

Wayne Jackson

D. CARTER.

Introduction

Concepts regarding aseptic techniques are not new. Literature references from various authors date back to the 19th century. These concepts are clearly still applicable today especially since the use of broad spectrum antibiotics which began in the late 1940's. The use of these agents resulted in the emergence of antibiotic - resistant bacterial strains which caused outbreaks of disease within the clinic or hospital. Aseptic techniques to control nosocomial or iatrogenic infections are clearly needed. According to James Shaffer, "The need for prevention of clinical - acquired infection is a recognized responsibility of the institution and its management."

Purpose

The purpose of this project is three-fold: 1) to set up a biodiagnostic laboratory and write a procedures manual 2) to allow for culturing and smears of ocular pathogens to aid in diagnosis and amelioration of patient related bacterial disease 3) to pave the way for future clinical bacterial evaluation and incorporation of aseptic tools and techniques to prevent the occurrence of outbreaks of iatrogenic diseases.

Materials Required

To adequately set up a biodiagnostic laboratory for cultures and smears, the following materials need to be obtained:

media:
5% TSA (blood) plates
chocolate plates
thioglycollate broth
oxidase reagent
optochin discs
coagulase plasma
sterile swabs
platinum loops
platinum wires
bacti-cinerator
gram stains:
235 ml gentian violet
235 ml safranin
25 ml iodine concentrate
225 ml iodine diluent
50 ml acetone
200 ml isopropyl alcohol
test tubes
biohazard bags
autoclave
frosted glass slides
wax pencils
immersion oil
tweezers or forceps
candle jar
candle
microscope
lens paper
35 - 37°C incubator
dropper bottles
disposable pipettes
iodine soap

recommended texts:

Atlas of Diagnostic Microbiology
by S. Stanley Schnelerson
Diagnostic Microbiology
by Bailey and Scott

The following is a list of laboratories from which materials

should be ordered:

Scientific Products 1-800-482-3740
Gibco 1-812-376-9081
Rupp Bowmann 1-313-478-9000

BIODIAGNOSTIC
LABORATORY
PROCEDURES
MANUEL

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Eye Cultures

Because tears constantly wash an eye and contain antibacterial components, the number of organisms recovered from many eye infections may be relatively low. A large inocula and variety of media must therefore be used to ensure a proper and sufficient culture. For optometric purposes material collected must be streaked on blood agar and chocolate plates and a tube of thioglycollate broth inoculated. These three media are sufficient to identify most organisms which ocularly, are of pathologic importance.

Eye secretions may have certain of the normal skin inhabitants in small number, such as Staph.epidermidis or diphtheroids, but cultures from the normal eye, including the conjunctiva, should be relatively free of microorganisms.

The following organisms are of pathologic importance:

- Diplococcus pneumoniae
- Streptococcus pneumoniae
- Hemophilus aegyptius (Kock-Weeks)
- Klebsiella pneumoniae
- Staphylococcus aureus
- Moraxella lacunata (Morax-Axenfeld)
- Pseudomonas aeruginosa
- Neisseria gonorrhoea
- Alpha and Beta hemolytic Strept.

Eye infections are accompanied by inflammation and

increased secretions which may become purulent. The most common bacteria are listed above, although the presence of any other bacteria in considerable numbers in infected eyes should be cause for suspicion. Viral infections must also be considered.

Successful Isolation

Factors contributing to successful isolation of causative agents include:

quantity - materials to be cultured should be gathered in sufficient quantity.

stage of disease - the stage of the disease at which the specimen is collected will determine the relative quantity of organisms present.

site - material should be collected where the expected organism is most likely to be found.

knowledge - the clinician must possess adequate knowledge to select appropriate media and techniques and identify causative organisms.

antimicrobial agents - always obtain material for culture before antimicrobial agents are administered.

time before culture - specimens should be cultured promptly whenever possible.

