LABORATORY METHODS
IN
ANAEROBIC BACTERIOLOGY
CDC LABORATORY MANUAL

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I. INTRODUCTION

The clinical importance of the anaerobic bacteria, particularly the toxigenic clostridia and some of the nonsporulating anaerobes is well recognized, however, our overall knowledge of these bacteria and their role in human and animal infections is quite limited. Fortunately, interest in the anaerobes has increased in recent years, and anaerobic methodology is now used routinely by most clinical and public health laboratories. This increase in interest has created an immediate demand for microbiologists and technologists who are familiar with the techniques of anaerobic bacteriology.

Since the establishment of the anaerobic bacteriology laboratory at the Center for Disease Control, a course in laboratory methods in anaerobe bacteriology has been offered for interested and qualified laboratory personnel. This manual was designed primarily as a guide for the laboratory portion of the course, and the material presented is supplemented with lectures. The manual can also be used as a laboratory reference for anaerobe techniques.

The media and techniques described are used routinely by the CDC Anaerobe Unit, and the reactions for the various organisms presented in the tables are based on data obtained with cultures studied by these methods. For comparative purposes it is necessary to study cultures under uniform conditions, and an effort should be made to standardize the media and techniques used as closely as possible.

Since this manual has been revised regularly, a number of people have contributed to its contents and organization. The authors would like to express their gratitude to Mrs. Frances Thompson, Mrs. Ann Armfield, Mr. David Whaley, and Mrs. Loretta McCroskey of the Anaerobe Unit staff and to Miss Gilda Jones and Mr. Bobby Strong of the Bacteriology Training Unit staff. Our particular appreciation goes to Dr. George Lombard, in charge of the CDC Anaerobe Unit, and to Dr. Lillian Holdeman, formerly in charge of the Anaerobe Laboratory at CDC.

II. ISOLATION OF ANAEROBIC BACTERIA FROM CLINICAL MATERIAL

A. The anaerobic bacteria can be isolated and studied quite readily provided certain cardinal principles of anaerobic bacteriology are rigidly applied. Four of the most important considerations in the cultivation of anaerobic bacteria are:

1. Proper collection and transport of the material to be examined.
2. Culture of the material as soon as possible after collection.
3. Use of freshly prepared and properly reduced media.
4. Proper anaerobic conditions.

Proper collection and transport of clinical specimens is of **primary** importance in recovery of anaerobes. The sample should be collected from the active site of infection and precautions should be taken to exclude surface contaminants and aeration of the sample. Whenever possible tissue samples or fluid aspirates should be collected rather than swab samples. The material on swabs should never be allowed to dry out. Specimens should be placed under anaerobic conditions immediately after collection for transport to the laboratory since some anaerobes are quite oxygen sensitive and will die rapidly in an aerobic environment.

Sterile rubber stoppered transport vials and tubes containing an oxygen free CO_2 atmosphere are available commercially. Specimens aspirated with a needle and syringe can be injected directly into the transport bottles; care must be taken to exclude any air. If necessary, a specimen tube can be opened in an upright position, the specimen or swab added, and the tube closed for transport to the laboratory. Since CO_2 is heavier than air, the CO_2 atmosphere is maintained in the transport tube. As a very minimum procedure, the material can be placed in a medium containing a reducing agent such as cysteine or thioglycollate at room temperature for a period not exceeding 2 hours. Samples should not be refrigerated since chilling is detrimental to some anaerobes, and oxygen absorption is greater at lower temperatures.

All clinical material except specimens likely to be contaminated with normal flora should be routinely cultured for anaerobes. Specimens that **should not** be cultured include nasal swabs, throat swabs, sputum, gastric contents, skin, feces, voided or catheterized urine, and vaginal swabs.

For isolation of **anaerobes** from blood specimens, 5-10 ml of blood should be inoculated into 50-100 ml of liquid media (10% V/V) and the blood cultures incubated up to 14 days. Broth media containing 0.025% sodium polyanethol sulfonate (liquoid) and an anaerobic or partial CO_2 atmosphere are commercially available. Tryptic soy broth, trypticase soy broth, thioglycollate medium and pre-reduced brain heart infusion broth designed for anaerobic blood culture all appear to be equally satisfactory.[1] Liquoid may prevent the growth of some anaerobic cocci and slow the growth of some strains of *Bacteroides melaninogenicus.*[2] Blood cultures should be subcultured to plating media whenever there is any obvious growth and blind subcultures made at least after 48 hours incubation and at the end of 14 days. In addition to plating on blood agar plates, it has been shown that subculturing to a selective plating medium will allow detection of anaerobes mixed with aerobic organisms in bacteremic infections.[3]

Ideally, specimens should be cultured as soon as possible after collection and every effort should be made to prevent exposure of culture media to molecular oxygen. Plating media for primary isolation should be prepared on the day it is used, or freshly prepared media should be placed under anaerobic conditions for a period no longer than 2 weeks. Plating media can be stored in an anaerobe jar, glove box, or in an air-tight cabinet containing an oxygen free CO_2 atmosphere such as the one used in the Mayo Clinic anaerobe laboratory.[4] Liquid media containing reducing agents should be stored in the dark at room temperature in tightly capped tubes for not longer than 2 weeks.

Provided the media is fresh and properly reduced, successful cultivation of anaerobes can be obtained by use of the GasPak anaerobe jar or by use of an anaerobe jar with a gas replacement method.[5,6,7] At the CDC, an anaerobe glove box system[8] is used routinely for primary isolation of the anaerobes from clinical specimens and for subculture of colony isolates. Two excellent methods for the cultivation of anaerobes are the glove box system and the roll-streak tube system in which prereduced anaerobically sterilized (PRAS) media are used as recommended by the VPI anaerobe laboratory.[9] A section covering the use of various anaerobe systems is included in this manual.

All specimens except blood should be Gram stained and cultured by both direct plating and enrichment procedures. All liquid or semi-solid media stored in an aerobic environment should be prereduced by heating the media for 10 minutes in a boiling water bath and cooling before inoculation. Since most clinical laboratories are not set up for the glove box or roll-streak tube systems, the following procedures are designed for use with the anaerobe culture jar.

B. Prepare and stain direct smears from each specimen.

In order to gain some insight into the quantity and type of organisms in the specimen, examine a gram stained smear. Examination of wet mounts of unstained material, acid fast stained smears, and Giemsa stained smears may also be helpful. Use capillary pipettes to prepare smears from liquid specimens or use swabs directly. Observe and record:

1. The gram reaction, size, shape and relative numbers of organisms present.
2. The presence of spores and their shape and position in the cell.
3. Any distinctive morphological features such as branching, pseudo-branching, chaining, filaments, spherical bodies, or minute granular forms.

C. Inoculate primary isolation media* as soon as possible after specimens are received.

Fluid specimens: Use a capillary pipette to inoculate liquid or semisolid media near the bottom with one to two drops of inoculum. Place one drop on each plating medium and streak for isolation.

Tissue or other solid specimens: Mince with sterile scissors, add sufficient pre-reduced broth to emulsify the specimen, add sterile sand as necessary, and grind with a mortar and pestle. Treat as a liquid specimen.

Swabs: Place swab directly into liquid media and use separate swab to streak plates. If necessary, an inoculating suspension can be prepared by gently scrubbing the inoculum off a swab in approximately 2 ml of prereduced broth.

1. Heat all liquid and semisolid media in a boiling water bath for 10 minutes and cool before inoculation.

2. Inoculate one tube each of thioglycollate and chopped meat-dextrose medium (for enrichment cultures) and two blood agar plates. Add 0.5 ml sterile rabbit serum to the thioglycollate medium **after** the medium has been heated and cooled. Laked blood agar plates are recommended for the isolation of *Bacteroides melaninogenicus.*

   a. If clostridia are suspected inoculate one egg yolk agar plate (EYA) or a plate of stiff blood agar (4% to 6% agar).

   b. If mixed bacterial populations are suspected, inoculate selective plating media, such as phenylethyl alcohol blood agar[10] and/or kanamycin-vancomycin-menadione blood agar.[11] Paromomycin or neomycin may be substituted for kanamycin.

3. Incubate cultures at 35-37°C as follows:

   a. Incubate chopped meat and thioglycollate media in an anaerobe jar for 24 - 48 hours. Loosen caps to allow exchange of gasses.

   b. Incubate one BAP in a candle jar to determine aerobic flora.

   c. Incubate one BAP, EYA plate, and selective plating media in an anaerobe jar for a minimum of 48 hours, preferably 3 to 5 days. To absorb excess moisture in plates, use glass Petri dishes (100 X 15 mm) with metal covers containing absorbent discs or:

      (1) Add 2-3 drops of glycerin to the lid of each plate.

      (2) Place a piece of filter paper in the lid.

      (3) Replace bottom of plate, pressing the filter paper into the dish top.

D. After 24 hours incubation, examine enrichment cultures. If no growth is apparent, reincubate cultures.

1. Prepare and examine Gram stained preparations from thioglycollate and chopped meat-dextrose cultures.

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*For isolation of *Bacteroides melaninogenicus,* 5.0 mg of hemin and 0.5 mg of menadione (filter sterilized) should be added to each liter of isolation culture medium. It has recently been found that liquid vitamin K_1 (Nutritional Biochemicals), which is heat stable, is more satisfactory than menadione (personal communication, Dr. M. Lev, Albert Einstein Medical Center, New York). Dr. Lev suggests preparing a stock solution containing 1 mg vitamin K_1 per 10 ml of absolute ethanol. Add sufficient stock solution to give 0.1 μg/ml in liquid media and 10.0 μg/ml in solid media.

2. Remove inoculum from near the bottom of the culture tubes with a capillary pipette and inoculate plating media for subculture.

   a. Inoculate two blood agar plates from either the thioglycollate or chopped meat-dextrose cultures.

      (1) If clostridia are suspected, inoculate an egg yolk agar[12] plate or stiff blood agar.

      (2) If the culture contains a mixture of organisms, inoculate selective plating media.

   b. Incubate plates in a candle jar or anaerobically as outlined in Section C, 3.

3. Reincubate enrichment cultures if organisms seen on direct smear are not present in cultures.

E. After incubation:

1. Examine anaerobic and CO_2 plates with a hand lens and a dissecting microscope.

   a. Observe and record the action on blood and egg yolk, and the size and shape of colonies.

      (1) Prepare Gram stained smears for comparison of colonies on the different plates. Record shape and location of any spores observed.

      (2) Colonies on egg yolk agar **may** be used to test for catalase by adding a drop of 3% H_2O_2 to a suspension of organisms on a slide. Expose the EYA plates to air for at least 30 minutes before testing for catalase. Do not use colonies from blood agar plates to test for catalase.

   b. Determine the number of different colony types on the anaerobe plates.

      (1) For each colony to be transferred, prereduce one tube of chopped meat-dextrose medium and one tube of thioglycollate medium by heating the media in a boiling water bath for 10 minutes. Cool before use.

      (2) Using a needle with a small loop or a heat sealed 9" Pasteur capillary pipette, fish each different colony and inoculate a tube of chopped meat-dextrose medium and a tube of thioglycollate medium. If anaerobes other than clostridia are suspected, add 0.5 ml sterile rabbit serum to the thioglycollate medium.

      Chopped meat medium is best for culturing the clostridia and the enriched thioglycollate medium is more suitable for the nonsporeforming anaerobes.

   c. Incubate chopped meat and thioglycollate media in an anaerobe jar for 24 - 48 hours.

2. Be sure to have at least one representative colony of each morphological type seen on the original smear. If necessary, restreak plating media to obtain isolated colonies.

F. Examine plates inoculated with enrichment cultures after incubation. Subculture any colony types not isolated from direct plates to prereduced chopped meat-dextrose and thioglycollate media.

G. Examine thioglycollate and chopped meat subcultures from isolated colonies. If pure, use these cultures to inoculate appropriate differential media for identification of the isolates.

III. DETERMINATION OF CULTURAL AND BIOCHEMICAL CHARACTERISTICS

A. The identification of anaerobic bacteria involves the determination of cellular morphology, colonial characteristics on blood agar, and biochemical characteristics. In addition, the clostridia are tested for toxin production and, where necessary, the toxin is identified by toxin neutralization tests.

B. Gram stain the 24 hour culture to be used and check for purity. Observe the size and shape of organisms and the presence and location of spores.

C. Inoculate differential media.

1. Basic set of differential media: chopped meat, fermentation base, glucose, mannitol, lactose, sucrose, maltose, salicin, thiogel, iron-milk, indol-nitrite (for indol), indol-nitrite (for nitrite), H_2S medium, motility, esculin, peptone yeast glucose (PYG) agar, and an infusion agar slant. A tube of PYG broth may be added to the basic set to determine metabolic products by gas liquid chromatography.

   Additions to basic set for gram-positive sporeformers: chopped meat-dextrose and urea.

   Additions to basic set for gram-negative rods: xylose, arabinose, rhamnose, trehalose, thioglycollate broth, and 20% bile in thioglycollate broth.

   Additions to basic set for gram-positive nonsporeforming rods*: glycerol, xylose, arabinose.

2. Heat all media, **except** the slants and PYG agar, in a boiling water bath for 10 minutes. Cool in tap water.

3. Melt the PYG agar in a boiling bath or autoclave and place in 48°C water bath.

4. Using a capillary pipette, inoculate the fluid and semisolid media near the bottom of the tubes with a drop of culture. Expel a small amount of inoculum up the "line of stab" as the pipette is withdrawn. Be sure to expel all air from the pipette before placing the pipette into the medium. One pipette may be used to inoculate several different media.

*To check for spores, inoculate chopped meat agar slants, incubate anaerobically at 30°C for 10-14 days, and perform gram or spore stains as required.

5. Mix the inoculum and PYG agar by carefully inverting the agar tube; allow the medium to harden.

6. Inoculate infusion agar slants with a drop of culture placed at the top of the slants. Be sure the inoculum runs over the surface of the slant, not down the edge.

7. Inoculate the milk last.

8. Include an uninoculated tube of thiogel in each day's run as a control for gelatin liquefaction.

9. Incubate infusion agar slants and tubes of PYG broth in an anaerobe jar for 48 hours. Incubate other differential media at 35-37°C in an aerobic atmosphere or anaerobically if necessary for fastidious organisms.

D. Streak two blood agar plates with the 24 hour culture. If clostridia are suspected, also streak an egg yolk agar plate.

1. Incubate one BAP in a candle jar.

2. With gram-negative rods, add a two unit penicillin disc in the area of heavy inoculum on the second blood agar plate. Incubate the blood agar plate and EYA plate in an anaerobe jar. Anaerobic plates should be incubated at least 48 hours before the plates are examined.

E. Reading of plates.

1. Check plates incubated in the candle jar and compare with growth on the anaerobic blood agar plates to determine the oxygen tolerance of the organism. Record as anaerobe (growth only in anaerobe jar), aerotolerant anaerobe (some growth in candle jar but better growth anaerobically), or facultative anaerobe (more or less equal growth).

2. Check the egg yolk agar plates for lecithinase, lipase, and proteolytic enzyme production.

3. Check the anaerobic blood agar plates for hemolysis and colony characteristics. On plates with penicillin discs, any size zone of complete inhibition around the disc indicates sensitivity to penicillin. Record as sensitive or resistant.

Colonial characteristics on pure cultures are quite useful in the identification of anaerobic bacteria provided they are accurately described. A good hand lens and a dissecting microscope are invaluable for the inspection of colonies. It is helpful to follow a standard procedure for the examination and description of colonies so that characteristics of various cultures can be compared and the description of a colony by one can be interpreted by others.

Listed below are the characteristics of colonies which should be observed and some of the more common terms which aid in the description of bacterial colonies (See Figure I).

Medium_____. Culture age_____. Temp_____ C.

Diameter in mm_____.

Color_____.

Surface (glistening, dull, other).

Density (opaque, translucent, other).

Consistency (butyrous, viscid, membranous, brittle, other).

FIGURE 1

Colony Characteristics and Description of Bacterial Colonies

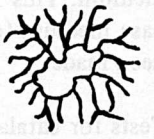
FORM

punctiform

irregular

circular

rhizoid



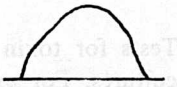
filamentous

spindle

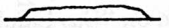
ELEVATION

flat

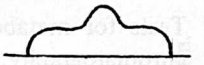
pulvinate



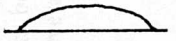
raised



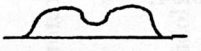
umbonate



convex



umbilicate



MARGIN

entire

erose

undulate

filamentous

lobate

curled

F. Reading of differential media.

Biochemical tests are routinely incubated up to 7 days after inoculation, but may be reported as early as 48 hours after inoculation if reactions allow identification of the isolate. Tubes are inspected daily and results recorded on the 1st, 2nd, and final day of incubation. All tests on fastidious or slow growing types may be held up to 3 weeks. Other exceptions to the routine are as follows:

1. Tests for indol and nitrite are made 24 hours after good growth is obtained in indol-nitrite medium. This is normally 48 hours after inoculation of the tubes. In addition, the fermentation base medium (control) can be tested for indol after the final reading of the fermentation test has been made.

2. Tests for catalase on infusion agar and hydrolysis of esculin are normally made at 48 hours, and the tubes are then discarded.

3. Thiogel may be incubated up to 1 month before it is reported as negative for liquefaction. Reactions are usually complete in 7 days, however.

4. Occasionally, determination of the motility of an organism in a semisolid motility medium will not be possible because of gas production. In this case motility can be checked by microscopic examination of wet mounts prepared from young (6-18 hour) broth cultures grown in a low-carbohydrate medium such as plain chopped meat medium.

5. Tests for toxin production in chopped meat-dextrose medium are normally made on 24 hour cultures. For exceptions and methods of testing see the section of "Detection of Clostridial Toxins, Toxin Neutralization Tests, and Pathogenicity Tests."

6. Tests for metabolic products are normally made on 48 hour PYG broth cultures by gas liquid chromatography. For the method of testing, see the section on "Gas Liquid Chromatography."

Interpretation of reactions given in differential media used for identification of anaerobes is given in Table I.

TABLE I

Interpretation of Reactions Given in Differential Media for Identification of Anaerobes

Medium	Purpose	Record
1. Chopped meat	**Proteolysis.** Digestion usually does not becoming apparent for several days.	1+B = sl black on edge of few particles 2+B = black on edge of most particles 3+B = 1/2 meat black 4+B = all meat black S = "sooty black" on tube D = digestion G = gas
2. Fermentation base	**Control for fermentation media.**	G = gas IR = indicator reduction*
3. Fermentation media	**Utilization of carbohydrates.**	A = acid (pH 6.0 or lower). – = no change IR = indicator reduction*
4. Thiogel	**Gelatin liquefaction.** Place cultures and control in beaker of cold water in the refrigerator. Check for liquefaction as soon as control is solid.	+ = liquefaction – = no liquefaction
5. Iron milk	**Proteolysis.**	C = clot G = gas D = digestion
6. Indol-nitrite	**Indol production.** Extract with one dropperfull of xylene. Add one dropper-full of Ehrlich's reagent.	+ = red with Ehrlich's – = no red with Ehrlich's

	Medium	Purpose
7.	Indol-nitrite	**Nitrate reduction.**** Add one dropperfull of solution A, 1/2 dropperfull of solution B. If no color, add Zn dust.
8.	H_2S	**H_2S production in lead acetate medium.**
9.	Urea	**Urease production.** If phenol red indicator is reduced, add a few drops dilute phenol red to culture last day of reading.
10.	Motility	**Motility.**
11.	Esculin	**Esculin hydrolysis.** Add one dropperfull 1% ferric ammonium citrate. Read the immediate reaction.
12.	PYG agar	**Gas production.**
13.	Infusion agar slant	**Catalase.** Expose slant to air for 30 minutes before testing. Add about 1 ml of 3% H_2O_2 to growth on slant. Gas evolution indicates presence of catalase.

Record	
+	= red with A and B
–	= red with Zn (may be slow)
NO_2+	= red with Zn
+	= obvious black color
tr	= slight black color
–	= no black color
+	= deep red color
–	= no color
IR	= indicator reduction
+	= motile
–	= non-motile
+	= brownish black
–	= no color change
+	= bubbles of gas
–	= growth with no gas
–	= no gas
4+	= bubbling to top of tube
3+	= immediate, many bubbles
2+	= delayed, considerable bubbles
1+	= delayed, few bubbles
tr	= few bubbles

Medium	Purpose	Record
14. Thioglycollate	**Growth characteristics and control for 20% bile broth.**	G = gas Describe growth
15. 20% bile in thioglycollate	**Growth in presence of 20% bile.** Compare to growth in thioglycollate broth.	S = stimulated N = no change I = inhibited
16. Chopped meat-dextrose	**Toxin testing on clostridia only.**	

* Some clostridia will reduce the indicator. If this occurs, prepare a dilute solution of bromthymol blue (BTB), using 2-3 drops 1% aqueous BTB per 30 ml water in 30 ml dropping bottles.

Put diluted indicator solution in depressions of plastic spot plates. With a capillary pipette, add a few drops of culture to the indicator in the spot plate and observe color. Record as acid or negative.

