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Rapid Micro-Carbohydrate Test for Confirmation of *Neisseria gonorrhoeae*

DAVID C. T. YONG* AND ANNE PRYTULA

Windsor Regional Public Health Laboratory, Laboratory Services Branch, Ontario Ministry of Health, Windsor, Ontario, Canada N9A 6S2

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A rapid carbohydrate utilization procedure for the confirmation of *Neisseria gonorrhoeae* and identification of other *Neisseria* species has been developed. This method utilizes both preformed enzymes, introduced in a heavy inoculum, and enzymes formed by the microorganisms as a result of growth in a small volume of super-enriched medium. Expected carbohydrate reactions were produced by 383 clinical isolates of neisseriae and were clearly visible within 4 h of incubation. The combined use of disposable glass tubes (6 by 50 mm) and micro-amounts of media (0.05 ml) make this method not only rapid, but also low in cost.

Laboratory identification of *Neisseria gonorrhoeae*, *N. meningitidis*, and other *Neisseria* species depends mainly on culture and subsequent carbohydrate degradation tests. Some laboratories identify *N. gonorrhoeae* by the fluorescent-antibody method (13, 17). However, there have been reports of cross-reactions between *N. gonorrhoeae* and nongonococcal neisseriae in the fluorescent-antibody technique (11, 18, 20, 21). *N. gonorrhoeae* has been isolated from nonurogenital sites, and *N. meningitidis* may be implicated in urogenital infections and cases of neonatal ophthalmia (2, 9, 10, 12). It is, therefore, useful for epidemiological, medicolegal, and clinical purposes to confirm the identity of neisseriae with carbohydrate tests.

Recently, numerous techniques for the demonstration of biochemical reactions by *Neisseria* species have been published (4, 8, 14, 15, 18, 19, 22, 23, 25, 26). This recent influx of new techniques is due primarily to the equivocal results obtained by many laboratories with the widely used cystine-Trypticase agar (CTA) method (24), in which a conclusive reaction is dependent on adequate growth of the test organism, which may take up to 48 h, or longer with more fastidious strains. It requires a prolonged incubation time, which may lead to nonspecific changes in the pH indicator (8, 19, 21, 25, 26). In addition, for those strains of *N. gonorrhoeae* which grow very poorly in the CTA medium, even "negative" results are unreliable. Various laboratories (5, 8, 15, 18, 19, 25) have reported anywhere from 60.9 to 96% reactivity with CTA sugars. Shtibel and Toma (21) obtained a 98.6% reactivity with a modified CTA method.

This report presents a rapid micro (RM)-carbohydrate utilization technique for the identification to species of *Neisseria* and the confirmation of *N. gonorrhoeae*, in which all test results are clearly readable after 4 h of incubation. The method utilizes both preformed enzymes and enzymes formed by the microorganisms as a result of growth in a small volume (0.05 ml) of super-enriched medium. The combined use of disposable glass tubes (6 by 50 mm) and micro-amounts of medium (0.05 ml) make this method not only rapid, but also low in cost.

MATERIALS AND METHODS

Organisms used. For this study, four stock strains of *Neisseria* spp. and one strain of *Branhamella catarrhalis* were obtained from the Central Public Health Laboratory, Ontario Ministry of Health, Toronto. The neisseria species consisted of *N. gonorrhoeae*, *N. meningitidis*, *N. sicca*, and *N. lactamicus*. In addition, 383 strains of *Neisseriae* isolated in our laboratory from clinical sources were included. The organisms were grown on chocolate agar (Difco GC base plus 5% sheep blood).

A Gram-stained smear and an oxidase test were performed on each of the *Neisseria* cultures, as well as a smear stained by the immunofluorescent staining technique as described by Kellogg and Deacon (13), using fluorescein-labeled *N. gonorrhoeae* antiserum (Difco). Control smears of *Escherichia coli*, *N. gonorrhoeae*, and *N. meningitidis* were included with every batch of test organisms stained with the antiserum.

