ATTENTION**

**VPM 201 WEB address: <http://people.upei.ca/jlewis/index.html>**

## II

### INTRODUCTION

The purpose of these laboratory exercises is to reinforce material discussed in lectures. The staining and culturing techniques demonstrated or used in these laboratory classes are not difficult. However, when done carefully and correctly, they provide valuable information. Whenever possible, case history should be considered before arriving at a diagnosis.

These laboratory experiences will also impress upon you the necessity of submitting proper specimens to the diagnostic laboratory to confirm or redirect your clinical plan. Laboratories also provide an understanding of the way submitted samples are processed and the time and techniques required to not only isolate and identify bacteria or fungi, but also carry out antimicrobial susceptibility testing.

**Please prepare for laboratories by reviewing web-based information prior to coming to the laboratory.** You are required to bring a hard copy of the lab exercise document to the lab unless directed otherwise. Additional support documents will be provided as needed. Relevant material presented in the laboratory will be made available on the web after laboratories have been delivered. Ultimately it is the student's responsibility to capture details on discussions in lab associated with questions, demonstration and student exercise material.

Students will normally work in pairs in the laboratory.

### OBJECTIVES

Upon completion of the laboratory component of VPM 201, the student is expected to be able to:

1. Employ proper and safe microbiology laboratory techniques for the diagnosis of bacterial and fungal infections in veterinary medicine.
2. Recognize the microscopic morphology of important bacterial and fungal pathogens in stained preparations from clinical material.
3. Culture clinical specimens successfully. Carry out Gram staining on clinical material and demonstrate familiarity with microscopic features of important bacterial and fungal pathogens..
4. Recognize bacterial or fungal colony features and other growth characteristics of important animal pathogens.
5. Understand the principles of antimicrobial susceptibility tests and correctly interpret these results.
6. Understand the habitat, major host(s) and zoonotic potential of important bacterial and fungal pathogen.

### III

#### **SAFETY PRECAUTIONS**

You must read, sign and return the Biohazard Awareness Document. You must be familiar with and employ the basic safety practices below.

1. **ABSOLUTELY NO** food, beverages or gum in the laboratory (215N).
2. A **laboratory coat** will be provided. This **laboratory coat is NOT to leave 215N**.
3. No open-toed shoes (i.e., sandals) are to be worn in the laboratory. No footwear or clothing worn in the barn or postmortem area is to be worn in the laboratory.
4. Students with **contact lenses** must **either**, wear biosafety glasses provided **or** remove contacts and wear prescription eyeglasses.
5. If you have long hair, please tie it back before starting the lab.
6. **Wash and disinfect hands** thoroughly before entering and prior to leaving the laboratory. Students with false nails or badly chipped nail polish must wear gloves provided.
7. **Wipe** the bench top thoroughly at the beginning and end of each laboratory period with the disinfectant provided.
8. Do not mouth pipette. Minimize the formation of droplets, spatters and splashes.
9. **Calmly report** any laboratory accident, spill or contamination to the students in your immediate vicinity **and** a laboratory coordinator or designate promptly.
10. Discard cultures (plates, tubes, etc.) and sharps (microscope slides are considered sharps) as directed by the laboratory coordinator or designate.
11. If you are immuno-modulated (splenectomy, pregnant etc.) see the laboratory coordinator or designate regarding any additional precautions.
12. You **must** read, sign and return a copy of the “**BioHazard Awareness Document**” provided at the beginning of the semester. Any student failing to practice these basic safety guidelines will be asked to leave the laboratory.

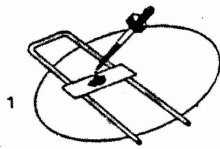
#### Additionally:

Microscopes are to be carried properly and cleaned as directed after each use. Notify your instructor if there is a problem with the microscope you are using.

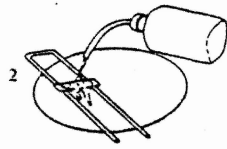
## APPENDIX A: Conventional (this page) and Chemical (page 2) Gram Stain

- if preparing a smear from colony place a **small drop of water on the slide** and disperse some colony collected with a sterile loop thoroughly.
- swabs that are moist can generally be applied to the slide surface after plating.
- you must **air-dry** and then gently **heat fix** your slides before staining

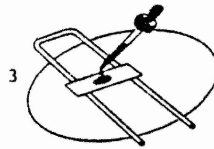
### GRAM STAIN PROCEDURE



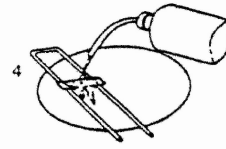
Crystal  
violet  
primary stain  
(1 minute)



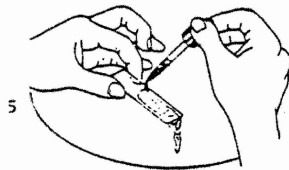
Wash with  
water



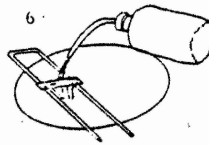
Gram's iodine  
mordant  
(1 minute)



