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Adaptation of the Minitek System for the Rapid Identification of *Neisseria gonorrhoeae*

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A suspending medium was developed for use with the Minitek system for the confirmatory identification of *Neisseria gonorrhoeae, N. meningitidis,* and *N. lactamica* based upon the production of acid from various carbohydrates. The addition of sodium bicarbonate to the medium made negative reactions easier to read. More isolates of *N. gonorrhoeae* were identified with the suspending medium in the Minitek system than with cystine-Trypticase agar media. With a suitable inoculum size, a positive identification could be made in less than 1 h; most isolates (90.8%) could be identified within 4 h of inoculation. The Minitek system is reliable and easy to use.

Both *Neisseria meningitidis* (16) and *N. lactamica* (8, 13) can be isolated from the genitourinary tract on selective media commonly used for the isolation of *N. gonorrhoeae* (8, 10). *N. meningitidis* can usually be differentiated from *N. gonorrhoeae* by the production of acid from maltose (21). However, Kingsbury (16) reported that 80% of meningococcal isolates obtained from transformation experiments and resistant to greater than 10 μg of sulfadiazine per ml were maltose negative when tested by conventional procedures and, thus, could not be differentiated from *N. gonorrhoeae* on the basis of sugar reactions alone. *N. lactamica* can be differentiated from *N. meningitidis* and *N. gonorrhoeae* by the production of acid from lactose or by the hydrolysis of *o*-nitrophenyl-β-D-galactopyranoside (5). In addition, many isolates of *N. lactamica* will grow on nutrient agar at 37 C, whereas *N. meningitidis* and *N. gonorrhoeae* will not (10).

The reported incidence of gonococcal pharyngitis has increased in recent years. Its frequency varies with the particular population group studied (6, 24). Hollis et al. (10) observed that 2% of a general civilian population were pharyngeal carriers of *N. lactamica*, whereas 1.5% were pharyngeal carriers of *N. meningitidis*. In another study of 2,224 patients at a venereal disease clinic, Wiesner et al. (25) isolated *N. meningitidis* from the posterior pharynx of 17.2% of the patients, *N. lactamica* from 1.9%, and *N. gonorrhoeae* from 5.6%. Thus, it becomes increasingly important to be able to accurately differentiate *N. gonorrhoeae* from *N. meningitidis* and *N. lactamica*.

There are several methods currently used to detect the production of acid from carbohydrates by *Neisseria* (1, 7, 14, 22). A number of strains of *N. gonorrhoeae* have recently been encountered that either fail to produce acid from glucose or do so slowly when tested in cystine-Trypticase agar (CTA) medium (5, 23). The Minitek (BBL) system has recently been introduced for the identification of various bacteria. Although this system has been found particularly useful in identifying members of the *Enterobacteriaceae* (15), the medium supplied is unsatisfactory for determining the production of acid from various carbohydrates by *N. gonorrhoeae, N. meningitidis*, and *N. lactamica*. The purpose of this study was to develop a suspending medium that could be used with the Minitek system for the rapid differentiation of *N. gonorrhoeae* from *N. meningitidis* and *N. lactamica*.

MATERIALS AND METHODS

Organisms. Primary isolates of *N. gonorrhoeae* were obtained from the Venereal Disease Clinic of the Multnomah County Health Department, Portland, Ore. Cultures were approximately 48 h old when received and had been presumptively identified as *N. gonorrhoeae* by direct fluorescent-antibody staining reaction and a positive oxidase test. Other clinical isolates of *N. gonorrhoeae* were obtained from Abdel Rashad, Clinical Microbiology Laboratory of the University of Oregon Health Sciences Center. *N. gonorrhoeae* CS-7 has been described previously (18). Representative strains of *N. meningitidis*, with known levels of resistance to sulfadiazine, from serogroups *A, B, C, X, Y, Z, 135,* and *29E* were obtained from Herman Schneider, Walter Reed Army Institute of Research, Washington, D.C. Other strains of *N. meningitidis* were

obtained from the American Type Culture Collection (19). Strains of *N. lactamica* were obtained from H. Schneider, Walter Reed Army Institute of Research, and from D. Kellogg, Jr., Center for Disease Control, Atlanta, Ga. Cultures of *Neisseria* spp. were stored as previously described (18). The identity of all cultures was confirmed on the basis of appearance in Gram-stained smears, oxidase reaction, and the production of acid from specific carbohydrates.