Preparation of media. The media for carbohydrate degradation tests were prepared as follows. (i) RM-carbohydrate base consisted of: Casamino Acids (Difco, certified), 2 g; L-cysteine-hydrochloride, 0.03 g; sodium sulfate (Baker), 0.03 g; neopeptone (Difco), 2.5 g; phenol red, 0.01 g; distilled water, 100 ml. (ii) For completed RM media, 2 g of carbohydrate (dextrose,

lactose, or sucrose) was added to 100 ml of base. Since some batches of maltose may contain small amounts of dextrose, which may interfere with the test, only 0.3% maltose was used instead of 2%. The pH was adjusted carefully to 7.2 to 7.25 with 1 N sodium hydroxide. The complete media were then sterilized by filtration through 0.45-μm membrane filters (Millipore Corp.). Autoclaving will precipitate some of the essential constituents in the media.

Initially, cystine (0.05 g/100 ml) and sodium sulfite (0.05 g/100 ml) were used in the RM base, and 333 cultures were tested with this medium. In the course of the study, these ingredients were substituted by L-cysteine and sodium sulfate, and an additional 50 cultures were tested.

From dropper bottles, approximately 0.05 ml (or a depth of 3 mm) of each of the sterile carbohydrate media was added to microtubes (disposable borosilicate glass, 6 by 50 mm; Kimble). The tubes were placed in microtiter V-plates (Cooke Engineering Co.) and allowed to warm to room temperature (22°C) before inoculation. Before use, the microtubes were placed, inverted, into screw-capped bottles and sterilized in a hot-air oven at 155°C for 5 h. Autoclaving is not recommended because alkaline substances may be released from the borosilicate glass and may affect some tests.

Method of inoculation. A 3-mm loopful of bacteria, subcultured once from the primary isolation medium and grown for 18 to 24 h on chocolate agar, was inoculated into each of the four carbohydrate-containing media (dextrose, maltose, sucrose, and lactose). The clumps of bacteria in the microtubes were dispersed by using a Vortex mixer. The inoculated medium looked turbid or "milky" after the mixing step. The tubes, placed in the microtiter plates, were incubated in a shallow water bath maintained at 36°C. The tests were read after 1, 2, 4, and 18 h of incubation, and a final reading was made at 48 h. A positive carbohydrate utilization reaction was indicated by a yellow color throughout the medium (or occasionally an orange-yellow color, depending on the strain of *N. gonorrhoeae*), and a negative reaction was indicated by a red color.

Parallel tests, using CTA medium, were inoculated with each of the organisms under study. For this study, the CTA medium was prepared as described by Vera (24). A loopful of organisms, from the chocolate agar plate, was inoculated onto the surface of the CTA semisolid medium by rimming along the edge of the medium. The inoculated medium was placed in a 36°C incubator. The tests were read after 1, 2, 4, 18, and 48 h. A positive carbohydrate utilization reaction was indicated by a yellow color, first appearing at the surface of the semisolid medium.

RESULTS

The results of carbohydrate utilization by 383 clinical isolates of neisseriae, using both the RM and CTA methods, are shown in Table 1. In the RM method, 197 of the cultures showed positive reactions after 2 h of incubation. The remaining 186 cultures showed completed reactions after 4 h. The 377 strains of *N. gonorrhoeae* utilized dextrose only, and six strains of *N. meningitidis* utilized dextrose and maltose. All of the gonococcal cultures were oxidase-positive, gram-negative diplococci and produced strong fluorescence when stained with fluorescein-labeled antigonococcal serum (Difco). The *N. meningitidis* isolates showed weak fluorescence when stained with the antigonococcal serum.

