

Carbohydrate Fermentation Plate Medium for Confirmation of *Neisseria* Species

YVONNE C. FAUR,* MARTIN H. WEISBURD, AND MARION E. WILSON

Public Health Laboratory Services, The City of New York, Department of Health, New York, New York 10016

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A carbohydrate fermentation plate medium is described for rapid and reliable confirmation of *Neisseria gonorrhoeae*, *N. meningitidis*, and other *Neisseria* species. The medium is based on a modification of NYC (U.S. Patent 3,846,241) medium, originally designed for the isolation of pathogenic *Neisseria* (3). A total of 715 clinical isolates were tested for their carbohydrate fermentation reactions on the medium, in parallel with cystine-Trypticase agar medium. In 82% of the strains tested, results were in agreement on both media and 18% gave conflicting results. The NYC modification provides rapid and accurate fermentation patterns for use in routine confirmation procedures for *Neisseria* species.

The confirmation of *Neisseria gonorrhoeae* and *N. meningitidis* in most clinical laboratories is made by sugar fermentation reactions. Numerous fermentation media have been developed for the identification of *Neisseria* species (1, 2, 4, 5, 6, 7, 9, 11, 12). Several of these medium formulations require the addition of carefully prepared, complex supplements, such as cocarboxylase, many of which are heat labile and have a limited shelf life. Other modifications require the addition of serum or other biological fluids which may interfere with pH indicator reactions.

An effective fermentation medium must support the growth of highly fastidious strains of *N. gonorrhoeae*, and *N. meningitidis*, produce a clear-cut, rapid color change upon fermentation of the carbohydrate, and yield adequate growth of tested strains when only a small inoculum is available. A semi-solid cystine-Trypticase agar base (CTA) to which carbohydrates are added is widely used but often gives equivocal results because of inadequate growth of fastidious strains of pathogenic *Neisseria*. It requires a heavy inoculum and prolonged incubation which may lead to nonspecific changes in the pH indicator.

In addition to its own volume of *Neisseria* isolates requiring full speciation, our laboratory receives a large number of isolated strains of suspected *N. gonorrhoeae* and *N. meningitidis* referred for confirmation from other laboratories experiencing difficulty with fermentation patterns. This problem, coupled with the inadequacy of CTA medium in our own hands, prompted us to investigate modification of NYC medium, originally formulated for the isolation

of pathogenic *Neisseria* (3), for use in identifying *Neisseria* isolates by their carbohydrate fermentation reactions.

MATERIALS AND METHODS

Medium formulation. The basal medium was prepared as previously described for NYC medium (3).

Basal medium. The formulation, per liter of base, was as follows. Solution 1 contained agar (Difco) (20 g) and double distilled water (400 ml). It was held in an autoclave (or Arnold) under flowing steam until the agar was completely dissolved. Solution 2 contained corn starch (U.S. Pharmacopeia) (1 g) and double distilled water (40 ml). It was thoroughly mixed on a magnetic stirring apparatus, then held in an autoclave (or Arnold) under flowing steam until it was homogeneous. Solution 3 contained: proteose peptone no. 3 (Difco), 15 g; dipotassium phosphate, 4 g; monopotassium phosphate, 1 g; sodium chloride, 5 g; and double distilled water, 200 ml. It was brought to a boil on a heated magnetic stirrer. To complete the base solutions 1, 2, and 3 were combined and thoroughly mixed on a heated magnetic stirrer. The completed base was then autoclaved at 15 lb/in² for 15 min at 121 C. After cooling it was stored at 4 C.

Complete medium. Complete fermentation medium was prepared as follows (all quantities are for 1 liter of medium): NYC basal medium, 640 ml; 0.5% hemolysed erythrocytes, 90 ml; yeast dialysate, 50 ml; 0.2% phenol red solution, 20 ml; and 10% carbohydrate solution, 200 ml (dextrose, maltose, sucrose, or levulose). (The erythrocytes, yeast dialysate, phenol red solution, and carbohydrate solution were added aseptically to basal medium at 55 C.)

