Rapid Carbohydrate Fermentation Test for Confirmation of the Pathogenic Neisseria Using a Ba(OH)$_2$ Indicator

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Gonorrhea is the most prevalent bacterial infectious disease at present. The incidence of this disease has almost doubled in the past 5 years (22). When underreporting and underdiagnosis of cases are considered, the true incidence of gonorrhea is estimated at 2.6 million cases each year (22).

Carbohydrate utilization patterns are generally distinctive for the differentiation of the pathogenic and saprophytic Neisseria. Currently, tests for the fermentation of carbohydrates by the Neisseria in clinical laboratories are widely performed with the application of various fermentation media and systems usually employing the pH indicator phenol red (1, 4, 11, 15, 16, 20, 22, 23, 26). A radiometric fermentation system is commercially available that measures the amount of 14CO$_2$ metabolized by Neisseria isolates from 14C-labeled carbohydrate test vials.

The present investigation concerns the development of a sensitive test procedure for the rapid identification of Neisseria species. This test utilized the capacity of these bacteria to ferment specific carbohydrates with the production of carbon dioxide. The carbon dioxide, in turn, is permitted to react with barium hydroxide, the indicator, to form barium carbonate, a visible white precipitate. Thus, the formation of the carbonate provides the basic principle of this indicator system.

MATERIALS AND METHODS

Bacterial strains. Stock cultures of N. gonorrhoeae, N. meningitidis, N. lactamica, N. sicca, N. flavescens, and N. subflava were maintained in Trypticase soy broth containing 15% (vol/vol) glycerol at -70°C (12). Primary isolates of N. gonorrhoeae were obtained from the Allegheny County Health Department, Pittsburgh, Pa. Freshly isolated clinical cultures of various Neisseria species were obtained from this laboratory.

The Ba(OH)$_2$ indicator system was demonstrated to be a practical procedure in assisting clinical bacteriologists in the accurate and rapid identification of the pathogenic Neisseria from clinical specimens. This system measured the release of CO$_2$, resulting from the metabolism of fermentable carbohydrate, as the precipitated BaCO$_3$, by means of a spectrophotometer. The method was uncomplicated and can be performed in most clinical bacteriology laboratories.

Basal fermentation medium. The basal fermentation medium contained 2% (wt/vol) proteose peptone no. 3 (Difco), 0.5% (wt/vol) soluble Lintner starch (Fisher Scientific Co., Fairlawn, N.J.), and 0.05% CaCl$_2$ (12). This solution was sterilized by autoclaving at 15 lb/in$^2$ for 15 min at 121°C. This basal medium was stored at 4°C and had a final pH of 7.1. The dextrose content of the basal medium was determined by the hexokinase-glucose-6-phosphate dehydrogenase method (18, 24).

Carbohydrate solutions. Solutions of 3% (wt/vol) carbohydrates (dextrose, maltose, lactose, sucrose, and levulose) (Difco), each prepared in double-distilled water, were sterilized by membrane filtration (0.45 μm; Nalge, Sybron Corp., Rochester, N.Y.) under negative pressure. These solutions were stored at 4°C.

Preparation of inoculum. A cell suspension from each Neisseria isolate was prepared by removing 4 to 5 loopfuls of cells with a 2-mm loop and suspending them in 3.0 ml of the basal medium to produce a turbid cell suspension. After gently mixing, 0.5 ml of the suspension was inoculated with a needle (26 gauge, ½ inches [ca. 0.96 cm]) and syringe into each of...
five sterile, glass serum vials (16 by 53 mm; Kimble, Neutraglas, Toledo, Ohio) fitted with red rubber serum stoppers. A 0.5-ml amount of the appropriate carbohydrate solution was then added to each of the vials. A light film of vacuum grease was applied to the top of each stopper to impede any diffusion of metabolized CO₂. The inoculated vials were incubated on a rotary orbital shaker (Lab-Line Instruments, Inc., Melrose-Park, Ill.) adjusted to 125 rpm for various periods at 35°C.

**Ba(OH)₂** indicator system. A saturated Ba(OH)₂ solution was prepared with double-distilled water and sterile filtered through a 0.45-μm filter. A 1-ml amount of the Ba(OH)₂ was inoculated into each of five evacuated glass tubes (103 by 10.25 mm; Vacutainer brand, B-D, Rutherford, N.J.) fitted with rubber stoppers. These tubes were each labeled as to the isolate number and carbohydrate that was to be tested.