In the CTA method, positive reactions were produced by eight isolates within 2 h. At 4 h only 88 additional isolates showed positive reactions, but these were weakly positive and occurred at the surface of the semisolid medium. The majority of positive reactions (258) were recorded after 18 h of incubation, although some of these might have been observed at 6 h, if readings had been made at that time. Four isolates did not show positive reactions until 48 h of incubation. Nineteen isolates of *N. gonorrhoeae* did not appear to utilize dextrose, even after 72 h of incubation. However, two of these finally produced an acid reaction in dextrose after they had been subcultured four times on chocolate medium. Two strains of *N. meningitidis* failed to produce acid from maltose except when calf serum (1%) was incorporated into the CTA medium. Two strains of *N. meningitidis* showed acid reactions after 4 h but reverted to alkaline reactions after 18 h of incubation. Thus, an overall 95% reactivity was observed with the CTA method.

The five stock strains, namely, *N. gonorrhoeae*, *N. meningitidis*, *N. sicca*, *N. lactami-*

TABLE 1. *Comparison of results of carbohydrate utilization in the CTA and RM methods*

Organism/method	Cultures that showed acid reaction(s) after incubation time (h) of:					No. of cultures tested	Cultures showing expected reactions	
	1	2	4	18	48		No.	%
N. gonorrhoeae								
CTA		8	88	258	4	377	358	95
RM	53	140	184			377	377	100
N. meningitidis								
CTA			2	2		6	4	67
RM	1	3	2			6	6	100

cus, and *B. catarrhalis*, produced the expected carbohydrate utilization patterns with both methods, within 4 h in the RM method and within 48 h in the CTA method. *N. gonorrhoeae* degraded dextrose, *N. meningitidis* degraded dextrose and maltose, *N. lactamicus* degraded dextrose, maltose, and lactose, *N. sicca* degraded dextrose, maltose, and sucrose, and *B. catarrhalis* did not produce acid from any of the four carbohydrates.

DISCUSSION

Kellogg and Turner (14) published a rapid utilization test for confirmation of *N. gonorrhoeae* which was later modified by Brown (4). This method had the advantage of being independent of growth. However, Young et al. (27) reported that both Kellogg-Turner's and Brown's buffer solutions were found to be unsatisfactory. When thick suspensions were used (as described by Brown) to obtain a rapid result, there was a tendency for the maltose to give a positive reaction, and when a thin suspension was used, changes in the glucose were slower and sometimes incomplete. Morse and Bartenstein (15) reported that the purity of the maltose source was important and that commercially prepared maltose was often contaminated with dextrose. Using commercially prepared carbohydrates for Brown's method, they found that 2 out of 20 *N. gonorrhoeae* isolates were incorrectly identified as *N. meningitidis* on the basis of positive glucose and apparently positive maltose reactions.

Pollock (18) stated that results of carbohydrate degradation by members of the *Neisseriaceae* may take 3 days or more, partly because the fastidiousness of many primary isolates prevents them from growing adequately in the semisolid CTA medium. Since the media devised by Young et al. (27), Brown (4) and Kellogg and Turner (14) do not support the growth of *Neisseria*, the tests are completely dependent on preformed enzymes and, therefore, inoculum size; and as Pollock (18) has pointed out, variations in the inoculum size, in a routine laboratory operation, may occasionally produce misleading negative results. In general, these methods should work, assuming that all strains of neisseriae produce equal, abundant amounts of preformed enzymes and every inoculum introduced by technologists in a routine laboratory contains sufficient preformed enzymes. However, this assumption is not always true. This is shown by the results obtained by Kellogg and Turner (14), Morse and Bartenstein (15), Brown (5), Pollock (18), Reddick (19), and Wallace et al. (25). When approximately equal numbers of gonococci were inoculated, the rates of the utilization of dextrose and the intensities of the yellow color were different. Since it is not possible to have prior knowledge of the nature of the *Neisseria* species in question, it is not possible to tailor the size of the inoculum to each strain. Consequently, negative results of the carbohydrate utilization test, depending on preformed enzymes alone, may not always be valid.