The final pH of the medium was adjusted to 7.1 ± 0.1 with 1 N NaOH. The medium was poured into petri plates (20 ml per plate), which were stored in plastic bags at 4 C. The plates can be maintained for at least 1 month under these conditions.

Preparation of supplements. (i) Yeast dialysate. Bakers' yeast (908 g) was carefully mixed to a smooth paste in 2.5 liters of double distilled water, autoclaved at 5 lb/in² for 10 min, and then dialyzed against 2 liters of double distilled water for 48 h at 4 C. The dialysate was autoclaved at 15 lb/in² for 15 min at 121 C and stored at -70 C.

(ii) Hemolysed erythrocytes. Fresh, packed horse erythrocytes were lysed in sterile double distilled water.

(iii) Carbohydrate solutions. The carbohydrate solutions were sterilized by membrane filtration (0.22 μ m; Millipore Corp.) under negative pressure.

Other media. Prepared, tubed CTA with carbohydrates was purchased from Baltimore Biological Laboratories, Inc.

The *O*-nitrophenyl- β -D-galactopyranoside medium for the β -D-galactosidase test was prepared according to the formulation of Lowe (8).

Neisseria strains. (i) Stock strains. NYC fermentation media were first tested with the following strains from our collection: 10 strains of *N. meningitidis*; 10 strains of *N. gonorrhoeae*, 6 strains of *N. lactamica*, and 6 strains of *N. pharyngis*.

(ii) Clinical strains. Strains of *Neisseria* were recovered from urogenital and pharyngeal specimens obtained from patients routinely screened for gonorrhea, or were received as isolated organisms recovered from various sources for identification and speciation in our bacteriology reference laboratory.

Organisms were identified as *Neisseria* by colonial morphology, Kovacs' oxidase test, and microscopy (Gram stain). In addition, all strains were inoculated onto Trypticase soy agar at room temperature and at 35 C in a candle extinction jar for 48 h. Colonies were subcultured for 20 h on NYC isolation medium and then were transferred to plates with NYC fermentation media and to CTA tubes. CTA tubes were inoculated as recommended by the manufacturer. A loopful of culture was inoculated onto the surface of the CTA medium without going into the depth of the tube. Up to six strains were inoculated on each NYC fermentation plate by rubbing each with the loop in a confluent circular zone of approximately 12 mm in diameter. Each tested organism was also inoculated onto a plate of NYC isolation medium without antibiotics as a control on its viability. NYC fermentation plates and CTA tubes with loosened screw caps were incubated for 20 h in a candle extinction jar at 35 C.

Positive fermentation reactions on NYC fermentation plates present a very distinct yellow zone around and beneath the area of growth. Negative reactions usually produce a dark red zone of alkalinity surrounding the colony. These reactions can be read and reported after 18 to 20 h of incubation.

CTA media with clear-cut reactions were also read after 20 h, but tubes with doubtful or negative reactions were incubated for another 24 h.

All strains presenting a fermentation pattern characteristic for *N. meningitidis* or *N. gonorrhoeae* on NYC media were further tested serologically, with meningococcal antisera or immunofluorescent gonococcal antiserum, respectively, by using colonies from the 20-h subculture on NYC isolation medium which

had served as the common inoculum for both sets of fermentation media. Sera were obtained from the Center for Disease Control, Atlanta, Ga. Stock cultures were used to control each lot of antiserum and each group of strains tested. A number of strains of *N. meningitidis* were also confirmed serologically by H. A. Feldman, Syracuse, N.Y.

RESULTS

Stock strains of *N. meningitidis*, *N. gonorrhoeae*, *N. lactamica*, and *N. pharyngis* gave characteristic reactions within 20 h of incubation on NYC fermentation media. The fermentation reactions of 715 *Neisseria* isolates of different sources (Table 1), studied simultaneously on NYC fermentation media and on CTA media, are presented in Table 2.

