Identification of Clinically Significant Anaerobic Bacteria

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ABSTRACT

By combining the various methodologies for anaerobic bacteriology, a scheme has been developed with four flow charts for the identification of the clinically significant anaerobic organisms. This scheme incorporates the use of gas chromatography and a micromethod biochemical system.

Anaerobic Identification Scheme

Many schemes for the identification of anaerobic bacteria have been developed over the past several years. The three major systems include those developed at: Virginia Polytechnic Institute (VPI), Wadsworth Veterans Administration Hospital in Los Angeles, and the Center for Disease Control (CDC). VPI, where much of the early research in anaerobic bacteriology was pioneered, developed methodology which includes the use of gas liquid chromatography (GLC). This system also employs the roll tube technique for bacterial isolation, which is quite effective but is generally too time consuming for most clinical laboratories. The CDC method uses GLC, but anaerobic identification is based primarily upon cellular morphology, colonial characteristics, and biochemical reaction patterns. The Wadsworth Scheme uses cellular morphology, Gram reaction and further emphasizes susceptibility to certain antibiotics. In our laboratory the three major schemes have been taken, using stock anaerobic organisms, and with modifications have been incorporated into one for the identification of clinically significant anaerobic bacteria. It has been used in our laboratory for four years and is revised as needed. This paper provides a summary of our procedure.

Materials and Methods

Specimens are submitted to the laboratory in appropriate anaerobic containers. Port-A-Cul, Anaerobic Specimen Collectors, and Anaswabs-Anaports have been used successfully. Purulent material is often transported and submitted directly in a stoppered syringe. In Table I are listed our criteria for specimen acceptability which must be met before specimens are

* BBL Cockeysville, MD
† Becton Dickinson, Rutherford, NJ
‡ Scott Laboratories, Fiskeville, RI
TABLE I
Acceptable and Nonacceptable Specimens* for Anaerobic Bacteriology

<table>
<thead>
<tr>
<th>Specimen</th>
<th>Acceptable</th>
<th>Nonacceptable</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sterile body fluids</td>
<td>Bile, blood, cerebrospinal, pleural, ascitic, synovial</td>
<td></td>
</tr>
<tr>
<td>Exudates</td>
<td>Aspirated pus from deep wounds or abscesses</td>
<td>Pus from superficial wounds</td>
</tr>
<tr>
<td>Genital</td>
<td>Bartholin’s gland, culdocentesis, endometrial, fallopian tube, placental, prostatic</td>
<td></td>
</tr>
<tr>
<td>Gastrointestinal</td>
<td>Appendix, abscesses, stool</td>
<td>Stool</td>
</tr>
<tr>
<td>Respiratory</td>
<td>Transtracheal aspirate</td>
<td>Bronchial washings, nasopharyngeal, sputum, throat</td>
</tr>
<tr>
<td>Urine</td>
<td>Suprapubic</td>
<td>Voided or catheterized</td>
</tr>
</tbody>
</table>

*Nonacceptable specimens are likely to contain normal or transient anaerobic flora.

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processed according to figure 1. A Gram stain is made from each specimen before culture. Media used for isolation include: (1) BBL Brucella or Columbia base agar plates supplemented with 5 percent defibrinated sheep blood cells, vitamin K₁ and hemin (An-BAP); (2) plates supplemented with laked blood (LKV-BAP) prepared in the same manner as the Brucella or Columbia plates, but with the addition of 100 μg per ml kanamycin and 7.5 μg per ml vancomycin; (3) BBL colistin nalidixic acid agar (CNA); (4) BBL fluid thioglycollate (THIO) supplemented with 50 μg per ml hemin; and (5) blood agar plates (BAP) made with Difco heart infusion agar base and 5 percent defibrinated sheep blood cells.