**Some bacteria reduce nitrate (NO_3) to nitrite (NO_2); others reduce nitrite to other products (N_2O, NH_2OH, NH_3).

In the nitrate test, solutions A (sulfanilic acid) and B (dimethyl-alpha-napthylamine) are used to indicate the presence of nitrite (NO_2 + A + B = red color).

Three types of reactions may be observed:

$NO_3 \xrightarrow[\text{(bacterial reduction)}]{\text{nitratase}}$ NO_2; add A and B; red color = + (bacteria reduced nitrate)

$NO_3 \xrightarrow[\text{(nonbacterial reduction)}]{\text{zinc}}$ NO_2; with A and B; red color = – (bacteria did not reduce nitrate)

$NO_3 \xrightarrow{\text{nitratase}} NO_2 \xrightarrow[\text{(bacterial reduction)}]{\text{dehydrogenases}}$ other end products; add A, B, Zn; no color = NO_2+; bacteria reduced NO_2

Glucose is added to the nitrate medium to serve as a hydrogen donor. In an agar-containing medium, nitrate reduction by zinc dust frequently takes place very slowly, sometimes requiring 15 minutes or more. *Any* development of red color after zinc is added is sufficient to indicate that the "culture is nitrate negative"; i.e., that zinc is reducing the nitrate in the medium.

IV. DETECTION OF CLOSTRIDIAL TOXINS, TOXIN NEUTRALIZATION TESTS, AND PATHOGENICITY TESTS

A. Tests for clostridial toxins.

1. All *Clostridium* cultures are routinely tested for toxin production. Although the conditions for toxin elaboration will vary with individual species, clostridial toxins can generally be detected in chopped meat-dextrose cultures incubated 18 hours at 35-37°C.

   If *C. novyi* A is suspected, chopped meat medium without dextrose should be used. Cultures suggestive of *C. botulinum* or *C. sporogens* should be grown in chopped meat-dextrose-starch medium and incubated at 30°C[13] (see section on detection of *C. botulinum* and botulinal toxins).

   Attention must also be given to the age of the culture. Some clostridia, e.g. *C. perfringens, C. histolyticum,* and *C. haemolyticum,* are more toxic in young cultures and others, e.g. *C. botulinum, C. novyi* and *C. tetani*, exhibit maximum toxicity after several days incubation.

2. Do routine tests for toxin on 18 hour chopped meat-dextrose cultures incubated at 35-37°C.

   a. Pipette 3.0 ml of culture supernate into a 12 ml plastic centrifuge tube.

   b. Centrifuge at 10,000 RPM (12,350 x g) for 10 minutes and decant the supernatant fluid in a clean test tube.

   c. Load a 1.0 ml syringe with 0.8 ml of the centrifuged culture fluid and inoculate two 15 to 20 gram mice I. P. with 0.4 ml each.

   d. Observe the mice for 4 days. Toxic clostridial cultures will usually kill mice within 24 hours after inoculation.

   e. The need for further toxin tests for which special media, cultural conditions, or inoculation procedures are used will depend on the overall results of the cultural, biochemical, and routine toxicity tests.

B. Identification of specific clostridial toxin.

1. When it has been demonstrated that a *Clostridium* culture fluid is toxic for mice, it is often helpful in the identification of the organism to perform animal protection tests with specific immune serum. Neutralization of toxic culture fluid is usually accomplished by mixing 1.2 ml of the centrifuged culture fluid with 0.3 ml of specific antitoxin, allowing the mixture to stand for 30 minutes at 37°C and inoculating two mice I. P. with 0.5 ml each of the material. Since toxicity of *Clostridium* culture filtrates may vary, it is advisable to test culture filtrates diluted 1:5 in gelatin diluent.

2. The cultural characteristics of *Clostridium septicum* and *Clostridium chauvoei* are essentially identical. Isolates resembling either of these organisms should be tested with both *C. septicum* and *C. chauvoei* antisera as well as normal rabbit serum.

3. The commercially available *Clostridium* antisera include:

   C. perfringens types A. B, C, D, and E
   C. novyi types A and B
   C. chauvoei
   C. septicum
   C. tetani
   C. botulinum types A, B, C, D, E, and F
   C. sordellii

C. Tests for pathogenicity.

1. Determination of the pathogenic properties of the anaerobic bacteria is quite useful in evaluating the role an organism may play in a pathological process. The type of laboratory animal to use and the conditions required for the demonstration of pathogenicity vary considerably and will depend upon the species being tested.

2. *Clostridium* cultures are routinely tested for pathogenicity by inoculating a guinea pig I.M. in the thigh with 0.5 ml of a mixture containing equal parts of an 18 hour chopped meat-dextrose culture and 10% calcium chloride.

3. Pathological changes in guinea pigs after injection with *Clostridium* cultures are quite varied and may include (1) hemorrhagic or gelatinous edema, (2) pockets of gas in the infected tissue, (3) necrosis, (4) digestion of muscle tissue, (5) rapid toxic death with little detectable pathology, and (6) paralysis and/or toxic muscular spasms[14]. The pathological changes usually occur within a period of 4-5 days; however, the guinea pigs should be held for a period of 2 weeks before the cultures are reported as nonpathogenic for guinea pigs.

V. TOXIN TYPING OF CLOSTRIDIUM PERFRINGENS

A. Most human *C. perfringens* infections or intoxications are due to type A strains. Types A, B, C, D, and E are responsible for a variety of diseases in animals. Type A strains produce a lethal alpha toxin and all other types produce at least one other major lethal toxin in additional to alpha toxin. Differentiation of types is based on the detection and specific neutralization of toxins from culture fluids. Tests for toxicity and serum neutralization for typing are done in mice.

C. perfringens types are characterized by the production of one or more of the following lethal toxins:

Type	A	B	C	D	E
Toxins	α	$\alpha\beta\epsilon$	$\alpha\beta$	$\alpha\epsilon$	$\alpha\iota$

The epsilon and iota toxins are activated by trypsin[15]. Alpha and beta toxins may be inactivated by trypsin.

B. Preparation of centrifuged culture fluid (CCF).

1. Use a 6 to 16-hour (no older) chopped meat-starch culture.
2. Centrifuge culture fluid in polyethylene centrifuge tubes at 10,000 RPM (preferably in the cold) for 10 minutes.
3. Pour CCF into a 15-x 125-mm tube.
4. For trypsinization mix one part 1% trypsin solution with nine parts CCF in a 15-x 125-mm tube and incubate the mixture for 30 minutes in a 37°C water bath.

C. Determination of toxicity.

1. Inoculate two mice I.P. with 0.5 ml each of untreated CCF.
2. Inoculate two mice I.P. with 0.5 ml each of trypsinized CCF.
3. Observe mice for 48 hours. Type A strains usually kill mice within 18 hours after inoculation.

D. Serum neutralization tests.

1. When working with toxic isolates from human specimen material, the routine neutralization procedure is to test with type A antiserum and normal rabbit serum control. This includes tubes 1,2, 5, and 6 in Table II. If no deaths occur in mice inoculated with either trypsinized or untrypsinized toxin neutralized with type A antiserum the culture is reported out as type A. Where deaths do occur the CCF is tested with antisera against the remaining *C. perfringens* types.

2. Mix 1.2 ml of CCF or trypsinized CCF in 12-x 75-mm tubes with 0.3 ml of the particular *C. perfringens* antisera (types A, B, C, D, or E) or normal rabbit antiserum as outlined in Table II.

3. Incubate the CCF-serum mixtures for 30 minutes in a 37°C water bath and inoculate two mice I.P. with each mixture.

TABLE II

Mouse Toxin Neutralization Tests of *C. perfingens* Centrifuged Culture Fluids (CCF)

Tube	Mixture Injected	Lethality for mice with *C. perfringens* type				
		A	B	C	D	E
1	Untreated CCF + normal rabbit serum	+	+	+	+	+
2	Untreated CCF + Anti A (anti alpha)	–	+	+	*–	±
3	Untreated CCF + Anti C (anti beta)	–	*–	–	*–	±
4	Untreated CCF + Anti B (anti alpha + anti beta + anti epsilon)	–	–	–	–	±
5	Trypsinized CCF + normal rabbit serum	±	+	**–	+	+
6	Trypsinized CCF + Anti A (anti alpha)	–	+	**–	+	+
7	Trypsinized CCF + Anti D (anti alpha + anti epsilon)	–	**–	**–	–	+
8	Trypsinized CCF + Anti B (anti alpha + anti beta + anti epsilon)	–	–	–	–	+
9	Trypsinized CCF + Anti E (anti alpha + anti iota)	–	+	**–	+	–
	Diagnostic toxin identified	α	$\beta+\epsilon$	β	ϵ	ι

* Usually negative, as epsilon in protoxin form.
** Usually negative, as beta toxin is destroyed by trypsin.

VI. IDENTIFICATION OF ANAEROBIC BACTERIA

A. Clinical isolates of anaerobic bacteria most frequently submitted to CDC for identification are listed in Table III. Table IV lists changes in the nomenclature of anaerobic bacteria since the 1968 edition of this manual was printed.

B. Identification of *Clostridium* species.

1. Sporeforming, catalase negative, anaerobic or aerotolerant bacilli are classified in the genus *Clostridium.* The majority of the clostridia are gram-positive, but certain species may appear gram-negative, particularly in older cultures. Some (e.g., *Clostridium perfringens*) rarely produce spores except in special media. Some particularly useful characteristics for identification of clostridia are aerotolerance, motility, position of the spore in the cell, lecithinase production, lipase production, fermentation of carbohydrates, proteolytic activity, urease activity, and toxicity for mice as noted in Table V. Other helpful characteristics for differentiation of the clostridia include reduction of nitrate to nitrite, indol production, hyrolysis of esculin, action on milk, determination of organic acids produced in PYG medium, and pathogenicity for guinea pigs. The characteristics of commonly encountered clostridia are listed in Table VI and data on rarely encountered species (less than five strains examined by CDC) are included in Table VII.

2. Commonly encountered pathogenic *Clostridium* species include *C. histolyticum, C. novyi* type A, *C. chauvoei, C. perfringens, C. septicum* and *C. tetani. Clostridium fallax, C. haemolyticum,* and *C. novyi* type B are also pathogenic for guinea pigs, but are rarely submitted to CDC for identification. Some strains of *C. sordellii* are pathogenic, but the majority of the strains tested at CDC were nonpathogenic for guinea pigs.

3. Some of the characteristics listed in the first edition of this manual, such as hemolysis, serum liquefaction, and starch hydrolysis, have been found to be less useful for identification of clostridia than those listed in Table VI. Determining the toxicity of *Clostridium* isolates is particularly important since some *Clostridium* species (e.g., *C. botulinum, C. chauvoei, C. septicum* and *C. tetani*) cannot be identified with certainty without performing toxin neutralization tests. When the identity of an isolate from a significant clinical source is in question, the culture should be submitted to a reference laboratory for identification.

TABLE III

Clinical Isolates of Anaerobic Bacteria Most Frequently Submitted to the CDC Anaerobe Unit

1. Clostridia

   C. bifermentans
   C. butyricum
   *C. cadaveris (C. capitovale)**
   C. innocuum
   C. limosum (Clostridium sp. CDC group P-1)
   C. perfringens
   C. ramosum (Catenabacterium filamentosum, Bacteroides terebrans)
   C. septicum
   C. sordellii
   C. sporogenes
   C. subterminale
   C. tertium

2. Nonsporeforming gram-positive bacilli

   Actinomyces israelii
   Actinomyces odontolyticus
   Actinomyces naeslundii
   Arachnia propionica (Actinomyces propionicus)
   Bifidobacterium eriksonii (Actinomyces eriksonii)
   Eubacterium alactolyticum (Ramibacterium species)
   Eubacterium lentum (Corynebacterium diptheroides)
   Eubacterium limosum
   Propionibacterium acnes (Corynebacterium acnes)
   Propionibacterium granulosum (Corynebacterium granulosum)

3. Nonsporeforming, gram-negative bacilli

   Bacteroides fragilis ssp. *fragilis (B. fragilis)*
   Bacteroides fragilis ssp. *thetaiotaomicron (B. variabilis)*
   Bacteroides fragilis ssp. *vulgatus (B. incommunis)*
   Bacteroides melaninogenicus ssp. *asaccharolyticus*
   Bacteroides melaninogenicus ssp. *intermedius*
   Fusobacterium mortiferum (Sphaerophorus ridiculosum)
   Fusobacterium necrophorum (Sphaerophorus necrophorus)
   Fusobacterium nucleatum (Fuosbacterium fusiforme)

4. Anaerobic cocci

   Peptococcus sp. CDC group 2
   Peptostreptococcus sp. CDC group 1
   Peptostreptococcus sp. CDC group 2
   Peptostreptococcus sp. CDC group 3
   Veillonella alcalescens
   Veillonella parvula

*Former name(s)

TABLE IV

List of Changes in the Nomenclature of Anaerobic Bacteria

Present Designation	Former Designation(s)
Arachnia propionica	*Actinomyces propionicus*
Bacteroides clostridiiformis ssp. *girans**	*Fusobacterium girans*
Bacteroides fragilis	
ssp. *fragilis*	*Bacteroides fragilis*
ssp. *distasonis*	*Bacteroides fragilis*
ssp. *vulgatus*	*Bacteroides incommunis*
ssp. *thetaiotaomicron*	*Bacteroides variabilis*
ssp. *ovatus*	*Bacteroides ovatus*
Bacteroides melaninogenicus	
ssp. *melaninogenicus*	*Bacteroides melaninogenicus*
ssp. *asaccharolyticus*	*Bacteroides melaninogenicus*
ssp. *intermedius*	*Bacteroides melaninogenicus*
Bacteroides oralis	
ssp. *oralis*	*Bacteroides oralis*
ssp. *elongatus*	*Bacteroides oralis*
Bacteroides pneumosintes	*Dialister pneumosintes*
Bifidobacterium eriksonii	*Actinomyces eriksonii*
Campylobacter sputorum ssp. *sputorum*	*Vibrio sputorum*
Clostridium cadaveris	*Clostridium capitovale*
Clostridium limosum	*Clostridium species* group P1
Clostridium ramosum	*Catenabacterium filamentosum, Bacteroides trichoides (B. terebrans)*
Eubacterium alactolyticum	*Ramibacterium alactolyticum, Ramibacterium pleuriticum*
Eubacterium lentum	*Corynebacterium diphtheroides* (CDC Manual), *Bifidobacterium cornutum, Corynebacterium* group 3
Fusobacterium necrophorum	*Sphaerophorus necrophorus*
Fusobacterium nucleatum	*Fusobacterium fusiforme*
Fusobacterium mortiferum	*Fusobacterium ridiculosum, Sphaerophorus ridiculosum*
Lactobacillus catenaforme	*Catenabacterium catenaforme*
Propionibacterium acnes	*Corynebacterium acnes*
Propionibacterium freudenreichii ssp. *freudenreichii*	*Propionibacterium freudenreichii*
Propionibacterium freudenreichii ssp. *shermanii*	*Propionibacterium shermanii*
Propionibacterium granulosum	*Corynebacterium granulosum*

*The validity of the species name *Bacteroides clostridiiformis* is presently in doubt. Some of the strains previously identified as this species have been shown to produce spores. It would be more correct to classify sporeformers in the genus *Clostridium*.

TABLE V

Characteristics of Commonly Encountered *Clostridium* Species Which Are Particularly Useful For Identification

CHARACTERISTIC	SPECIES
Aerotolerant	*C. histolyticum, C. tertium*
Nonmotile	*C. innocuum, C. perfringens, C. ramosum*
Terminal spores	*C. cadaveris, C. innocuum, C. paraputrificum, C. tertium, C. tetani*
Produce lecithinase on egg yolk agar	*C. bifermentans, C. limosum, C. novyi, C. perfringens, C. sordellii, C. subterminale*
Produce lipase on egg yolk agar	*C. botulinum, C. novyi* type A, *C. sporogenes*
Asaccharolytic	*C. histolyticum, C. limosum, C. subterminale, C. tetani*
Urease positive	*C. sordellii*
Nonproteolytic (do not hydrolyze gelatin)	*C. butyricum, C. innocuum, C. paraputrificum, C. ramosum, C. tertium*
Toxic for mice	*C. botulinum, C. chauvoei, C. histolyticum, C. novyi* type A, *C. perfringens**, *C. septicum, C. tetani*

*The majority of the *C. perfringens* isolates from human sources tested at CDC were nontoxic for mice when tested as described in the section on "Detection of Clostridial Toxins, Toxin Neutralization Tests, and Pathogenicity Tests."

TABLE VI
Differential Characteristics of Commonly Encountered *Clostridium* Species

SPECIES	NUMBER OF STRAINS	AEROBIC GROWTH	SPORES	MOTILITY	LECITHINASE	LIPASE	GLUCOSE	MANNITOL	LACTOSE	SUCROSE	MALTOSE	SALICIN	GLYCEROL	XYLOSE	ARABINOSE	NITRATE REDUCTION	INDOL	ESCULIN HYDROLYSIS	GELATIN HYDROLYSIS	MILK	TOXICITY FOR MICE	ORGANIC ACIDS DETECTED BY GLC*	OTHER
C. bifermentans	119	–	ST	+	+	–	A	–	–	–	A	V	V	–	–	–	+	V	+	CD	–	A,P,IB,(B),IV,IC	Urease negative
C. botulinum A	24	–	ST	+	–$^{+}$	+	A	–	–	–	A^{-}	–^{A}	V	–	–	–	–	+	+	CD	+		Toxin neutralization ‡
B	14	–	ST	+	–	+	A	–	–	–^{A}	A	–^{A}	–^{A}	–	–	–	–	+$^{-}$	+	(C)(D)	+		Toxin neutralization ‡
C	14	–	ST	+	–	+	A	–	–	–^{A}	V	–	–^{A}	–	–	–$^{+}$	–	–	V	NC(C)	+		Toxin neutralization ‡
D	5	–	ST	+	–	+	A	–	–	–	V	–	V	–	–	–	–	V	V	NC	+		Toxin neutralization ‡
E	10	–	ST	+	–$^{+}$	+	A	–	–	A	A	–	V	–	–	–	–	–	–$^{+}$	NC	+		Toxin neutralization ‡
F	7	–	ST	+	–	+	A	–	–	V	A	V	V	–	–	–	–	V	V	(C)(D)	+		Toxin neutralization ‡
C. butyricum	74	–	ST	+	–	–	A	–	A	A	A	A	A^{-}	A	A^{-}	–$^{+}$	–	+	–	CG	–	A,(P),B	
C. cadaveris †	45	–	T	+	–	–	A	–	–	–	–	–	–	–	–	–	+	–	V	CG	–	A,(IB),B,IV	
C. chauvoei	13	–	ST	+	–	–	A	–	A	A^{-}	A	–	–	–	–	+$^{-}$	–	–$^{+}$	+$^{-}$	(C)	V	A,B	Toxin neutralization ‡
C. difficile	10	–	ST	+	–	–	A	A	–	–	–	A^{-}	–	V	–	–	–	+	V	NC	V	A,P,IB,B,IV,V,IC	
C. histolyticum	6	+	ST	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	CD	V	A,L	Pathogenic for guinea pig
C. innocuum	84	–	T	–	–	–	A	A	–^{A}	A^{-}	–^{A}	A	–	–	–	–	–	+	–	NC	–	A,B	
C. limosum †	47	–	ST	+	+	–	–	–	–	–	–	–	–	–	–	–	–	–	+	CD	–	A	
C. novyi A	13	–	ST	+	+	+	A	–	–	–	A^{-}	–	A	–	–	–$^{+}$	–	V	+	(C)(G)	V	A,P,B	Toxin neturalization ‡
C. paraputrificum	29	–	T	+	–	–	A	–	A	A^{-}	A	A	–	–	–	V	–	+$^{-}$	–	(C)(G)	–	A,(P),B,(V)	
C. perfringens	678	–	ST	–	+	–	A	–	A	A	A	V	A	–	–	+	–	V	+	CG	V	A,(P),B	Spores seldom observed
C. ramosum †	61	–	T	–	–	–	A	A^{-}	A	A	A	A	–	–	–	–	–	+	–	(C)(G)	–	A,L,S	Frequently gram negative
C. septicum	83	–	ST	+	–	–	A	–	A	–	A	A	–	–	–	+$^{-}$	–	+	+	(C)(G)	+	A,B	Toxin neutralization ‡
C. sordellii	88	–	ST	+	+$^{-}$	–	A	–	–	–	A	–	A^{-}	–	–	–	+	–	+	CD	–	A,(P),(IB),(IV),(IC)	Urease positive
C. sphenoides	9	–	ST	+$^{-}$	–	–	A	V	A^{-}	V	A^{-}	A	–^{A}	A^{-}	A^{-}	–$^{+}$	V	+	–$^{+}$	(C)(G)	–	A,L,S	Usually appear gram negative
C. sporogenes	132	–	ST	+	–	+	A	–	–	–^{A}	A	V	V	–	–	–	–	+	+	CD	–	A,(P),(IB),(B),IV,(IC)	
C. subterminale	53	–	ST	+	–$^{+}$	–	–	–	–	–	–	–	–	–	–	–	–	–$^{+}$	+	CD	–	A,(P),IB,B,IV	
C. tertium	103	+	T	+	–	–	A	A	A	A	A	A	–	A^{-}	–	+$^{-}$	–	+	–	(C)(G)	–	A,B	
C. tetani	52	–	T	+	–	–	–	–	–	–	–	–	–	–	–	–	V	–	+	NC	+	A,P,B	Toxin neutralization ‡

*GLC (gas liquid chromatography) analysis was not performed on all of the strains listed.
+ = positive reaction for 90-100% of strains.
– = negative reaction for 90-100% of strains.
A superscript indicates the reaction shown in 11-25% of the strains.
V = variable reaction
( ) = variable
A = acid reaction (yellow color with bromthymol blue indicator, pH less than 6.0)

C = coagulated
D = digested
G = gas
NC = no coagulation
ST = subterminal
T = terminal
A = acetic acid

P = propionic acid
IB = isobutyric acid
B = butyric acid
IV = isovaleric acid
V = valeric acid
IC = isocaproic acid
L = lactic acid

S = succinic acid
† = *C. cadaveris* was formerly listed as *C. capitovale*, *C. limosum* includes organisms formerly identified as *Clostridium* species CDC Group P-1, and *C. ramosum* includes organisms previously listed as *Catenabacterium filamentosum* and *Bacteroides terebrans*.
‡ = Toxin neutralization test is required for specific identification.