The collection needle at one end of the shutoff valve was inserted into the culture vial. Subsequently, the delivery needle at the other end of the valve was introduced into the vacutainer tube containing the Ba(OH)₂. Figure 1 shows the components of the system described above. The collection needle was removed from the vacutainer after a few seconds, when the diffusion of CO₂ was complete.

The reaction was allowed to continue to completion by placing the tubes on an aliquot mixer (Ames Co., Elkhart, Ind.) for 15 min. The optical density (OD) of the BaCO₃ precipitate was read at 650 nm in a spectrophotometer (Spectronic 10, Bausch & Lomb, Inc., Rochester, N.Y.). Controls consisted of inoculated carbohydrate-free basal medium and uninoculated basal medium containing the test carbohydrates.

**RESULTS**

Carbon dioxide production as a function of time. The amount of CO₂ produced by the six *Neisseria* species employed in this investigation for each of the five carbohydrates in basal medium and the basal medium without the carbohydrates was determined spectrophotometrically for BaCO₃ formation.

Carbon dioxide formation from the dextrose-containing vials with *N. gonorrhoeae* and *N. meningitidis* generally increased in a linear manner at each 0.5-h incubation period that was examined. The OD values for maltose fermentation increased at each examination period only for *N. meningitidis*. OD values from maltose fermentation with *N. gonorrhoeae* were obtained. However, the latter OD values were always lower than the OD values obtained for dextrose fermentation by either *N. gonorrhoeae* or *N. meningitidis*. The OD from the vials containing sucrose, lactose, or levulose yielded values for both *Neisseria* species similar to those obtained for *N. gonorrhoeae* for maltose. The inoculated controls of basal medium yielded BaCO₃ with both of these species of *Neisseria*. The OD values in these instances were again similar to those obtained with *N. gonorrhoeae* when maltose was employed as the test carbohydrate in the basal medium. Control uninoculated basal medium containing the test carbohydrates gave OD values close to 0. These results are shown in Fig. 2. Analysis of the basal medium did not reveal the presence of dextrose.

To avoid the OD values due to the background fermentation of the basal medium, an OD breakpoint of 0.4 for BaCO₃ formation at 2.5 h was arbitrarily chosen to indicate any specific carbohydrate utilization. The carbohydrate utilization responses of the six *Neisseria* species examined were in agreement with established
Practical identification of the Neisseria species depends upon the carbohydrate utilization patterns developed by growth (1) or acid production (2) in the inoculated basal medium (6). The visible color phenomenon is due to the presence of dextrose, sucrose, lactose, maltose, and levulose. The visible color change in the medium is a function of the concentration of carbohydrate. A small amount of Neisseria gonorrhoeae may only affect the pH indicator with the commonly employed color indicator used in this study (6). Furthermore, the value of the inoculated basal medium was always below the breakpoint value and did not obscure the delineation of the specific carbohydrate patterns. These data are shown in Table 1.

**Table 1. Comparison of CO₂ production from various carbohydrates and basal medium by six Neisseria species, using the Ba(OH)₂ indicator system**

