Comparison of Atmospheric Conditions for Culture of Clinical Specimens of *Neisseria gonorrhoeae*

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We cultured 55 clinical specimens of *Neisseria gonorrhoeae* in the following atmospheric conditions: (i) 10% carbon dioxide in a CO₂ incubator; (ii) a candle extinction jar; (iii) an air convection incubator; and (iv) an anaerobic jar without added CO₂. The number and size of colonies growing on modified Thayer-Martin medium were evaluated after incubation of cultures for 24 and 48 h at 36 C. After 24 h, the specimens from the candle extinction jar had the greatest number and size of colonies, but after 48 h growth was approximately equal for specimens from the candle jar and the CO₂ incubator. Only 19 of 55 specimens grew in the air convection incubator. None of 55 clinical specimens or of 10 laboratory strains grew anaerobically. Development of colonial morphology for colony types 1, 2, 3, and 4 was studied at 24 h on a base medium that contained no hemoglobin. The relative numbers of the four colony types in specimens were comparable after 24 h of incubation in any of the three atmospheric conditions under which growth occurred, but the different types were distinguished most readily when grown in the candle extinction jar.

What are the best atmospheric conditions for isolating *Neisseria gonorrhoeae* from patients? Authors of two recent papers (7, 8) raised this question, and the answer is of concern because of the rising incidence of gonorrhea. Although Ferguson (3) examined the effect of various atmospheric conditions on growth of the gonococcus, his studies were done before the introduction of media now recommended for isolation of *N. gonorrhoeae* (9, 10).

To resolve the question, we compared various atmospheric conditions for their effect on growth of strains of *N. gonorrhoeae* taken directly from patients. Cultures were incubated in a CO₂ incubator containing 10% CO₂ in air, a candle extinction jar, an air convection incubator, and an anaerobic jar. In addition to evaluations for the amount of growth, we also determined the effect of the atmospheric conditions on development of colony types (4, 5). Growth under anaerobic conditions was of particular interest because of the statement in *Bergey's Manual* that ability to grow anaerobically is a characteristic of *N. gonorrhoeae* (1).

**MATERIALS AND METHODS**

**Sample collection and processing.** Strains of *N. gonorrhoeae* were isolated from males reporting to the Social Hygiene Clinic of the Houston City Health Department (HCHD) with symptoms of gonorrhea. The 55 strains were identified as *N. gonorrhoeae* by the HCHD laboratory. Laboratory strains F62, Ngc98, Ngc111, Ngc113, Ngc115, Ngc117, obtained from the Center for Disease Control, Atlanta, Ga., and WP, WOR, VJ, and EE, from the HCHD, were used in some anaerobic experiments.

Samples of exudate were obtained from the urethral orifice on two sterile cotton swabs. One swab was inoculated directly on a plate of modified Thayer-Martin (T-M) medium and sent to the HCHD laboratory for confirmation of infection with *N. gonorrhoeae*. The second swab was placed in 0.5 ml of Trypticase soy broth (Baltimore Biological Laboratory) in a test tube. Within 15 min after collection of the specimen, the test tube with the swab was brought to the laboratory. The tube and its contents were agitated on a Vortex Jr. mixer (Scientific Industries, Inc.), and as much fluid as possible was expressed from the swab, which was then discarded. Four sterile swabs then were inserted into each tube to absorb all broth. After each swab was rolled over half of the surface of an agar
plate, the inoculum was spread evenly by streaking that portion of the plate with a sterile loop, and then was streaked over the other half of the plate, again using a sterile loop, to give well-separated colonies. This method was used for inoculation of all media.

All cultures were incubated at 36°C under four different atmospheric conditions: (i) candle extinction jar containing 3 to 4% CO₂ (3); (ii) CO₂ incubator containing 10% CO₂ in air; (iii) air convection incubator containing 0.03% CO₂ in air (2); and (iv) anaerobically in an anaerobic jar (Gas-Pak, BBL). Moisture was added to the containers, either by a pan of water or by saturated cotton in a petri dish, to insure a comparable relative humidity in all four environments.

Growth was evaluated at 24 and 48 h. We rotated the plates at various angles to a 75-W light source, so that even very small colonies were visible. Each specimen was then assigned a numerical rating from 1, minimal number and size of colonies, to 4, maximal number and size of colonies (Fig. 1).

Twelve strains selected at random were grown on GC base medium for colony typing. Approximately 100 colonies from each plate were typed by the method of Kellogg et al. (5).

Clostridium sporogenes was used as a control for anaerobic conditions. Anaerobes from the urethral exudate, which were not N. gonorrhoeae, grew under these conditions as natural controls.

**Media.** Trypticase soy broth was used as the transfer fluid for maintaining the viability of N. gonorrhoeae on collection swabs en route to the laboratory. For determination of the extent of growth and colony size, T-M medium (9; 36 g/liter of GC base, 1% IsoVitalex, 2% powdered hemoglobin, 1% VCN, all from BBL) was modified by addition of 5 μg of trimethoprim lactate per ml (6, 10). GCB medium (GC base plus 1% IsoVitalex, BBL) without added hemoglobin was used for typing colonies (4, 5).

