

## New System for Cultivation of *Neisseria gonorrhoeae*

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A modified Thayer-Martin medium, containing 2.0% agar, 0.25% dextrose, and 5.0  $\mu\text{g}$  of trimethoprim per ml was compared to Thayer-Martin medium for growth of stock cultures of *Neisseria gonorrhoeae* and for isolation of *N. gonorrhoeae* from clinical specimens. Stock cultures and male urethral cultures grew equally well on both media, but the modified Thayer-Martin medium detected 10% more female patients positive for gonococci. A description is given of the use of a citric acid-sodium bicarbonate tablet for generating carbon dioxide in a sealed plastic bag containing a culture plate. Equivalent results were obtained on duplicate plates of 336 cultures and specimens when this procedure was compared with the standard candle jar extinction method of incubation. The tablet and plastic bag system has the advantages of economy, ease of use, and safety.

The importance of culture methods in the diagnosis of gonorrhea is well established. Although today's culture media and procedures have evolved through many stages, the basic constituents were introduced in 1891 by Werthheim (17). Early modifications of his medium included heating blood agar for 5 min at 80 C, which resulted in "chocolate agar" (9), the use of commercial peptone (10), and replacement of animal fluids with yeast extracts (1). Recent modifications have involved the introduction of antibiotics into the chocolate agar base and the resulting development of a selective medium (Thayer-Martin medium) for the isolation of pathogenic *Neisseria* (14, 16). Later, the antibiotic combination was altered and the yeast extract was replaced with a defined supplement; these changes improved the selectivity and sensitivity of this medium for isolation of gonococci (6, 15). Although selective media are not absolutely selective, the high degree of specificity and sensitivity of Thayer-Martin (TM) agar permitted presumptive positive identification of *Neisseria gonorrhoeae* on the basis of cultural characteristics, oxidase tests, and Gram stains (2, 8). More recently, a modified TM medium (MTM) was employed in a transport system (Transgrow) (7). This medium contained twice as much agar as did TM medium, additional dextrose, and 5.0  $\mu\text{g}$  of trimethoprim per ml.

The importance of a partial carbon dioxide environment for enhancing primary growth of the gonococcus was first reported by Wherry

and Oliver (18). Chapin (3) introduced the candle jar extinction method for providing this atmosphere, and Spink and Keefer (13) confirmed that this procedure supplied adequate carbon dioxide for primary growth of gonococci. Although various methods for providing carbon dioxide in a closed container were shown to be as effective as the candle jar (4), this procedure is the one most widely used today for incubating primary cultures of gonococci. Unfortunately, the candle jar, although inexpensive, is cumbersome, easily breakable, and requires a large amount of incubator space. This restricts its use by the average practitioner or small laboratory. Obviously, another method of providing the required carbon dioxide atmosphere is needed—one that would be as effective and inexpensive as the candle jar without having its disadvantages.

This is a preliminary report describing the use of a citric acid-sodium bicarbonate tablet for generating carbon dioxide in a sealed plastic bag containing MTM agar plates. This procedure is compared to the standard candle jar extinction method. In addition, the MTM medium is compared to regular TM medium for isolation of gonococci.

### MATERIALS AND METHODS

TM medium was prepared by supplementing GC agar base (BBL) with an equal volume of 2% hemoglobin solution, 1% (vol/vol) of a defined supplement (IsoVitaleX, BBL), and antimicrobials. The final concentrations of antimicrobials were: vancomycin

(vancomycin sulfate, Eli Lilly & Co., 962 U/mg), 3.0 U/ml; colistin (colistimethate sodium, Warner-Lambert Research Institute Inc., 414 µg/mg), 7.5 µg/ml; nystatin (E. R. Squibb & Sons, Inc., 500,000 U/vial), 12.5 U/ml.

MTM medium was prepared as above, except that final concentrations of 2% agar, 0.25% dextrose, and 5.0 µg of trimethoprim (trimethoprim lactate, Burroughs-Wellcome Co., 760 µg/mg) per ml were also included. All media were stored at room temperature in plastic bags and were used within 2 weeks.

Carbon dioxide generators (TABCO<sub>2</sub>) were supplied by the Ames Co., Division of Miles Laboratories, Inc., Elkhart, Ind. Each 100-mg tablet contained a mixture of citric acid and sodium bicarbonate. Polyethylene plastic bags employed for determining optimum thickness required were obtained from the Marion Manufacturing Co., Atlanta, Ga., in 0.001-, 0.002-, 0.003-, and 0.004-inch thicknesses, and were 5 by 8 inches in size. The bags used in the clinical evaluation of the system were 0.003-inch-thick, 18-oz. "Whirl-Pak" bags produced by the Nasco Co., Atkinson, Wis.