Preparation of inoculum. One-half of a plate of gonococcal (GC) agar (Difco) containing a growth factor supplement identical in composition to IsoVitaleX enrichment (BBL) and 0.5% glucose was inoculated with the test organism and incubated overnight in a CO_2 incubator (5% CO_2) at 37 C. Inocula were prepared by removing several (2 to 3) loopfuls of cells with a 2-mm (ID) loop and suspending them in 0.5 ml of medium to produce a turbid suspension containing approximately 5×10^8 colony-forming units/ml. The suspending medium contained 1.5% (wt/vol) proteose peptone no. 3 (Difco) sterilized by autoclaving. Before use, filter-sterilized $NaHCO_3$ (4.2%, wt/vol) was freshly added. Unless otherwise mentioned, the final concentration of $NaHCO_3$ was 210 μg/ml. In some experiments, polypeptone (BBL) and Trypticase peptone (BBL) were substituted for the proteose peptone no. 3.

Minitek system. The Minitek system consists of a covered, rectangular, plastic plate containing 12 wells. The manufacturer also supplies single- or multiple-disk dispensers, a pipetter with sterile disposable tips, and a humidified chamber (humidor) for incubation. For use, the disks were dispensed into individual wells; three or four isolates were identified on a single Minitek plate. The disks employed in our study were dextrose-nitrate, dextrose, maltose, lactose, and *o*-nitrophenyl-β-D-galactopyranoside. As *Neisseria* are aerobic organisms, mineral oil was not used as an overlay.

Determination of acid production from carbohydrates. Minitek disks containing glucose, maltose, sucrose, lactose, fructose, and *o*-nitrophenyl-β-D-galactopyranoside and corresponding CTA medium (BBL) deeps containing 1% carbohydrate were inoculated with 1 drop of the inoculum suspension dispensed from the Minitek automatic pipetter. GC agar plates were simultaneously inoculated with 1 drop of inoculum, and Gram-stained smears were prepared to monitor the initial purity of the bacterial suspensions. In later studies, inoculation of CTA medium tubes and the preparation of smears were omitted. The inoculated CTA medium tubes and Minitek plates (placed in the Minitek humidor) were incubated at 37 C without additional CO_2 and read at hourly intervals. The GC agar plates were incubated overnight at 37 C in a CO_2 incubator (5% CO_2).

Determination of inoculum size. The Minitek automatic pipetter was calibrated with an aqueous solution of ^{14}C-labeled sodium bicarbonate (0.2 μCi/ml). Single drops dispensed by the pipetter were added to scintillation vials containing 1 ml of absolute ethanol. The vials were counted after the addition of 15 ml of scintillation fluid, which contained 0.4% 2,5-diphenyloxazole and 0.1% 1,4-bis-[2]-(5-phenyloxazolyl)benzene in toluene. Analysis of the data revealed that each drop delivered by the pipetter contained 49.66 $\pm$ 0.06 μl. Viable counts were determined as previously described (18).

Rapid fermentation procedure. The Brown modification (4) of the rapid fermentation procedure of Kellogg and Turner (14) was used for comparison. The procedure was as described by Brown (4), except that the concentration of phenol red in the buffered salt solution diluent was doubled (W. J. Brown, personal communication). The increased phenol red concentration made the interpretation of the reactions easier. Initially, we experienced difficulty with our source of maltose. During early incubation, the phenol red would indicate an acid reaction; continued incubation often resulted in a reversion to basic pH. Brown (4) has reported a similar finding and attributed it to the contamination of maltose with low concentrations of glucose. This problem was overcome when we obtained a sample of pure maltose from W. J. Brown, Center for Disease Control, Atlanta, Ga.