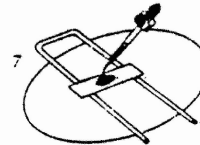
Wash with  
water



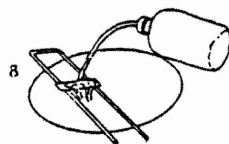
Decolorize with  
acetone/alcohol  
until no further  
color washes out  
of smear



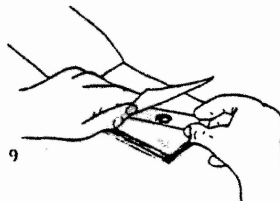
Wash with  
water



Safranin counterstain  
(30 seconds-1 minute)



Wash gently with  
water



Blot dry gently

10 Examine under oil  
immersion lens

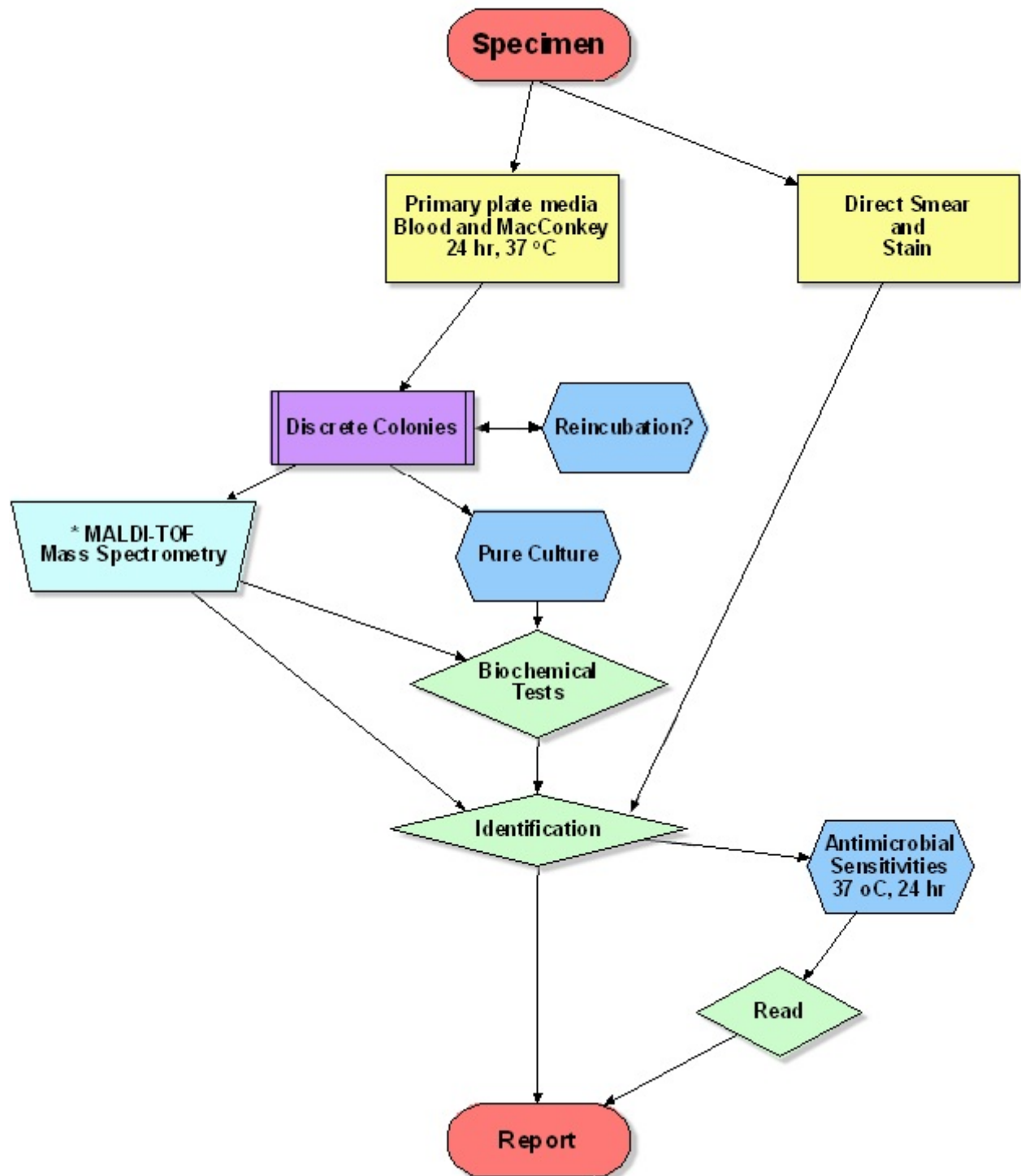
**Chemical Gram Stain : KOH Test**

This is a simple test to presumptively distinguish gram-positive from gram-negative bacteria. Place a drop of 3% KOH on a clean slide. Take a loopful of the culture and thoroughly mix with KOH. Lift the loop at intervals to see if a gel is forming. Gram-negative bacteria form a viscous gel in a minute or so. Gram-positive bacteria don't (except some *Bacillus* sp.).