TABLE VII

Differential Characteristics of *Clostridium* Species Infrequently Isolated from Clinical Materials

SPECIES	NUMBER OF STRAINS	AEROBIC GROWTH	SPORES	MOTILITY	LECITHINASE	LIPASE	GLUCOSE	MANNITOL	LACTOSE	SUCROSE	MALTOSE	SALICIN	GLYCEROL	XYLOSE	ARABINOSE	NITRATE REDUCTION	INDOL	ESCULIN HYDROLYSIS	GELATIN HYDROLYSIS	MILK	TOXICITY FOR MICE	ORGANIC ACIDS DETECTED BY GLC*	OTHER
C. barati	1	–	ST	–	+	–	A	–	A	A	A	A	–	–	–	–	–	+	–	NC	–	A,P,B	
C. cochlearium	1	–	ST	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	(C)	–		
C. glycolicum	2	–	ST	+	–	–	A	–	–	–	A	–	V	V	–	–	–	–	–	NC	–	A,P,IB,IV	
C. hemolyticum	2	–	ST	+	+	–	A	–	–	–	–	–	–	–	–	–	V	+	+	D	V		Pathogenic for guinea pig
C. malenominatum	2	–	T	+	–	–	–	–	–	–	–	–	–	–	–	–	+	–	–	NC	–	A,B	
C. perenne	1	–	T	–	+	–	A	–	A	A	A	A	–	–	–	+	–	+	–	C	–	A,P,B	
C. pseudotetanicum	2	–	T	–	–	–	A	–	A	A	A	A	–	V	–	V	–	+	–	CG	–	A,P,(IB),(IV)	
C. scatologenes	2	–	T	+	–	–	A	–	–	–	–	–	–	–	–	–	+	–	–	(C)	–	A,P,B	

*GLC (gas liquid chromatography) analysis was not performed on all of the strains listed.
+ = positive reaction for 90-100% of strains
– = negative reaction for 90-100% of strains
A superscript indicates the reaction shown in 11-25% of the strains
V = variable reaction
( ) = variable

A = acid reaction (yellow color with bromthymol blue indicator, pH less than 6.0).
C = coagulated
D = digested
G = gas
NC = no coagulation
ST = subterminal

T = terminal
A = acetic acid
P = propionic acid
IB = isobutyric acid
B = butyric acid
IV = isovaleric acid

C. Identification of gram-negative nonsporeforming anaerobic bacteria:

1. Gram-negative nonsporeforming anaerobes are presently classified in twelve genera:

Acidaminococcus	*Leptotrichia*
Bacteroides	*Selenomonas*
Borrelia	*Succinimonas*
Butyrivibrio	*Succinivibrio*
Campylobacter	*Treponema*
Fusobacterium	*Veillonella*

Key characteristics for differentiation of these genera are shown in Figure 2. The gram-negative nonsporeforming anaerobes include microorganisms which are indigenous in the oral cavity, intestinal tract, genitourinary tract, or skin of man and/or lower animals and in the rumen of herbivorous animals. Only members of the genera *Bacteroides, Fusobacterium,* and *Veillonella* are commonly isolated from humans with clinically significant infections, and of these, the *Bacteroides* and *Fusobacterium* are most important. This does not imply that members of other genera are not pathogenic for humans.

FIGURE 2

Differentiation of Gram-Negative, Nonsporeforming Anaerobic Bacteria to the Genus Level

I. Rods or spiral forms	
A. Nonmotile or peritrichous flagella if motile	
1. Butyric acid is a major metabolic product	*Fusobacterium*
2. Lactic acid is only major metabolic product	*Leptotrichia*
3. No butyric acid produced in absence of iso-acids	*Bacteroides*
B. Motile with polar flagella	
1. Asaccharolytic	*Campylobacter*
2. Saccharolytic	
a. Produce butyric acid	*Butyrivibrio*
b. Produce succinic acid	
(1) Spiral forms	*Succinivibrio*
(2) Ovoid cells	*Succinimonas*
c. Tuft of flagella on concave side of cell	*Selenomonas*
d. Spiral forms with axial filaments	*Treponema* and *Borrelia*
II. Cocci	
A. Produce propionic and acetic acids	*Veillonella*
B. Produce butyric and acetic acids	*Acidaminococcus*

2. The genus names *Dialister, Sphaerophorus,* and *Vibrio* as listed in the 1968 edition of this manual are no longer recognized. *Dialister pneumosintes* is now classified as *Bacteroides pneumosintes,* members of the former genus *Sphaerophorus* as *Fusobacterium* or *Bacteroides,* and *Vibrio sputorum* is now called *Campylobacter sputorum* ssp. *sputorum.*[9]

3. Differential characteristics of commonly isolated *Bacteroides* and *Fusobacterium* species from clinical materials are listed in Table VIII. The cultural and biochemical characteristics of *Bacteroides clostridiiformis* ssp. *girans* are quite similar to those of *Clostridium sphenoides* (see Table VI). The differential characteristics of other anaerobic, nonsporeforming, gram-negative bacilli isolated from non-human sources or of which less than five strains have been examined at CDC are shown in Table IX. Some of these, e. g. *Bacteroides oralis* and *Bacteroides fragilis* ssp. *ovatus* are present in the oral cavity and/or the intestinal tract of man, but are rarely if ever recovered from clinical materials not contaminated with normal flora. Differential characteristics for the *Veillonella* are shown in Table XIII with those of other anaerobic cocci. Differential characteristics for the subspeciation of *Bacteroides fragilis* are shown in Table X.

D. Identification of gram-positive, nonsporeforming, anaerobic bacteria.

1. Gram-positive, nonsporeforming anaerobes include members of the following genera:

Actinomyces	*Peptococcus*
Arachnia	*Peptostreptococcus*
Eubacterium	*Ruminococcus*
Lachnospira	*Sarcina*
Lactobacillus	

Key characteristics for differentiation of these genera are shown in Figure 3. The genus names *Catenabacterium, Cillobacterium,* and *Ramibacterium* are no longer recognized and species formerly classified as *Corynebacterium* are now included in the genera *Eubacterium* or *Propionibacterium* (see Table IV).

2. Differential characteristics of anaerobic, nonsporeforming, gram-positive bacilli commonly isolated from clinical materials are listed in Table XI, and characteristics of species rarely encountered in infected humans and those from non-human sources are shown in Table XII. Differential characteristics of anaerobic cocci are presented in Table XIII. At the present time there is little agreement on the classification of the anaerobic gram-positive cocci. For this reason the cocci are listed by CDC groups rather than species.

E. Colonial characteristics of some representative anaerobes are shown in plates 1 and 2. Plates 3, 4, and 5 are photomicrographs of anaerobic bacteria in smear preparations from broth or agar media that show some of the wide variety of morphological characteristics.

TABLE VIII

Differential Characteristics of *Bacteroides* and *Fusobacterium* Species Commonly Isolated from Clinical Materials

SPECIES OR GROUPS	NUMBER OF STRAINS	AEROBIC GROWTH	BLACK COLONIES, BLOOD AGAR	MOTILITY	GLUCOSE	MANNITOL	LACTOSE	SUCROSE	MALTOSE	SALICIN	XYLOSE	ARABINOSE	ESCULIN HYDROLYSIS	GELATIN HYDROLYSIS	NITRATE REDUCTION	INDOL	MILK	ORGANIC ACIDS DETECTED BY GLC*	OTHER
Bacteroides																			
B. corrodens	8	–	–	–	–	–	–	–	–	–	–	–	–	–	+–	–	NC	A	Pitted colonies on blood agar.
CDC Group F-1	37	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	NC	A	Colonies not pitted on blood agar.
CDC Group F-2	30	–	–	–	–	–	–	–	–	–	–	–	–	–	+	–+	NC	A,(P),(L),(S)	Colonies not pitted on blood agar.
B. clostridiiformis ssp. girans	7	–	–	+	A	–	A–	A	A	A–	A	A	–+	–	+	–	C	A	Peritrichous flagella.
*B. fragilis ssp. distasonis***	11	–	–	–	A	–	A	A	A	V	A–	–A	+	–	–	–	C	A,P,(IV)	Not inhibited by 20% bile or penicillin (2 unit disc).
*B. fragilis ssp. fragilis***	376	–	–	–	A	–	A	A	A	–	A	–	+	–	–	–	C	A,P,(IV)	"
*B. fragilis ssp. thetaiotaomicron***	84	–	–	–	A	–	A	A	A	V	A	A	+	–	–	+	C	A,P,(IV)	"
*B. fragilis ssp. vulgatus***	46	–	–	–	A	–	A	A	A	V	A	A	+–	–	–	–	(C)	A,P	"
B. melaninogenicus ssp. asaccharolyticus	12	–	+	–	–	–	–	–	–	–	–	–	–	+	–+	V	CD	A,(P),(IB),(B),(IV)	Most strains require vitamin K as well as hemin.
B. melaninogenicus ssp. intermedius	5	–	+	–	A	–	–	V	V	–+	–	–	–	+	–+	+	CD	A,(IB),(IV)	"
Fusobacterium																			
F. mortiferum	20	–	–	–	A	–	A	A	A	A–	–	–	+–	–	–	–	(CG)	A,P,B	Highly pleomorphic, swollen bodies common.
F. necrophorum	46	–	–	–	A	–	–	–	A–	–	–	–	–	–	–	+	NC	A,P,B	Cells quite variable in length and width.
F. nucleatum	78	–	–	–	A–	–	–	–	–	–	–	–	–	–	–	+	NC	A,(P),B	Usually thin, uniform filaments with or without pointed ends.

*GLC (gas liquid chromatography) analysis was not performed on all of the strains listed.
**See Table X which lists key characteristics for sub-specialization of *Bacteriodes fragilis*.
\+ = positive reaction for 90-100% of strains.
– = negative reaction for 90-100% of strains.
Superscript indicates the reaction shown in 11-25% of the strains.
V = variable reaction
( ) = variable.

A = acid reaction (yellow color with bromthymol blue indicator, pH less than 6.0).
C = coagulated
D = digested
G = gas
NC = no coagulation
A = acetic acid

P = propionic acid
IB = isobutyric acid
B = butyric acid
IV = isovaleric acid
L = lactic acid
S = succinic acid

TABLE IX

Characteristics of Anaerobic, Nonsporeforming Gram-Negative Bacilli Infrequently Isolated from Clinical Materials

SPECIES	AEROBIC GROWTH	BLACK COLONIES ON BLOOD AGAR	MOTILITY	GLUCOSE	MANNITOL	LACTOSE	SUCROSE	MALTOSE	SALICIN	XYLOSE	ARABINOSE	ESCULIN HYDROLYSIS	GELATIN HYDROLYSIS	NITRATE REDUCTION	INDOL	MILK	ORGANIC ACIDS DETECTED BY GLC*	OTHER
Bacteroides																		
B. biacutus	–	–	–	A	–	A	A	A	–	A	A	+	–	–	–	C	A,L,S	Motile strains have been reported (VPI).
B. clostridiiformis ssp. clostridiiformis	–	–	–	A	–	A	A	A	A	A	A	+	–	–	–	NC	A	
B. fragilis ssp. ovatus	–	–	–	A	A	A	A	A	–	A	A	+	–	–	+	C	A,P	See Table X for sub-speciation of *B. fragilis.*
B. hypermegas	–	–	–	A	A	A	A	A	A	A	A	+	–	–	–	C	A,P	Found in intestine of chickens.
B. melaninogenicus ssp. melaninogenicus	–	+	–	A	–	A	A	A	–	–	–	+	+	–	–	C	A,IB,IV	Most strains require vitamin K as well as hemin.
B. oralis	–	–	–	A	–	A	A	A	–	–	–	+	–	–	–	C	A,L,S	Inhibited by 20% bile and penicillin (2 unit disc).
B. pneumosintes	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	NC	A	Very short rods, < 0.3 microns in length.
Fusobacterium																		
F. necrogenes	–	–	–	A	–	–	–	–	–	–	–	+	–	–	–	NC	A,P,B	Found in intestine of chickens.
F. novum	–	–	–	A	–	A	A	A	A	A	A	+	–	–	+	NC	A,P,B	Cells quite variable in length and width.
F. russii	–	–	–	A	–	–	–	–	–	–	–	–	–	–	–	NC	A,P,B	Similar to *F. nucleatum.*
F. varium	–	–	–	A	–	–	–	–	–	–	–	–	–	–	+	NC	A,P,B	Not inhibited by 50 mcg/ml of Rifampicin (Rifampin).
Campylobacter																		
C. sputorum ssp. sputorum	–	–	+	–	–	–	–	–	–	–	–	–	–	+	–	NC	A	Polar flagella.

*GLC (gas liquid chromatography) analysis was not performed on all of the strains listed.
+ = positive reaction for 90-100% of strains.
– = negative reaction for 90-100% of strains.
V = variable reaction.
A = acid reaction (yellow color with bromthymol blue indicator, pH less than 6.0).

C = coagulated.
NC = no coagulation.
A = acetic acid.
P = propionic acid.
IB = isobutyric acid
B = butyric acid

IV = isovaleric acid.
L = lactic acid.
S = succinic acid

TABLE X

Key Characteristics for Sub-speciation of *Bacteroides fragilis* *

Subspecies	Indol	Mannitol	Trehalose	Rhamnose
distasonis	–	–	A	V
fragilis	–	–	–	–
ovatus	+	A	A	A
thetaiotamicron	+	–	V	A
vulgatus	–	–	–	A

*Strains of *B. fragilis* may be encountered which cannot be subspeciated with these characteristics.

FIGURE 3

Differentiation of Gram-Positive Nonsporeforming Anaerobes to the Genus Level

I. Rods

A. Produce propionic acid

1. Catalase usually produced — *Propionibacterium*
2. Catalase not produced — *Arachnia*

B. Propionic acid and catalase not produced

1. Ratio of lactic to acetic acid produced greater than 1:1
   a. Lactic acid only major product — *Lactobacillus*
   b. Succinic acid is a major product — *Actinomyces**
2. Ratio of lactic to acetic acid produced less than 1:1
   a. Produce butyric acid plus other acids or no major acids — *Eubacterium* and *Lachnospira*
   b. Butyric acid not produced — *Bifidobacterium*

II. Cocci

A. Cubical packets of cells formed — *Sarcina*

B. Cubical packets of cells not formed

1. Produce catalase — *Peptococcus*
2. Catalase not produced
   a. Require fermentable carbohydrate — *Ruminococcus*
   b. Fermentable carbohydrate not required — *Peptostreptococcus*

**Actinomyces viscosus* is catalase positive. However, this organism is a facultative anaerobe.

TABLE XI

Characteristics of Anaerobic, Nonsporeforming Gram-positive Bacilli Commonly Isolated from Clinical Materials

SPECIES	NUMBER OF STRAINS	OXYGEN TOLERANCE	MOTILITY	GLUCOSE	MANNITOL	LACTOSE	SUCROSE	MALTOSE	SALICIN	GLYCEROL	XYLOSE	ARABINOSE	ESCULIN HYDROLYSIS	GELATIN HYDROLYSIS	NITRATE REDUCTION	INDOL	MILK	CATALASE	ORGANIC ACIDS DETECTED BY GLC*	OTHER
Actinomyces israelii †		M or An	–	A	V	A^-	A	A	V	–	A^-	V	+−	–	V	–	(C)	–	A,L,S	No DAP in cell wall
Actinomyces naeslundii †		F	–	A	–	A^-	A^-	A^-	V	V	–	$-^A$	+−	–	+−	–	(C)	–	A,L,S	No DAP in cell wall
Actinomyces odontolyticus †		M or An	–	A	–	A^-	A^-	A	A^-	A^-	V	V	V	–	+	–	(C)	–	A,L,S	No DAP in cell wall
Arachnia propionica †		M or An	–	A	A	A	A	A	$-^A$	$-^A$	–	–	$-^+$	$-^+$	+	–	(C)	–	A,P,L,S	DAP in cell wall
Bifidobacterium eriksonii ‡	74	An	–	A	V	A	A	A	A	–	A	A	+−	–	–	–	(CG)	–	A,L,S	
Eubacterium alactolyticum	20	An	–	A	A^-	–	–	–	–	–	–	–	–	–	–	–	NC	–	A,B,C	
Eubacterium lentum	61	An	–	–	–	–	–	–	–	–	–	–	–	–	V	–	NC	–	A,L,S	
Eubacterium limosum	21	An	–	A	A^-	–	–	–	–	–	–	–	V	–	–	–	NC	–	A,B	
Propionibacterium acnes ‡	484	An	–	A	V	–	–	–	–	A	–	–	–	+−	+	+−	C(G)	+	A,P,(IV)	
Propionibacterium granulosum	20	An or F	–	A	–	–	A	A	$-^A$	A^-	–	–	–	V	–	–	(C)	+−	A,P,(IV)	

*GLC (gas liquid chromatography) analysis was not performed on all of the strains listed.
+ = positive reaction for 90-100% of strains.
– = negative reaction for 90-100% of strains.
A superscript indicates the reaction shown in 11-25% of the strains.
V = variable reaction.
( ) = variable
A = acid reaction (yellow color with bromthymol blue indicator, pH less than 6.0).
C = coagulated
G = gas
NC = no coagulation
A = acetic acid
P = propionic acid
B = butyric acid
IV = isovaleric acid
C = caproic acid
L = lactic acid
S = succinic acid
M = microaerophilic
F = facultative
An = anerobic
† = Reactions for the *Actinomyces* and *Arachnia* species are based primarily on data from the Mycology Unit, Mycology Section, CDC. (Georg, L.K. Diagnostic Procedures for the Isolation and Identification of the Etiologic Agents of Actinomycosis. Proceedings – International Symposium on Mycoses. PAHO Publication No. 205: 71-81, 1970 and Li, Y.Y.F and L.K. Georg. Differentiation of *Actinomyces propionicus* from *Actinomyces israelii* and *Actinomyces naeslundii* by gas chromatography. Canad J. Microbiol 14: 749-753, 1968.)
‡ = Rare strains of bacteria with similar cell morphology and physiological characteristics but capable of growth in a candle jar have been received. Data on these are not included in this table.
DAP = Diaminopimelic acid.

