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Simple Disk-Plate Method for the Biochemical Confirmation of Pathogenic *Neisseria*

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The carbohydrate fermentation test in cystine trypticase agar (BBL)-tubed medium was compared with a simple method using commercially available carbohydrate impregnated disks on culture inoculated Thayer-Martin medium with and without (vancomycin, colistimethate, and nystatin) inhibitors. There was 100% agreement between the two methods when a limited sample of clinical isolates of *Neisseria gonorrhoeae* and *Nesseria meningitidis* were tested.

The conventional differentiation of the organisms which comprise the genus *Neisseria* includes the determination of carbohydrate fermentation patterns by the sugar-tube fermentation test utilizing 0.5 to 2% concentration of the "sugars" glucose, maltose, lactose, sucrose, and levulose in cystine trypticase agar base (CTA) to which phenol red is added as an indicator of pH change.

However, a number of investigators including White and Kellogg (7), Beno et al. (1), Carifo and Catlin (2), and recently Faur et al. (4) have noted deficiencies in the method because of poor growth, delayed result, and atypical biochemical patterns in a significant proportion of tests with *Neisseria gonorrhoeae* and *Neisseria meningitidis*. Further, the use of the conventional method requires an increased inventory of reagent grade sugars, media, glassware, sterilization equipment, and technologist involvement which makes it a costly and time-consuming procedure. The problems in its use are further compounded especially if an adequate check is to be made on a number of suspect isolants from pharyngeal and rectal sites in cases of gonorrhea. The very economics of the test might have a chilling effect on the microbiologist attempting to make an adequate check in such a situation.

We, therefore, attempted to devise a simplified procedure by utilizing comparatively inexpensive, commercially available sugar disks and Thayer-Martin medium in a modified disk-plate technique (5) and compared the results on a limited number of isolates with the conventional method. (This work was presented in part at the 74th Annual Meeting of the American Society for Microbiology, Chicago, Ill., 12-17 May 1974.)

MATERIALS AND METHODS

Plates of Thayer-Martin (TM) medium were either prepared with vancomycin (3 μg/ml), colistimethate (7.5 μg/ml), and nystatin (12.5 U/ml) inhibitors according to commercial manufacturers instructions or purchased from BBL. In addition, plates and slants of enriched chocolate agar (ECA) (which consisted of basal TM without antibiotics) were prepared. Carbohydrate disks were obtained from Difco. Blank (sugar-free) disks were obtained from BBL and steam sterilized at 20 pounds/30 min. Andrade indicator was used to detect pH change and was prepared as follows: 0.5 g of acid-fuchsin was dissolved in distilled water with the aid of approximately 16.0 ml of 1 N NaOH, final volume 1 liter, pH 7.0. It was steam sterilized at 10 pounds/10 min and allowed to age for at least 2 days at room temperature before use. (The sterile, tubed indicator may be left at room temperature for up to 2 months.)

All media passed usual quality control tests for sterility and growth support of typical *Neisseria* species; quality control was also run on each (combination) lot of disks, medium, and indicator: positive fermentation control was accomplished by using a strain of *Neisseria sicca* and *Neisseria lactamica* by the described method on ECA medium; negative control consisted of using a *Neisseria catarrhalis* strain which was able to grow on TM medium.

Wild-type *N. gonorrhoeae* (35 strains) and *N. meningitidis* (10 strains) were isolated initially on TM, the former from urethral, vaginal, rectal, and pharyngeal specimens, and the latter from rectal crypt sites. All isolants were purified initially on ECA and characterized as *Neisseria* by typical colonial growth, morphology, and oxidase characteristics. The pure cultures on ECA slants were utilized as follows: 1.0 ml of heart infusion broth (sugar-free) was added to each slant, the growth was suspended and swabbed onto seven quadrants of two TM plates (Quadri-Petri dishes, 100 by 15 mm, Falcon, are preferable) so as to form a circle about the size of a U.S. nickel (2 cm in diameter). Each disk was placed

eccentrically on an inoculated site to detect possible growth inhibition by the sugar diffusing out of it. One inoculated site received a blank disk which acted as a sugar-free control. The remaining inoculated and uninoculated sites served as contamination and uninoculated controls, respectively (optional). Since only pure cultures and sterile media, disks, and indicator should be tested as in this study, these two controls are unnecessary and could alternatively be used as follows. Inoculate both quadrants and place a duplicate glucose and maltose disk as described; should the 24-h result on glucose and/or maltose appear questionable on plate no. 1, and the culture found not to be contaminated, the second plate may be tested after 48 h of incubation.