Chemicals and radioisotopes. The 2,5-diphenyloxazole and 1,4-bis-[2]-(5-phenyloxazolyl)benzene were products of Fisher Scientific Co. ^{14}C-labeled sodium bicarbonate (specific activity, 54.5 mCi/mmol) was obtained from International Chemical and Nuclear Corp. (Irvine, Calif.). All other reagents were of analytical grade.

RESULTS

Determination of optimal bicarbonate concentration. Sodium bicarbonate was added to the solution of proteose peptone no. 3 (1.5% wt/vol) to facilitate the differentiation of positive from negative reactions. The optimal $NaHCO_3$ concentration was determined by observing changes in the color of the phenol red indicator in the carbohydrate disks caused by *N. gonorrhoeae* CS-7. Table 1 shows the effects of various $NaHCO_3$ concentrations on the color changes in the indicator observed with glucose, maltose, and sucrose disks. The addition of $NaHCO_3$ decreased the time required to obtain a positive glucose reaction. At $NaHCO_3$ concentrations ranging from 26 to 105 μg/ml, the production of acid from glucose was ascertained by observing a change in the indicator color within 2 h after inoculation. However, a definite negative reaction with the maltose disk was delayed. The reading of a negative reaction was made easier by increasing the sodium bicarbonate concentration to 210 μg/ml. Therefore, this concentration was used in all subsequent experiments. Concentrations of $NaHCO_3$ greater than 420 μg/ml delayed or inhibited the appearance of a positive reaction. Substitution of polypeptone or Trypticase peptone for the proteose peptone no. 3 had no appreciable effect upon a positive glucose or maltose reac-

tion. However, the ability to discern a definite negative reaction was hampered in medium containing either polypeptone or Trypticase peptone.

Effect of cell concentration. Table 2 shows that a critical cell concentration was required to effect a positive reaction within 24 h. An increase in the cell concentration shortened this time. At very high cell densities (>5.0 × 10^9 colony-forming units/ml), positive reactions could be read within 30 min. The concentration of organisms contained in 2 to 3 loopfuls of cells suspended in 0.5 ml of medium was determined for several strains. The results (not shown) indicated that these suspensions contained 0.5 × 10^9 to 4.0 × 10^9 colony-forming units/ml. Therefore, with a sufficiently high concentration of cells, it should be possible to determine the carbohydrate reactions within 4 h. The effect of incubation time was examined on 196 clinical isolates of *N. gonorrhoeae*. The results (Table 3) showed that 90.8% of the isolates produced detectable acid from glucose within 4 h. All isolates were identified by 6 h. The 18 isolates that failed to give a positive reaction within 4 h also exhibited sparse growth on GC agar plates. Twelve of these isolates were later retested at a higher cell concentration. All

TABLE 1. *Effect of sodium bicarbonate concentration on Minitek reactions*[a]

Hour	Carbohydrate disk	Sodium bicarbonate concn (μg/ml)						
		420[b]	210	105	52.5	26.2	13.1	6.6
1	Glucose	R/O	R/O	R/O	R/O	R/O	R/O	R/O
	Maltose	R	R	R/O	R/O	R/O	R/O	R/O
	Sucrose	R	R	R	R	R	R	R
2	Glucose	O	O	Y/O	Y/O	Y/O	O	O
	Maltose	R	R	R/O	R/O	R/O	R/O	R/O
	Sucrose	R	R	R	R	R	R	R
3	Glucose	Y/O	Y/O	Y	Y	Y	Y/O	Y/O
	Maltose	R	R	R	R/O	R	R/O	R
	Sucrose	R	R	R	R	R	R	R
4	Glucose	Y	Y	Y	Y	Y	Y/O	Y/O
	Maltose	R	R	R	R	R	R	R
	Sucrose	R	R	R	R	R	R	R
5	Glucose	Y	Y	Y	Y	Y	Y	Y
	Maltose	R	R	R	R	R	R	R
	Sucrose	R	R	R	R	R	R	R

[a] Y, Yellow (positive reaction); Y/O, yellow-orange (positive reaction); O, orange (intermediate reaction); R/O, red-orange (negative reaction); R, red (negative reaction).