TABLE XII

Characteristics of Anaerobic, Nonsporeforming Gram-positive Bacilli Infrequently Isolated from Clinical Materials

SPECIES	NUMBER OF STRAINS	OXYGEN TOLERANCE	MOTILITY	GLUCOSE	MANNITOL	LACTOSE	SUCROSE	MALTOSE	SALICIN	GLYCEROL	XYLOSE	ARABINOSE	ESCULIN HYDROLYSIS	GELATIN HYDROLYSIS	NITRATE REDUCTION	INDOL	MILK	CATALASE	ORGANIC ACIDS DETECTED BY GLC*
Bifidobacterium breve	5	An	–	A	A	A	A	A	A	–	–	–	V	–	$-^{+}$	–	C(G)	–	A,L,S
Bifidobacterium longum ssp. longum	5	An	–	A	–	A	A	A	–	–	A	A	V	–	–	–	C(G)	–	A,L,(S)
Lactobacillus catenaforme	4	An F	–	A	–	V	A	V	V	–	–	V	$-^{+}$	–	–	–	NC	–	A,L
Propionibacterium arabinosum	9	or An	–	A	A	V	A	A	V	A	V	A	+	–	V	–	NC	V	A,P
Propionibacterium avidum	2	An F	–	A	–	A	A	A	V	A	–	V	+	+	V	–	(C)	V	A,P
Propionibacterium freudenreichii ssp. freudenreichii	8	or An F	–	A	–	–	–	$-^{A}$	V	A	$-^{A}$	A^{-}	V	–	–	–	NC	+	A,P,(B)
Propionibacterium freudenreichii ssp. shermanii	18	or An F	–	A	–	A	–	–	$-^{A}$	A	V	V	$-^{+}$	–	–	–	(C)	+	A,P

*GLC (Gas liquid chromatography) analysis was not performed on all of the strains listed.
+ = positive reaction for 90-100% of strains.
– = negative reaction for 90-100% of strains.
A superscript indicates the reaction shown in 11-25% of the strains.
V = variable reaction
( ) = variable

A = acid reaction (yellow color with bromthymol blue indicator, pH less than 6.0).
C = coagulated
G = gas
NC = no coagulation
A = acetic acid

P = propionic acid
B = butyric acid
L = lactic acid
S = succinic acid
F = facultative
An = anaerobic

TABLE XIII
Differential Characteristics of Anaerobic Cocci Commonly Isolated from Clinical Materials

GROUPS	NUMBER OF STRAINS	GRAM STAIN REACTION	OXYGEN TOLERANCE	MOTILITY	GLUCOSE	MANNITOL	LACTOSE	SUCROSE	MALTOSE	SALICIN	GLYCEROL	XYLOSE	ARABINOSE	ESCULIN HYDROLYSIS	GELATIN HYDROLYSIS	NITRATE REDUCTION	INDOL	MILK	CATALASE	ORGANIC ACIDS DETECTED BY GLC*	OTHER
Peptococcus																					
Peptococcus CDC Group 2	10	+	An	–	A	–	–	–	–	–	A	–	–	–	–	+	–	NC	+	A	*Peptococcus saccharolyticus* – VPI
Peptostreptococcus																					
Peptostreptococcus CDC Group 1	23	+	An	–	–	–	–	–	–	–	–	–	–	–	–	–	+	NC	–	A,B	*Peptococcus asaccharolyticus* – VPI
Peptostreptococcus CDC Group 2	83	+	An	–	–	–	–	–	–	–	–	–	–	–	–	–	–	NC	–	A,(P),(B)	*Peptococcus prevotii* – VPI
Peptostreptococcus CDC Group 3	42	+	An	–	A	–A	–A	V	A–	–	–	–	–	–	–	–+	–	NC	–	A,(P),(IB),(B),(IV),(IC)	*Peptostreptococcus anaerobius* and others – VPI
Peptostreptococcus CDC Group 4**	5	+	An	–	A	V	A	A	A	A	–	V	V	+	–	–	–	(C)	–	(A),L	*Peptostreptococcus intermedius* and others – VPI
Sarcina																					
Sarcina – unspeciated	12	+	An	–	–	–	–	–	–	–	–	–	–	–+	–	–	–+	NC	–	A	
Veillonella																					
Veillonella alcalescens	14	–	An	–	–	–	–	–	–	–	–	–	–	–	–	+	–	NC	+	A,P	
Veillonella parvula	7	–	An	–	–	–	–	–	–	–	–	–	–	–	–	+	–	NC	–	A,P	
Veillonella CDC Group 3	13	–	An	–	–	–	–	–	–	–	–	–	–	–	–	–	–	NC	–	A,P	

*GLC (gas liquid chromatography) analysis was not performed on all of the strains listed.
+ = positive reaction for 90-100% of strains.
– = negative reaction for 90-100% of strains.
A superscript indicates the reaction shown in 11-25% of the strains.
V = variable reaction.
( ) = variable.
A = acid reaction (yellow color with bromthymol blue indicator, pH less than 6.0).
C = coagulated
NC = no coagulation
An = anaerobic
A = acetic acid
P = propionic acid
IB = isobutyric acid
B = butyric acid
IV = isovaleric acid
IC = isocaproic acid

**The characteristics of *Peptostreptococcus* CDC Group 4 are more closely related to the genus *Streptococcus* than the genus *Peptostreptococcus* according to the classification schema described by Rogosa, M. (*Peptococcaceae*, A New Family To Include the Gram-Positive, Anaerobic Cocci of the Genera *Peptococcus, Peptostreptococcus,* and *Ruminococcus.* International Journal of Systematic Bacteriology: 21: 234-237. 1971).

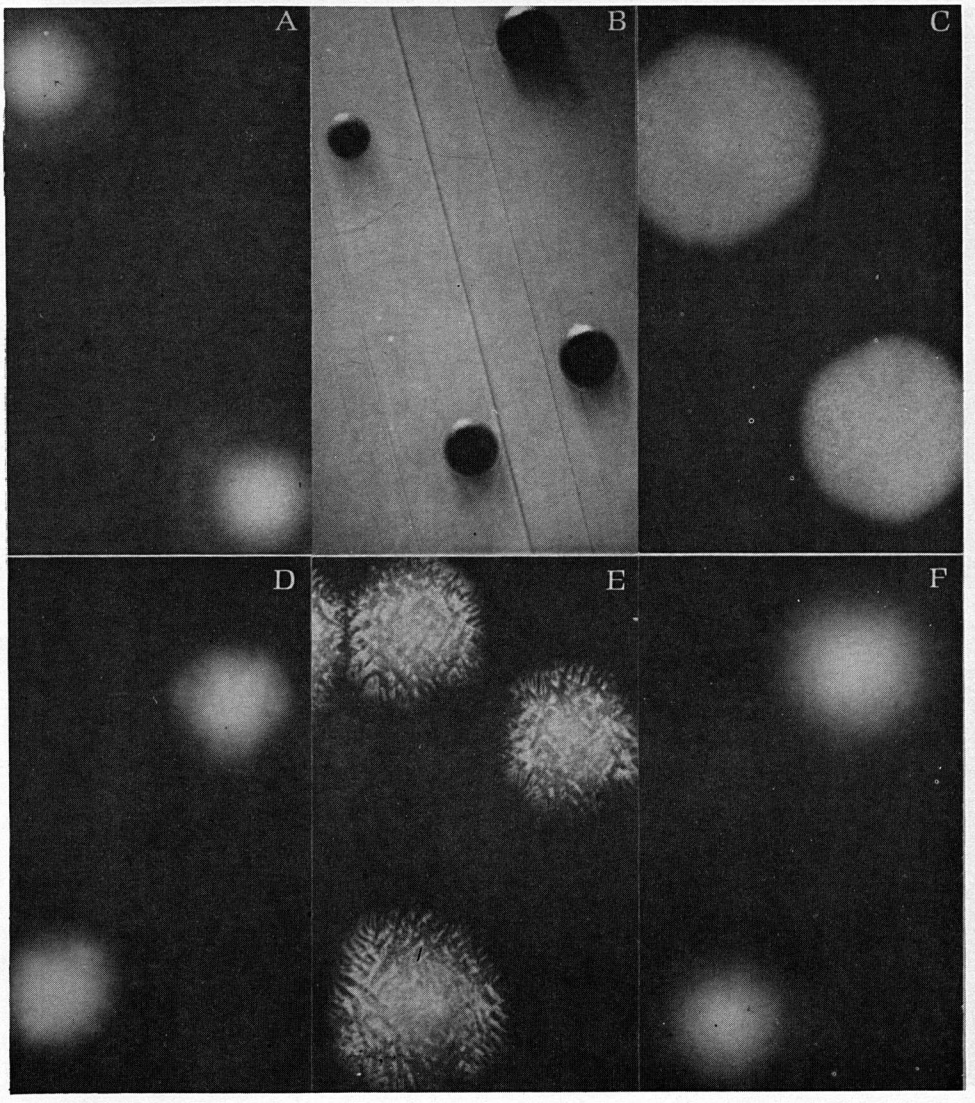


PLATE 1. Representative colonies of anaerobic bacteria.

A. *F. necrogenes,* 48 hr blood agar, x 37.
B. *B. melaninogenicus,* 5 day blood agar, x 15.
C. *B. fragilis* ssp. *fragilis,* 48 hr blood agar, x 22.
D. *F. necrophorum* 48 hr blood agar, x 37.
E. *F. nucleatum,* 48 hr blood agar x 22.
F. *B. fragilis* ssp. *distasonis*, 48 hr blood agar, x 37.

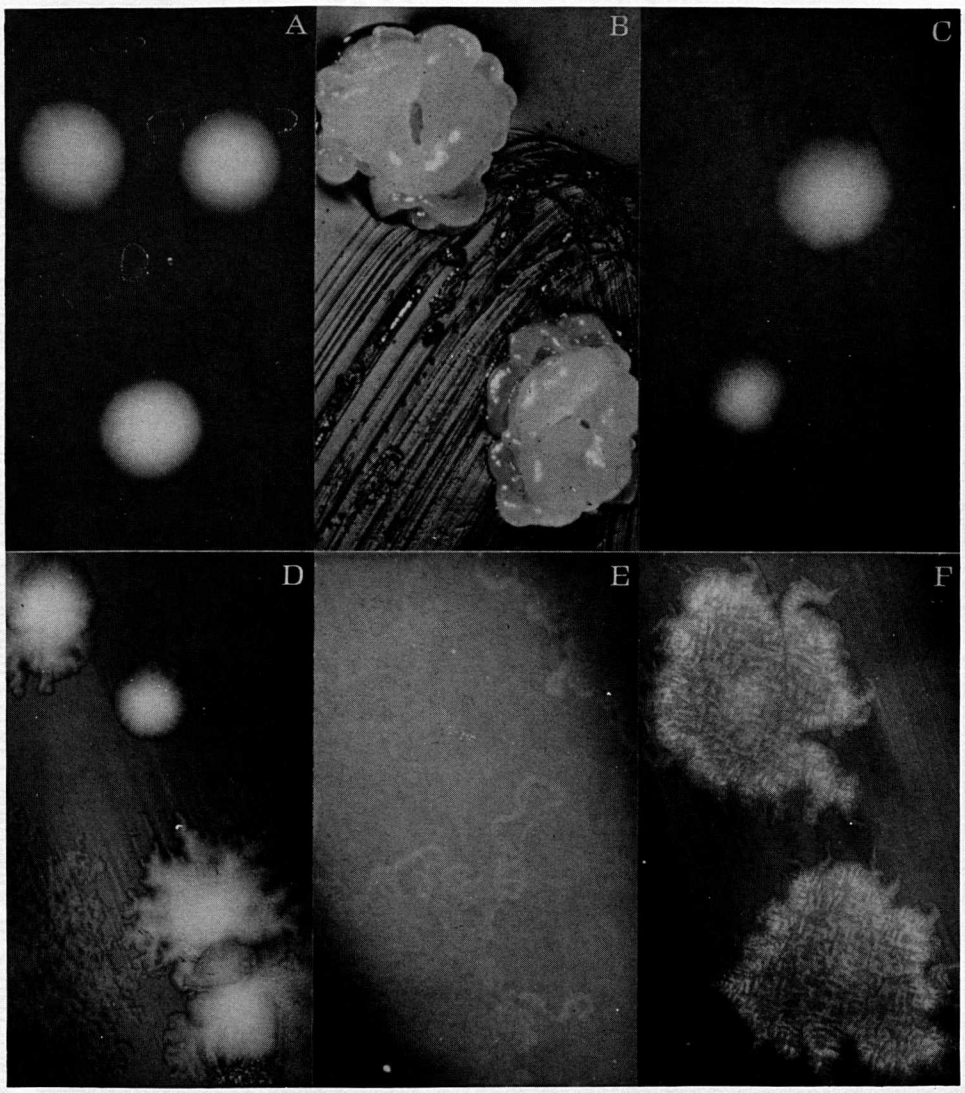


PLATE 2. Representative colonies of anaerobic bacteria.

A. *Eubacterium lentum,* 72 hr blood agar, x 7.5.
B. *Actinomyces israelii,* 7 day brain heart infusion agar, x 37.
C. *C. perfringens* (smooth colony), 48 hr blood agar, x 4.8.
D. *C. perfringens* (dissociated), 48 hr blood agar, x 3.
E. *C. septicum* (spreading edge), 48 hr blood agar, x 15.
F. *C. novyi* type A, 48 hr blood agar, x 5.2.

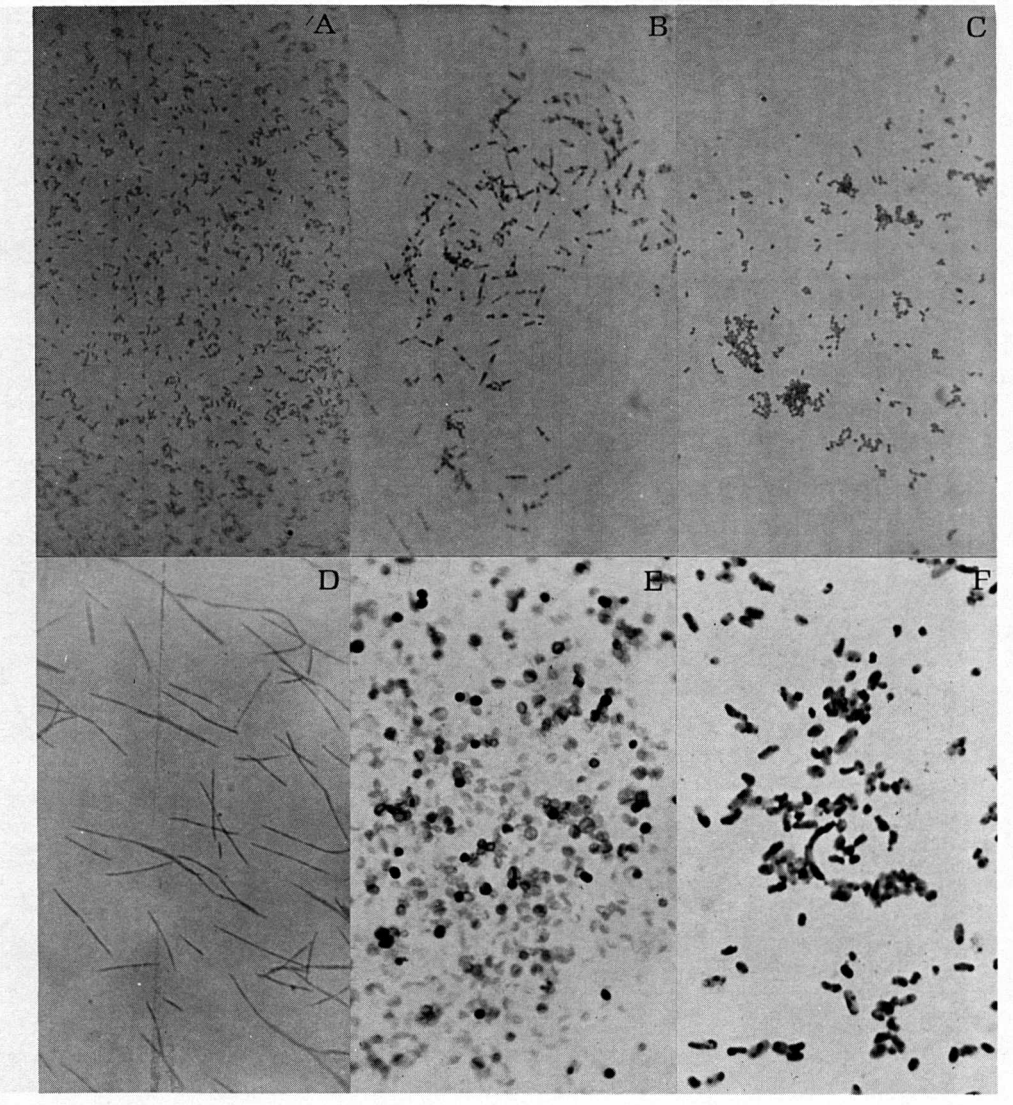


PLATE 3. Representative photomicrographs of anaerobic bacteria.

A. *B. fragilis* ssp. *fragilis,* 48 hr blood agar, x 950.
B. *B. fragilis* ssp. *fragilis*, 48 hr thioglycollate. x 950.
C. *B. melaninogenicus,* 48 hr blood agar, x 950.
D *F. nucleatum,* 48 hr blood agar, x 950.
E. *F. necrophorum,* 48 hr blood agar, x 950.
F. *F. mortiferum,* 48 hr thioglycollate, x 950.

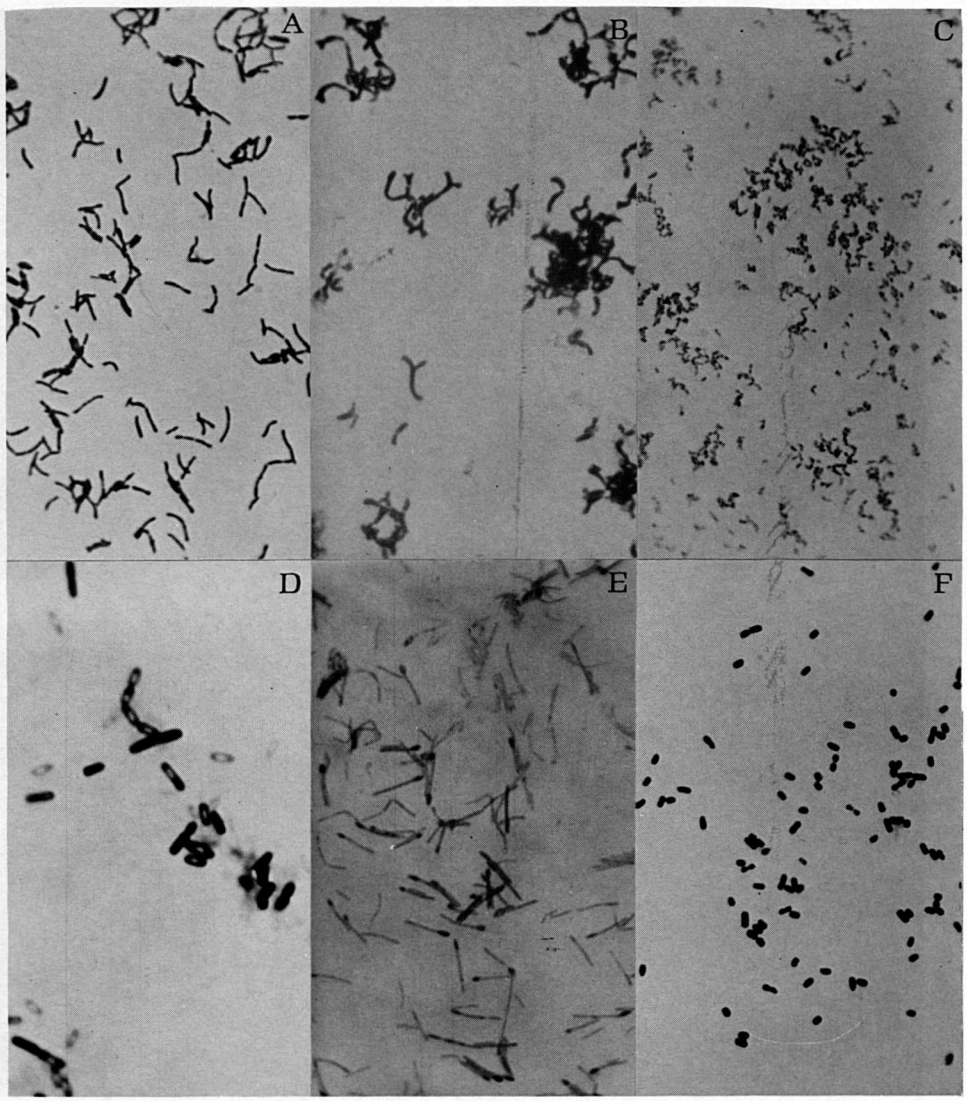


PLATE 4. Representative photomicrographs of anaerobic bacteria.

A. *B. eriksonii,* 48 hr blood agar, x 950.
B. *A. israelii,* 48 hr brain heart infusion agar, x 950.
C. *P. acnes,* 48 hr blood agar, x 950.
D. *C. bifermentans,* 24 hr chopped meat, x 950.
E. *C. innocuum,* 24 hr thioglycollate, x 950.
F. *C. perfringens,* 24 hr chopped meat, x 950.

