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Carbohydrate Fermentation Patterns of *Neisseria meningitidis* Determined by a Microtiter Method

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A carbohydrate fermentation technique has been developed and compared to the standard fermentation test with cystine-Trypticase-semisolid agar for the identification of *Neisseria meningitidis*. This new method utilizes Mueller-Hinton broth as a basal substrate and is performed with microtiter methods. By using Mueller-Hinton broth with and without the addition of antibiotics, the method can be adjusted to test the fermentation patterns of all of the *Neisseria* including *N. gonorrhoeae*.

The need for a rapid, more reliable carbohydrate fermentation test for the genus *Neisseria* has been indicated by several workers (1, 2, 6–9). Fermentation tests for *Neisseria* are complicated by certain characteristics of these aerobic bacteria. A soft-agar basal medium, such as cystine-Trypticase-agar (CTA) with carbohydrates added, is now favored for this test. Strains which metabolize glucose do so primarily by an oxidative pathway rather than by fermentation; consequently, the production of acid may be rather slight. During the growth of *Neisseria*, other enzymes degrade peptone and produce alkaline products which tend to neutralize the acid and may cause reversion to an alkaline *p*H. Therefore, many positive fermentations will appear to be negative (2). Hajek et al. (6) showed that it is possible to obtain a different set of biochemical data by using different basal medium. In some instances, they observed a variation in the fermentation of a specific carbohydrate in the same basal medium by a given culture. White and Kellogg (9) showed that some strains of *N. gonorrhoeae* fail to grow or grow poorly, whereas others fail to ferment carbohydrates with the CTA fermentation method. Beno et al. (1) demonstrated the inadequacy of the CTA method for determining fermentation patterns of *N. meningitidis* and found Mueller-Hinton (MH) broth to be a more reliable substrate. For these reasons, together with the fact that CTA may require at least 48 hr of incubation before the reactions are complete, our laboratory has developed a sensitive, rapid, and reliable microtiter method with MH broth for determining the fermentation patterns of the genus *Neisseria*.

MATERIALS AND METHODS

Bacterial cultures. Strains of *N. meningitidis* were isolated from nasopharyngeal cultures obtained from Marine Corps trainees at Camp Stone Bay and Camp Geiger, Camp Lejeune, N.C. The isolation and identification techniques used are basically those of Devine et al. (5), which were recently adopted by this laboratory for the definitive identification of *N. meningitidis*. The scheme utilizes the following identification criteria: (i) typical colony morphology on Thayer-Martin or MH agar or both, (ii) positive oxidase test, (iii) failure to grow on nutrient agar (as distinguished from 1.5% nutrient agar, which has NaCl added), and (iv) agglutination in group-specific antisera by using the slide agglutination method. All group-specific rabbit antisera were prepared in this laboratory.

Lyophilized cultures of *N. flava*, *N. catarrhalis*, *N. subflava*, *N. sicca*, and *N. flavescens* were obtained from the Neisseria Repository, Naval Medical Research Unit No. 1, Naval Supply Center, Oakland, Calif. *N. gonorrhoeae* cultures were obtained from M. C. Shepard of the Naval Medical Field Research Laboratory, Camp Lejeune, N.C. A total of 163 of the *N. meningitidis* isolates and the above *Neisseria* species were grown for testing in MH broth, both with and without antibiotics added. The MH broth with antibiotics contained 5 μg of ristocetin and 12.5 units of polymyxin B per ml (half of the strength used for preparing Thayer-Martin agar, used for initial isolation). Of the *N. meningitidis* isolates 306 were grown for testing on MH agar plates without antibiotics. All cultures were incubated at 37 C in an atmosphere of 8% CO_2 before fermentation testing.

Fermentation reagents. Tubes (13 by 100 mm) containing 3 ml of Cystine Trypticase Agar (BBL) with 1% carbohydrate (reference 3; dextrose, maltose, sucrose, lactose, fructose, and mannitol) were prepared and sterilized by autoclaving for 15 min at 188 C (12 lb of pressure).