TABLE 2. *Effect of cell concentration on production of acid from glucose by N. gonorrhoeae CS-7*[a]

Hour	Cell concn (colony-forming units/ml[b])							
	4.7 × 10^9 (2.4 × 10^8)	2.3 × 10^9 (1.2 × 10^8)	1.2 × 10^9 (5.9 × 10^7)	5.9 × 10^8 (2.9 × 10^7)	2.9 × 10^8 (1.5 × 10^7)	1.5 × 10^8 (7.3 × 10^6)	7.3 × 10^7 (3.7 × 10^6)	3.7 × 10^7 (1.8 × 10^6)
1	Y/O	O	R/O	R/O	R	R	R	R
2	Y	Y/O	Y/O	O	R/O	R	R	R
3	Y	Y	Y	Y/O	O	R/O	R/O	R
4	Y	Y	Y	Y/O	O	O	R/O	R/O
5	Y	Y	Y	Y	O	O	R/O	R/O
6	Y	Y	Y	Y	Y/O	O	O	R/O
11	Y	Y	Y	Y	Y/O	Y/O	O	O
24	Y	Y	Y	Y	Y	Y/O	O	O

[a] Y, Yellow (positive reaction); Y/O, yellow orange (positive reaction); O, orange (intermediate reaction); R/O, red-orange (negative reaction); R, red (negative reaction).

[b] Values in parentheses are the numbers of colony-forming units per well.

TABLE 3. *Effect of incubation time on the identification of Neisseria gonorrhoeae, N. meningitidis, and N. lactamica*

Organism	No. of strains tested	Cumulative no. (%) of strains showing positive reactions at:					
		1 h	2 h	3 h	4 h	5 h	6 h
N. gonorrhoeae	196	38 (19.4)	117 (59.7)	166 (84.7)	178 (90.8)	191 (97.4)	196 (100)
	12[a]	5 (41.7)	11 (91.7)	12 (100)			
N. meningitidis[b]	32	16 (50.0)	25 (78.1)	29 (90.5)	31 (96.7)	31 (96.7)	32 (100)
N. lactamica[c]	8	2 (25.0)	8 (100)				

[a] Repeat of strains that failed to produce acid from glucose within 4 h.
[b] Identification as *N. meningitidis* requires positive glucose and maltose reactions.
[c] Identification as *N. lactamica* requires positive glucose, maltose, and lactose reactions.

isolates produced acid from glucose within 3 h and showed good growth on GC agar plates.

Strains of *N. meningitidis* and *N. lactamica* were also identified by the Minitek procedure. The results (Table 3) indicated that 97% of the *N. meningitidis* strains and 100% of the *N. lactamica* strains could be identified within 4 h. With strains of *N. meningitidis*, the maltose disk always gave a positive reaction before the glucose disk. With *N. lactamica*, the *o*-nitrophenyl-β-D-galactopyranoside reaction was more rapid than the production of acid from lactose.

Effect of nitrate on the production of acid from glucose by N. gonorrhoeae. The suitability of using dextrose (glucose) disks containing nitrate in place of disks containing only glucose was examined with 87 isolates of *N. gonorrhoeae*. The results (Fig. 1) show that the presence of nitrate in the disk markedly inhibited the production of acid from glucose by *N. gonorrhoeae*. With disks containing nitrate, only 56 of 87 isolates (64.4%) gave a positive reaction within 4 h. Eleven isolates (12.6%) failed to give a positive reaction after 24 h of incubation. By comparison, 84 of 87 isolates (96.6%) gave a positive reaction with glucose disks without nitrate. Only one isolate failed to give a positive reaction within 24 h.