The same culture was also tested by the described procedure using ECA which acted as an antibiotic-free control as well as by the conventional CTA sugar-tube method (1% sugars). All plates were incubated in the inverted position at 35 C in a candle-extinction jar or CO_2-incubator. Since there is a small but distinct possibility of isolating *N. meningitidis* from pharyngeal and rectal sites, these culture plates were taped with Micro-pore tape (3M Company, Minneapolis, Minn.) which allows gaseous diffusion. The CTA fermentation tubes were incubated at 35 C for up to 48 h without CO_2.

At the end of 24 h the growth of the test organisms on TM plates was compared to that on the ECA control. If adequate and comparable growth (closely opposed colonies or lawn of growth) occurred, two drops of Andrade indicator were added to each disk via Pasteur pipette, the plates were gently inverted and an interval timer immediately was set for 10 min. Results were read not later than 10 min (Important: at about 15 min some lots of TM may show a light pink coloration of the dye and so confuse the novice) as follows. The negative (sugar-free) control disk was read first and if colorless the remainder of the disks were observed for a color change. A positive result (denoting acidity or fermentation of a sugar) was recorded if a dark-red or red-violet coloration was noted on any part of a disk and/or medium in its immediate periphery. A colorless or faint pink color was noted as negative.

Results of the conventional sugar-tube method were read at 24 and 48 h.

RESULTS AND DISCUSSION

Results indicated that all strains of *N. gonorrhoeae* (35/35) and *N. meningitidis* (10/10) tested by the disk-plate technique gave unequivocal fermentation patterns at 24 h both on TM and ECA. Apparently, therefore, the concentration of vancomycin, colistimethate, and nystatin in TM did not affect the biochemical differentiation of the strains tested. A qualitative check on the adequacy of growth indicated that the strains grew equally well on TM and ECA at 24 h. We recommend the use of TM for the pathogenic *Neisseria* for the above reasons and its commercial availability, at least in the U.S. and Canada, but clinical microbiologists are cautioned in the use of a modification called Trans-grow medium or Lester-Martin medium which contains a higher concentration of glucose as well as trimethoprim and has not been evaluated for use with this method at present.

The rich growths as well as excellent results on ECA might recommend its use for the biochemical differentiation of the great majority of *Neisseria* (including rare strains of *N. gonorrhoeae*) which are inhibited on TM, as well as for testing other fastidious organisms.

When the results were read with the conventional method, only 27 out of 35 *(N. gonorrhoeae)* gave definitive results at 24 h. The remaining eight strains had to be incubated a further 24 h before becoming definitive.

Therefore, there was some indication that the described procedure was more rapid than the conventional one.

Although this exploratory study encompassed an admittedly small sample of test organisms, it was brought to the author's attention since presenting this paper (J. Valu, Abstr. Annu. Meet. Am. Soc. Microbiol. 1974, M345, p. 123) that other investigators evaluating this method for their own in-house use have obtained high correlations on large samples of *N. gonorrhoeae* and that at least one research facility has incorporated it in its daily routine.

The need to develop simplified procedures to differentiate the pathogenic *Neisseria* has been recognized for some time. One such method, that of Taylor and Keys (6) attempted to identify *N. gonorrhoeae* on two plates of CTA-based medium, one containing glucose and the other a mixture of maltose, sucrose, and lactose. Although this method could reportedly identify, with few exceptions, *N. gonorrhoeae* strains, the methodology would preclude a definitive differentiation of *N. meningitidis*. The use of nutritionally deficient CTA in that test would, presumably, make it as unsuitable as the CTA-based conventional method.

On the other hand, judicious choice of nutritious TM or ECA by the described procedure would allow the clinical microbiologist to arrive at a definite differentiation of the pathogenic *Neisseria* and/or other opportunistic *Neisseria* sometimes associated with diseased states (see reference 3). An added asset in the use of the disk-plate method is reduced cost; we estimate a 40 to 60% savings may be achieved over the conventional method, depending on whether the culture media are purchased or prepared in-house.

We, therefore, believe that the wider application of a simple and rapid procedure such as the

disk-plate method would be advantageous from an economic as well as logistic point of view in the continued fight against near epidemic gonorrheal disease in the western hemisphere.

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