Additional media are: (1) egg yolk agar plates (EYA) prepared by using 7.5 percent McClung-Toabe base and Difco egg yolk enrichment; (2) nutrient gelatin plates prepared with nutrient agar and 0.4%

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FIGURE 1. Scheme for processing specimens for anaerobic bacteriology.
percent gelatin; and (3) motility tubes consisting of 1.0 percent casitone, 0.5 percent sodium chloride, 0.3 percent yeast extract, and 0.3 percent agar. Commercially prepared prereduced peptone-yeast-glucose broth (PYG) and chopped meat carbohydrate broth (CMC) are obtained from Scott Laboratories and provide a liquid growth medium for GLC and spore testing, respectively. Two-unit penicillin discs are obtained from BBL. For biochemical testing, the BBL Minitek System or the API 20 Anaerobic system is used.

Primary inoculation media are incubated for 48 hours in an anaerobic environment at 35°C and observed for growth. GasPak jars (BBL) or a glove box are used to provide the anaerobic environments necessary. Suspect colonies are subcultured on an An-BAP and PYG broth, both incubated anaerobically, and a BAP under 10 percent CO₂ incubation. After 18 to 24 hours of incubation, both plates are observed; if no growth is detected on the BAP, the organism is considered to be an anaerobe. Exceptions include microaerophilic streptococci and lactobacilli, and facultatively anaerobic propionibacteria and certain *Clostridium* spp.

Organisms in the PYG broth are Gram stained and microscopic morphology and Gram reaction recorded. The PYG broth is then inoculated to an An-BAP for isolation and a BAP to insure contamination with aerobes has not taken place. If *Bacteroides* spp. are suspected, a two unit penicillin disc is placed on the An-BAP in the area of heaviest inoculation. From this plate organisms are used for biochemical and spore testing as necessary. The heat shock treatment using CMC as a medium, in which cultures are exposed to 80°C for 10 minutes provides a reliable spore test. Alternatively, alcohol treatment is also used for spore testing. If required, a catalase test should be performed directly in the PYG broth by adding hydrogen

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**Figure 2. Flow chart for identification of Gram positive bacilli.**

<table>
<thead>
<tr>
<th>Major Volatile Acids</th>
<th>Acetic &amp; Propionic</th>
<th>Acetic &amp; Butyric or no Acids</th>
</tr>
</thead>
<tbody>
<tr>
<td>+ Cnelalase</td>
<td>+ Spore Test</td>
<td>+ Clostridium sp.* Eubacterium sp.*</td>
</tr>
<tr>
<td>Propionibacterium sp.*</td>
<td>Arachnia propionica* or Propionibacterium sp.</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Major Non-volatile Acids</th>
<th>Lactic &amp; Succinic</th>
<th>Lactic &lt; Acetic</th>
<th>Lactic &gt; Acetic</th>
</tr>
</thead>
<tbody>
<tr>
<td>+ Actinomyces sp.</td>
<td>Speciate by Immunofluorescence</td>
<td></td>
<td></td>
</tr>
<tr>
<td>+ Growth in 10% CO₂</td>
<td>+ Lactobacillus sp.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>+ Spore Test</td>
<td>+ Bifidobacterium sp.* or Eubacterium sp.*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>+ Clostridium sp.*</td>
<td>+ Lactobacillus sp.</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Biochemical testing is needed for further identification.
peroxide dropwise and observing for gas production.

GLC, as described by Holdeman and Moore, is performed where indicated in figure 1. The PYG broth is aliquoted into one and two ml portions, each of which is acidified with 0.04 ml of 50 percent sulfuric acid and stoppered. The two ml aliquot is used for the volatile acid (ether) extraction procedure, while the one ml portion is used for the nonvolatile acid (methylated) extraction procedure. For our purpose, it is much easier to do both procedures on all organisms. However, in some cases only the volatile extraction is required for identification. This could provide a feasible economic short cut for some laboratories. An Anabac gas chromatograph* is used for the chromatography studies.