Bacterial cultures used were stock cultures of *N. gonorrhoeae* recently received for antibiotic susceptibility testing and cultures freshly isolated from venereal disease clinic patients. Stock cultures were grown for 20 h on TM agar slants, and the growth was washed off with Trypticase soy broth (TSB). This suspension was adjusted to produce 45% light transmittance in a B&L Spectronic 20 spectrophotometer at 530 nm and then diluted 1:1,000. A 3-mm loopful of this diluted suspension was used to inoculate agar plates.

Fresh clinical specimens were obtained from male and female venereal disease clinic patients in Atlanta and Baltimore. All specimens were taken on sterile cotton or calcium alginate swabs and were either suspended in 1.0 ml of sterile TSB (all specimens from males and specimens from Baltimore females) or inoculated directly on agar plates (specimens from Atlanta females). In the Baltimore study, the broth suspensions were mixed by pipetting or agitation; a sterile swab was inserted, rolled against the side of the tube, and used to inoculate a small area of each agar plate. In the Atlanta study, two plates were inoculated directly with duplicate specimens from the same patient. All agar plates were cross-streaked with a

sterile loop and incubated at 35 to 37 C. After 18 to 24 h of incubation, each plate was examined for *N. gonorrhoeae* and the colonies were counted. Plates were also examined for spreading *Proteus*. Presumptive identifications of gonococci were based on colony morphology, Gram stains, and oxidase tests.

Candle jars were handled according to normal procedures: plates were inserted, the candle was lit, and the closed jar was placed in an incubator. A TABCO<sub>2</sub> tablet was placed into a plastic bag, an inoculated agar plate was added, and the bag was closed with the built-in closure and placed in an incubator.

Carbon dioxide determinations were performed by standard gas chromatography methods. Sealed bags containing a TABCO<sub>2</sub> tablet and an inoculated MTM plate were sampled after various times of incubation.

## RESULTS

**Comparison of TM and MTM media.** Table 1 shows results of the comparative study of specimen cultures grown on TM and MTM media in a candle jar. Stock cultures of *N. gonorrhoeae* grew equally well on both media. In the aggregate, the same number of male urethral specimens yielded gonococci on both media, but 10% more specimens from the female cervical os were positive for gonococci on MTM than on TM media. When compared individually, 167 clinical specimens grew gonococci on both media, 1 specimen was positive on TM medium only, and 13 specimens were positive on MTM medium only. This difference in the ability of the two media to detect gonococci is statistically significant ( $P < 0.001$ ) by the binomial method. As expected, incorporation of trimethoprim into the MTM medium completely eliminated spreading by *Proteus*, whereas 23 cultures from clinical specimens were overgrown with spreading *Proteus* on TM agar plates. Trimethoprim had no apparent effect on the amount of growth from 228 specimens (including stock cultures) which were positive on both media, as judged by numbers of

TABLE 1. Results obtained with *Neisseria gonorrhoeae* specimens cultured on TM and MTM media in candle jars

Cultures or sites	No. of cultures	Total positive	TM agar		MTM agar	
			Gonococci	Spreading <i>Proteus</i> spp.	Gonococci	Spreading <i>Proteus</i> spp.
Stock cultures of <i>N. gonorrhoeae</i> . . . . .	61	61	61 (100) <sup>a</sup>	0	61 (100)	0
Male urethral specimens . . . . .	129	58	58 (100)	4 (6.9)	58 (100)	0
Female cervical specimens . . . . .	328	123	110 (89.4)	19 (15.4)	122 (99.2)	0
Total . . . . .	518	242	229 (94.6)	23 (9.5)	241 (99.5)	0

<sup>a</sup> Numbers in parentheses are percentages.

colonies observed. As a result of these findings, MTM medium was used exclusively in subsequent work.

**Development of the plastic bag-carbon dioxide generator.** Sixteen stock cultures of *N. gonorrhoeae* were used to determine the minimal thickness of plastic required to prevent excess diffusion and loss of carbon dioxide. Replicate cultures incubated in bags 0.004-, 0.003- and 0.002-inches thick showed good and equivalent growth. However, only 9 of the 16 cultures grew in bags 0.001-inches thick, and in only 1 of these 9 was growth comparable to that on control plates. Thus, although 0.002-inch-thick bags gave satisfactory growth, 0.003-inch-thick bags were used to provide a margin of safety.