Parallel results were obtained with 586 (82%) of *Neisseria* strains tested, but with 129 (18%) strains the results were conflicting. Using serologic testing as a final criterion for identification of *N. meningitidis* and *N. gonorrhoeae*, all strains giving parallel typical fermentation reactions for either of these organisms on both media were confirmed. Among those giving conflicting fermentation results, serologic tests confirmed all identifications of meningococci and gonococci, as indicated by carbohydrate reactions on NYC fermentation media. All other strains with fermentation patterns different from pathogenic *Neisseria* were further confirmed by inhibited growth on the selective medium, growth on simple nutrient agar at 35 and 22 C, colonial morphology, and in some cases pigmentation.

Of the 129 conflicting strains, 96 gave the characteristic pattern of *N. meningitidis* on NYC fermentation media and were further confirmed serologically, but none of these would have been identified as *N. meningitidis* on CTA media. On CTA media, 44 of these strains gave negative reactions to all the sugars (all negative tubes were held for a total of 48 h), whereas eight fermented all three carbohydrates. Forty-one of these strains were positive on CTA dextrose only and might therefore have been

TABLE 1. *Clinical sources of Neisseria isolates*

Source of specimen	Number
Uro-genital	226
Pharyngeal	460
Blood	10
Sputum	9
CSF ^a	5
Synovial fluid	3
Eye	2

^a CSF, Cerebral-spinal fluid.

TABLE 2. Reactions of *Neisseria* isolates on NYC and CTA fermentation media

Results	NYC			CTA			Total	Percent
	Dextrose	Maltose	Sucrose	Dextrose	Maltose	Sucrose		
Parallel	+	+	-	+	+	-	253	35.40
	+	-	-	+	-	-	293	40.98
	-	-	-	-	-	-	31	4.33
	+	+	+	+	+	+	9	1.26
Conflicting	+	+	-	-	-	-	44	6.15
	+	+	-	+	+	+	8	1.11
	+	+	-	+	-	-	41	5.73
	+	+	-	-	+	-	3	0.42
	+	-	-	-	-	-	25	3.50
	+	-	-	+	-	+	1	0.14
	+	+	+	-	-	-	3	0.42
	+	+	+	-	+	+	4	0.56

erroneously speciated as gonococci, if serologic confirmation was not available. Three strains that fermented CTA maltose, but did not ferment CTA dextrose, could not have been speciated according to these reactions.

In the same group of 129 strains giving conflicting results, 25 of 26 strains which fermented dextrose only on NYC fermentation media, and were confirmed as *N. gonorrhoeae* by the immunofluorescent test, did not ferment dextrose or the other sugars on CTA media, whereas one fermented both dextrose and sucrose.

The remaining seven strains gave clear-cut reactions for *N. pharyngis* on NYC medium, but on CTA medium three were completely negative, whereas four fermented maltose and sucrose and therefore could not be classified as any known *Neisseria* species.

DISCUSSION

NYC fermentation media have been developed to fill our increasing need for accurate speciation of *Neisseria* by biochemical tests. They have been in routine use in our laboratory for the past 2 years, giving results which have been confirmed by serology.

The major changes in the formulation of the medium as prepared for isolation of pathogenic *Neisseria* include omission of horse plasma and antibiotics, reduction of the erythrocyte component, a doubling of the aliquot of yeast dialysate, and the addition of specific carbohydrate solutions together with a pH indicator. These modifications provide sensitivity to pH changes, while ensuring adequate growth of fastidious pathogenic *Neisseria*.

Neisseria organisms initially recovered on

NYC isolation medium need no adaptation period when transferred to NYC fermentation plate media, as the essential growth factors are the same in both media. The amounts of carbohydrates (2%) and indicator used, together with the pH of the media, have contributed to the clear-cut, easy-to-read reactions.

Many *Neisseria* strains were referred to us from other laboratories where they had been isolated on either chocolate agar or Thayer-Martin medium. Difficulties encountered in these laboratories in confirming identification with CTA media prompted the referrals, and our results corroborated the CTAs inconsistencies. It may also be noted that a few other laboratories that use Thayer-Martin or chocolate agar for isolation of *Neisseria* have run fermentation tests on NYC fermentation plates and have paralleled our findings. For these reasons, we believe the NYC fermentation media are reliable regardless of the isolation medium used.

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