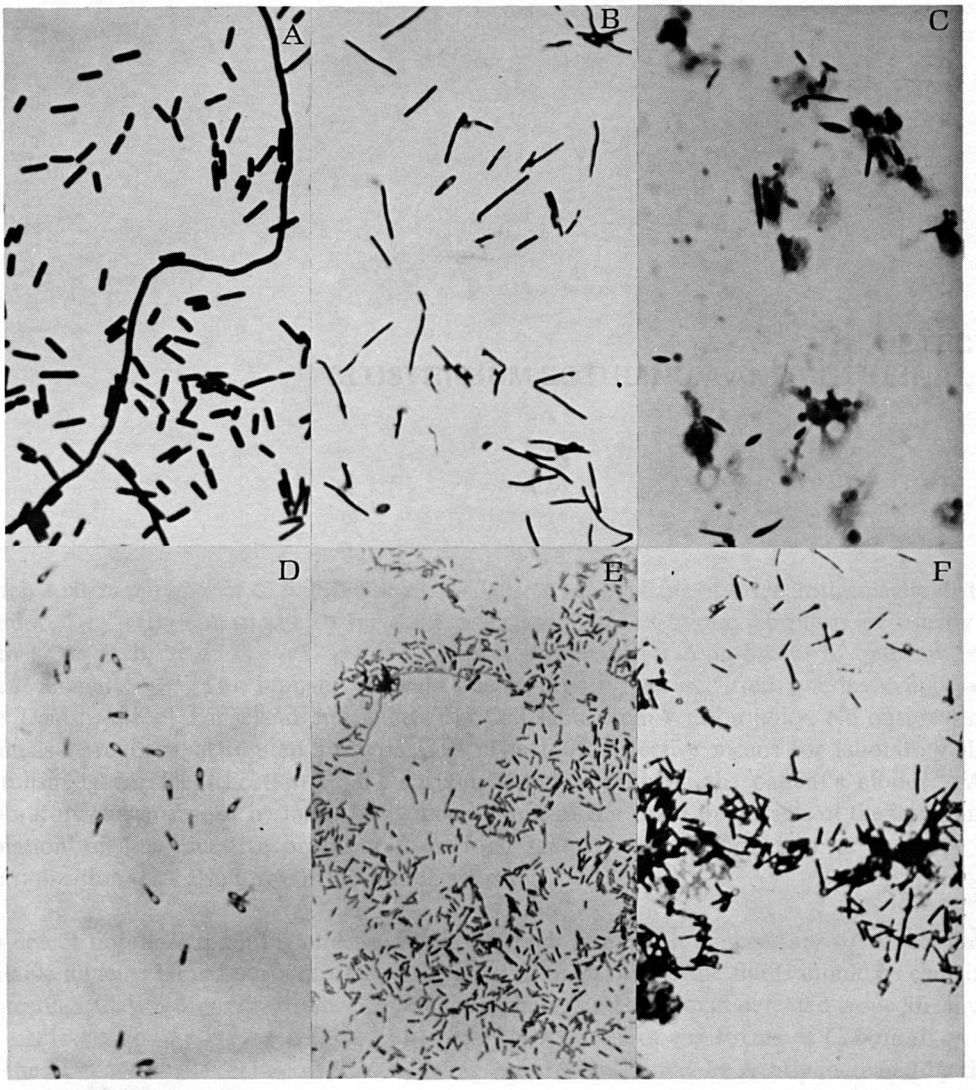
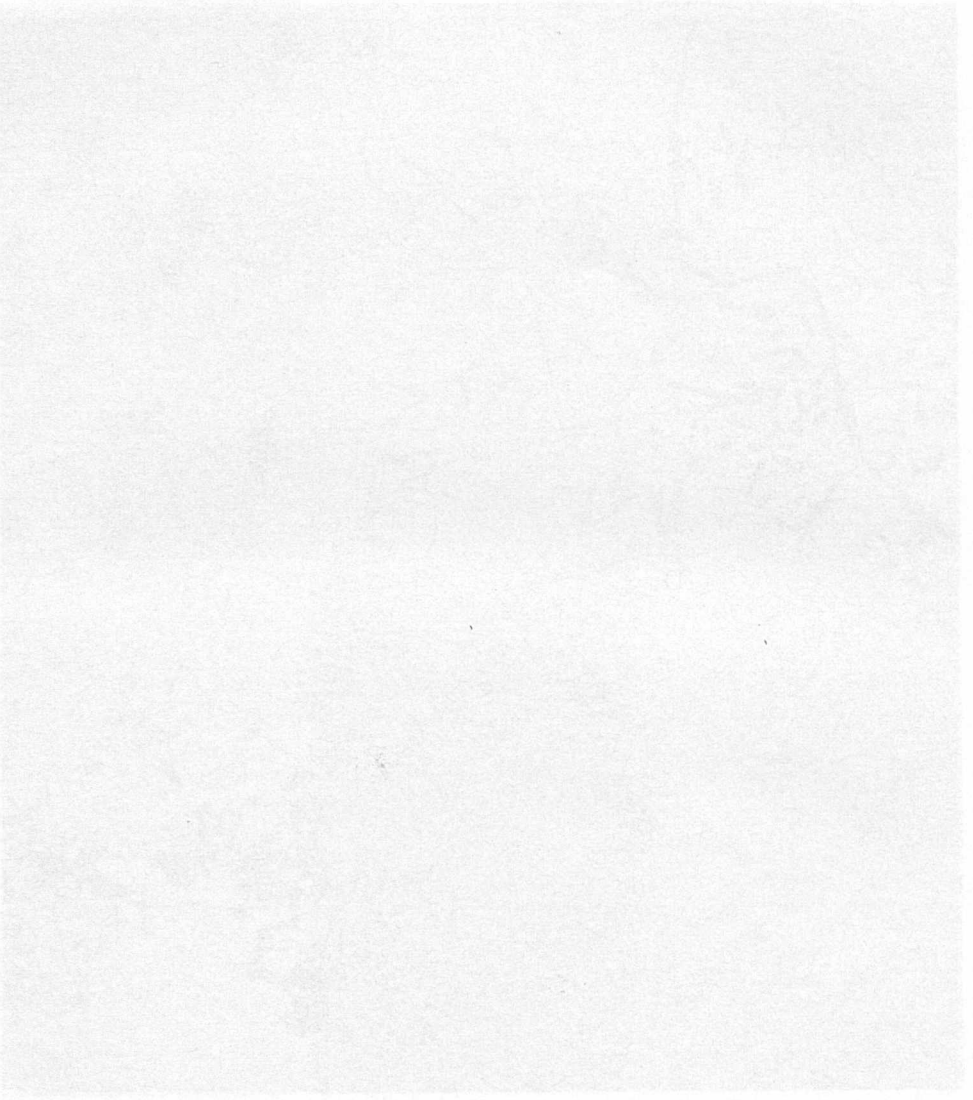


PLATE 5. Representative photomicrographs of anaerobic bacteria.

A. *C. perfringens,* 24 hr thioglycollate, x 950.
B. *C. septicum,* 24 hr chopped meat, x 950.
C. *C. chauvoei,* 24 hr chopped meat, x 950.
D. *C. subterminale,* 48 hr blood agar, x 950.
E. *C. ramosum,* 48 hr blood agar, x 950.
F. *C. tetani,* 72 hr chopped meat, x 950.



VII. DETECTION OF CLOSTRIDIUM BOTULINUM AND BOTULINAL TOXINS

A. Seven toxigenic types of *C. botulinum* are recognized on the basis of seven antigenically distinct toxins produced by different strains of the same organism. Cases of human botulism are usually associated with types A, B, or E. Types C and D are frequently involved in outbreaks of botulism in birds and other animals[13,14]. Two human outbreaks due to type F have occurred, one involving liver paste on the Danish Isle of Langeland and another in California from venison jerky. No outbreaks in man or animals have been attributed to type G[16]. The most effective means for laboratory diagnosis of botulism is the identification of the specific botulinal toxin in the patient's blood[17]. An indirect laboratory diagnosis can be made by demonstration of the toxin in extracts of the incriminated food. Isolation of the causative organism from food samples and confirmation of the type by mouse neutralization tests also prove helpful in diagnosis.

To detect botulinal toxin in solid food samples, such as meat, it is necessary to extract the toxin in a suitable diluent. Liquid portions of canned foods, extracts, or culture fluids should be centrifuged prior to testing. Clarified materials are inoculated into mice and any toxin detected is specifically identified by neutralization with type specific antitoxin. The toxicity of the toxins of *C. botulinum* type E and of the nonproteolytic strains of *C. botulinum* types B and F may be greatly increased by the addition of trypsin[18,19]. For this reason, toxin testing and mouse neutralization should be performed with trypsinized as well as nontrypsinized material. Trypsinization of serum samples is not necessary to activate the toxins[17].

B. Preparation of food extract.

1. Record all identifying information with food.
2. If canned foods are to be tested, wipe the top of the can with a solution of 10% Roccal and 70% isopropanol (1:1) and place the can in a large plastic bag before opening it to prevent the formation of an aerosol.
3. Record condition of the food (gassy, dark, putrid, etc.) and remove a small sample for a pH determination.
4. Grind food in a sterile, chilled mortar (pre-weighed).
   a. Place food in mortar, weigh, and record amount of food used. If sufficient material is available, 50 gm samples are a convenient size to work with.

b. Add 1 to 2 gm of sterile sand.

c. Add a small amount (5 ml) of cold gelatin diluent and grind with a sterile pestle until a homogenous suspension is obtained. In some cases where food is extremely dry, it may be necessary to add additional gelatin diluent in order to grind the specimen.

d. After grinding, add sufficient diluent to give a volume of diluent equal to the grams of food employed (V/W).

5. Prepare smear and gram stain. Note size of rods, approximate number and types of organisms present, and presence and location of spores.

C. Culture of food sample.

1. Alcohol treatment.

a. Using a safety Pro-Pipette or a "broken tip" capillary pipette, place approximately 0.5 ml of the food suspension in a 13-x 100-mm sterile screwcap tube.

b. Add an equal volume of absolute alcohol and incubate at room temperature for 1 hour; mix at approximately 15 minute intervals. The alcohol treatment kills vegetative cells but spores should remain viable.[13]

2. Heat five tubes of chopped meat-dextrose-starch medium in a boiling water bath for 10 minutes. Transfer three tubes to a 70°C water bath, and cool the other two tubes in cold water.

3. Inoculate one of the cooled tubes of chopped meat-dextrose-starch medium with an untreated food suspension and the second with the alcohol treated food. Inoculate with 0.5 to 1.0 ml near the bottom of the tubes with a capillary pipette. Try to avoid introducing air bubbles into the medium.

4. After equilibration to temperature, inoculate the three tubes of chopped meat-dextrose-starch medium in the 70°C water bath. Start timing. After 10 minutes, remove one tube to cold water, and transfer the other two tubes to an 80° water bath. After 10 minutes at 80°C , cool one tube and transfer the remaining tube to a boiling water bath for an additional 10 minutes before cooling.

5. Incubate the chopped meat-dextrose-starch medium cultures in an anaerobic jar at 30°C. Some types of *C. botulinum* produce little or no toxin at a temperature above 30°C.[19] Maximum toxin production usually occurs after 3 to 5 days incubation.[13]

6. Isolation and identification. To obtain pure cultures of *C. botulinum,* subculture from the chopped meat-dextrose-starch tubes to blood and egg yolk agar plates. Streak to obtain isolated colonies. Incubate plates in an anaerobic jar at 35-37°C for 48 hours. Pick isolated colonies to tubes of chopped meat-dextrose-starch medium, and incubate at 30°C. Establish the identity of pure cultures by conventional cultural and biochemical procedures and the toxin type with the mouse toxin neutralization test.

D. Typing of *C. botulinum* toxin from food or cultures.

1. Clarification.

a. Food suspension. Press fluid from food particles in the mortar, and transfer the fluid suspension to a suitable plastic centrifuge tube.

b. Cultures. Transfer the liquid portion from chopped meat-dextrose-starch medium cultures (30°C incubation) to plastic centrifuge tubes.

c. Centrifuge food suspension or culture fluids at 12,350 x g for 10 minutes (preferably in the cold). A slower centrifugation for a longer period of time may also be used.

d. Pour supernatant fluid into 15-x 125-mm tubes for testing. In some cases it may be necessary to clarify with a second centrifugation.

2. Trypsinization. Mix 4.5 ml of the food extract or culture fluid with 0.5 ml of trypsin solution and incubate at 37°C for 45 minutes.

3. Mouse inoculations.

   a. Label 11 (15-x 85-mm) tubes and prepare the various mixtures as shown in Table XIV.

   b. Inoculate two mice intraperitoneally with each test mixture (0.5 ml). One 1-ml syringe can be used to inject two mice.

   c. Observe mice for at least 4 days. Deaths from botulism usually occur within 6-24 hours in mice; however, occasionally death is delayed. Symptoms of botulism in mice often appear within 4 hours after inoculation and include ruffling of the fur, laboring "bellows" type breathing, and paralysis of the limbs[17].

   d. If botulinal toxin is present in the food or culture sample in sufficient quanitities to be detected under the test conditions, mice receiving the untreated sample should die, and mice receiving the heated material should survive. Since the toxin should be neutralized by the specific antitoxin, the type involved will be indicated by the type of antitoxin mixed with the sample. If mice are not protected by the antitoxins used, the toxin may be type C, D or a potent toxin of one of the types tested. To check for type C or D, repeat the test using untrypsinized extract and the appropriate antitoxins along with the heated and unheated controls. To test for a potent toxin, dilute the extract in gelatin diluent and repeat the neutralization test. In working with specimens from animals, types C and D antitoxin should be included in the neutralization test routine.

TABLE XIV

Detection of *C. botulinum* Toxin in Food Extracts or Culture Fluids

Tube No.	Extract or culture fluid	Serum or antitoxin*	Treatment
1	1.2 ml plain	0.3 ml NRS**	37°C for 30 min.
2	1.2 ml plain	0.3 ml NRS after boiling and recooling	Plug tube with cotton-boiling H_2O 10 min.
3	1.2 ml plain	0.3 ml Anti A	37°C for 30 min.
4	1.2 ml plain	0.3 ml Anti B	37°C for 30 min.
5	1.2 ml plain	0.3 ml Anti E	37°C for 30 min.
6	1.2 ml plain	0.3 ml Anti F	37°C for 30 min.
7	1.2 ml trypsinized	0.3 ml NRS	37°C for 30 min.
8	1.2 ml trypsinized	0.3 ml NRS after boiling and recooling	Plug with cotton -boiling H_2O for 10 min.
9	1.2 ml trypsinized	0.3 ml Anti B	37°C for 30 min.
10	1.2 ml trypsinized	0.3 ml Anti E	37°C for 30 min.
11	1.2 ml trypsinized	0.3 ml Anti F	37°C for 30 min.

*No diagnostic antitoxin is available for type G at the present time.
**NRS = normal rabbit serum

e. Reconstitute the respective *C. botulinum* diagnostic antitoxins as directed by the manufacturer. Instructions for reconstituting diagnostic antitoxins prepared at CDC are printed on the labels of the vials.

In the neutralization test as described, the antitoxin is diluted so that one international unit is present in 0.1 ml; therefore, each mouse will receive one international unit of antitoxin. One unit of antitoxin neutralizes 10,000 mouse IP LD_{50} doses of toxin in the case of types A, B, C, D, and F, or 1,000 mouse IP LD_{50} doses of type E toxin.

E. Identification of *C. botulinum* toxin in blood serum.

1. Blood samples should be obtained as soon as possible after the onset of symptoms and at intervals during the acute and convalescent stages of illness. The quantity of serum injected into mice should not exceed 1.0 ml per mouse since excessive amounts of normal human serum can cause death[17].

2. Toxin detection and neutralization tests are carried out as indicated in table XIV with the following exceptions:

   a. Patient's serum is used in place of extract or culture fluid.

   b. Only tubes 1,3,4,5, and 6 are used since heating in boiling water will coagulate the serum and trypsinization is not necessary to activate the toxin.

3. To conserve patient's serum, it is sometimes advisable to use polyvalent mixtures of the antitoxins for the preliminary screening before using the specific antitoxins.

F. Detection of wound botulism.

1. Several cases of wound botulism have been reported in the United States during the last few years. To establish the diagnosis of this illness, it is necessary to demonstrate the presence of botulinal toxin in the serum of the patient and isolate *C. botulinum* from the wound if possible.

2. After grinding with a mortar and pestle, culture biopsied material from wounds in chopped meat-dextrose-starch medium; follow the recommendations in section C-6 for isolation and identification of the organisms.

G. Detection of botulinal toxin in feces.

1. Recently it has been demonstrated that botulinal toxins can be detected in the feces of patients with botulism. For this reason stool specimens as well as blood samples should be collected from all suspect cases.

2. Mix 50 gm of feces 1:1 with gelatin diluent in a mortar as described for food in section B and extract overnight at 4°C.

3. Centrifuge the extract at 12,350 x g in a refrigerated centrifuge for a minimum of 20 minutes.

4. Do the toxin neutralization test as described for food or culture samples in section D.

5. Feces may be cultured for *C. botulinum* as described in section C on culture of food sample.

VIII. EXAMINATION OF FOODS AND FECES FOR CLOSTRIDIUM PERFRINGENS

A. To show that *C. perfringens* is the causative agent in an outbreak of foodborne disease, examine both suspect foods and the feces of patients if possible. Quantitative *C. perfringens* colony counts should be performed on the food samples.[20] At least three colony isolates of *C. perfringens* from the food and three colony isolates of *C. perfringens* from the feces of each patient should be examined serologically to establish the serotype involved. In large outbreaks, sets of isolates should include three or more strains from the incriminated food when the food is available and three or more fecal isolates from each of 10 different patients.

B. Enumeration of *C. perfringens* in food samples.

1. Weigh 50 gm of food sample in a mortar. To prepare an initial 1:10 food dilution, grind and dilute sample in 450 ml buffered dilution water as follows*:

   a. Add 1-2 gm of sterile sand to the contents of the mortar.

   b. Add a portion (15-20 ml) of the buffered dilution water to contents of mortar and grind until a homogenous suspension is obtained.

   c. Transfer the ground sample to a wide-mouth bottle; use the remaining buffered dilution water to rinse any adherent food from the mortar into the bottle.

   d. Mix the contents of the bottle by inverting 50 times.

   e. Prepare a smear of the food suspension for gram staining.

2. Prepare tenfold dilutions (10^{-2} through 10^{-6}) by serial transfer of 1 ml of diluted sample in 9 ml of buffered dilution water.

3. Plate the 10^{-3}, 10^{-4}, 10^{-5}, and 10^{-6} dilutions in duplicate or triplicate in sulfite-polymixin-sulfadiazine (SPS) agar[20] as follows:

   a. Pipette 1 ml of well mixed dilution into a Petri dish.

*The food sample can also be homogenized and diluted in 450 ml of buffered dilution water in a blender at low speed (8000 RPM) for 2 minutes.

b. Add 15 ml of melted agar medium; mix well, and allow the agar to solidify.

c. When agar is solid, overlay with an additional 4-5 ml of SPS agar.

4. Incubate the plates for 24 hours in an anaerobe jar at 35-37°C.

5. After incubation, open the anaerobe jar and count black colonies in all plates containing 30-300 black colonies. To facilitate counting, use a Quebec colony counter with a piece of white tissue paper over the counting area.

6. *C. perfringens* confirmation.

a. Subculture at least 10 representative black colonies from each culture to tubes of chopped meat-dextrose medium (or thioglycollate medium).

b. Incubate chopped meat medium for 4 hours at 46°C or overnight at 35-37°C.

(1) Prepare Gram stains from each chopped meat medium and look for typical gram positive bacilli with blunt ends and the absence of spores (usually).

(2) Prepare wet mounts and check for motility.

(3) Using a capillary pipette, inoculate a tube of lactose-motility medium up the "line of stab" with each chopped meat medium culture. Incubate at 35-37°C.

c. Examine lactose-motility medium at 24 and 48 hours. Reactions are usually complete within 24 hours.

Clostridium perfringens is nonmotile, rarely produces demonstrable spores in chopped meat or thioglycollate media, and produces acid and gas from lactose medium.

7. Calculate the number of viable *Clostridium perfringens* per gram of food sample.

Average plate count x ratio *C. perfringens* to black colonies x dilution = total count/gram of food.

Note: If plate counts with 30-300 black colonies are obtained at two dilutions, calculate total count for each dilution and average.

8. Save at least three different colony isolates of *C. perfringens* from the food in chopped meat medium for serological typing.

C. Isolation of *Clostridium perfringens* from feces.

1. Inoculate two 25 ml tubes of thioglycollate broth with approximately 1 gm of feces or 5 ml of fecal suspension.

a. Heat one tube in an 80°C water bath for 15 minutes and cool in tap water.

b. Incubate both tubes for 4 hours at 46°C or 18-24 hours at 35-37°C.

2. After incubating the tubes, subculture each thioglycollate broth culture to blood and egg yolk agar plates. Transfer the growth from near the bottom of the broth tubes using a capillary pipette. Place one drop on each plate and streak for isolation. Incubate plates for 24 hours in an anaerobe jar at 35-37°C.

3. Remove plates from the incubator and allow jar to set at room temperature for 2 hours (or in refrigerator at 4°C for 30 minutes) to allow full development of hemolytic patterns.

4. Open anaerobe jar and subculture at least one colony of every type resembling *C. perfringens* to freshly heated and cooled tubes of chopped meat-dextrose medium (or thioglycollate medium). Incubate tubes for 4 hours at 46°C or overnight at 35-37°C.

5. Confirm *C. perfringens* isolates morphologically and biochemically as in step B-6.

6. Save at least three different isolates of *C. perfringens* from each fecal specimen examined for serological typing.

D. Serological identification of *Clostridium perfringens.*

1. Using a capillary pipette, inoculate a tube of *C. perfringens* antigen medium near the bottom with approximately 0.5 ml of an 18-24 hour chopped meat-dextrose culture. Try to avoid the introduction of meat particles. Incubate the antigen medium for 18 hours at 35-37°C.