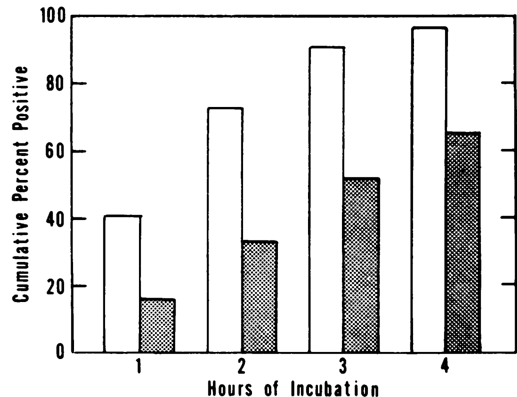

FIG. 1. *Effect of nitrate on the production of acid from glucose by clinical isolates of Neisseria gonorrhoeae. Open bars, Glucose disks without nitrate; stippled bars, glucose disks containing nitrate.*

Validity of the Minitek system. The validity and sensitivity of the Minitek procedure for the identification of pathogenic species of *Neisseria* was compared with CTA medium and the Brown modification (4) of the rapid fermentation test. For the first comparison, glucose, maltose, sucrose, and lactose disks and CTA medium containing the corresponding sugars were inoculated with 1 drop (49.66 μl) of the cell suspension dispensed from the Minitek pipetter. (Conditions of incubation are given above.) Minitek reactions were determined within 6 h; reactions in CTA medium were determined at 24 h. The results of this comparison are shown in Table 4. All isolates identified with CTA medium were also identified by the Minitek system. In addition, the Minitek procedure identified more isolates of *N. gonorrhoeae* and *N. meningitidis* than were identified with CTA medium. The same two isolates of *N. gonorrhoeae* were not identified by either procedure; this was attributed to a low density of inoculum cells. However, since both procedures used the same inoculum, it is unlikely that the 17 strains not identified by CTA medium could be attributed to low inoculum density. It was of interest that all strains of *N. meningitidis* tested in CTA medium produced acid from glucose; only eight strains

TABLE 4. *Comparison of Minitek and CTA procedures*

Organism	No. of strains examined	No. (%) of strains identified by:	
		Minitek	CTA
Neisseria gonorrhoeae	110	108 (98)	91 (82.7)
N. meningitidis	13	13 (100)	8 (61.4)[a]
N. lactamica	8	8 (100)	8 (100)

[a] No strains resistant to ≥8 μg of sulfadiazine per ml were identified by the CTA procedure.

produced acid from maltose. The five strains that failed to produce acid from maltose were also resistant to >8.0 μg of sulfadiazine per ml. All of these strains of *N. meningitidis* produced acid from maltose in the Minitek system.

Table 5 shows the results of the comparison of the Minitek and rapid fermentation procedures. The same isolates were tested by each procedure. When a glucose-free maltose source was used, the rapid fermentation test gave a positive identification in less time. Both procedures correctly identified all isolates. The purity of the maltose source was very important. Two isolates were incorrectly identified as *N. meningitidis* because the early acid reaction with maltose failed to revert. In addition, the early positive maltose reactions delayed the correct identification of many isolates.

TABLE 5. *Comparison of Minitek and rapid fermentation procedures for identification of Neisseria gonorrhoeae*

Method	No. of strains tested	Cumulative no. (%) of strains showing positive reactions at:			
		1 h	2 h	3 h	4 h
Minitek	20	2 (10)	12 (60)	19 (95)	20 (100)
Rapid fermentation[a]	20	18 (90)	20 (100)		
Rapid fermentation[b]	20	9 (45)	18 (90)	18 (90)	18[c] (90)

[a] Sugars obtained from W. J. Brown, Center for Disease Control, Atlanta, Ga.

[b] Sugars obtained from commercial sources.

[c] Two isolates misidentified as *N. meningitidis* on the basis of positive glucose and maltose reactions.