General Instructions

1. Specimens should be processed as soon as possible.
If a specimen can not be cultured immediately, it should be refrigerated with the following exception. Cultures suspected of containing Neisseria gonorrhoea should not be refrigerated but should be cultured immediately and incubated in an atmosphere of CO₂.
2. Media should be stored in a refrigerator and must be warmed to room temperature before being inoculated. Chocolate agar or other material which may be used for Neisseria gonorrhoea or other fastidious organisms should be warmed to 37°C before inoculation.
3. Check incubator temperatures daily. Deviations in temperature will hinder the growth of microorganisms.
4. If organisms are seen on the initial gram stain and no growth occurs within 48 hours, keep cultures for a longer time to insure growth of very fastidious organisms.
5. Petri dishes are incubated upside down. Proper identification of petri dishes should include the patients name, date and source of the specimen.

6. Media to be inoculated with microorganisms should be held at an angle to avoid air contamination from falling dust and bacteria.
7. Use a loop for initial streaking of plates. Use a needle for subculturing isolated colonies and for inoculating biochemical tests.
8. Flame inoculating loops before and after coming in contact with a culture. Cool inoculating loops and needles prior to placing them in media to avoid creating aerosols.

Eye Culture Technique

1. Swab the inflamed area or collect purulent material on a sterile cotton swab (culturette). Make smears for staining.
2. Streak the material on blood and chocolate plates using the prescribed technique below.
3. Incubate the blood and chocolate plates in a candle jar placed inside a 35 - 37°C incubator. Environmental cultures do not have to be placed inside a candle jar.
4. Inoculate the thioglycollate broth by swirling the swab in the broth. Place inside a 35 - 37°C incubator.
5. Examine the plates for growth after 24 and 48 hours incubation.

Smears - Gram Stain Procedure

1. Roll the swab firmly over a small area of a clean glass slide and allow it to dry. The dried slide should be rapidly passed through a flame to heat-fix the material to the slide. The frosted portion of the slide should contain the patients name, date and the source of the specimen.
2. Stain the smear 1 minute with crystal violet solution.
3. Wash briefly in tap water.
4. Add iodine solution and allow it to stand for 1 minute.
5. Wash briefly in tap water.
6. Decolorize with acetone until the solvent flows colorlessly from the slide.
7. Counterstain 10 seconds with safranin.

Note: ()



The streaked plates are incubated in an inverted position, media side up, at 35 - 37°C and examined at 24 and 48 hour intervals.

Broth media is used for maintenance of cultures. They are inoculated with a swab or transfer of colonies by a loop. Propagation of bacteria is indicated by cloudiness. Gram stains should be performed on all broth cultures.

Incubation in an Atmosphere of Increased CO₂

To obtain an atmosphere of increased CO₂, the simplest and most useful method is to use a candle jar. This is a jar with a tightly fitting top into which petri dishes can be

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8. Wash briefly in tap water.

9. Dry and Examine.

Results - gram positive organisms stain blue; gram negative organisms stain red.

Isolation of Bacteria by Streaking

The isolation of bacteria from specimens is almost invariably accomplished by streaking on the surface of an agar plate. The purpose of streaking is to spread an inoculum so as to insure the appearance of isolated colonies on incubation. Most such isolated colonies will be pure cultures of an organism and may be picked for the next step - identification.

A recommended technique ----- with a sterile inoculating loop place a loopful of the material near the edge of the plate or roll the swab containing the inoculum firmly over a small area near the edge of the plate. The loop should then be sterilized in the flame and allowed to cool. Next, the loop is applied to the material on the plate and streaked, using a gentle pressure in the manner illustrated, being careful to flame and cool the loop between each series of parallel strokes.

placed along with a smokeless candle. The candle is lit and the lid tightly sealed. When the candle burns out, the atmosphere contains approximately 3 to 5% CO₂. Place the jar with the cultures in an incubator and examine at 24 and 48 hour intervals for growth.

Coagulase Plasma Procedure

1. Place .5 ml of coagulase plasma (human or rabbit) in a sterile test tube.
2. Inoculate the plasma with a large loopful of the suspected colony.
3. Place the test tube in a 35 - 37°C incubator for 3 hours.