## **APPENDIX B: FLOW CHARTS FOR IDENTIFICATION OF COMMON PATHOGENS**

<b>FLOWCHARTS</b>	<b>PAGE</b>
General Overview of Bacterial Isolation and Identification Procedures	4
Simplified Flow Chart for Identification of Gram-positive Aerobic Bacteria	5
Simplified Flow Chart for Identification of Gram-negative Rods	6
Simplified Flow Chart Illustrating Clinically Important Anaerobic Rods	7

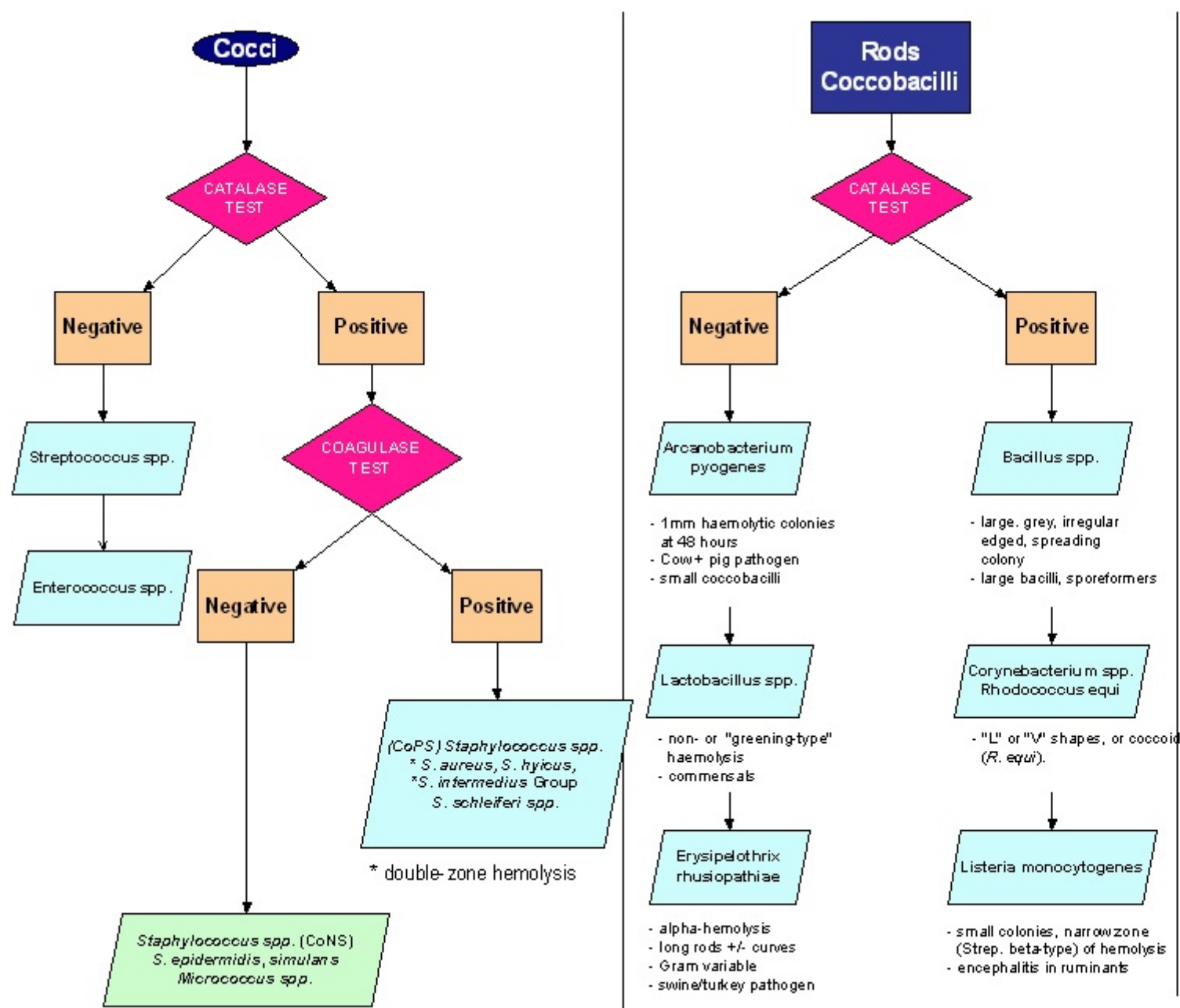
# Isolation and Identification of Bacteria from Clinical Specimens



