Neisseria Confirmation by an Enriched, Bicarbonate-Containing Carbohydrate Medium

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A sugar fermentation medium for the confirmation of Neisseria and related species was developed. The medium contained a commercial supplement and a hemoglobin source prepared from lysed sheep erythrocytes. Bicarbonate in the medium substituted for a CO₂-supplemented atmosphere. The medium was dispensed into screw-capped tubes. This medium was compared to cystine-Trypticase agar (CTA) with added carbohydrates and the modified rapid fermentation test in the confirmation of Neisseria species. Performance of the new medium was equivalent to that of the modified rapid fermentation test, but cystine-Trypticase agar failed to confirm a significant number of clinical isolates of Neisseria gonorrhoeae.

In recent years much emphasis has been placed on development of reliable systems for the biochemical confirmation of Neisseria species. Most of these have been based on the detection of acid products from carbohydrate degradation. Cystine-Trypticase agar (CTA) with added carbohydrates has been generally considered the standard medium for this purpose since its introduction in 1948 (19), and it is still used by many laboratories for confirmation of Neisseria gonorrhoeae.

However, this medium has proved inadequate in certain cases due to the failure of occasional isolates of N. gonorrhoeae to metabolize glucose (1). One study indicated that approximately 9% of 267 fresh gonococcal isolates and 39% of 124 referred strains tested were glucose negative using CTA (3).

Several alternate methods have been proposed. Of these, perhaps the most commonly used are the Minitek system (14, 17), the NYC fermentation medium (6), and the modified rapid fermentation test (MRFT) (2–4, 13). Several inherent disadvantages exist with each of these. The Minitek procedure is a commercial system and requires purchase of the manufacturer's equipment and reagents. The NYC medium requires horse blood and the preparation of a yeast dialysate. The MRFT has the advantage of low cost and ready availability of materials. However, it requires a heavy and adequately suspended inoculum that is occasionally difficult to obtain with older cultures and certain mucoid strains.

The purpose of this study was to devise an easily prepared, growth-type medium giving accurate fermentation patterns when used in confirmation of Neisseria species. One important consideration was the requirement by many fastidious gonococcal strains for certain nutritional supplements.

Although recent studies have suggested the elimination of hemoglobin from gonococcal media (9), others have noted that the amount of growth may be reduced with hemoglobin-free media (8). Powdered hemoglobin, however, imported a dark, opaque color to a medium, masking color changes of indicators and limiting its usefulness with fermentation media. Faur et al. (6) met this problem by using a lysed erythrocyte solution that gave a translucent medium. They also investigated the substitution of cow and sheep blood in NYC isolation medium and reported that sheep blood was unsatisfactory due to delayed and inconsistent growth of some N. gonorrhoeae strains (7). Their test involved the standard NYC formulation containing both lysed erythrocytes and plasma, and did not include the NYC fermentation medium in which the plasma is eliminated.

A nutritional supplement is generally added to basal media for isolation of gonococci. Supplements also have been used in confirmation media for Neisseria (6, 20). A commercially available supplement is IsoVitaleX (BBL). However, IsoVitaleX contains glucose, making it unsuitable for use in fermentation media. Since the glucose is contained in the reconstituting fluid, substituting sterile, distilled water will eliminate this problem.

Another component generally used in the isolation and culture of gonococci is an atmosphere supplemented with carbon dioxide. CO₂ apparently is essential for certain strains of gonococci,
particularly on initial isolation on solid media (10, 16). Recent investigators have used bicarbonate in isolation media as a replacement for gaseous CO2 (5, 11, 18) and to enhance phenol red color reactions of Neisseria in fermentation tests using the Minitek system (14).

MATERIALS AND METHODS

Medium formulation. GC medium base (Difco) was chosen as the basal medium, with phenol red as the pH indicator and IsoVitaleX, reconstituted with sterile water, as the nutritional supplement. For a hemoglobin source we modified the method of Peizer (15) as subsequently used by Faur et al. (6, 8) by substituting lysed sheep erythrocytes. In our study only the lysed erythrocytes were utilized, with plasma discarded. Rather than utilize a candle jar or CO2 incubator, we included bicarbonate in our medium. An important factor in this method of CO2 replacement is the use of sealed containers (18), so we chose to dispense the medium into screw-capped tubes. In our formulation, the NaHCO3 concentration was 0.02%, and the air volume of a typical tube containing the medium was about 6 ml. Test sugars were glucose, maltose, sucrose, and lactose.

Preparation of medium. The hemoglobin solution was made prior to preparation of the final medium. A quantity of hemoglobin solution sufficient to prepare about 30 to 35 sets of media was made by centrifuging defibrinated sheep blood, removing the serum with a sterile Pasteur pipette, and adding 2 ml of the packed erythrocytes to 80 ml of sterile, distilled water. This solution was stored at 4°C until use.

The medium was made up in sets of glucose, maltose, sucrose, and lactose tubes. The basal medium for each sugar consisted of 3.6 g of GC medium base (Difco) suspended in 50 ml of distilled water, to which 2 ml of a 0.2% phenol red solution was added. The mixture was heated to boiling, sterilized by autoclaving at 121°C for 15 min, and cooled to 50°C in a water bath.

Twenty milliliters of the hemoglobin solution, 25 ml of an 8% filter-sterilized solution of the appropriate sugar, 0.5 ml of a sterile 4% NaHCO3 solution, and 1 ml of IsoVitaleX (reconstituted with sterile, distilled water instead of the supplied diluent) were added to the cooled basal medium. The medium was adjusted to approximately pH 7.5 with sterile 0.2 N NaOH. The final medium (designated as Gonococcus Identification medium or GCID) was dispensed aseptically in 3-ml amounts into sterile screw-capped tubes (100 by 13 mm). The tubes were allowed to cool in a slanted position and stored at 4°C.