Results - Most positive strains will coagulate the plasma within 1 hour. Those not clotting within 3 hours should be incubated and reexamined in 18 hours. Any degree of clotting is considered positive.

Oxidase Procedure

The oxidase test is useful in identifying colonies of Neisseria in mixed cultures. The oxidase reagent must first be prepared before the test can be performed;

Dimethyl paraphenylene diamine HCL - 1.0 gm
Fresh distilled water - 100 ml

The test is performed by placing a drop of the oxidase reagent on the suspected colonies. Oxidase positive colonies become black rather rapidly. All Neisseria are oxidase positive but there are certain other microorganisms which are also positive. Thus, it is necessary to do a gram stain of the colony to make sure it is a gram negative diplococcus.

Optochin Disc Procedure

The optochin disc procedure is a method of differentiating Diplococcus pneumoniae from other alpha hemolytic strep bacteria. The test is performed by placing a disc on the media during the incubation period. Diplococcus pneumoniae is inhibited by optochin (ethylhydrocupreine hydrochloride), and a zone of inhibition will be seen around the disk after incubation. Incubation under increased CO₂ may decrease the size of the inhibition zone produced by the optochin disk. For this reason it is preferable to incubate the plates in a room-air incubator.

A heavy inoculum of the suspected organism should be streaked on a small area on a 5% TSA plate and an optochin disk placed on top of the area. The plate is incubated for a period of 24 hours and examined for a zone of inhibition. A zone of inhibition indicates a positive test.