The utilization of dextrose by *N. gonorrhoeae* occurs in two stages. First, dextrose is dissimilated to acetate and carbon dioxide during active growth of the culture. Second, the acetic acid is oxidized after the depletion of dextrose in the medium (16). In media used for the dextrose utilization test, the change in the indicator to acid reflects the production of acetic acid, and possibly a small amount of lactic acid, during the dissimilation of dextrose. The oxidation of acetic acid, after the depletion of dextrose, combined with the formation of alkaline products due to enzymatic degradation of peptone appear to be causes of the reversion of an acid reaction to an alkaline reaction on prolonged (more than 6 h) incubation of the carbohydrate degradation tests (6, 7, 21). In the CTA method, we have experienced pH reversions by *N. gonorrhoeae* isolates, similar to that reported by Shtibel and Toma (21). Two strains of *N. meningitidis* showed acid reactions in both the CTA-dextrose and CTA-maltose tests after 4 h but reverted to alkaline reactions after 18 h of incubation. One of these strains also showed reversion in the RM-maltose test. Since it is not necessary to incubate the RM tests longer than 4 h, this pH reversion does not present a problem in this method. The RM tests should, therefore, be read after 4 h of incubation. If they cannot be read at 4 h, they can be stored, in a moist container, in the refrigerator after the initial incubation period of 4 h and then read the next day.

It is apparent that the optimal method for testing carbohydrate utilization by *Neisseria* spp. should incorporate the combined action of both preformed enzymes and those formed during growth. To demonstrate that growth of the organisms does occur in the RM-medium and has a significant effect on the rapidity of the reaction, a strain of *N. gonorrhoeae* was tested with RM medium from which neopeptone and Casamino Acids were omitted. It failed to degrade the dextrose to acid within 4 h, using one loopful of the culture. When a larger inoculum was used, thereby introducing a larger quantity of preformed enzymes, acid was produced in the dextrose test within 4 h. On the other hand, with neopeptone and Casamino Acids added, even half a loopful of the same strain of *N. gonor-*

• *rhoeae* was sufficient to produce an acid reaction in the dextrose test within 4 h. This suggests that for this strain of *N. gonorrhoeae*, growth and enzyme synthesis are required for the rapid reaction in the RM test. Our results therefore show that the combined action of both sources of enzymes produced results which were not affected by small variations in either the inoculum size or the growth requirements of individual strains. Acid reactions were clearly indicated by a distinct yellow color throughout the medium, making the tests easy to read.

As reported by other investigators (5, 8, 15, 18, 19, 25), we also encountered strains of *N. gonorrhoeae* that did not appear to degrade dextrose in the CTA tests. However, these 19 strains did produce clear-cut positive reactions with the RM method (Table 1).

Vera (24) reported that sodium sulfite and cystine enhanced the growth of *N. gonorrhoeae* in CTA medium. Beno et al. (3) and Wallace et al. (25) found that the Mueller-Hinton base was a more reliable substrate for the growth of *N. gonorrhoeae*. Since Casamino Acids are the main nutrients in the Mueller-Hinton base, we initially combined cystine, sodium sulfite, and Casamino Acids as ingredients of our RM base. However, during the course of our study, Baron and Saz (1) reported that certain concentration ratios of cystine and sodium sulfite were inhibitory to the growth and/or dextrose metabolism of some strains of *N. gonorrhoeae*. By substituting these ingredients with L-cysteine and sodium sulfate, they were able to eliminate this inhibitory effect. Although we had not encountered this inhibitory effect in the RM method with 333 strains of neisseriae, we nevertheless incorporated L-cysteine and sodium sulfate, in place of cystine and sodium sulfite, into our medium. In 50 gonococcal cultures subsequently tested, we observed consistently rapid reactions, all of which were completed within 2 h.

As shown by our data, the RM method should be of significant practical importance to clinical microbiology laboratories for the rapid confirmation of *N. gonorrhoeae*.

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