2. After 18 hours' incubation, examine antigen medium cultures.

   a. Prepare Gram stain and check for purity.

   b. Centrifuge the culture in a plastic centrifuge tube for 10 minutes at 12,350 x g and discard the supernatant fluid.

   c. Add sufficient 0.4% formalinized 0.85% saline (0.5 to 1 ml) to the sediment to prepare a turbid suspension suitable for slide agglutination.

      Note: It may be necessary to filter the antigen through nonabsorbent cotton wrapped around the tip of a capillary pipette to obtain a homogenous suspension.

3. Slide agglutination test.

   a. With a wax pencil, mark a slide into five or six equal parts.

   b. Place a drop of each *C. perfringens* antiserum (Hobbs types 1-13)[21] near the top of one area. Include a drop of normal rabbit serum as a control.

   c. Add a drop of antigen near the bottom of each area and mix the reagents by tilting the slide.

   d. Record reaction in 30 seconds as:

Neg. = no agglutination
1+ = 25% clumping
2+ = 50% clumping
3+ = 75% clumping
4+ = 100% clumping

The CDC Anaerobic Bacteriology Laboratory presently has 74 specific *C. perfringens* antisera in addition to the 13 Hobbs serotypes used routinely in the examination of *C. perfringens* cultures from foodborne disease outbreaks.

IX. STORAGE AND SHIPMENT OF ANAEROBES

The nonsporeforming anaerobes are maintained at the CDC by suspending growth from slants in blood, quickfreezing the suspensions at -70°C, and storing the blood suspensions at -42°C or lower. *Clostridium* species are grown in brain storage medium and the cultures are stored at -20°C or at -42°C. Lyophilization of anaerobic bacteria is also a satisfactory storage procedure.

A. Storage of nonsporeforming anaerobes.

1. Transfer actively growing chopped meat-dextrose or thioglycollate cultures to infusion agar slants and incubate the slants at 35-37°C in an anaerobe jar for 48 hours or until confluent growth is obtained.

2. With a capillary pipette, add approximately 0.35 ml of sterile defibrinated rabbit blood to each slant, suspend growth in the blood, and transfer the blood suspension to a sterile cotton plugged 6-x 50-mm pyrex tube (Corning #9820 is satisfactory). Cut off excess cotton and flame the lip of the freezing tube.

3. Quickfreeze the blood suspension in a 95% alcohol-dry ice bath and store at -42°C in a suitable deepfreeze.

4. To recultivate the frozen organisms, hold the frozen tube at the meniscus of the blood and gently rotate the tube until only a small portion has thawed. Aspirate the thawed portion with a capillary pipette to freshly heated and cooled chopped meat-dextrose or thioglycollate medium and return the frozen tube **immediately** to the freezer for future use. Incubate reisolation media in an anaerobe jar.

B. Storage of sporeforming anaerobes.

1. Using capillary pipettes, transfer actively growing chopped meat-dextrose cultures to brain storage media, and incubate the storage media at 35-37°C.

2. Prepare Gram stained smears daily and check for spores. Cultures are stored in the freezer at -20 or at -42°C as soon as spores develop or after 5 days incubation if no spores are observed.

3. To recultivate the frozen organisms, thaw the storage medium and transfer a few drops of culture to the bottom of freshly heated and cooled chopped meat-dextrose medium. Incubate the chopped meat medium in an anaerobe jar.

C. Lyophilization of anaerobe cultures.

1. Select a smooth colony on blood agar, inoculate thioglycollate medium (BBL 135C or equivalent), and incubate anaerobically at 35-37°C until good growth is obtained.

2. Check the purity of the culture with a Gram stained smear.

3. If the culture is pure, inoculate a blood agar slant, and incubate anaerobically at 35-37°C for 48 hours.

4. Recheck the purity of the culture with a Gram stained smear.

5. Suspend the growth on the slant in 0.5 ml to 0.75 ml of sterile skimmed milk and dispense approximately 0.25 ml quantities of the suspension into sterile 1.0 ml lyophilization ampoules (Virtis). These ampoules have a long tapered stem to allow heat sealing and are prescored for easy opening.

6. Freeze the suspensions rapidly by swirling the tubes in a "slush" mixture of ethanol and dry ice (−70°C), and hold the tubes in a freezer (−20°C or lower) until lyophilized.

7. Before placing the ampoules on the freeze dryer, pull a vacuum of 5 microns or less (as measured with a McLeod gauge) with a heavy duty vacuum pump (70-140 liters per minute) and fill the trap with a mixture of dry ice and ethanol. A vacuum of 1.2 mm or less must be maintained throughout the lyophilization procedure, otherwise the suspensions will thaw.

8. After 5-6 hours on the lyophilization unit, seal the ampoules under vacuum using a torch fueled with a mixture of illuminating gas and air.

9. Check the vacuum in the ampoules with a high frequency induction coil to insure proper sealing.

10. Store lyophilized cultures at ambient temperature or under refrigeration.

D. Shipment of anaerobic cultures.

Anaerobic cultures can be shipped to a reference laboratory for identification in tubes of liquid or semisolid media. Plates or slants are not satisfactory. Cultures should be purified before shipment.

Cultures can best be shipped in a carbohydrate free medium containing 0.3 to 1% agar such as motility medium. The medium should be freshly prepared and tubed 2 to 3 inches deep in screwcap tubes. *Clostridium* cultures in plain chopped meat media or cultures of the nonsporeformers in thioglycollate media can also be used for shipment.

Before shipment, a ¾ to 1 inch overlay of melted paraffin or 5% agar should be added to actively growing cultures in either semisolid or liquid media. The screwcaps should be tightened and sealed with waterproof tape.

E. Collection, handling, and shipment of specimens for detection of botulinal toxin and *C. botulinum.*

Botulinal toxins are extremely poisonous. Therefore, all materials suspected of containing botulinal toxin should be handled with maximum precaution and only experienced personnel, preferably immunized with botulinal toxoid, should perform laboratory tests.

1. Food samples. Laboratory testing includes extraction and identification of botulinal toxin and isolation of the causative organism. Suspect foods should be examined as soon as possible after they are collected. Leave unopened containers sealed until ready to be examined in the laboratory.

Otherwise, collect food samples in sterile containers. Place specimens to be sent to a distant laboratory in a leakproof container, wrap with a cushioning material, pack with ice in a second leakproof, insulated shipping container, and ship by the most rapid means. Notify the recipient laboratory in advance when and how the specimens are being shipped, when they should arrive, and what the waybill or shipping number is.

2. Body fluids and tissues. Blood samples should be collected before the patient is treated with botulinal antitoxin and as soon as possible after botulism is suspected. Collect enough blood to provide at least 10 ml (preferably 15-20 ml) of serum for toxin neutralization tests. Collect serum, gastric contents, feces, and autopsy specimens in sterile containers and arrange for examination as soon as possible after collection. Do not ship whole blood samples. Centrifuge and remove serum to a sterile tube and seal cap with waterproof tape. Pack and ship specimens referred to a distant laboratory as described for food samples above. Consultation and laboratory assistance in cases of suspected botulism as well as therapeutic trivalent (ABE) botulinal antitoxin can be obtained from CDC on a 24-hour basis. A description of the emergency assistance available as well as information on the epidemiology, diagnosis, and therapy of botulism is provided in the CDC publication **Botulism in the United States Review of Cases, 1899-1969 and Handbook for Epidemiologists, Clinicians, and Laboratory Workers,** U.S. Department of Health, Education, and Welfare, Center for Disease Control, Atlanta, Georgia.

X. MEDIA FOR ISOLATION AND CHARACTERIZATION OF ANAEROBIC BACTERIA

Media preparation is quite important in the cultivation of fastidious anaerobic bacteria. The following precautions should be closely observed:

1. Avoid excessive heating. Autoclave media no longer than 15 minutes.
2. Prepare media from recently opened bottles or use preweighed packets of dehydrated media.
3. Store media in the dark at room temperature in tightly sealed tubes. Biochemical media in cotton plugged tubes should be stored in the refrigerator to prevent evaporation.
4. Do not use media containing reducing agents that have been stored longer than 14 days under aerobic conditions.

A. PLATING MEDIA

1. **Blood agar.**
   a. Base
      (1) Add 0.5 gm yeast extract to 100 ml trypticase soy agar base.
      (2) Adjust pH to 7.3 – 7.5.
      (3) Autoclave at 121°C for 15 minutes.
      (4) Cool to 48°C.
   b. Add 1.0 ml sterile vitamin K-hemin solution per 100 ml (see section on Reagents).
   c. Add 5.0 ml sterile, defibrinated rabbit or sheep blood per 100 ml. Mix and pour plates.

2. **Modified McClung and Toabe egg yolk agar.***

   a. Base

   (1) Trypticase (BBL) 20 gm
   Na_2HPO_4 2.5 gm
   NaCl 1 gm
   $MgSO_4$, 5% aqueous solution 0.1 ml
   Glucose 1 gm
   Agar 12.5 gm
   Distilled water 500 ml

   (2) Adjust pH to 7.3 – 7.4.

   (3) Autoclave at 121°C for 15 minutes.

   (4) Cool to 60°C.

   b. Add one egg yolk, mix, pour plates (eggs must be free of antibiotics).

   Egg shells are decontaminated before the yolk is separated by immersing the eggs in a beaker of 95% alcohol for 1 hour.

3. **Phenylethyl alcohol blood agar.**

   Add 5 ml defibrinated rabbit or sheep blood to 100 ml phenylethyl alcohol agar base (BBL) which has been autoclaved as directed and cooled to 48°C in a water bath. After cooling, also add 1.0 ml of vitamin K-hemin solution to allow isolation of *B. melaninogenicus.*

4. **Kanamycin-vancomycin-menadione blood agar.**

   a. Prepare blood agar base as described above (1,a).

   b. After the medium has been autoclaved and cooled, add aseptically to each 100 ml of base:

   (1) 1.0 ml vitamin K-hemin solution.

   (2) 10 mg kanamycin (Bristol Laboratories).

   (3) 0.75 mg vancomycin (Eli Lilly & Co.).

   (4) 5 ml sterile defibrinated blood.

   c. Mix and pour plates.

Note: Either paromomycin (Parke Davis & Co.) or neomycin (Eli Lilly & Co.) in a final concentration of 100 mcg/ml may be substituted for kanamycin.

*Ingredients (basal medium and an egg yolk suspension) for preparation of egg yolk agar are available commercially (Difco Laboratories).

B. DIFFERENTIAL MEDIA

1. **Thioglycollate.**

   Thioglycollate medium (BBL 135-C) with dextrose but without resazurin indicator. Use 8 ml in 16-x 125-mm screwcap tubes.

2. **Fermentation base.***

   Thioglycollate medium without dextrose or indicator. For each 1000 ml medium, add:

Yeast extract (Difco)	2 gm
Bromthymol blue (1% aqueous solution)	1 ml

   Dispense 8 ml in 15-x 125-mm tubes with fermentation vials.

   *Available commercially as Bacto CHO base (Difco C841-01).

3. **Glucose.**

   Add 6 gm glucose to 1000 ml fermentation base before dispensing and autoclaving. Use 8.5 ml in 15-x 125-mm tubes with fermentation vials.

4. **Arabinose, Glycerol, Lactose, Maltose, Mannitol, Rhamnose, Sucrose, Trehalose, Xylose.**

   To 8.0 ml autoclaved fermentation base in 15-x 125-mm tubes, add 0.5 ml Seitz filtered aqueous solution of 10% carbohydrate. Use sterile technique.

5. **Salicin.**

   To 8.0 ml autoclaved fermentation base in 15-x 125-mm tubes, add 1.0 ml Seitz filtered aqueous solution of 5% salicin. Use sterile technique.

6. **Indol-nitrite (BBL).**

   Prepare as directed by manufacturer. Dispense 8.0 ml in 15-x 125-mm tubes.

7. **Chopped meat medium.**

Ground meat (fat free)	500 gm
Distilled water	1000 ml
1 normal NaOH	25 ml

   Use lean beef or horse meat. Remove fat and connective tissue before grinding. Mix meat, water and NaOH and bring to a boil while stirring. Cool, refrigerate overnight, and skim off any remaining fat. Filter the mixture through two layers of gauze, and spread the meat particles out to partially dry.

   Add sufficient distilled water to the filtrate to restore 1 liter original volume and add:

Trypticase or peptone	30 gm
Yeast extract	5 gm
Potassium phosphate	5 gm

   Adjust pH to 7.8 with 1 N NaOH.

   Dispense meat particles in 16-x 125-mm screwcap tubes with a small scoop and add the enriched filtrate. Use about one part meat particles plus three to four parts liquid (v/v) per tube. Add a few iron filings to each tube. Tubes should be over half full (about 8 ml fluid). Autoclave at 121°C for 15 minutes.

8. **Chopped meat-dextrose.**

Add 3 gm glucose per liter chopped meat filtrate before dispensing.

Chopped meat-dextrose-starch.

Add 2 gm soluble starch per 1000 ml chopped meat-dextrose filtrate before dispensing.

Chopped meat-starch.

Add 2 gm soluble starch per 1000 ml chopped meat filtrate before dispensing.

9. **Chopped meat agar slants for spores.**

Add 15 gm agar per 1000 ml chopped meat medium. Boil to melt agar, dispense in tubes, autoclave and slant.

10. **Iron milk.**

Place a few iron filings in the bottom of 15-x 125-mm test tubes. Add 8 ml whole, nonhomogenized milk to each tube. Autoclave at 121°C for 15 minutes.

11. **H_2S.**

Trypticase (BBL)	1.00 gm
Yeast extract	0.50 gm
Agar	0.20 gm
Lead acetate, 10% solution	0.20 ml
Glucose	0.50 gm
Distilled water	100.00 ml

Adjust pH to 7.2 – 7.3. Dispense 8 ml in 15-x 125-mm tubes. Autoclave at 121°C for 15 minutes.

12. **Thiogel (BBL).**

Prepare as directed by manufacturer. Dispense 9.0 ml in 15-x 125-mm tubes.

13. **Urea.**

Add sufficient distilled water to 31 gm Urea Broth (Difco) to make a total volume of 100 ml. Seitz filter. Add 1.0 ml to 8.0 ml autoclaved fermentation base without the bromthymol blue indicator.

14. **Lactose-Motility medium.**

Rehydrate LM agar (Difco) as directed. Dispense 8 ml in 15-x 125-mm tubes. Autoclave at 121°C for 10 minutes.

15. **Motility medium.**

Use Motility Test Medium (BBL) or Motility Medium (Difco) diluted to a final concentration of 0.4% agar as follows:

Motility medium (Difco)	16.0 gm
Nutrient broth	4.0 gm
NaCl	1.0 gm
Distilled water	1000.0 ml

Dispense 8 ml in 15-x 125-mm tubes. Autoclave at 121°C for 15 minutes.

16. **Esculin broth.**

Heart infusion broth (Difco)	25.0 gm
Esculin	1.0 gm
Agar	1.0 gm
Distilled water	1000.0 ml

Adjust pH to 7.0. Dispense 8 ml in 15-x 125-mm tubes. Autoclave at 121°C for 15 minutes.

17. **20% bile broth.**

Oxgall	40.0 gm
Distilled water	100.0 ml

Autoclave at 121°C for 15 minutes. Store in the refrigerator at 4°C.

Add 0.4 ml of 40% oxgall to 8 ml of thioglycollate medium (BBL 135-C) as needed.

18. **Peptone-yeast extract (PY) medium.**

Peptone	20.0 gm
Yeast extract (Difco)	10.0 gm
Salts solution*	40.0 ml
Cysteine HCl	0.5 gm
Resazurin solution**	4.0 ml
Distilled water	1000.0 ml

Adjust pH to 7.2, dispense 8 ml in 15-x 125-mm screwcap tubes, and autoclave at 121°C for 15 minutes.

*Salts solution

$CaCl_2$ (anhydrous)	0.2 gm
$MgSO_4$	0.2 gm
K_2HPO_4	1.0 gm
KH_2PO_4	1.0 gm
$NaHCO_3$	10.0 gm
NaCl	2.0 gm

Mix $CaCl_2$ and $MgSO_4$ in 300 ml distilled water until dissolved. Add 500 ml water, and while swirling slowly add remaining salts. Continue swirling the salts until all are dissolved. Add 200 ml distilled water, mix, and store at 4°C.

**Resazurin solution:

Dissolve one resazurin tablet (ca. 11 mg Allied Chemical catalog #506) in 44 ml distilled water.

19. **Peptone-yeast extract-glucose (PYG) medium.**

Add 1.0 gm glucose to 100 ml of PY medium. Adjust to pH 7.2, dispense, and autoclave at 121°C for 15 minutes.

20. **Infusion agar slants.**

Use blood agar base or another comparable medium. Dispense in 15-x 125-mm tubes. Autoclave and slant.

21. **Infusion agar-glucose slants.**

Add 1 gm glucose per 100 ml infusion agar. Autoclave and slant.

22. *C. perfringens* antigen medium.[22]

Nutrient broth	1.6 gm
Glucose	0.2 gm
Cysteine HCl, 10% solution	0.5 ml
Phosphate buffer solution*	2.4 ml
Distilled water	100.0 ml

Adjust pH to 7.4. Dispense 5 ml in 13-x 100-mm screwcap tubes. Autoclave at 121°C for 15 minutes.

*Phosphate buffer solution. Dissolve 14 gm $Na_2HPO_4 . 12H_2O$ and 1 gm $NaH_2PO_4 . H_2O$ in 60 ml distilled water.

23. **Flagella broth.**

Bacto tryptose	10 gm
NaCl C.P.	2.5 gm
K_2HPO_4	1.0 gm
Distilled water	1.0 liter

Adjust pH to 7.0. Dispense in 15-x 125-mm tubes, 8.0 ml per tube. Autoclave at 121°C for 15 minutes.

C. STORAGE MEDIA

1. **Brain Storage Medium**

   Add small amount of water to beef or calf brains and mix in a blender.

   Prepare peptone solution, using 20 gm peptone in 1000 ml distilled water. Adjust pH to 7.2 – 7.4.

   Dispense in 13-x 100-mm screwcap tubes. Use one part of brain and two parts peptone solution. Tubes should be over half full.

   Autoclave at 121°C for 15 minutes.

2. **Maintenance Medium for Actinomyces**[2 3]. (Actinomyces Broth, BBL)

   a. Part I:

   (1) Salt solution 250.0 ml

KH_2PO_4	60.00 gm
$(NH_4)_2SO_4$	4.00 gm
$MgSO_4.7H_2O$	0.80 gm
$CaCl_2$	0.08 gm

   Dissolve in one liter distilled water.

   (2) Casitone, U.S.P., pancreatic digest 4.0 gm

   (3) Cysteine HCl 1.0 gm

   (4) Yeast extract 2.0 gm

   (5) Brain heart infusion broth (Difco) 25.0 gm

   Adjust medium to pH 6.5 with 20% KOH, dilute to 500 ml and sterilize by filtration.

   b. Part II:

Distilled water	500.0 ml
Glucose	5.0 gm
Agar	7.0 gm
Starch – Baker's purified potato	1.0 gm
Oleic acid	10.0 ml

   100 mg/100 ml distilled water, neutralized to pH 7.0 with 1.0 N NaOH.

   Prepare by suspending the starch in 50 ml cold water, then pour the mixture into 450 ml boiling water and add the remaining components.

   Autoclave Part II and mix with Part I while it is still hot for a final volume of one liter.

   Dispense in sterile test tubes plugged with cotton. After inoculation of the medium seal the tube with pyrogallol-sodium carbonate and a rubber stopper as follows:

   Pyrogallol – 100 gm/150 ml distilled water

   Sodium carbonate – 10% solution

   Push in cotton plug and cover with a wad of absorbent cotton, add five drops of each of the above solutions, and plug tube with a rubber stopper.

XI. REAGENTS

A. **Bromthymol blue 1%.**

Dissolve 1 gm bromthymol blue in 20 ml 0.1 N sodium hydroxide. Add 80 ml distilled water.

B. **Buffered Dilution Water.**

1. Stock phosphate buffer solution:

KH_2PO_4	32 gm
Distilled water	500 ml

Adjust pH to 7.2 (usually requires about 175 ml of 1 N sodium hydroxide.)

Add distilled water to make total of 1000 ml.

2. Working buffered dilution water:

Stock phosphate buffer solution	1.25 ml
Distilled water to make	1000 ml

Dispense in 450 ml amounts in 32 oz. prescription bottles or in 9 ml amounts in 16-x 150-mm screwcap tubes for dilution blanks.

Autoclave at 121°C for 15 minutes.

C. **Carbol fuchsin 1%.**

Dissolve 1 gm basic fuchsin in 10 ml 95% alcohol. Add 5 ml phenol and 85 ml distilled water.

Dilute 1:2 with 95% alcohol for use.

D. **Ehrlich's reagent for indol testing.**

Dissolve 4 gm para-dimethylamino-benzaldehyde in 380 ml 95% ethyl alcohol and add 80 ml concentrated hydrochloric acid.