**RESULTS**

Initial evaluation of growth in air, CO₂ incubator, or candle jar was made at 24 h (Fig. 2). A majority of all the cultures (66%) showed grade 4 growth after incubation in candle jars, as opposed to 36% of those from the CO₂ incubator. Colonies were often significantly larger, as well as more numerous, in specimens from candle jars (Fig. 3). More of the specimens from the CO₂ incubator showed growth of grades 2 or 3 than did those from the candle jar. One of the 55 strains did not produce visible colonies under any atmospheric condition at 24 h, and one strain produced colonies only in the candle extinction jar. Only eight specimens grew in air at 24 h. No growth of N. gonorrhoeae was observed on plates incubated anaerobically, although controls of C. sporogenes and anaerobes from the urethral exudate of patients showed luxurious growth.

Colony types were evaluated for strains grown on GCB medium. No significant differences were observed in the number of the various colony types of strains grown under different atmospheric conditions (Table 1). In addition, there was no discrepancy between the colony types of a single specimen incubated under various conditions. Colonies grown in the candle conditions.
**Fig. 3.** Two representative samples of the same clinical isolate of *N. gonorrhoeae* grown on T-M medium and incubated for 24 h under two different atmospheric conditions: 10% CO₂ (CO₂ incubator) and 3 to 4% CO₂ (candle jar).

**Table 1.** Growth of colonies of each colony type from 12 freshly isolated strains of *N. gonorrhoeae* grown on GCB medium under three atmospheric conditions

| Colony type | Growth under each atmospheric condition (%) | CO₂ incubator | Candle extinction jar | Air |
|-------------|---------------------------------------------|---------------|-----------------------|-----|
| 1           |                                             | 70            | 62                    | 67  |
| 2           |                                             | 20            | 17                    | 17  |
| 3           |                                             | 9             | 17                    | 16  |
| 4           |                                             | 1             | 4                     | >1  |

* No growth was obtained under anaerobic conditions.

jar had, however, an appearance more typical of the characteristics of the four colonial types defined by Kellogg et al. (4, 5) for any period of incubation from 18 to 30 h. The immature appearance of the colonies incubated in 10% CO₂ (Fig. 4a) and in air (Fig. 4c) is evident when compared with colonies of the same age and strain incubated in 3 to 4% CO₂ (Fig. 4b). Colonial types 1 and 2 are readily recognizable in Fig. 4b but cannot be identified with accuracy in Fig. 4a and c.

After 48 h, growth of gonococci was approximately equal whether incubated in 10% CO₂ or 3 to 4% CO₂ (Fig. 5). All 55 strains produced visible colonies, and 95% of all the specimens showed growth of grade 3 or 4. There was an increase in percentage of specimens growing in air from 12 to 36%.

No colonies appeared on T-M medium under anaerobic conditions after 48 h (Fig. 5), nor did prolonged incubation, up to 100 h, result in growth. To determine whether the antibiotics in the T-M medium could have prevented growth, 20 of the 55 strains were cultured on chocolate agar and GCB media. No colonies developed on these plates. Ten laboratory strains were also incubated anaerobically on these three types of media, and none showed any growth.

**DISCUSSION**

*N. gonorrhoeae* grew best at 24 h in candle extinction jars containing 3 to 4% CO₂. We used
fresh clinical isolates of *N. gonorrhoeae* to determine appropriate atmospheric condition because laboratory strains, commonly used in this type of study, have been transferred numerous times on artificial media, and their requirements may be less stringent than those of isolates taken directly from the patient. Colony types of these latter isolates, either in morphology or relative numbers, are not affected significantly by CO₂ levels. However, the 6-h lag in development of typical colonial morphology makes difficult the typing of colonies at 18 to 24 h when grown in air or 10% CO₂.

At 48 h, specimens in candle jars or in a CO₂ incubator were approximately equal in growth. The strains which did grow in air developed like those in 10% CO₂ and reached a comparable level of growth after 48 h.

Since burning a candle not only increases the amount of CO₂ in the atmosphere but also decreases the oxygen tension, one might question which condition of the atmosphere is most important. Ferguson found that simply reducing oxygen tension did not permit the growth of gonococci in 25% of strains as compared with 100% enhancement of growth by increased CO₂ (3). A CO₂ content of 3 to 4% apparently permits cultures of *N. gonorrhoeae* to mature in 24 h. In contrast, higher or lower levels of CO₂ do not enhance growth during the first 24 h, although continued incubation does permit the gonococci to reach similar maturity in 48 h.

*Bergey’s Manual* (1) states that the ability to grow anaerobically is a characteristic that differentiates the gonococcus from the menin-gococcus. Our data do not support this statement. Under anaerobic conditions, neither 55 fresh clinical isolates nor 10 laboratory strains grew on either T-M, chocolate, or GCB media.

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