**Results and Discussion**

Based on Gram stain results and the GLC analysis, flow charts were developed for the identification of the more commonly isolated anaerobic bacteria. In figure 2 is shown the chart for the nonsporing Gram positive bacilli. Owing to the inherent difficulties of identifying the Gram positive bacilli by morphology and biochemical reactivity, GLC is useful for identification of this group of organisms. Although *Arachnia propionica* is included as a possible isolate, it has not been isolated in the four year period for which data are known by us. The *Actinomycetes* can be identified to the genus level using Gram stain results and GLC. However, identification of *Actinomyces israelii*, the principal pathogen in the genus and other *Actinomyces* sp., must be done by immunofluorescence. Spore tests must be performed where indicated to separate clostridia from other Gram positive bacilli. Because some species of clostridia do not readily sporulate, an initial negative heat-shock spore test may place a spore-forming organism into this chart. If colonial morphology and Gram stain indi-
cata a possible clostridia, additional efforts should be made to demonstrate spores via heat-shock and alcohol treatment.

The anaerobic Gram negative bacilli, which include Bacteroides and Fusobacterium spp., are identified in figure 3. The Bacteroides fragilis group of organisms includes B. distasonis, B. fragilis, B. ovatus, B. thetaiotamicron, and B. vulgatus. This group of organisms along with certain Bacteroides spp. are resistant to a two-unit penicillin disc and require biochemical testing for speciation. Bacteroides spp. susceptible to the penicillin disc are usually not speciated. Bacteroides melaninogenicus is typically characterized by its black pigmentation after two to six days growth. The young colonies of B. melaninogenicus fluoresce red when exposed to a Wood’s lamp. However, this cannot be used for definitive identification since other anaerobes, particularly Gram negative cocci, may also exhibit this characteristic. Presumptive identification of B. melaninogenicus and B. fragilis may be determined by immunofluorescent testing* of a smear from a patient’s specimen. Bacteroides corrordes is characterized by a pitting of the agar beneath the colony. Immunofluorescent testing for identification of the B. fragilis group of organisms would be especially useful for smaller laboratories where more extensive biochemical testing is not routinely carried out. Certain clostridia, particularly Clostridium ramosum and Clostridium malenomatum, do not retain crystal violet and appear as Gram negative bacilli. Therefore, spore tests are performed where indicated on the chart. Fusobacterium spp. may be indicated by long, thin, slightly staining, pointed rods.

* Pfizer, New York, NY.
CLOSTRIDIA

Major Non-volatile Acids

Acetic & Butyric
Acetic and/or Lactic
Acetic, Butyric, Isovaleric

Clostridium sp.*

*Biochemical testing is needed for further identification.

**Figure 5. Flow chart for identification of clostridia.**

The anaerobic cocci are identified using figure 4. Many of the cocci can be identified using only chromatographic data. The microaerophilic streptococci however, are speciated by biochemical testing.

In figure 5 is contained the chart for identifying the Gram variable spore-forming bacilli, the clostridia. These organisms are grouped into three major divisions using GLC. Biochemical testing is used to identify further the clostridia, excluding *Clostridium perfringens*. This organism can be identified by the typical double zones of hemolysis on blood agar, a positive spore test, lecinthinase production on egg yolk agar, negative motility, and positive gelatinase activity. Owing to the large number of species and biochemical variability of this genus, it is unlikely that more than 50 percent of the clostridia can be speciated without being sent to a reference laboratory.

No identification system is of value if improper specimens, improperly collected specimens, or improperly transported specimens are accepted for culture. Specimens from body sites where anaerobic microorganisms colonize as normal flora and specimens submitted in aerobic containers should be discouraged.

Strict attention to proper specimen collection and transportation will increase the chances of viability and ultimately aid in the isolation of significant anaerobic organisms. Exposure of most organisms to air during collection, inoculation, and certain testing procedures will not destroy viability to a great extent as long as the organisms are promptly returned to an anaerobic atmosphere. The use of a glove box enables cultures to be examined after 24 hours without exposing them to air, while cultures in GasPak jars should routinely be incubated for 48 hours before examination. If GasPak jars are used, the catalyst must be reactivated after each use by heating at 160°C for two hours.

The scheme devised considers most major accepted techniques and can be easily modified or expanded based on the needs of the individual laboratory. Constant monitoring of the literature as well as the needs of the clinician are necessary to keep the scheme current and relevant.

**References**