Test procedure. Each GCID set was inoculated by streaking the slants with a small amount of growth from a 14- to 18-h culture on chocolate agar, using a 2-mm bacteriological loop. Caps were securely tightened. Tubes were incubated at 35°C and read at 24 and 48 h. A change in the medium from deep red to yellow or gold along the slant was considered a positive reaction. An uninoculated control tube was included with each test for color comparison.

Test organisms. A comparison of the GCID medium with CTA and the direct MRFT (4) was conducted using 50 presumptive isolates of N. gonorrhoeae, 7 laboratory strains of N. gonorrhoeae (including 1 confirmed CTA-negative strain), laboratory strains of several other Neisseria species (including 2 atypical N. meningitidis strains), and 2 Branhamella catarrhalis strains. The gonococcal isolates, six N. gonorrhoeae strains, one N. meningitidis strain, and one N. sicca strain were provided by the Mississippi State Public Health Laboratory, Jackson. All others were laboratory strains provided by the Center for Disease Control, Atlanta, Ga.

The 50 gonococcal isolates were randomly selected from positive patient specimens from the urogenital tract. Presumptive identification consisted of isolation on modified Thayer-Martin medium, along with confirmation of typical colonial and microscopic morphology, Gram stain, and catalase reaction. These strains were subcultured on chocolate agar and tested immediately. No attempts were made to characterize these strains by colony type or to maintain them after completion of testing. Laboratory strains used in these tests were subcultured on chocolate agar from quick-frozen stocks maintained at −60°C.

RESULTS

Results of the comparison of the new medium with CTA and the MRFT are shown in Table 1.

**Table 1. Comparison of methods for confirmation of Neisseria and related species**

<table>
<thead>
<tr>
<th>Organism</th>
<th>Typical carbohydrate pattern*</th>
<th>No. of strains tested</th>
<th>No. of strains with typical pattern on:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>G</td>
<td>M</td>
<td>S</td>
</tr>
<tr>
<td><em>N. gonorrhoeae</em></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Clinical isolates</td>
<td>+</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>Laboratory strains</td>
<td>+</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td><em>N. meningitidis</em></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Laboratory strains</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><em>N. subflava</em></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Laboratory strains</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><em>N. lactamica</em></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Laboratory strains</td>
<td>+</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td><em>N. sicca</em></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Laboratory strains</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><em>N. mucosa</em></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Laboratory strains</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><em>N. flavescens</em></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Laboratory strains</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><em>B. catarrhalis</em></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Laboratory strains</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

* G, Glucose; M, maltose; S, sucrose; L, lactose.
All gonococcal strains gave the expected pattern with GCID, and most were positive within 24 h. Only one gonococcal strain failed to yield the expected pattern with the MRFT. However, six gonococcal isolates (12%) were negative for all sugars on CTA. One laboratory strain of N. gonorrhoeae was glucose negative with CTA but positive with the other procedures. One laboratory strain of N. meningitidis was negative for all sugars with all three methods, and a second strain was maltose negative with CTA. All other strains gave expected patterns with each of the three procedures.

**DISCUSSION**

In this study the new medium performed as well as the MRFT in testing gonococcal isolates, laboratory strains of N. gonorrhoeae, and other Neisseria and related species. The only difficulties encountered were with one atypical N. meningitidis strain that gave aberrant results with both methods. In contrast, while performing well with other Neisseria, CTA failed to give expected results with 12% of the gonococcal isolates and two of three N. meningitidis strains.

Sheep cells were chosen for use in our medium, since sheep blood is commonly used and readily available in most microbiology laboratories. Although use of sheep blood has been reported as unsatisfactory for gonococci in NYC medium (7), its use in fermentation media was not reported. In our study, 50 clinical isolates and 7 laboratory strains of N. gonorrhoeae were tested on GCID medium, and all grew sufficiently to give positive glucose reactions. It should be noted that the medium contained lysed sheep erythrocytes, but not plasma, and that a direct inoculum from colonial growth (about 10⁵ cells) was utilized. It is possible that certain strains not encountered in this study may not grow on this medium. In this case either cow or horse erythrocytes probably could be substituted.

GCID medium was found to support adequate growth of all strains tested without incubation in CO₂. Other studies indicate that the concentration of NaHCO₃ necessary to support growth of gonococci is dependent on the air space of the container and the amount of inoculum (11, 18). Our ability to obtain satisfactory growth of all gonococcal strains tested is probably related to the presence of a limited air space (6 ml) within the screw-capped tubes and the use of a relatively heavy inoculum.

The new medium, in this limited test, was equivalent in performance to the MRFT for identification of Neisseria, whereas CTA was less satisfactory. Laboratories that prefer a growth-type medium may desire to conduct further tests to determine if this new medium meets their requirements.

**ACKNOWLEDGMENTS**

We thank the staff of the Mississippi State Public Health Laboratory for supplying cultures, other materials, and technical advice. The CTA-negative strain of N. gonorrhoeae and suggestions concerning the MRFT procedure, provided by W. J. Brown of the Center for Disease Control, were appreciated. Thanks are also due Robert Weaver, K. H. Wong, and Charles W. Griffin of the Center for Disease Control for assistance in obtaining laboratory strains of Neisseria.

**LITERATURE CITED**