Carbohydrate solutions (4%) for the microtiter technique were prepared in distilled water sterilized under the same conditions as above, and stored at 4 C.

MH broth containing 0.02% phenol red was prepared and sterilized by autoclaving for 15 min at 121 C (15 lb of pressure). When used, antibiotics were added to the sterile broth aseptically.

Disposable "U" microtiter plates were sterilized by exposure to ultraviolet irradiation for a minimum of 12 hr.

Fermentation tests. Fermentation patterns with CTA were determined by inoculating one loopful of organisms from those grown in MH broth (or organisms picked from a single colony from those cultures on MH agar plates) onto the surface of the CTA-carbohydrate-semisolid agar. The surface of the media was massaged with the inoculating loops so that some of the inoculum was placed subsurface. The inoculated CTA tubes were incubated at 37 C and were read at 24 hr and again at 48 hr. "KAP-UTS" plastic tube closures (Bellco) were used on all CTA and broth tubes.

The microtiter fermentation test for broth cultures was carried out as follows. (i) Two drops of MH broth (with or without antibiotics) containing phenol red were added to six wells across a microtiter plate. (ii) One drop of the appropriate carbohydrate solution was added per well. (iii) One drop of an 18- to 24-hr MH broth culture was then added as inoculum to all six wells. (iv) The plates were sealed with microtiter pressure-stick adhesive covers and incubated for 18 to 24 hr at 37 C. CO_2 incubation could not be used as an acid reaction would occur in all wells.

The fermentation test for solid substrate cultures was carried out as follows. (i) Sufficient growth was removed from the MH subculture plates (pure cultures incubated for 18 to 24 hr) to give a discernible cloud when swirled in a 1-dram vial containing 2 ml of sterile antibiotic containing MH broth with phenol red. (ii) Three drops of the then inoculated broth were placed in each of six wells across a microtiter plate. (iii) Carbohydrate solutions were added as in the broth method. (iv) The plates were sealed and incubated as previously described.

Interpretation. The determinations were read in the conventional manner of *p*H-dependent colorimetric tests. The development of a yellow color indicated an acid reaction and fermentative utilization of the specific carbohydrate, whereas no change in color or a deepening of the color indicated the absence of fermentation. Only rarely was a borderline reaction encountered, and in every case it was due to a slow fermenter and was noted in both the CTA and microtiter determination.

Fermentation of only dextrose and maltose of the six carbohydrates used was considered as the typical *N. meningitidis* fermentation pattern. If only dextrose was fermented, slide agglutination tests were performed for specific differentiation of aberrant strains of *N. meningitidis* from *N. gonorrhoeae*.

TABLE 1. *Comparison of typical fermentation patterns in the genus Neisseria with the cystine-Trypticase-agar (CTA) method and the microtiter plate technique with broth-grown inocula*

Strain *N. meningitidis*	Microtiter plate method		CTA method
	Mueller-Hinton broth with antibiotics	Mueller-Hinton broth without antibiotics	
Group B	36/36[a]	26/26	32/36
Group C	35/36	28/28	36/36
Group Y	30/31	25/26	29/31
Other[b]	60/60	48/48	55/60
Total *N. meningitidis*	161/163	127/128	152/163
N. flava	0/1	1/1	1/1
N. catarrhalis	0/1	1/1	1/1
N. subflava	0/1	1/1	1/1
N. sicca	0/1	1/1	1/1
N. flavescens	0/1	1/1	1/1
N. gonorrhoeae	1/6	4/5	2/5

[a] Number of typical fermentation patterns/total number of isolates tested.

[b] Consisted of 29-E, W-135, X, 737 (nongroupable strain, supplied by NAMRU-4, Great Lakes, Ill.), RAS′ (5), RAS-10 (4), Lac 25 (7, 8), autoagglutinating, and nongroupable strains.