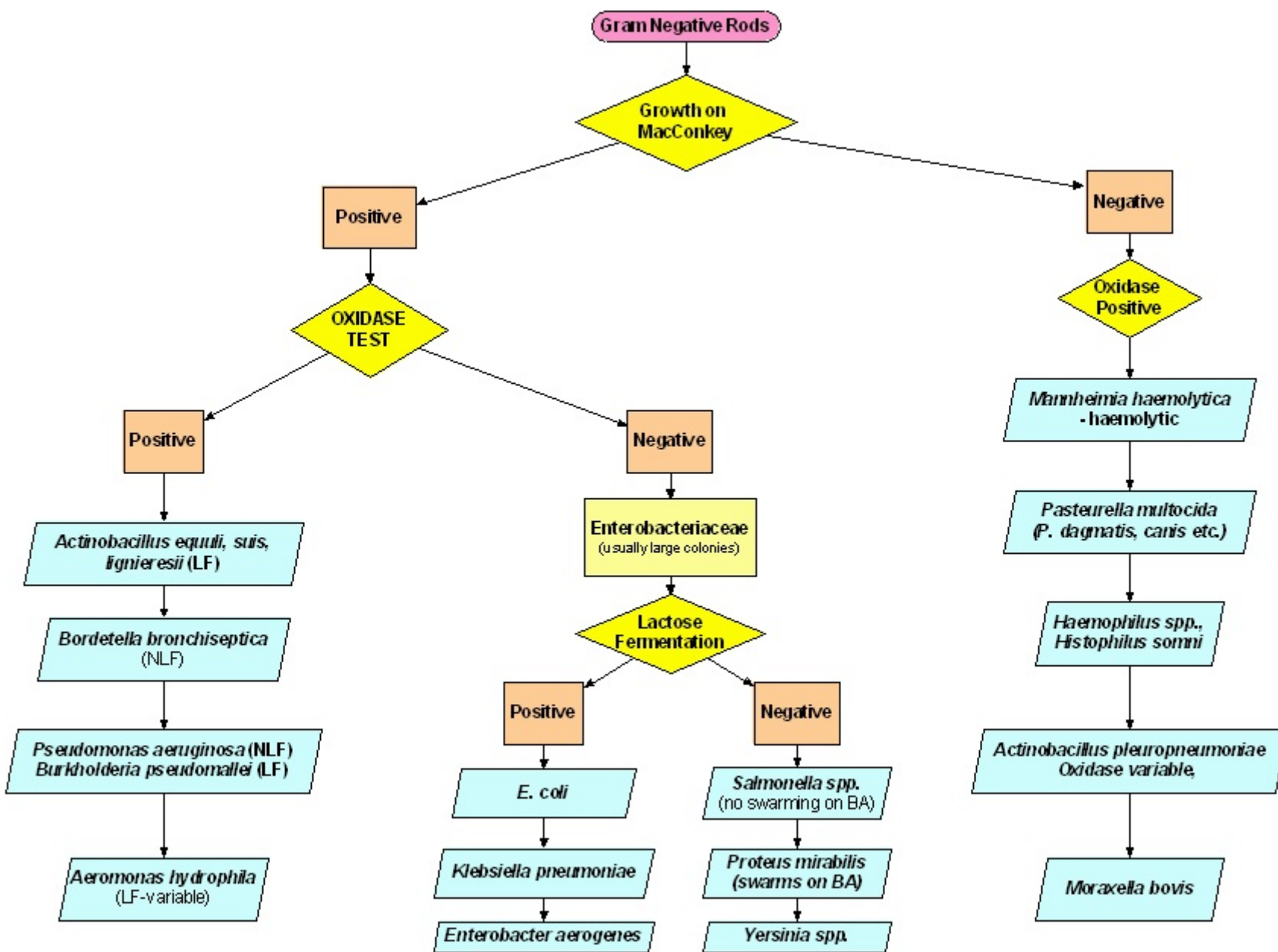
Matrix Assisted Laser Desorption/Ionization - Time of Flight mass spectrometry.