Primary Bibliography
Characteristics

Secondary Bibliography
Characteristics

Organic
negative

Organic
negative
analytic

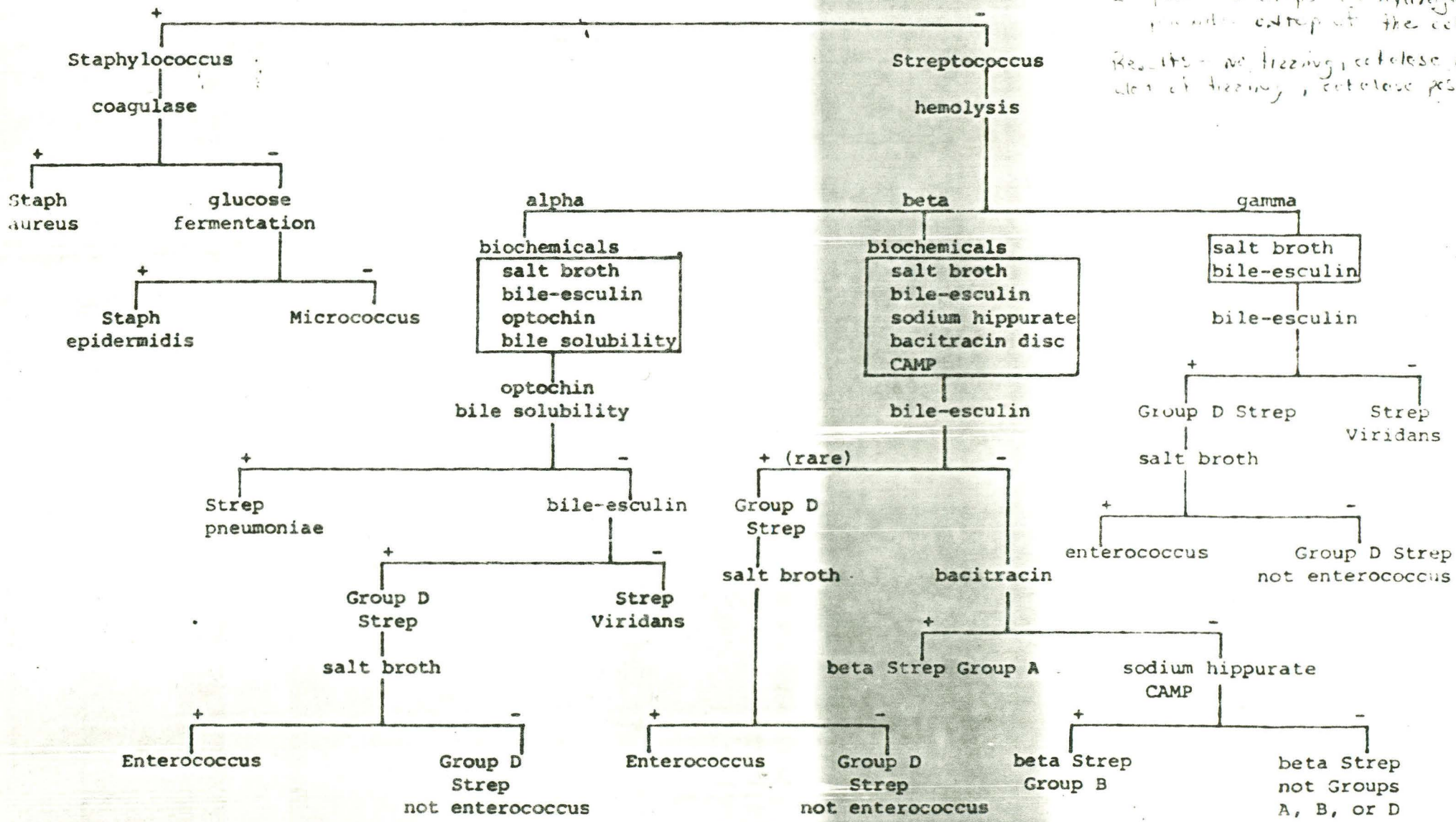
Organic
negative
analytic

Organic
negative
analytic

Organic
negative
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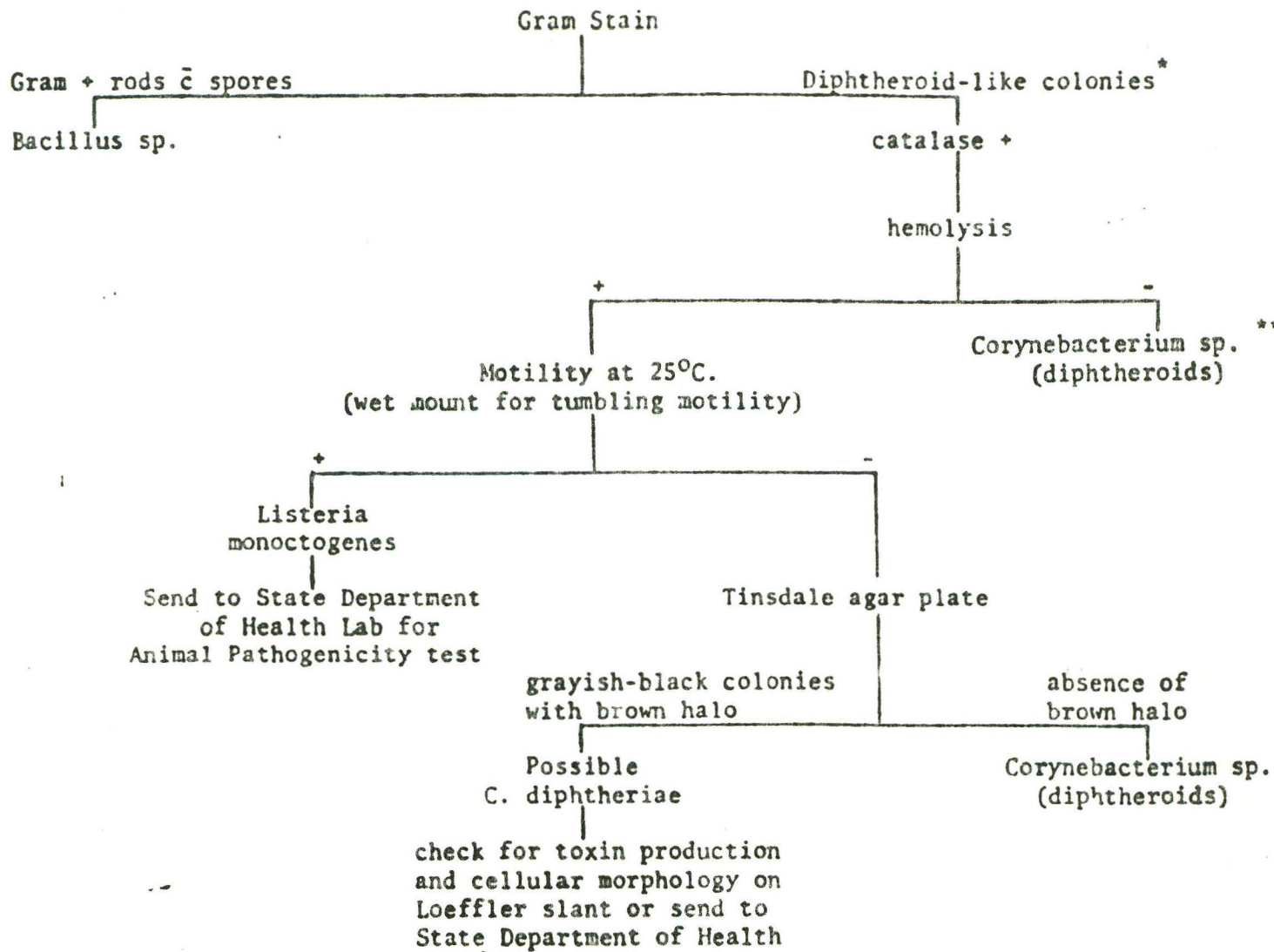
Gram Positive Cocci