E. **Ferric ammonium citrate 1%.**

Dissolve 1 gm ferric ammonium citrate in 100 ml distilled water.

F. **Gelatin diluent.**

Gelatin	2 gm
Na_2HPO_4	4 gm
Distilled water	1000 ml

Adjust to pH 6.2 with HCl. Dispense into screwcap bottles and autoclave at 121°C for 15 minutes.

G. **Methylene blue indicator.**

$NaHCO_3$	400 gm
Glucose	100 gm
Methylene blue chloride	trace

Mix thoroughly. Immediately before use, mix about 2 gm indicator and about 8 ml tap water in a test tube. The mixture should be light blue when oxidized and colorless when reduced.

H. **Nitrate A.**

Dissolve 5 gm sulfanilic acid in 1000 ml 5 N acetic acid.

I. **Nitrate B.**

Dissolve 5 gm dimethyl-alpha-naphthylamine in 1000 ml 5 N acetic acid.

J. **Phenol red, 1%.**

Dissolve 1 gm phenol red in 30 ml 0.1 N sodium hydroxide. Add 70 ml distilled water.

K. **Trypsin, 1%.**

Dissolve 1 gm trypsin 1:250 (Difco) in 100 ml distilled water. Store in refrigerator. Prepare fresh weekly.

L. **Vaspar.**

Melt together equal portions (W/W) vaseline and paraffin. Mix. Dispense in screwcap Erlenmeyer flask, and autoclave at 121°C for 30 minutes.

M. **Vitamin K-hemin solution.**

1. Stock hemin solution:

   Dissolve 50 mg hemin in 1 ml of 1 N sodium hydroxide. Add 100 ml distilled water. Autoclave at 121°C for 15 minutes.

2. Stock menadione solution:

Menadione	100 mg
95% ethyl alcohol	20 ml

   Sterilize by filtration.

3. Working vitamin K-hemin solution:

   Add 1 ml sterile menadione solution to 100 ml sterile hemin solution.

4. For use add 1 ml vitamin K-hemin solution per 100 ml sterile medium or 0.08 ml per 8 ml tubed medium.

XII. STAINING PROCEDURES

A. **Gram stain.**

Staining Schedule:

1. Fix smears with gentle heat by passing them through a flame.
2. Flood slides 1 minute with a mixture of equal parts Hucker's crystal violet solution and 1% sodium bicarbonate. Wash them off briefly in tap water (not over 5 seconds).
3. Flood slides with Gram's iodine solution for 1 minute and wash them off in tap water.
4. Flood slides with 95% alcohol and pour it off immediately. Reflood slides with 95% alcohol for 10 seconds, and wash them off in tap water.
5. Flood slides with Hucker's safranin solution for 1 minute and wash them off in tap water.

B. **Spore stains.**[24]

1. Schaeffer-Fulton (1933) modification of the Wirtz (1908) method.

   Staining Schedule:

   a. Prepare smears and heat fix.
   b. Flood slides 30-60 sec. with 5% aqueous malachite green.
   c. Heat to steaming three or four times.
   d. Wash off the excess stain in tap water.
   e. Stain 30 sec. in 0.5% aqueous safranin 0.
   f. Wash smear in running water.
   g. Blot dry and examine.

   Results:

   Spores – Green
   Rest of cell – Red

   Note: Trouble is sometimes experienced with the green fading after the slides have stood a few days. Apparently this is due to an alkaline reaction and can be prevented by acid cleaning the slides before preparing smears. (The alkalinity of the slides may be due to an invisible film of soap or washing powder.)

2. Bartholomew and Mittwer's "cold" method.

   Staining Schedule:

   a. Fix air-dried smear by passing it through a flame 20 times.

   b. Stain 10 minutes with saturated aqueous malachite green (approximately 7.6%) without heat.

   c. Rinse the slides with tap water for about 10 seconds.

   d. Stain 15 seconds in 0.25% aqueous safranin.

   e. Rinse the slides with tap water and blot dry.

   Results:

   Spores – Green
   Rest of cell – Red

C. **Leifson's Flagellar Stain.**

Solution A:

Basic fuchsin (certified for flagellar staining)	0.6 gm
Ethyl alcohol, 95%	50.0 ml

Shake and let stand overnight to dissolve.

Solution B:

Distilled water	100.00 ml
Sodium chloride	0.75 gm
Tannic acid	1.50 gm

Combine solutions A and B and mix thoroughly. The stain is ready for use immediately and should remain satisfactory for about 1-2 months when stored at 4-5°C. A precipitate develops during storage which should not be disturbed when the stain is used.

Staining Schedule:

1. Smears for flagella staining may be made from growth on plates by making a light suspension in distilled water, taking care that only bacterial growth and none of the agar is carried into the suspension. More satisfactory preparations may be obtained with a formalinized, washed cell suspension of the organisms from an overnight culture in a nondextrose containing broth, e.g., flagella broth. To prepare the cell suspension, add 0.25 ml formalin to 5 ml of an overnight broth culture, mix, and allow to stand for 15 minutes. Add an equal portion of distilled water to the tubes, mix and centrifuge. Decant the supernatant carefully and wipe off the lip of the tube. Add distilled water, mix, and recentrifuge. Decant as before, resuspend the organisms in 1-2 ml distilled water, and then dilute to a barely turbid suspension.

2. It is essential that clean and grease-free slides be used for flagella staining. Many workers use concentrated sulfuric acid saturated with potassium dichromate to clean the slides; however, new and unopened slides that have been precleaned at the time of manufacture will give satisfactory results. Immediately before they are used, the slides must be polished with a soft cotton cloth and then be well flamed in the **blue** flame of a Bunsen burner with the side to be used next to the flame. A smoky or yellow flame ruins the slides. Draw a line with a wax pencil from the top to the bottom of each slide near the frosted end. The slides should have been flamed uniformly and sufficiently to slightly melt this wax line.

3. To prepare a smear for staining, place the frosted end of a prepared slide on an applicator stick to tilt it slightly. Place a 3 mm loopful of suspension near the wax line and allow the drop to run to the other end of the slide. The drop will run quite freely if the slide is satisfactory and has been well cleaned. Allow the slide to air dry.

4. Place the slide on a staining rack and add 1 ml of stain warmed to room temperature. Staining is not complete until a metallic sheen has formed on the surface of the stain. This may vary from 6-12 minutes depending on the age of the stain. It is advisable to stain a control slide to determine the staining time; prepare a fresh stain if the staining time exceeds 12 minutes.

5. Wash the stained slide with tap water without tilting the slide to drain the stain off. Air dry and examine the slide.

6. If a counterstain is desired, use a 1:10 dilution of Loeffler's methylene blue after washing off the flagellar stain.

Flagella broth

Bacto tryptose	10 gm
NaCl C.P.	2.5 gm
K_2HPO_4	1 gm
Distilled water	1 liter

Adjust pH 7.0
Tube 8.0 ml in 15-x 125-mm tubes.
Autoclave at 121°C for 15 minutes.

XIII. ANAEROBIC SYSTEMS

Systems currently used for isolation of anaerobic bacteria include anaerobic jars, the Hungate roll-tube or roll-streak tubes of prereduced anaerobically sterilized (PRAS) media, and anaerobic glove boxes. Although there are advantages and disadvantages to each system, recent studies have shown that all of the systems are suitable for isolation of the commonly encountered anaerobes responsible for human infections if certain principles of anaerobic bacteriology are followed.

1. Specimens must be properly collected and handled to exclude atmospheric oxygen.
2. Fresh or prereduced media must be used.
3. The anaerobic system must be properly used by providing an active catalyst in the system to allow effective removal of residual oxygen.

A. Use of the anaerobe culture jar.

There are a number of anaerobe culture jars (Brewer, Torbal, GasPak, etc.) commonly used which rely on the same general principle for removal of oxygen. Addition of hydrogen allows reduction of oxygen to form water as follows:

$$2H_2 + O_2 \rightleftharpoons 2H_2O$$

Each system uses a catalyst to accelerate the rate of oxygen reduction. The palladium catalyst in the lid of the Brewer jar (modified McIntosh – Fildes jar) requires heating with an electric current to be fully active, but the catalysts used in the more modern jars (Torbal, GasPak) do not require heating.

It is important to keep the lids of anaerobe jars clean and dry when not in use to prevent inactivation of catalyst. The catalyst (palladium coated alumina pellets) used with the GasPak system is known to be inactivated ("poisoned") by hydrogen sulfide, chlorine, and sulfur dioxide gases. Therefore, the pellets must be replaced at frequent intervals (preferably each time the jar is used) with new or "rejuvenated" pellets. The activity of used catalyst can be restored "rejuvenated" by heating the pellets in a dry heat oven at 160-170°C for 2 hours. After rejuvenation, store the pellets in a clean, dry container away from contaminating gases until they are used.

Either an evacuation-replacement technique or the GasPak technique can be used with anaerobe culture jars to produce anaerobic conditions. Both techniques are effective but the evacuation-replacement method is more economical and produces anaerobic conditions more rapidly. When the evacuation-replacement method is used, any container (Brewer jar, Torbal jar, vented GasPak jar, Case Jar, vacuum desiccator, modified pressure cooker, etc.) which can be evacuated and replaced with a gas mixture, e.g., 80% N_2 + 10% CO_2 + 10% H_2, can serve as an effective anaerobic system if a catalyst is provided to allow removal of residual oxygen. The use of a gas replacement procedure without a catalyst is not recommended. If the container to be used is not equipped with a catalyst, addition of a cotton gauze packet of palladium coated alumina pellets (Englehard Inudstries, East Newark, New Jersey 07029) (quantity determined by volume of container) will allow reduction of residual oxygen.

1. Evacuation-replacement technique.

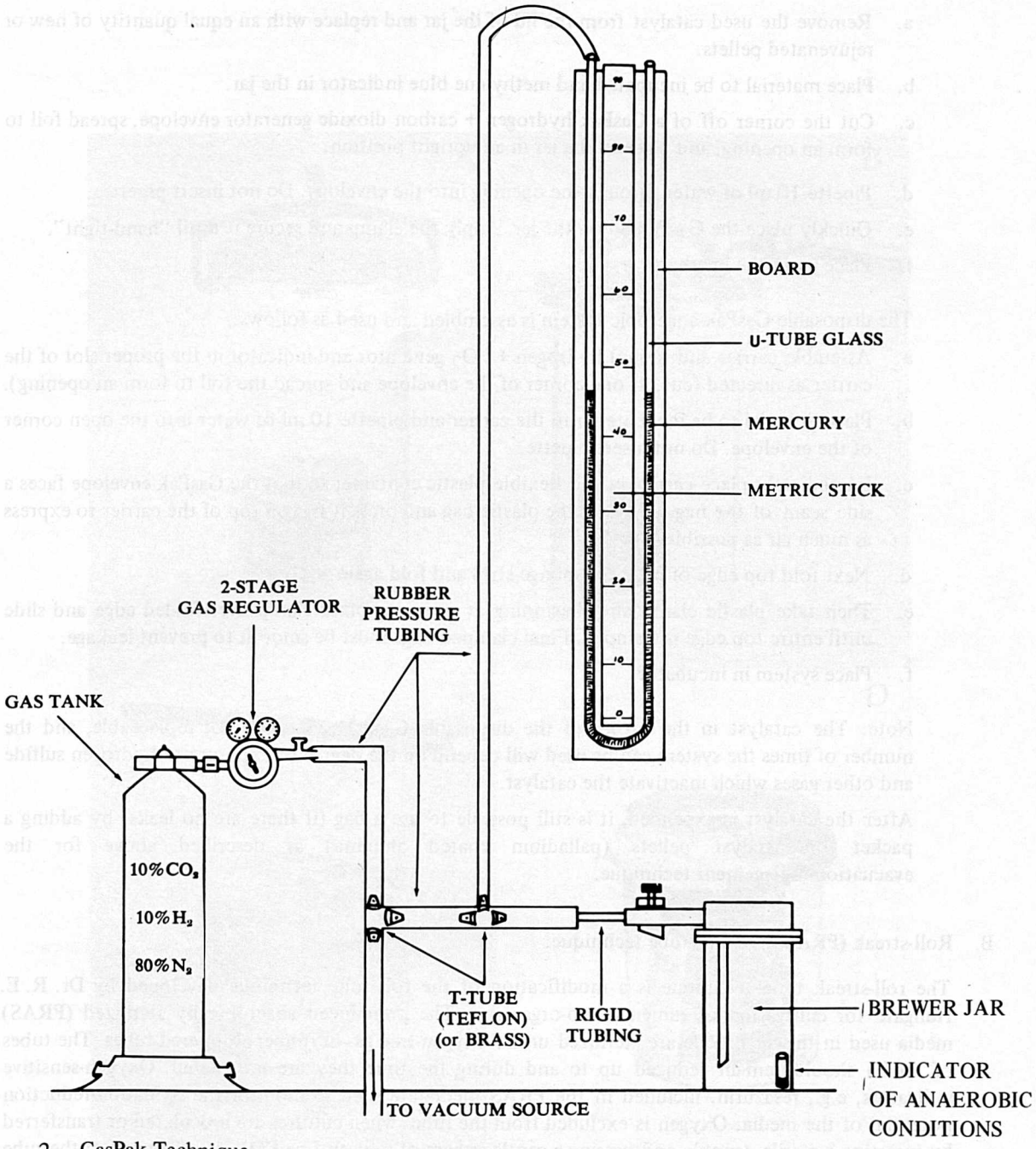
   Figure 4 illustrates the evacuation-replacement technique in which a Brewer jar is used. If a manometer is not available, a balloon can be connected to the system to serve as a pressure indicator during the evacuation and replacement procedure. The procedure is performed as follows:

   a. Place material to be incubated inside the jar.
   b. In a 15-x 25-mm test tube, place methylene blue – $NaHCO_3$ – glucose mixture* (see Reagents) to a depth of 0.5 inch. Fill the tube to 1/2 volume with water and mix by inverting. Place tube in jar. The GasPak disposable anaerobic indicator strips (BBL) are also suitable.
   c. Seal lid to jar with plasticine or use stop-cock lubricant such as "Cello Seal" (BBL) and fasten lid with a clamp.
   d. Connect outlet on the jar lid to vacuum-gas-manometer assembly.
   e. Evacuate to 30 cm mercury.
   f. Clamp off tubing to vacuum pump and turn off vacuum pump motor.
   g. Slowly fill the jar with gas mixture (80% N_2, 10% CO_2, 10% H_2; available from Matheson Co., East Rutherford, New Jersey 07030 and branch outlets). A full tank of gas has approximately 1500 pounds pressure, therefore, tanks must be securely fastened to a post or wall bracket and a reducing valve used that has been adjusted to 4 to 5 pounds pressure.
   h. Remove clamp on tubing to vacuum pump.
   i. Repeat steps e, f, g, h.
   j. Repeat steps e, f, g.
   k. Tighten clamp on jar outlet tubing.
   l. Disconnect jar from vacuum gas assembly.
   m. Remove clamp on tubing to vacuum pump.
   n. Connect electrical element and heat for 10 minutes.
   o. Disconnect electrical element and place jar in incubator.

   The evacuation-replacement technique is used with other types of containers (Torbal, vented GasPak jar, vacuum dessicator, etc.) exactly as described for the Brewer jar except step n is omitted.

*If anaerobic conditions are achieved, the methylene blue indicator solution will be colorless after overnight incubation, otherwise, the solution will be blue.

FIGURE 4. Evacuation–Replacement System



2. GasPak Technique.

Three types of anaerobic systems are available as shown in plate 6. (1) GasPak anaerobic system (polycarbonate jar)*, (2) GasPak anaerobic system, vented (polycarbonate jar), and (3) GasPak disposable anaerobic system. Each of these is a self-contained anaerobic system in which hydrogen and carbon dioxide are supplied from a disposable GasPak hydrogen + carbon dioxide generator envelope. A room temperature catalyst is used to accelerate reduction of oxygen. As previously mentioned, the evacuation-replacement technique can also be used with the vented system.

*A larger version of this jar which will accommodate twelve 150-mm Petri dishes is now available from BBL. Because of the larger volume, three GasPak hydrogen + carbon dioxide generator envelopes are required to provide anaerobic conditions in the jar. An adaptor is also available from BBL which permits evacuation and replacement of this system as described in step A-1 above.

The polycarbonate GasPak systems (vented or unvented) are used as follows:

a. Remove the used catalyst from the lid of the jar and replace with an equal quantity of new or rejuvenated pellets.

b. Place material to be incubated and methylene blue indicator in the jar.

c. Cut the corner off of a GasPak hydrogen + carbon dioxide generator envelope, spread foil to form an opening, and place in the jar in an upright position.

d. Pipette 10 ml of water through the opening into the envelope. Do not insert pipette.

e. Quickly place the GasPak lid on the jar. Apply the clamp and secure it until "hand-tight".

f. Place jar in the incubator.

The disposable GasPak anaerobic system is assembled and used as follows:

a. Assemble carrier and mount hydrogen + CO_2 generator and indicator in the proper slot of the carrier as directed (cut off one corner of the envelope and spread the foil to form an opening).

b. Place material to be incubated into the carrier and pipette 10 ml of water into the open corner of the envelope. Do not insert pipette.

c. Immediately place carrier in the flexible plastic container so that the GasPak envelope faces a side seam of the bag, fold over the plastic bag and press it flat on top of the carrier to express as much air as possible.

d. Next fold top edge of bag over sponge strip and fold again.

e. Then take plastic clamp and, beginning at one end, place clamp over folded edge and slide until entire top edge is clamped. Final clamped edge must be smooth to prevent leakage.

f. Place system in incubator.

Note: The catalyst in the carrier of the disposable GasPak system is not replaceable, and the number of times the system can be used will depend on the degree of exposure to hydrogen sulfide and other gases which inactivate the catalyst.

After the catalyst is expended, it is still possible to use a bag (if there are no leaks) by adding a packet of catalyst pellets (palladium coated alumina) as described above for the evacuation-replacement technique.

B. Roll-streak (PRAS medium) tube technique.

The roll-streak tube technique is a modification of the roll tube technique developed by Dr. R. E. Hungate for cultivation of rumen micro-organisms. The prereduced anaerobically sterilized (PRAS) media used in this technique are sterilized under oxygen-free gas in rubber stoppered tubes. The tubes of media should remain reduced up to and during the time they are inoculated. Oxygen-sensitive indicators, e.g., resazurin, included in the PRAS media are used to monitor the oxidation-reduction potential of the media. Oxygen is excluded from the tubes when cultures are inoculated or transferred by inserting a sterile cannula and passing a gentle stream of oxygen-free CO_2 into the neck of the tube until the stopper is replaced. This maintains anaerobic conditions in the tube and allows each tube to serve as an anaerobic culture system which can be incubated in an ordinary incubator and inspected at any time without exposure to atmospheric oxygen. PRAS media can readily be prepared in the laboratory if time and available personnel permit or may be obtained commercially (Robbin Laboratories, Division of Scott Laboratories, Inc., Fiskeville, Rhode Island 02823; Highland Division of Travenol Laboratories, Costa Mesa, California 92626).

1. The following is an outline of the procedures recommended by the VPI Anaerobe Laboratory for preparation of PRAS media. A more detailed description is given in the VPI Anaerobe Laboratory Manual.[9]

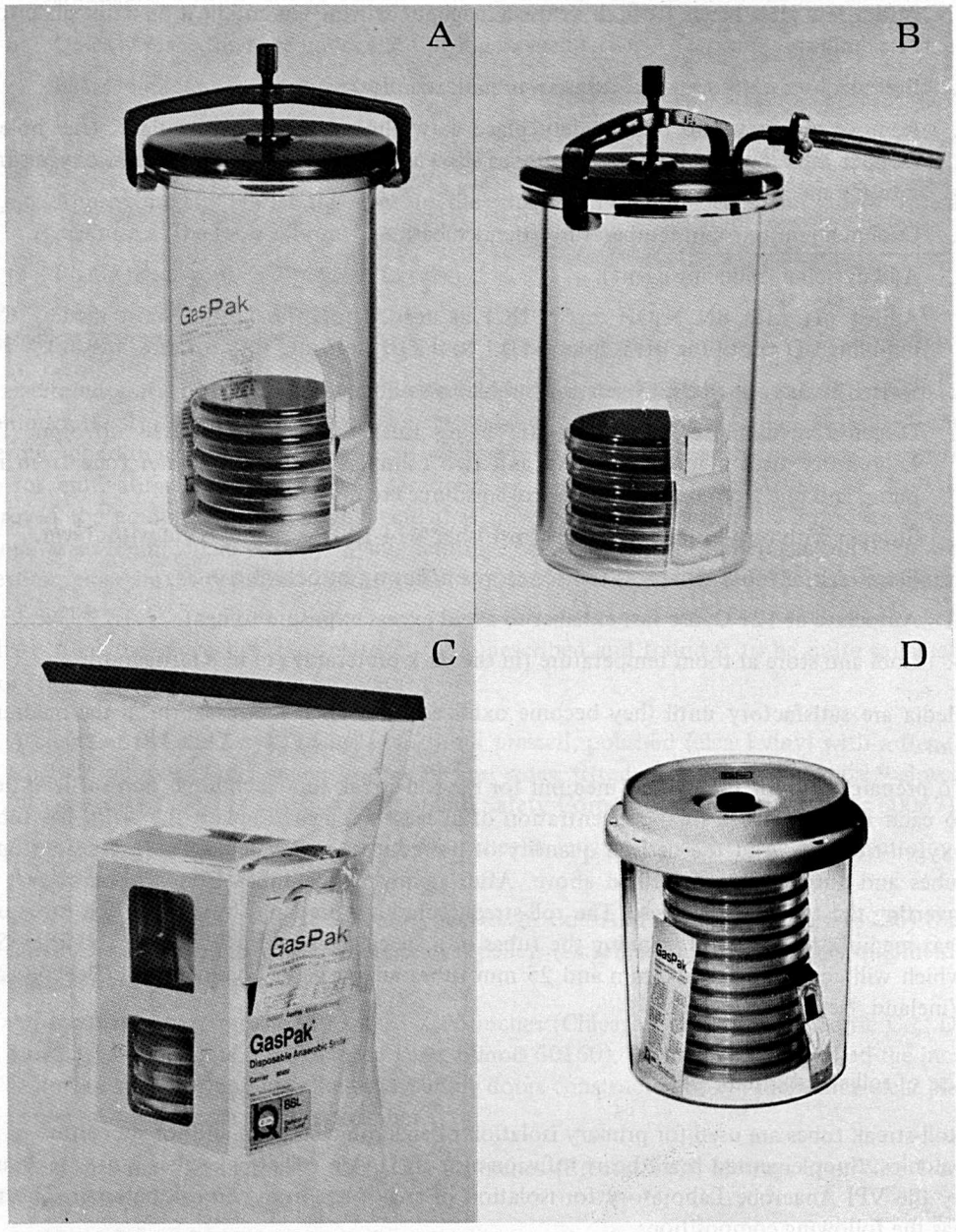


PLATE 6. Anaerobic Systems.