TABLE 2. *Comparison of typical fermentation patterns in the genus Neisseria with the cystine-Trypticase-agar (CTA) method and the microtiter plate Technique with solid substrate-grown inocula*

Strain *N. meningitidis*	Microtiter plate method	CTA method
Group B	112/112[a]	97/112
Group C	71/72	65/72
Group Y	92/97	81/97
Other[b]	25/25	24/25
Total	300/306	267/306

[a] Number of typical fermentation patterns/total number of isolates tested.

[b] Consisted of 29-E, W-135, X, 737 (nongroupable strain, supplied by NAMRU-4, Great Lakes, Ill.), RAS′ (5), RAS-10 (4), Lac 25 (7, 8), autoagglutinating, and nongroupable strains.

RESULTS

Broth cultures. Table 1 shows that the microtiter plate fermentation method with MH broth-grown cultures (with or without antibiotics) gives a significantly (P = 97.5) higher rate of positive fermentation patterns for *N. meningitidis* than the CTA method. The microtiter method (with antibiotics) revealed two isolates which did not ferment the sugars in a typical manner. Without antibiotics added to the microtiter media, only one isolate failed to show a typical fermentation pattern. In contrast, the CTA method yielded 11 isolates which gave an atypical fermentation pattern. Seven of these failed to ferment either dextrose or maltose, two did not ferment any sugar, and two fermented other sugars in addition to

dextrose and maltose. MH broth with antibiotics was unsatisfactory for determining fermentation patterns in all species other than *N. meningitidis* (Table 1). No difference was noted between the microtiter method (without antibiotics) and the CTA method regarding the nonmeningitidis strains, except for *N. gonorrhoeae* with which the microtiter plate method may be superior.

Solid substrate cultures. Table 2 shows that only *N. meningitidis* isolates were tested from MH agar plates. The microtiter technique again gave a significantly (P = 97.5) higher rate of positive fermentation patterns than that employing CTA tubes. Of the six strains which did not conform to a typical fermentation pattern by the microtiter technique, four fermented no carbohydrate, whereas two fermented carbohydrates in addition to dextrose and maltose. The CTA method demonstrated atypical fermentation patterns for 39 strains of *N. meningitidis*. Of these, 19 failed to show fermentation of any carbohydrate, 7 failed to ferment either dextrose or maltose, and 13 fermented sugars in addition to dextrose and maltose.

Finally, to test the reliability of the microtiter plate technique, repeat tests were performed with 519 isolates. We tested 270 twice, whereas 249 were tested three times. The interval between testing ranged from 1 to 36 days. Of the 768 retests, 16 or 2.1% failed to duplicate the original results. In all instances, a contaminate was found to be responsible.

DISCUSSION

A carbohydrate fermentation technique has been developed and compared to the standard fermentation test utilizing cystine-Trypticase-semisolid agar for the genus *Neisseria*. Technique variations have been presented to accommodate identification procedures in which the organisms are carried in broth or on solid agar substrate.

The microtiter plate method was found to be superior to the more standard CTA technique in several respects. The first and most important of these is the significantly (P = 97.5) higher rate of typical carbohydrate fermentation patterns demonstrated by the microtiter technique. This permits more isolates to be congruously identified both serologically and biochemically. In addition, the results of the microtiter method are available in 18 to 24 hr, whereas 48 hr is often necessary for conclusive readings with the CTA medium. Since developing this test, an additional 1,053 groupable strains of *N. meningitidis* have been tested with 1,029 showing typical fermentation patterns. Six isolates of the proposed strain *N. lactamicus* that were originally identified as *N. meningitidis* were also tested and differentiated with the microtiter technique. These isolates cross-reacted in our group B or C antisera as also reported by others (7, 8).

By utilizing MH broth with and without the addition of antibiotics, the method can be adjusted to test the fermentation patterns of all of the *Neisseria* including *N. gonorrhoeae*.

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