catalase



cat. test procedure
 1. remove colony from agar plate
 2. place a drop of hydrogen peroxide on top of the colony
 Results = no fizzing, catalase negative
 lots of fizzing, catalase positive

SUBCULTURE AND IDENTIFICATION OF GRAM POSITIVE BACILLI



*If Erysipelothrix is suspected, inoculate a KIA and observe for H₂S production.

**Non-hemolytic gram-positive rods exhibiting "diphtheroid-like" morphology when isolated from CSF or Blood should be inoculated onto motility media and incubated at room temperature and 35°C.

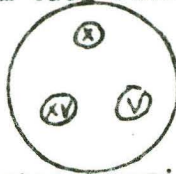
SUBCULTURE AND IDENTIFICATION OF HAEMOPHILUS

I. METHOD USING X AND V DISCS

Materials: Brain Heart Infusion Agar (BHI)
Bacto-Differentiation Discs (Difco)
BV, BX, BVX

Procedure:

1. Streak the suspected Haemophilus colony with a swab uniformly over the BHI plate.
2. Aseptically (with cooled forceps) place each one of the discs equidistant from each other as shown below:



3. Incubate the plates overnight at 35°C. The following day observe which discs the organisms grew around indicating the required factor(s). Growth over the entire plate would indicate that the organism is not a Haemophilus.

	<u>X Factor</u>	<u>V Factor</u>
<u>H. influenza</u>	+	+
<u>H. aegypticus</u>	+	+
<u>H. haemolyticus</u>	+	+
<u>H. parainfluenzae</u>	-	+
<u>H. parahemolyticus</u>	-	+
<u>H. hemoglobinophilus</u>	+	-
<u>H. aphrophilus</u>	+	-

Notes:

1. H. influenza and H. aegypticus both require X and V factors. H. aegypticus is associated with conjunctivitis ("pink eye"); whereas, H. influenza is the causative agent of influenza but can also be isolated from cases of conjunctivitis. The two species can be distinguished serologically but this is not usually done routinely. Thus, in an eye specimen, a Haemophilus which requires both X and V factors should be reported as "Haemophilus influenza or Haemophilus aegypticus". In any specimen other than an eye culture, the Haemophilus should be reported as Haemophilus influenza.
2. H. haemolyticus also requires both X and V factors and is distinguished by its beta hemolytic colonies on blood agar. H. influenza and H. aegypticus do not hemolyze blood.

