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

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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.

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 ^{14}CO$_2$ metabolized by *Neisseria* isolates from ^{14}C-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 iso-

lated clinical cultures of various *Neisseria* species were obtained from this laboratory.

When the stock cultures were required for use, they were thawed at room temperature and inoculated on chocolate agar. All primary isolates grown on Thayer-Martin medium were subcultured onto chocolate agar plates; this was considered necessary since the antibiotics in Thayer-Martin medium can influence the fermentation patterns of the bacteria (10). The inoculated chocolate agar plates were then incubated for 18 to 20 h in a CO$_2$ incubator (6% CO$_2$) at 35°C.

Organisms were identified as *Neisseria* by typical colonial morphology, the Kovacs oxidase test, and Gram stain characteristics. The radiometric system employing the BACTEC unit, model 301 (Johnston Laboratories, Inc., Cockeysville, Md.), was used to primarily establish the carbohydrate utilization of the *Neisseria* isolates in accordance with the instructions of the manufacturer.

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, ^{3}/$_8$ inch [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 CO_2. The inoculated vials were incubated on a rotory 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 CO_2 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 BaCO$_3$ 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 CO_2 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 BaCO$_3$ 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 BaCO$_3$ with both of these species
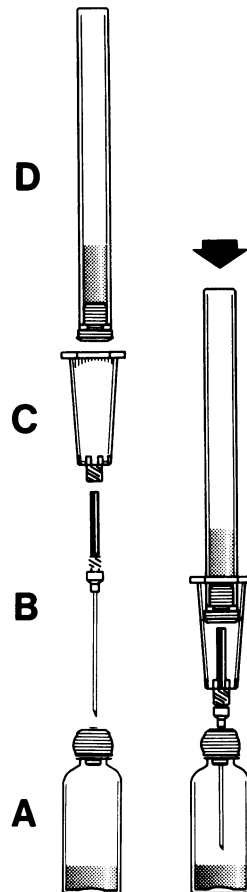

FIG. 1. *Components of the Ba(OH)$_2$ indicator system. (A) Rubber-stoppered glass serum vial containing medium and inoculum; (B) collection needle with shutoff valve; (C) vacutainer holder; (D) vacutainer containing Ba(OH)$_2$. The diagram on the right shows the needle in the vial. The vacutainer is then pushed downward (arrow), permitting CO_2 to pass into the vacutainer.*

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 BaCO$_3$ 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
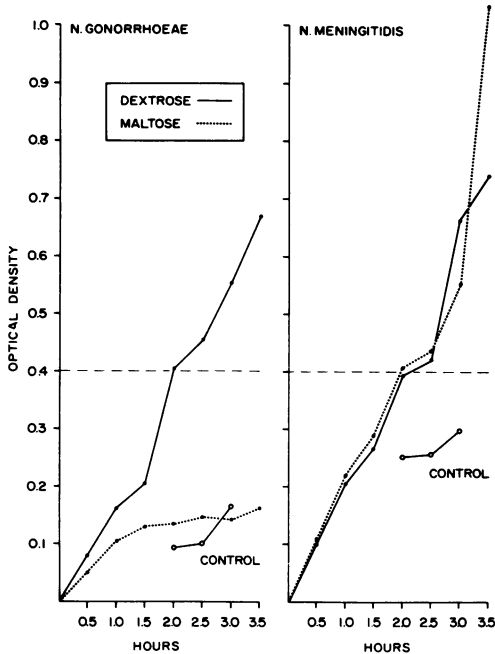
FIG. 2. *Analysis of carbon dioxide production, using the* $Ba(OH)_2$ *indicator system, as a function of time. The threshold value of 0.4 OD denotes the arbitrary minimal value indicating specific carbohydrate utilization. The controls consisted of inoculated basal medium without added carbohydrate.*

patterns of these *Neisseria*. At 2.5 h of incubation, all positive carbohydrate utilizations were observed to be over an OD of 0.4, whereas negative carbohydrate utilization responses were below this breakpoint value. Background OD values of the inoculated basal medium were always below the breakpoint value and did not obscure the delineation of the specific carbohydrate patterns. These data are shown in Table 1.

DISCUSSION

Practical identification of the *Neisseria* species depends upon the carbohydrate utilization patterns (2) developed by growth (11) or acid production (1) in the presence of dextrose, maltose, lactose, sucrose, and levulose. The visibility of the color reaction of these generally employed fermentation tests is influenced by the pH indicator used (1, 11), the buffering properties of the fermentation medium (1), inoculum size (6, 11), and contamination of maltose with a small amount of dextrose (1, 11). Some strains of *N. gonorrhoeae* may only effect weak or no visible color changes with the commonly employed phenol red indicator (1). Furthermore, during growth of *Neisseria*, certain intrinsic

TABLE 1. *Comparison of* CO_2 *production from various carbohydrates and basal medium by six Neisseria species, using the* $Ba(OH)_2$ *indicator system*

Organism	No. of strains tested	Basal Medium		Dextrose		Maltose		Lactose		Sucrose		Levulose	
		Range	Mean	Range	Mean	Range	Mean	Range	Mean	Range	Mean	Range	Mean
N. gonorrhoeae	65	0.087–0.129[b]	0.102[b]	0.435–0.470	0.455	0.134–0.162	0.148	0.172–0.202	0.183	0.125–0.143	0.133	0.122–0.126	0.124
N. meningitidis	75	0.248–0.283	0.260	0.416–0.426	0.420	0.420–0.450	0.435	0.267–0.286	0.278	0.248–0.254	0.250	0.252–0.269	0.260
N. lactamica	5	0.111–0.172	0.148	0.456–0.545	0.498	0.470–0.490	0.483	0.435–0.455	0.446	0.153–0.172	0.162	0.152–0.153	0.152
N. sicca	3	0.141–0.153	0.147	0.509–0.523	0.514	0.428–0.480	0.460	0.139–0.154	0.146	0.465–0.481	0.471	0.452–0.460	0.457
N. subflava	2	0.182–0.185	0.183	0.487–0.500	0.492	0.432–0.439	0.433	0.166–0.169	0.168	0.413–0.429	0.423	0.441–0.465	0.451
N. flavescens	2	0.103–0.119	0.109	0.126–0.134	0.131	0.165–0.180	0.174	0.120–0.123	0.121	0.142–0.156	0.148	0.190–0.200	0.195
None				0.018–0.022	0.020	0.026–0.031	0.027	0.031–0.032	0.032	0.024–0.026	0.025	0.031–0.032	0.031

[a] Values are OD units obtained turbidometrically at 650 nm.
[b] 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 $^{14}CO_2$ 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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