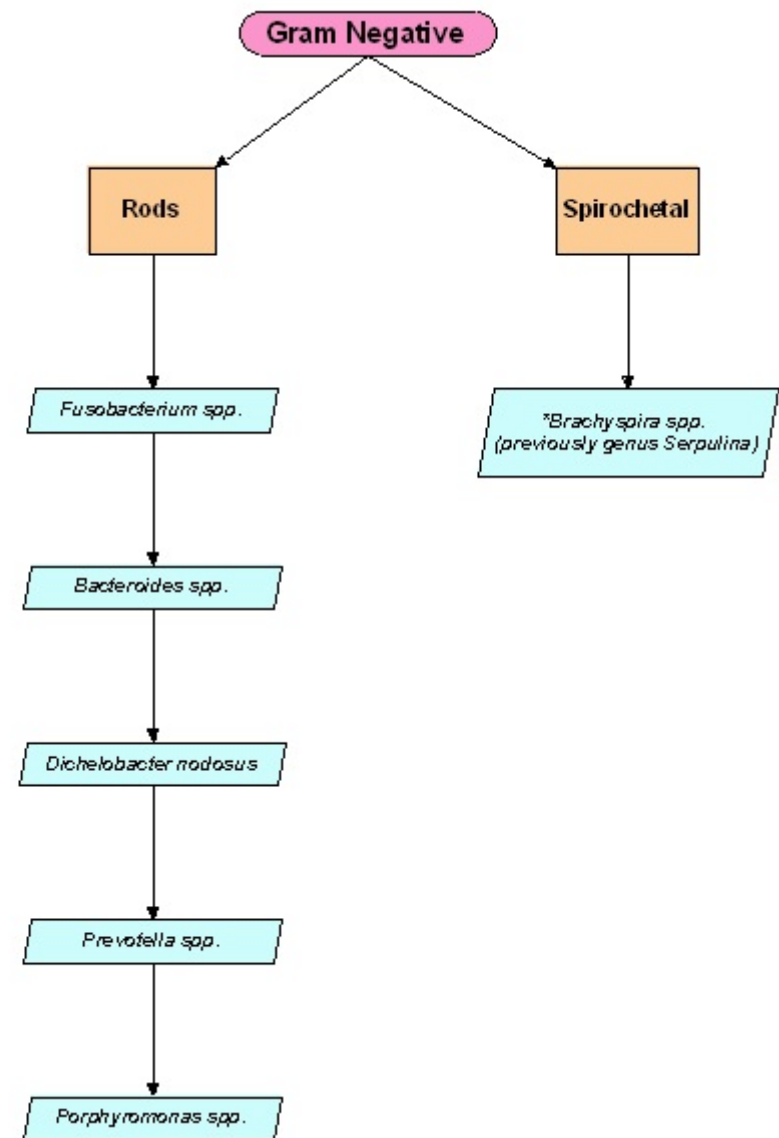
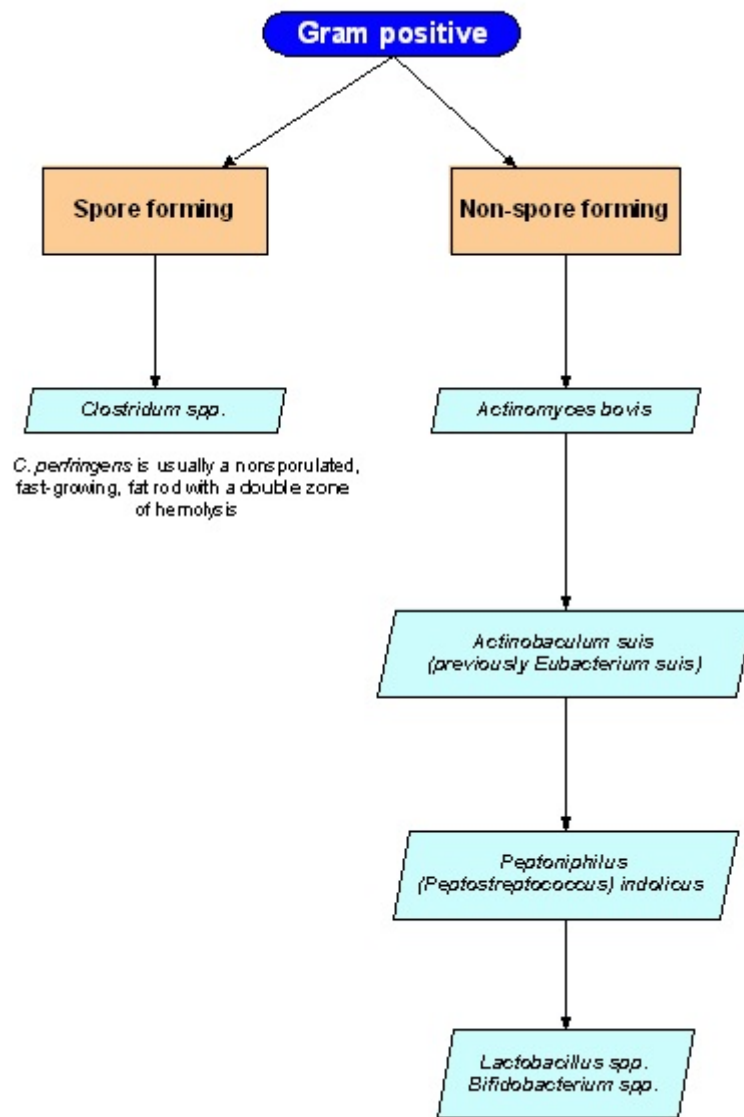
## Simplified Flow Chart for Identification of Gram-Positive Aerobic Bacteria



**Simplified Flow Chart for Identification of Gram-Negative Rods** Most gram-positive organisms **cannot** grow on MacConkeys. Lactose Fermenters (LF) show colonies that are pink or red, Non-Lactose Fermenters (NLF) are colorless.



## Flow Chart Illustrating Clinically Important Anaerobic Rods



## APPENDIX C: SELECT CULTURE MEDIA

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7.	Modified Semi-solid Rappaport Vassiliadis Medium (MSRV) . . .	11
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## 1. BLOOD AGAR

This media consists of Columbia agar base with 5% sheep blood added. It is a widely used general purpose media. The Columbia agar base is a basal medium to which blood may be added for isolating fastidious organisms, or to which other enrichments may be added for special purposes. It contains two types of peptones to obtain a fast and abundant growth, give sharply defined hemolytic reactions, typical colonial morphology and improved pigment production.

## 2. CAMPYLOBACTER MEDIA

### a. Modified Preston Agar

This medium is a selective medium for the isolation of campylobacters. It contains charcoal, ferrous sulfate and sodium pyruvate to enhance growth.

Sodium deoxycholate and cefoperazone are added as selective agents. Feces are inoculated directly onto "Campy" medium. Plates are incubated at 37°C or 43°C for 24 - 48 hours. Selectivity for *Campylobacter jejuni* is increased and quicker results obtained by incubating at 42° - 43°C, but to the exclusion of *Campylobacter fetus* subsp. *intestinalis* and *fetus*. Plates are incubated in an atmosphere of 6% oxygen 10% CO<sub>2</sub> and 84% nitrogen. This is provided by a gas generating envelope being placed in a jar.