DISCUSSION

The increased isolation of *N. gonorrhoeae* from nongenital sites, as well as the isolation of *N. meningitidis* and *N. lactamica* from genital sites (3, 6, 9, 12, 17, 25), presents a problem in the clinical laboratory. All of these species of *Neisseria* will grow on media used for the isolation of *N. gonorrhoeae* (8). Therefore, isolates must be further characterized to ascertain the identity of the organism. The presumptive identification of an isolate as *N. gonorrhoeae* is based upon typical oxidase-positive colonies of a gram-negative diplococcus (2). Local policy and special situations will determine the need for a confirmatory identification of the isolate as *N. gonorrhoeae*. Confirmatory identification consists of either direct fluorescent-antibody staining or the production of acid from specific carbohydrates. Many laboratories use both methods. Current tests for acid production from carbohydrates may give variable results due to inoculum size (4), age of inoculum, inhibitory substances in the peptone (E. S. Baron and D. Lowry, Abstr. Annu. Meet. Am. Soc. Microbiol., p. 39, 1975), or the contamination of maltose with small amounts of glucose (4).

The present study has described a rapid method for the identification of *Neisseria* based upon the production of acid from various carbohydrates. This method is faster and more sensitive than results obtained with CTA medium. With an identical cell concentration, the identity of 91% of the isolates could be determined with as little as 4 h of incubation in comparison with the 24 to 48 h of incubation often required with CTA medium. Furthermore, the Minitek procedure correctly identified 15% more isolates; no false-positive maltose reactions were observed.

The false-negative reactions occasionally observed were often due to an inoculum with a low cell density. False-negative reactions have also been encountered when inocula were prepared directly from Transgrow medium or the Thayer-Martin plate used in the initial isolation of the organism. This potential problem has been overcome by restreaking isolates on one-half of a GC agar plate. After overnight incubation, the cells can be harvested for preparation of the inoculum. In spite of this extra step, a confirmatory identification of an isolate as *N. gonorrhoeae* can be made in less time than required with conventional CTA medium.

When a glucose-free maltose source is used, the rapid fermentation procedure (4) will give

faster identification of *N. gonorrhoeae* (2 versus 4 h). However, the difficulty in obtaining glucose-free maltose coupled with the absence of false-positive maltose reactions with Minitek disks make the Minitek procedure a viable alternative method.

Glucose is metabolized in growing cells of *N. gonorrhoeae* by a combination of the Entner-Doudoroff and pentose phosphate pathways (20). Carbon dioxide and acetic acid are the major end products of glucose metabolism during aerobic growth (20). No growth occurs anaerobically but small amounts of acetic and lactic acids are produced from glucose (20). The presence of these acids effects a color change in the phenol red indicator contained in the Minitek disks and CTA medium, thereby indicating a positive reaction.

The addition of $NaHCO_3$ to the resuspending medium affected the appearance of both positive and negative reactions. At high concentrations, HCO_3^- may act as a buffer and slow the appearance of a positive reaction. At optimal HCO_3^- concentrations, a negative reaction is easier to read as evidenced by the bright red color of the disk. In the absence of a utilizable carbohydrate, *Neisseria* spp. can deaminate amino acids in proteose peptone no. 3 producing an alkaline pH (11, 12). The pH may be increased further by the production of CO_3^{2-} from HCO_3^-. Other effects, such as the utilization of HCO_3^- as a growth factor (S. A. Morse and L. Bartenstein, Abstr. Annu. Meet. Am. Soc. Microbiol., p. 44, 1972), cannot be eliminated at this time.

The Minitek procedure can also detect acid production from maltose by sulfadiazine-resistant strains of *N. meningitidis*. These sulfadiazine-resistant meningococci presumably lack maltose permease activity (16). However, the concentration of maltose in the Minitek disks (3 mg of maltose per disk; 6% wt/vol, final concentration) may be sufficiently high that maltose enters the cells by passive diffusion.

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