<table>
<thead>
<tr>
<th>Organism</th>
<th>No. of strains tested</th>
<th>Basal Medium</th>
<th>Dextrose</th>
<th>Maltose</th>
<th>Lactose</th>
<th>Sucrose</th>
<th>Levlulose</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td>Range</td>
<td>Mean</td>
<td>Range</td>
<td>Mean</td>
<td>Range</td>
<td>Mean</td>
</tr>
<tr>
<td>N. gonorrhoeae</td>
<td>65</td>
<td>0.087-0.129</td>
<td>0.102*</td>
<td>0.345-0.470</td>
<td>0.455</td>
<td>0.134-0.162</td>
<td>0.148</td>
</tr>
<tr>
<td>N. meningitidis</td>
<td>75</td>
<td>0.248-0.283</td>
<td>0.260</td>
<td>0.416-0.426</td>
<td>0.420</td>
<td>0.420-0.450</td>
<td>0.435</td>
</tr>
<tr>
<td>N. lactamica</td>
<td>5</td>
<td>0.111-0.117</td>
<td>0.114</td>
<td>0.456-0.545</td>
<td>0.498</td>
<td>0.470-0.490</td>
<td>0.483</td>
</tr>
<tr>
<td>N. sicca</td>
<td>3</td>
<td>0.141-0.153</td>
<td>0.147</td>
<td>0.509-0.523</td>
<td>0.514</td>
<td>0.428-0.480</td>
<td>0.460</td>
</tr>
<tr>
<td>N. subflava</td>
<td>2</td>
<td>0.182-0.185</td>
<td>0.183</td>
<td>0.487-0.500</td>
<td>0.492</td>
<td>0.422-0.439</td>
<td>0.433</td>
</tr>
<tr>
<td>N. flavescens</td>
<td>2</td>
<td>0.103-0.119</td>
<td>0.109</td>
<td>0.126-0.134</td>
<td>0.131</td>
<td>0.165-0.180</td>
<td>0.174</td>
</tr>
<tr>
<td>None</td>
<td></td>
<td>0.018-0.022</td>
<td>0.020</td>
<td>0.026-0.031</td>
<td>0.027</td>
<td>0.031-0.032</td>
<td>0.032</td>
</tr>
</tbody>
</table>

* Values are OD units obtained turbidometrically at 650 nm.
* Values are uncorrected for the background values of basal medium.
enzymes can degrade peptones and produce alkaline products that tend to neutralize the acid formed and cause reversion to an alkaline pH (4).

The evolution of $^{14}\text{CO}_2$ from radioactively labeled carbohydrates is established as an index of microbial metabolism (5, 7). Presently, this procedure of radiorespirometry is being employed as a means of detecting microbial life on Mars and other planets (13, 14). A radiometric system is presently available for the detection of bacteremia from clinical specimens (5, 21).

Recently, this system was shown to be applicable for the differentiation and confirmation of Neisseria isolates with speed and accuracy. The only noted disadvantage of this system was related to cost (T. Foley and R. Broman, Abstr. Annu. Meet. Am. Soc. Microbiol. 1975, C27, p. 31; S. Rapacz and J. Kasper, Abstr. Annu. Meet. Am. Soc. Microbiol. 1976, C76, p. 38).

Ba(OH)$_2$ has long been known to absorb CO$_2$ rapidly and to readily form the white precipitate BaCO$_3$ (3, 17, 19). Recently, a Ba(OH)$_2$ indicator system was applied for the determination of CO$_2$ evolution as a means of examining the biodegradability by microorganisms (8) and the dextrose fermentation products of various bacteria (25).

The present investigation is the first to establish a Ba(OH)$_2$ indicator system as a means of elucidating the carbohydrate utilization patterns of clinically isolated Neisseria species. The Ba(OH)$_2$ indicator system appears to be clearly as effective as that described in the reported data concerning the radiometric system and those systems employing the phenol red indicator. The present system permitted an effective and rapid means for differentiating all of the Neisseria isolates employed in this investigation.

Contamination of commercial carbohydrates with other fermentable carbohydrates (1) could have been the factor that yielded the background effect in the basal medium. However, other fermentable factors may be present to augment the background. As our data indicate, however, the relatively smaller amount of CO$_2$ production formed from the basal medium alone did not hinder the system in clearly presenting the correct carbohydrate utilization patterns of the Neisseria species examined in this investigation.

The suspending basal medium did not contain NaCl since an inhibitory effect of this salt on gonococci was previously reported (12). The incorporation of CaCl$_2$ in the formulation of the basal medium was made due to its growth potential for gonococci (Leonard J. LaScolea, Jr., personal communication). Starch was incorporated to adsorb any possible toxic components present in the basal medium (9).

The principle of both the Ba(OH)$_2$ indicator system and the radiometric system is similar in that they both indicate the release of CO$_2$ resulting from the metabolism of fermentable carbohydrate. The Ba(OH)$_2$ indicator system, however, does not require a radioactive detector, radioactively labeled carbohydrates, or a pH indicator. The methods described herein are uncomplicated and can be performed in most clinical bacteriology laboratories. Only a minimal amount of equipment is required for the technique, and this equipment is usually available within hospital facilities. The Ba(OH)$_2$ indicator system clearly fulfills the diagnostic need for a reliable and rapid carbohydrate utilization test for the differentiation of the Neisseria species.

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LITERATURE CITED