### RESULTS:

*Campylobacter* species appear as a flat, grey colony with an irregular edge or as raised and round with a mucoid appearance. Some strains may appear tan or slightly pink. Swarming or spreading may be seen on moist surfaces.

## 3. CITRATE MEDIUM

This medium is used to determine if an organism can use citrate as its sole source of carbon and inorganic ammonium salts as its sole source of nitrogen. If the organism can use citrate and ammonium salts for growth the medium becomes alkaline and the indicator bromothymol blue changes from the original green to a blue color. The original green color indicates a negative test.

RESULTS: Positive = Blue color (ie. *K. pneumoniae*); Negative = Green color (ie. *E. coli*)

#### 4. EDWARD'S AGAR

This is a selective medium with a blood agar base to which the inhibitory compounds crystal violet and thallous sulphate and the differential agent esculin have been added. This is used as a selective medium for the isolation of streptococci and is commonly used in mastitis labs.

##### RESULTS:

*Strep. uberis* - hydrolyses esculin to produce grey colonies.

*Strep. agalactiae* - appear as small, transparent, bluish-grey (hemolytic or non-hemolytic) colonies.

*Strep. dysgalactiae* subsp. *dysgalactiae* - may appear as  $\beta$ -,  $\alpha$ - or non-hemolytic colonies.

Enterococci such as *E. bovis*, *E. durans*, *E. faecalis* and *E. equinus* hydrolyse aesculin and appear as dark colonies (some are black).

NOTE: Staphylococci **do not** grow on this media at 24 h.

#### 5. MacCONKEY AGAR (MAC)

This is a differential and selective medium. The differential action of MacConkey agar is based on the fermentation of lactose. Colonies capable of fermenting lactose produce a localized drop in pH, which, followed by the absorption of neutral red from the media, gives the colony a pink color. A zone of precipitated bile may also be seen due to the drop in pH. Colonies that do not ferment lactose are translucent and colorless. Selectivity of the media is due to the presence of crystal violet and bile salts which almost completely inhibit growth of gram-positive organisms.

##### RESULTS:

Lactose fermenters - pink colony - *E. coli*, *Klebsiella pneumoniae*.

Non-Lactose fermenters - colorless colony - *Salmonella*, *Proteus*.

6. RAPPAPORT BROTH ( used in combination with MSRV - see # 7)

This broth is recommended as the selective enriched medium when isolating *Salmonella* from food and environmental specimens. This media was developed to meet four characteristics unique to *Salmonella* when compared with other gram-negatives in the family Enterobacteriaceae.

- a. The ability to survive at relatively high osmotic pressure.
- b. The ability to multiply at relatively low pH values.
- c. The ability to be more resistant to malachite green.
- d. The ability to have less demanding nutritional requirements.

To use: Place a small portion of feces in Rappaport broth. Incubate overnight at 42°C and plate it to MRSV agar the next day.

7. MODIFIED SEMI-SOLID RAPPAPORT - VASSILIADIS MEDIUM (MSRV)

MSRV is a semi-solid medium for detection of motile *Salmonella* species. Selection for *Salmonella* species is achieved by the addition of malachite green, magnesium chloride and novobiocin. The low concentration of agar (0.27%) produces a semi-solid gel that allows motile *Salmonella* species to migrate ahead of other motile bacteria.

RESULT: Motile *Salmonella* species produce opaque halos of growth.

8. SABOURAUD DEXTROSE AGAR

This medium uses a neopeptone as its source of nitrogen and 4% dextrose for nutrients. It is a good routine medium for the primary isolation and growth of fungi. For primary isolation, bacterial inhibitors such as antibiotics can be added to the medium before pouring. This is not usually necessary as the acid pH inhibits most bacteria.

9. MYCOSEL™ AGAR

This is a selective medium containing cycloheximide and chloramphenicol and is recommended for the isolation of pathogenic fungi (including dermatophytes) from samples containing large amounts of nonpathogenic fungal and bacterial flora. Cycloheximide inhibits the growth of most saprophytic molds.

10. SIM MEDIUM - (SULPHIDE, INDOLE, MOTILITY)

This medium was designed to determine three characteristics: (1) hydrogen sulphide production (2) indole production and (3) motility.

Tryptone is incorporated into the medium.

Ferrous ammonium sulphate is incorporated into the medium to show hydrogen sulphide production. H<sub>2</sub>S will be seen along the line of inoculation as a black precipitate.

After incubation, indole can be detected as described on page 18 (note there is no glucose in this medium as glucose fermentation can give false-positive indole reactions).

A lower concentration of agar (0.35%) in the medium gives it a semi-solid consistency good for motility detection. Motile organisms will grow away from the stab line while the non-motile organisms grow only on the stab line.