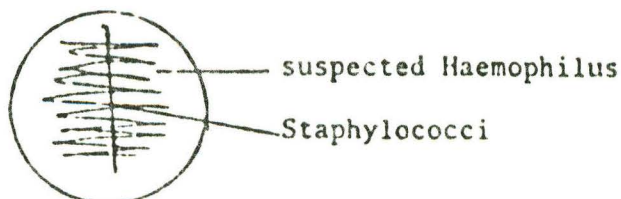
3. H. parainfluenza and H. parahemolyticus both require only factor V. They are distinguished on the basis of the production of beta hemolytic colonies by H. parahemolyticus and non-hemolytic ones by H. parainfluenza.
4. H. hemoglobinophilus and H. aphrophilus both require only factor X. H. aphrophilus grows as fluffy clumps on the inner walls of tubed media. H. hemoglobinophilus does not exhibit this growth characteristic. Both organisms are not very common pathogens.

II. METHOD USING STAPHYLOCOCCI STREAKING

Materials: Brain Heart Infusion Agar (BHI)
Sheep Blood Agar (SB)
Pure culture of Staphylococci

Procedure:

1. Streak the suspected Haemophilus colony on the BHI and SB plates as shown below.
2. Using a straight wire, streak the Staphylococci perpendicular to the previously inoculated areas as shown below.



3. Incubate the plates overnight at 35°C.
4. Examine the plates for satellite growth along the staphylococcal streak.

The sheep blood provides factor X, the Staphylococcus provides V factor. Thus, the sheep blood plate provides both X and V while the BHI plate provides only V.

	<u>Satellitng on</u>	
	<u>SB</u>	<u>BHI</u>
H. influenza	+	-
H. aegypticus	+	-
H. haemolyticus	+	-
H. parainfluenza	+	+
H. parahemolyticus	+	+
H. hemoglobinophilus	+	-
H. aphrophilus	+	-

Reference:

Bailey, W.R., and E.G. Scott. Diagnostic Microbiology, 4th Ed., 1974, pp. 172-175

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SUBCULTURE AND IDENTIFICATION OF NEISSERIA

Subculture Protocol:

1. Inoculate warm CTA sugars (dextrose, maltose, sucrose, lactose) by making several short stabs into the upper portion of the tubes.
2. Incubate tubes in regular atmosphere for 24-48 hours or until good growth is observed.
3. Observe tubes for growth as well as any color change. A yellow color at the top of the tube indicates acid production from the oxidation of the sugar.
4. Gram stains should be made on positive tubes to check for purity.
5. Agglutination tests should be performed on all cultures positive for *N. meningitidis* using specific antiserum, if available, or cultures should be sent to the State Department of Health laboratories for grouping.

IDENTIFICATION OF NEISSERIACEAE

Organisms	Yellow Pigment	Carbohydrate Rxns.				ONPG	Growth			Oxidase
		Glu	Mal	Suc	Lac		On TM	On Nut. Agar	At 22°C	
<i>N. gonorrhoeae</i>	-	A	-	-	-	-	+	-	-	+
<i>N. meningitidis</i>	-	A	A	-	-	-	+	-	-	+
<i>N. sicca</i>	V	A	A	A	-	-	-	+	+	+
<i>N. subflava</i>	+	A	A	V	-	-	-	+	+	+
<i>N. flavescens</i>	+	-	-	-	-	-	-	+	+	+
<i>N. mucosa</i>	-	A	A	A	-	-	-	+	+	+
<i>N. lactamica</i>	+	A	A	-	A	+	-	+	+	+
<i>Branhamella</i>	-	-	-	-	-	-	-	+	+	+
<i>Moraxella</i>	-	-	-	-	-	-	-	+	+	+
<i>Acinetobacter</i>	-	V					-	+	+	-

References:

- Bailey, W.R., and E.G. Scott. Diagnostic Microbiology, 4th ed., 1974, p. 131
- Lennette, E.H., E.H. Spaulding, and J.P. Truant. Manual of Clinical Microbiology, 2nd ed., 1974, Chapter 10.
- Bergey's Manual of Determinative Bacteriology, 8th ed., R.E. Buchanan, and N.E. Gibbons (editors), 1974.