A. GasPak 100 system.
B. GasPak 100 system, vented.
C. GasPak disposable system.
D. GasPak 150 system.

Courtesy of Baltimore Biological Laboratories, P.O. Box 175, Cockeysville, Maryland 21030.

a. Weigh out dry ingredients.

b. Place ingredients in flask and add water, salts solution, and resazurin. The flask should be slightly larger than volume of medium to minimize air space above medium.

c. Fit a removable chimney (e.g., a modified polypropylene bottle) to the flask to prevent medium from boiling over.

d. Add a few glass beads to flask or use a magnetic stirring bar and heat on a hot plate to obtain even boiling.

e. Boil medium until resazurin turns from pink to colorless.

f. Remove chimney and immediately place a two-hole stopper in the flask. One hole of the stopper must be fitted with a cannula to allow addition of a gentle stream of oxygen-free CO_2 into the medium to exclude air.

g. Cool medium to room temperature in an ice bath.

h. Add cysteine (reducing agent).

i. Adjust pH with 8N NaOH or 5N HC1 as needed (pH 7.0-7.1 for most media). Continue bubbling CO_2 until the pH is lowered 0.1 to 0.2 of a pH unit (6.9 for most media).

j. Switch to oxygen-free nitrogen and bubble to exclude air.

k. Dispense media into tubes that are being flushed with oxygen-free nitrogen. The VPI Laboratory uses a medium pump and two cannulas soldered together (one from medium pump and one from oxygen-free nitrogen source) to dispense medium.

l. Stopper with butyl rubber stoppers (or Fisher #14-130) as cannulas are withdrawn.

m. Place rack of tubes in a press to hold stoppers during autoclaving.

n. Autoclave at 121°C; use fast exhaust to avoid excess exposure to heat.

o. Cool and store at room temperature (in the dark preferably) or in a refrigerator.

Media are satisfactory until they become oxidized, i.e., when the resazurin in the medium turns pink.

To prepare tubes of PRAS agar medium for the roll-streak tube technique, add sufficient dry agar to each tube to give a final concentration of at least 2% agar. Replace the air in the tubes with oxygen-free N_2. Add the desired quantity of prereduced broth medium to the agar, stopper the tubes and autoclave as described above. After removing the tubes from the autoclave, mix by inverting the tubes in the press. The roll-streak tubes are prepared by cooling the tubes of PRAS agar medium to 45°C and spinning the tubes in a special spinner until the agar solidifies. Spinners which will accommodate 10 mm and 25 mm tubes are available commercially (Bellco Glass, Inc., Vineland, New Jersey 08360).

2. Use of roll-streak tubes.

Roll-streak tubes are used for primary isolation of anaerobic bacteria and for subculture of isolated colonies. Supplemented brain heart infusion agar (BHIA) in roll-streak tubes is now recommended by the VPI Anaerobe Laboratory for isolation of anaerobes from clinical materials. This medium has the following composition:

Brain heart infusion broth (dehydrated)	3.7 gm
Yeast extract	0.5 gm
Distilled water	100.0 ml
Resazurin solution	0.4 ml
Vitamin K-hemin solution	1.0 ml

Boil, cool, and add 0.05 gm cysteine. Dispense under oxygen-free N_2 in 10 ml quantities into tubes containing 0.25 gm agar, stopper the tubes and then autoclave them.

A multipurpose unit for inoculation of PRAS media in roll-streak tubes or broth is available commercially from Bellco Glass, Inc., as shown in plate 7. Stainless steel or platinum wire loops are recommended for inoculation since other types of wire may oxidize the media. Roll-streak tubes are inoculated to obtain isolated colonies as follows:

a. Remove rubber stopper with flame sterilized 6-inch hemostat which has been bent to fit the stopper.

b. Quickly place the tube under the flowing oxygen-free CO_2 gas cannula of the streaker.

c. Place loop containing microorganisms or clinical material against the agar at the bottom of the tube.

d. Start motor to rotate the tube; carefully draw loop straight up the tube.

e. Approximately 1/4 of the way up the tube, turn loop on edge and streak with decreasing pressure as the loop is brought towards the top.

f. Flame the stopper and replace in tube.

C. Anaerobic glove box techniques.

An anaerobic glove box is a self contained anaerobic system. With the glove box, most bacteriologic techniques involved in the isolation and identification of anaerobic bacteria can be performed under anaerobic conditions without exposing the microorganisms to air. The first practical, inexpensive glove box for cultivation of anaerobes was described by Aranki et al.[25] Since then Dr. Rolf Freter has improved the equipment considerably and it is now possible to obtain the improved glove boxes in various sizes commercially (Coy Manufacturing Co., Ann Arbor, Michigan 48104). There were anaerobic glove boxes described before 1969, but these were constructed of rigid materials and were quite expensive.

We have used the glove box (see plate 7) to be described and found it to be quite satisfactory. The major components of the system include:

1. A chamber (84 x 32 x 40 inches) of 20 mil pressed, polished (clear) vinyl with a floor of 50 mil frosty vinyl extended 2 inches up on all four sides, fitted with two pairs of size 9 gloves with GL 20 sleeves (G-F Supply Division of Standard Safety Corporation, Palatine, Illinois 60067).
2. A metal entry lock (12 inches I.D. x 18 inches in length) from the Coy Manufacturing Co., Ann Arbor, Michigan 48104.
3. Two unheated catalyst boxes (Prototype Design Co., Ann Arbor, Michigan 48106) for use with DEOXO palladium-coated alumina catalyst pellets (Engelhard Industries, Gas Equipment Division, East Newark, New Jersey 07029).
4. An incubator that measures 23 x 23 x 25 inches (Chicago Surgical and Electric Co., Division of Lab-Line Instruments, Inc., Melrose Park, Illinois 60160). We have since modified the incubator by removing the door and installing two sliding doors constructed of 1/4 inch thick clear plexiglass to conserve working space in the chamber.
5. Metal shelving that measures 12 x 32 x 36 inches (Erecta Shelf, Metropolitan Wire Goods Corp., Wilkes-Barre, Pennsylvania 18701).
6. An inner floor of 1/4 inch thick clear plexiglass acrylic sheet with cross strips of the same material (1 x 1/4 inch) mounted in the bottom of the chamber to allow free circulation of gasses.
7. A Bacti-Cinerator (Aloe Scientific, St. Louis, Missouri 63155).
8. A vacuum pump (Precision Scientific Co., Chicago, Illinois 60647) Model 150.

After the glove box is assembled and sealed, the chamber is partially evacuated and filled 10 times with technical grade nitrogen and 10 times with a gas mixture of 5% CO_2 (bone dry), 10% H_2, and 85 N_2 (Matheson Co., East Rutherford, New Jersey 07073). Approximately one pound of catalyst pellets in each of the catalyst diffusion boxes aids in the removal of residual oxygen.

The relative humidity of the chamber is regulated with a drying agent "Tel-Tale" silica gel, desiccant grade H type IV, Davison Commercial Chemical Corp., Baltimore, Maryland 21223) which is reusable by heating in a dry heat oven at 160°C to remove moisture. Anaerobic conditions are maintained in the glove box by replacing the catalyst pellets with new or "rejuvenated" catalyst at frequent intervals (at least once per week).

The oxygen concentration can be monitored, if desired, with an oxygen analyzer (Lockwood-McLorie Model GP) available from Lockwood and McLorie, Inc., Horsham, Pennsylvania 19044. We maintain the oxygen concentration in glove boxes used in our laboratory at less than 10 parts per million. Use of an oxygen analyzer is not absolutely necessary if the catalyst is changed frequently and oxygen sensitive indicators are used to monitor anaerobic conditions.

When material is passed into the chamber the lock is evacuated and replaced two times with nitrogen and once with the gas mixture (5% CO_2, 10% H_2, 85% N_2) before the inner door is opened. The same procedure in reverse is used when removing materials from the chamber. Media prepared in the usual manner are passed into the chamber and reduced 48 hours before they are used. PRAS media can also be used in the glove box if desired. Cultures are incubated within the chamber and can be inspected and subcultured at any time without exposure to air.

The use of an anaerobic glove box has several advantages:

1. Either conventional media (prereduced in the chamber) or prereduced anaerobically sterilized media can be used.
2. Clinical specimens are not exposed to aerobic conditions during culture procedures.
3. The need for boiling liquid media just before use is eliminated.
4. Conventional plating techniques can be used.
5. Cultures can be incubated under anaerobic conditions, inspected and subcultured at any time without exposing the bacteria to air.
6. Using the system is quite economical. The only major operating cost is for gases that are used when materials are passed into and out of the chamber.

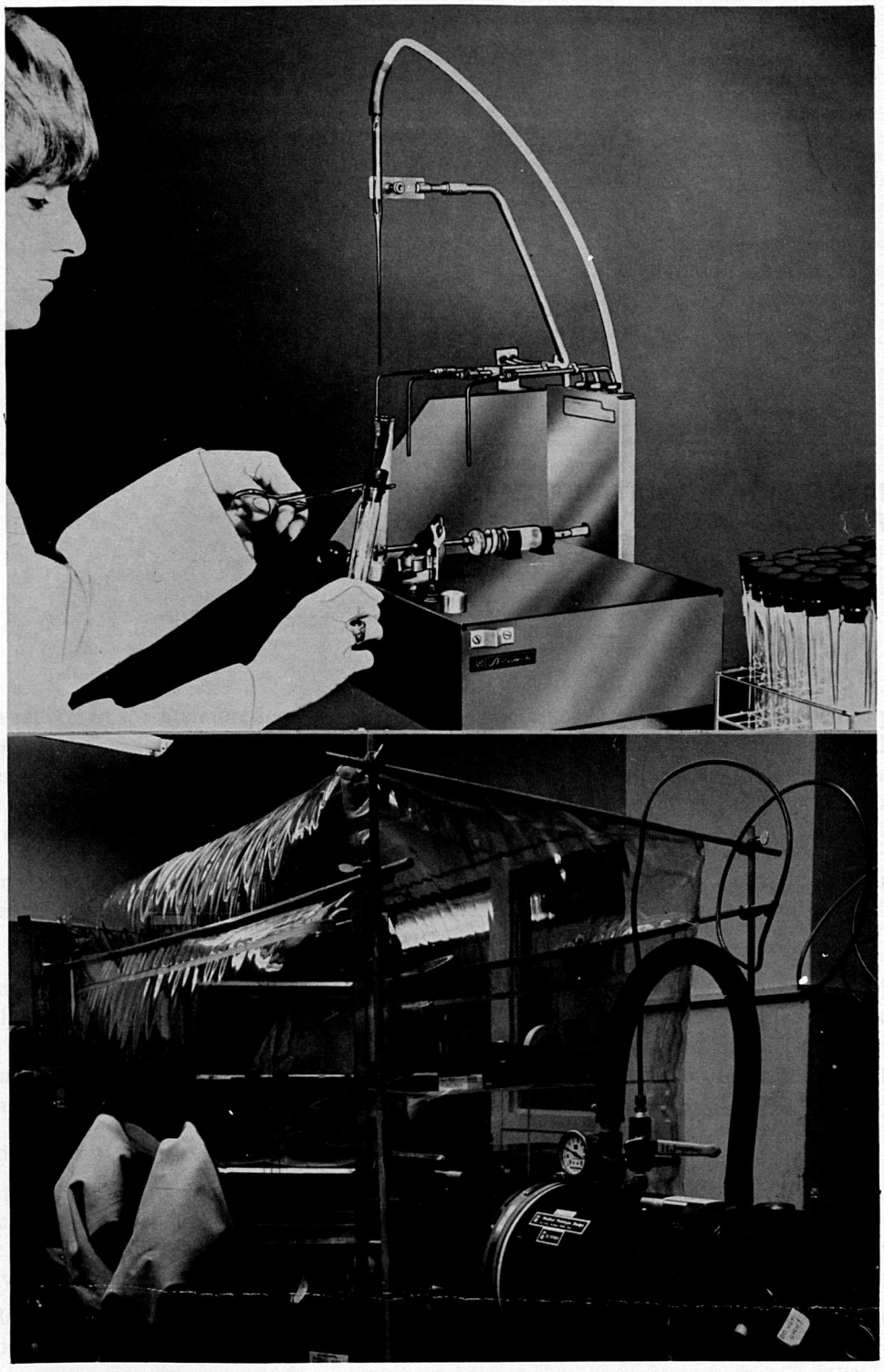


PLATE 7. Anaerobic Systems.

A. VPI anaerobic culture system; courtesy Bellco Glass, Inc., 340 Edrudo Rd., Vineland, N.J. 08360.

B. Anaerobic glove box system.

XIV. IDENTIFICATION OF ACID METABOLIC PRODUCTS BY GAS LIQUID CHROMATOGRAPHY

Numerous reports in the literature have emphasized the importance of determining the metabolic products (particularly organic acids) of anaerobic bacteria as an aid to their classification and identification. Practical procedures, using gas liquid chromatography (GLC), are now available which allow rapid identification of metabolic products in a clinical microbiology laboratory. The GLC procedures can be performed with a relatively inexpensive chromatograph (available from various scientific instrument manufacturers).

The information provided by GLC examination allows more rapid and accurate identification of anaerobes with fewer differential tests. This actually results in decreasing the costs for accurate identification of these bacteria.

Rapid and accurate identification of anaerobic bacteria is quite important in the management of patients, particularly those with life threatening infections. Therapy required for some infections caused by anaerobic bacteria may be quite different from that for infections caused by faculative or aerobic microorganisms. Also, treatment of infections caused by certain anaerobic bacteria must sometimes be radical, and its effectiveness decreases with delay.

A. Identification of volatile acids.

This procedure allows the identification of a number of volatile acids that are soluble in ether (e.g., acetic, propionic, isobutyric, butyric, isovaleric, valeric, isocaproic, and caproic acids), but pyruvic, lactic, and succinic acids are not detected. These nonvolatile acids are identified by the examination of methyl derivatives (Section B).

1. Inoculate 8 ml tubes of prereduced Peptone-Yeast Extract-Glucose (PYG) medium with a few drops (0.05-0.1 ml) of an actively growing broth culture.
2. Incubate under anaerobic conditions for 48 hours or until adequate growth is obtained.
3. Acidify cultures to pH 2.0 or below by adding 0.1-0.2 ml of 50% v/v aqueous H_2SO_4.
4. Transfer 4.0 ml of acidified culture to a 16-x 125-mm screwcap tube. (Save remainder of culture for methylation procedure, if necessary.)
5. Add 1 ml of ethyl ether, tighten cap, and mix by inverting tube gently about 20 times.
6. Centrifuge briefly in a clinical centrifuge (1500-2000 RPM) to break the ether-culture emulsion.

7. Place the ether-culture mixture in a freezer (minus 10°C or lower) and leave until the aqueous portion (bottom) is frozen. Pour off the ether layer into a small (13 x 100 mm) screwcap tube, add anhydrous $MgSO_4$ to equal about one-half the volume of ether extract, tighten the cap, and let stand at least 10 minutes to allow removal of water from the extract. (Extracts not chromatographed on the day of preparation should be held in a freezer to prevent evaporation of ether.)
8. Inject 14 microliters (μl) into the column.
9. Identify volatile acids by comparing elution times of products in extracts with those of known acids chromatographed on the same day.
10. Examine a tube of uninoculated PYG medium in the same manner since some lots of peptone and yeast extract have been shown to contain significant quantities of these acids. The medium should be as free of contaminating acids as possible; however, most will contain a trace amount of acetic acid.

B. Analysis of methylated products is required if no volatile acids are detected or if only acetic acid is detected. Currently either of two methods may be used for the preparation of methyl derivatives.

1. Boron trifluoride-methanol method.
   a. Transfer 1 ml of acidified culture to a 13-x 100-mm screwcap tube.
   b. Add 1 ml of boron trifluoride-methanol (14% v/v) and let stand at room temperature for at least 4 hours (preferably overnight), or heat in a waterbath at 100°C for 5 minutes or at 70°C for 30 minutes.
   c. Add 0.5 ml of chloroform and mix by inverting the tube gently about 20 times. It may be necessary to centrifuge the tube slowly to break an emulsion in the chloroform layer.
2. Methanol method.
   a. Transfer 1 ml of the original acidified culture to a 13-x 100-mm screwcap tube.
   b. Add 2 ml of methanol and 0.4 ml of 50% H_2SO_4 (v/v). Place the tube in a 55°C water bath for 30 minutes or hold overnight at room temperature.
   c. Add 1 ml of distilled water and 0.5 ml of chloroform. Mix by inverting the tube gently about 20 times. It may be necessary to centrifuge the tube slowly to break an emulsion in the chloroform layer.

Regardless of the method used to prepare the methyl derivatives, the chloroform extracts are all tested in the manner shown below.

1. Fill a syringe with the chloroform extract after placing the tip of the needle beneath the aqueous layer.
2. Wipe off the outside of the needle with a clean tissue, and inject 14 μl of the chloroform extract into the column. After testing about 15-20 methylated samples, recondition the column by injecting 14 μl of methanol.
3. Do the analyses of chloroform extracts with the same column and conditions as used for volatile acids.
4. Identify the methylated acids by comparing elution times of products in extracts with those of known methylated acids.
5. Test an uninoculated medium by one of the two methods described above to detect the presence of nonvolatile acids (especially lactic and succinic acid). If these acids are present in any amount, corrections must be made or the medium discarded.

C. Chromatograph and operating conditions which have been found to be satisfactory at CDC:

Chromatograph	Beckman Model GC2A
Type of Detector	Thermal Conductivity
Type of Column	6-foot x 1/4-inch stainless steel Resoflex LAC-1-R-296 standard concentration (P), Burrell Corporation, Fifth Avenue, Pittsburg, Pennsylvania 15219.
Operating Conditions	1X attenuation, 200 milliamps, 149°C column temperature, 198°C heated inlet temperature and a helium gas flow rate of about 120 ml/minute or 32 lbs. pressure.
Recorder	Beckman 10 inch laboratory potentiometer recorder.

D. Standard solutions.

1. Volatile acid standard. To 100 ml of distilled water, add:

acetic	0.057 ml
propionic	0.075 ml
isobutyric	0.092 ml
butyric	0.091 ml
isovaleric	0.127 ml
valeric	0.125 ml
isocaproic	0.126 ml
caproic	0.126 ml

The prepared standard solution contains about 1 meq of each acid depending on the purity of the reagent. To use, acidify 4 ml of standard and proceed as for culture.

2. Nonvolatile acid standard. To 100 ml of distilled water add:

pyruvic acid	0.068 ml
lactic acid (85%)	0.084 ml
succinic acid	0.06 gm

The prepared standard contains about 1 meq of each acid depending on the purity of the reagent. To use, methylate 1 ml of the standard and proceed as for culture.

E. Approximate elution time for standard acids in minutes:

1. Volatile acids.

acetic	4.5
propionic	6.0
isobutyric	6.5
butyric	8.5
isovaleric	10.0
valeric	13.0
isocaproic	17.0
caproic	20.0

2. Nonvolatile acids.

methyl-pyruvate	3.0
methyl-lactate	3.5
methyl-succinate	12.0

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