RESULTS:

- a. Hydrogen Sulphide Production - black precipitate along stab line - *Erysipelothrix rhusiopathiae*
- b. Motility - Positive - growth away from stab line - *Enterobacter*  
Motility - Negative - growth along stab line only - *Klebsiella*
- c. Indole - Positive - pink layer after addition of Kovacs reagent - *E. coli*  
Indole - Negative - No color or pale yellow - *Klebsiella*

11. TSI (TRIPLE SUGAR IRON)

This peptone medium is commonly used for differentiating gram-negative enteric bacteria according to their ability to ferment lactose (1%), sucrose (1%) and dextrose (0.1%) and to produce hydrogen sulfide. The pH indicator phenol red is included in the medium and is yellow at acidic pH and red at alkaline pH.

Carbohydrate fermentation is indicated by the production of gas and a change in the color of the pH indicator from red to yellow. To facilitate the detection of organisms that only ferment dextrose, the dextrose concentration is one-tenth the concentration of lactose or sucrose. The small amount of acid produced in the slant of the tube during dextrose fermentation oxidizes rapidly, causing the medium to remain red or revert to an alkaline pH. In contrast, the acid reaction (yellow) is maintained in the butt of the tube because it is under lower oxygen



tension. After depletion of the limited dextrose, organisms able to do so will begin to utilize the lactose or sucrose.<sup>2</sup>

If dextrose is utilized the agar will turn yellow (acid), but when incubated overnight and being exposed to oxygen and the lessening of the dextrose concentration, the slant will turn alkaline (red) and the butt stays acid (yellow), giving an alkaline over acid reaction (K/A).

If lactose and/or sucrose are used the slant turns yellow (acid) giving an acid over acid A/A. If these are not used the slant will be red (alkaline).

Hydrogen sulfide (H<sub>2</sub>S) is indicated by blackening in the butt due to ferrous sulfide production and gases are indicated by splitting in the agar. This is important in screening intestinal pathogens that do not ferment lactose and/or sucrose.

INTERPRETATION: A - acid, K - alkaline, G-gas, H<sub>2</sub>S - hydrogen sulfide production

1. A/A +/- G : *E. coli*, *Klebsiella*
2. K/A + H<sub>2</sub>S : *Salmonella* species
3. K/A eg. *Shigella* species
4. K/K or no change - Oxidizers eg. *Pseudomonas*

## 12. UREA MEDIUM

This medium contains urea as the sole source of nitrogen and is used to determine whether an organism produces the enzyme urease.

If the enzyme is present, urea will be hydrolyzed with the resulting production of ammonia, thus creating an alkaline condition and changing the medium from light yellow to bright pink. The indicator used is phenol red.

RESULTS:

Positive - bright pink - *Proteus* spp., *Klebsiella* spp.

Negative - light yellow - *E. coli*

- ## 13. MUELLER-HINTON AGAR : A medium utilized for testing the sensitivity of clinically important pathogens towards antibiotics. Fastidious bacteria are tested on Mueller-Hinton agar supplemented with blood.

## APPENDIX D: SELECT BIOCHEMICAL TESTS

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11. Indole Test . . . . .	18

1. AGGLUTINATION TEST

Antibodies can cross-link particulate antigens, resulting in their clumping or agglutination. Agglutination may be produced by mixing antigenic particles such as bacteria with specific antisera. IgM are more effective agglutinators than IgG or IgA. Slide agglutination tests are used frequently in bacteriology to identify certain organisms.

2. CALIFORNIA MASTITIS TEST (CMT)

This is a test that can be carried out at the side of the cow. About 2 ml of foremilk is drawn from each of the four quarters of the mammary gland into a paddle containing four plastic cups. To this milk an equal volume of reagent is added. Mix using a gentle circular motion of the paddle. A positive test is indicated by a gel formation.

The CMT reaction of mastitic milk is due to deoxyribonucleic acid (DNA) from the nuclei of leucocytes in milk. The cells are ruptured by the CMT reagent (essentially a detergent) resulting in the release of DNA and gel formation as the reagent reacts with the released DNA. The CMT reagent consists of triethanolamine sulfonate available in a thick liquid form containing 60% of the active ingredient. It is diluted with water to 3%. Bromocresol purple is added as an indicator and the pH is adjusted to 7.0-7.5.

The CMT reaction is graded from 0-4, and is dependent on the number of inflammatory cells in the milk being tested.

3. CAMP TEST (named after Christie, Atkins, and Munch-Peterson - researchers who first described this lytic phenomenon in 1944)

This test is used mainly for the presumptive identification of *Streptococcus agalactiae*. This pathogen produces a “CAMP factor” which is able to complete (synergistic hemolysis) the **partial** lysis of red cells produced by the  $\beta$ -hemolysin of a *Staphylococcus* species.

PROCEDURE:

1. Streak a  $\beta$ -hemolytic Staph across the center of a blood plate.
2. Streak the Strep to be tested at right angles up to, but not touching, the Staph line.
3. Incubate the plate at 35°C for 18-24 hours.

A positive CAMP reaction is noted by the appearance of a zone of clear hemolysis in the partial zone of  $\beta$ -hemolysis produced by the Staph. Positive reaction hemolysis zones can have a characteristic shape that aids in identification.