Carbon dioxide concentrations in sealed bags containing an inoculated MTM agar plate and TABCO<sub>2</sub> are shown in Table 2. Bags that had been incubated for 1 h averaged 1.48% carbon dioxide; the average amount of carbon dioxide present during the first 8 h of incubation varied from 1.30 to 2.23%. After 24 h of incubation, 2.91% carbon dioxide was present. This increase was probably due to the production of carbon dioxide by the metabolizing gonococci. The levels of carbon dioxide found during the 24-h incubation period are well within the range that provides for initial growth of the gonococcus (5).

**Comparison of candle jar and plastic bag-carbon dioxide systems.** Sixty-one stock cultures of *N. gonorrhoeae* and 275 specimens from patients were cultured in duplicate on MTM agar plates. One plate of each duplicate culture was incubated in a candle jar, and the other plate was incubated in the plastic bag-carbon dioxide system, all at 35 to 37 C. Table 3 summarizes the data. Results obtained with candle jars and with plastic bags were identical for stock cultures and cultures obtained from males. In addition, equivalent numbers of colonies grew on the duplicate plates under these

TABLE 3. Results obtained with *Neisseria gonorrhoeae* specimens cultured on MTM medium in candle jars and in plastic bags

Cultures or sites	No. tested	Total positive cultures	No. positive cultures in	
			Candle jar	Plastic bag-tablet
Stock cultures . .	61	61	61	61
Male				
Urethra . . . . .	129	58	58	58
Anal canal . . .	1	1	1	1
Female				
Cervix . . . . .	145	30	29	29

two test conditions. Cultures of secretions from the female cervix produced identical numbers of positive isolations of gonococci, but each system detected a single positive culture that was negative in the alternate system. In both of these cases, colony counts were less than 10.

## DISCUSSION

MTM medium has been in use in the "Trans-grow" system since 1971 (7, 19). The increased agar content produces a more rigid transport medium; the increased dextrose content permits optimum growth of certain gonococci; the addition of trimethoprim (5.0 µg/ml) suppresses growth and spreading by frequently found *Proteus* species. Previous reports on the comparative sensitivity of TM versus MTM media are difficult to interpret because times of incubation, methods of carbon dioxide generation, and concentrations of trimethoprim varied (7, 12). In the present study, trimethoprim reduced the number of cultures containing spreading *Proteus* from 23 to 0, and this was accompanied by a statistically significant increase in isolations of gonococci (10%). This increase may be underestimated, however, since only one rectal culture was obtained, and these more often contain *Proteus*. In addition, we were unable to detect any inhibition of growth of gonococci due to the presence of trimethoprim. However, there has been a report that a 5.0 µg/ml concentration of trimethoprim inhibited growth of 12 to 22% of the gonococcal strains tested (11).

Routine incubation of cultures in the plastic bag with the TABCO<sub>2</sub> tablet produced an increase in humidity within the bag, and this catalyzed the generation of carbon dioxide. Laboratory tests of the plastic bags with carbon dioxide-generating tablets demonstrated that adequate levels of carbon dioxide were produced to permit initial growth of gonococci.

TABLE 2. Gas chromatography measurements of carbon dioxide in plastic bag-TABCO<sub>2</sub> system at selected incubation times

Incubation time (h)	Range <sup>a</sup> (%)	Avg <sup>a</sup> (%)
0	0.00-0.03	0.02
1	1.25-1.75	1.48
2	1.85-2.55	2.23
4	1.40-1.85	1.60
8	1.05-1.65	1.30
24	1.80-3.40	2.91

<sup>a</sup> Four to five determinations per time interval.

Clinical trials of the plastic bag with the carbon dioxide tablet showed that this incubation system is equally as effective as the candle jar. However, the plastic bag and tablet system has the advantage of requiring less incubator space, being far less cumbersome, and being relatively unbreakable. In addition, the system has potential as an alternate transport system for gonococci and might be applicable to other pathogenic bacteria that prefer special conditions of incubation, e.g., streptococci or meningococci.

The small-scale clinical trials reported here may be representative of the performance to be expected of the plastic bag-tablet system, but this can only be accurately determined by field trials in large epidemiological surveys for carriers of gonococci. If these trials are successful, use of this system with MTM medium may overcome some of the technical problems associated with the isolation and transport of gonococci.

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