Some CAMP-positive bacteria: *Streptococcus agalactiae* (arrow-head zone of hemolysis), *Listeria monocytogenes*, *Rhodococcus equi* (shovel-shaped zone of hemolysis), *Arcanobacterium pyogenes*, *Corynebacterium renale* etc..

#### 4. CARBOHYDRATE FERMENTATION: (TREHALOSE AND SORBITOL)

The ability of bacteria to utilize a particular subset of carbohydrates can allow differentiation of genera and species.

For testing the fermentative reactions of bacteria, carbohydrates (ie. trehalose, sorbitol) are added to a sugar-free medium at a concentration of 0.5% to 1%. The medium is inoculated with a test organism and incubated at 35°C. The tubes are read after 18-24 hours.

We use semisolid medium "Bacto Cystine Tryptic Agar", which incorporates a pH indicator, phenol red.

#### RESULTS:

Uninoculated tube is red.

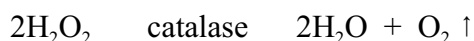
Negative reaction - tube remains red.

Positive reaction - tube turns yellow.

#### 5. CATALASE TEST

This is a test for the presence of the enzyme catalase. This test is useful for differentiating Streptococci (-ve) from Staphylococci (+ve), *Erysipelothrix* (-ve) from *Listeria* (+ve) and *Corynebacterium* (+ve) except *Arcanobacterium pyogenes* (recall that this organism was previously called *Corynebacterium pyogenes*).

Pick a portion of a colony with a wire loop, smear onto a glass slide, add a drop of hydrogen peroxide (3%). Immediate bubbling is indicative of a positive test.



## 6. COAGULASE TEST (CO)

This is a test of the ability of an organism to clot rabbit plasma through the action of the enzyme coagulase. This test is used to differentiate coagulase-positive staphylococci (CoPS) from coagulase-negative staphylococci (CoNS).

Two tests are used.

### a. SLIDE TEST: (bound coagulase)

Emulsify a colony in a drop of rabbit plasma on a microscope slide and mix slowly. A positive test is indicated by clumping within 10 seconds. A negative test result when the mixture remains homogenous (no signs of clumping).

### b. TUBE TEST: (free coagulase)

Mix 0.5 mL of undiluted plasma with a loopful of bacteria and incubate at 37°C for 4 hours. Examine for a coagulum (solid clot). Negative tubes should be left at room temperature overnight and then re-examined.

Results:

CoNS : *S. epidermidis*

CoPS : *S. aureus*, *S. intermedius* Group, \**S. schleiferi* spp. \**S. hyicus*.

\* Note that these are not necessarily positive in both Slide and Tube tests.

## 7. CYTOCHROME OXIDASE (CO) TEST (OXIDASE TEST)

This test is used primarily with Gram-negative bacteria. The test is positive for bacteria containing cytochrome C as a respiratory enzyme.

Add a drop of reagent (tetramethyl-p-phenylenediamine) to a piece of filter paper. Remove some growth from the culture with a loop and rub into the moistened area of the paper. A purple color change in 1 minute indicates a positive reaction. The use of an iron containing loop may give a false positive reaction, so the use of a platinum or a plastic loop is recommended.

RESULTS:

Positive - purple reaction, e.g. *Pseudomonas* species, *Pasteurella*, *Bordetella*

Negative - colorless, e.g. *E. coli*

8. HIPPURATE TEST

This test is used to detect the hydrolysis of sodium hippurate by beta hemolytic Streptococci and by *Campylobacter jejuni*. The principle of this test is that one of the products of hydrolysis of sodium hippurate is the amino acid glycine. This can be detected by adding ninhydrin.

One to three colonies of the organism to be tested are emulsified in 0.1 ml of sterile water. A hippurate disc is placed in the tube and the mixture is incubated for 2 hours at 35°-37°C.

Following incubation add 2 drops of ninhydrin reagent to the tube and re-incubate for up to 30 minutes. A positive test is indicated by a purple color. A negative test will be colorless or light grey.

9. INDIA INK WET MOUNT (Negative Stain)

This method is used to demonstrate the capsule present on some organisms. It is most useful for demonstrating the large capsule of *Cryptococcus neoformans*.

Clinical material or organisms from cultures are mixed on a slide in a drop of distilled water and India ink. A coverslip is added and the preparation is examined.

10. MODIFIED ACID-FAST STAIN

Heat fixed smears are stained for 10 minutes with carbol-fuchsin, washed with tap water and decolorized with 0.5% acetic acid for 20-30 seconds. Counterstain with methylene blue for 20 seconds. Slides are washed with tap water and blotted dry.

Acid fast organisms will be red against a blue background. This test is particularly useful to demonstrate *Brucella abortus* from stomach fluid of an aborted bovine fetus.

11. INDOLE TEST

The Indole test is used for the determination of the organism's ability to produce indole (results from the deamination of the amino acid tryptophan by tryptophanase). Indole can be detected by a red dye complex reaction with "Kovacs Reagent" (amyl alcohol, paradimethylaminobenzaldehyde and concentrated hydrochloric acid). A simplified Indole Test can be carried out using an overnight culture grown in TSB (Tryptone Soy Broth) followed by the addition of Kovac's reagent for detection of indole in the liquid media.