Anaerobic Survival of Clinical Isolates and Laboratory Strains of *Neisseria gonorrhoeae*: Use in Transfer and Storage

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Eleven laboratory strains and 67 clinical isolates of *Neisseria gonorrhoeae* were tested for the ability to survive during anaerobic incubation. The survival of the laboratory strains was dependent on auxotype, temperature, and cell density on agar plates. For both the laboratory strains and the clinical isolates, anaerobic survival was better at lower temperatures. We concluded that anaerobic incubation, for as long as 7 days, is useful when transporting or storing *N. gonorrhoeae*.

Neisseria gonorrhoeae is an organism that survives and proliferates in the genitourinary tract in a milieu of strict anaerobic organisms (20). Therefore, the oxygen tension of both the transport medium and the culture medium could be critical to the successful cultivation of this microbe. There are two limitations to detecting *N. gonorrhoeae* in clinical specimens: (i) the gonococci may not survive during transportation from the collection site to the laboratory; and (ii) the examination of specimen cultures must be made after an optimum length of incubation, usually 48 h. Several systems, developed for the inoculation of the exudate into a culture transport system (13-15) immediately after collection have, in a clinic or office setting, significantly increased the yield of positive cultures. However, substantial loss of positive results may still occur if transport or incubation is delayed or if there is poor environmental temperature control (2-4, 12, 22).

It has long been recognized that the optimum length of incubation for cultures of *N. gonorrhoeae* is 30 to 48 h (6, 10, 16, 17), and the problem of autolysis of the organism was noted in 1919 by Maitra (11). Morse and Bernstein (18) observed that the onset of autolysis is not due to a decrease in pH but rather to the depletion of the energy source. Because extensive autolysis occurs during long periods of incubation, the possibility that the subculture will be viable for test confirmation is decreased. Furthermore, the problem of arranging the optimum time for examination of the cultures can be complicated by work schedules in laboratories and particularly by weekend and holiday staffing.

Buchanan reported that oxidation, not drying, was detrimental to bacterial cells (1); he protected the gonococci during transport by including a reducing agent, sodium thioglycolate, in the

transport medium of Stuart (21) and by adding a nitrogen gas atmosphere to the vial of medium (8). The use of a 2% agar deep tube to exclude oxygen from the specimen has also been reported (7).

The need for improved methods of transport that would maintain the viability of the cultures and enable the optimum time for culture examination to be controlled is evident. This report deals with the survival of laboratory strains and clinical cultures under strict anaerobic conditions and the feasibility of using anaerobic storage to delay the initiation of incubation, either to make the time for examination more practical or to facilitate the transport of the culture.

MATERIALS AND METHODS

Laboratory strains. Laboratory strains of *N. gonorrhoeae* to be studied for survival after anaerobic incubation were randomly selected from collections of strains that had been suspended in Trypticase soy broth (BBL Microbiology Systems) containing 15% (vol/vol) glycerol and frozen at -70°C. Strains were plated from frozen stock onto GCI agar plates containing GC Medium Base (Difco Laboratories) supplemented with 1% IsoVitaleX (BBL). The cultures were grown aerobically for 18 h at 37°C with 5% CO₂. All media used for the anaerobic study were allowed to prereduce in an anaerobic chamber (Coy Laboratory Products, Inc.) for 48 h before being used; the atmosphere in the chamber consisted of 10% hydrogen, 5% carbon dioxide, and 85% nitrogen. *N. gonorrhoeae* strains grown aerobically were placed in the anaerobic chamber, and a sterile Dacron-tipped swab was used to inoculate 5.0 ml of a prereduced minimal salts solution (9) to an approximate turbidity of 50 Klett units (Klett-Summerson colorimeter, 54 filter). Dilutions of 10⁻⁴, 10⁻⁵, and 10⁻⁶ were inoculated onto multiple (10 each) GCI agar plates to determine viability after different conditions and lengths of incubation. Cultures were incubated anaerobically at 37°C in an incubator within the chamber or were placed within

TABLE 1. Survival of laboratory strains of *N. gonorrhoeae* after anaerobic incubation

Strain	Auxotype ^a	% Survival at ^b :			
		27°C		37°C	
		48 h	96 h	48 h	96 h
RUG 1004 ^c	Arg ⁻ Hyx ⁻ Ura ⁻ Val ⁻	96	96	52	44
CDC 005 ^d	Arg ⁻ Hyx ⁻ Ile ⁻ Ura ⁻ Val ⁻	76	71	32	45
CDC 293	Arg ⁻ Hyx ⁻ Ura ⁻	90	80	11	16
CDC 226	wt	86	75	7.9	5.0
CDC 217	wt	80	80	1.0	10 ⁻²
CDC 229	wt	81	33	10 ⁻²	10 ⁻²
CDC 230	wt	21	14	9.8	10 ⁻²
CDC 224	wt	47	13	10 ⁻²	10 ⁻²
ATCC 19424	Met ⁻	11	6.7	10 ⁻²	10 ⁻²
CDC 225	Pro ⁻	95	1.9	0.3	10 ⁻²
CDC 221	wt	1.7	10 ⁻²	1.0	10 ⁻²

^a Strains were auxotyped by using gonococcal genetic medium (9, 19).

^b Percent survival was determined by using control plates, which had not been incubated anaerobically before being incubated aerobically as described in the text.

^c RUG, Rochester University Gonococcus.

^d Strains designated CDC were obtained from the Centers for Disease Control (500 series).

the chamber proper, where the ambient temperature was 27°C. The remainder of the original 5.0-ml suspension was retained in the chamber and assayed for viability after 24 h of incubation. Two sets of dilution plates were removed immediately from the chamber. One set was immediately incubated at 37°C with CO₂ to determine the viable count at zero time. The second set of inoculated plates was held at 22°C without CO₂ for 24 h before being incubated at 37°C with CO₂ to test for viability after low-temperature, non-CO₂ incubation. The remaining sets of plates were incubated in the anaerobic chamber at 37 or 27°C and, after 24, 48, 72, and 96 h, were aerobically incubated at 37°C with CO₂.

Because some gonococcal strains exhibit a sensitivity to the oxygen indicator resazurin, the agar slants used in the study were prepared in two stages. GCI agar with 1 µg of resazurin per ml was poured to form a butt in a screw-capped tube (15 by 125 mm) and allowed to solidify, and a slant of GCI agar was placed on top of the butt. The agar in the butt of the tube provided an indicator for oxygen contamination, but the resazurin-free slant did not interfere with the growth of the organism tested. The prepared slants, with screw caps loosened, were allowed to prereduc in the chamber for 48 h. In the anaerobic atmosphere of the chamber, aerobically grown cultures were inoculated onto the slants, and the caps were tightened and sealed with a double layer of parafilm before the cultures were removed from the chamber. The inoculated slant cultures were incubated at 37, 22, and 4°C. Any tubes exhibiting O₂ contamination, determined by a change in the resazurin indicator, were discarded. At the desired time, the slant culture was opened to allow the tube to be aerated before being aerobically incubated at 37°C with CO₂ for 48 h, thus allowing the organism to grow.

Clinical strains. Cervical and urethral exudates from female and male patients at the Monroe County Health Department Venereal Disease Clinic, Rochester, N.Y., were collected on sterile Dacron-tipped swabs. The swabs were immediately inoculated onto multiple

Jembec plates (15) containing Martin-Lewis agar (Scott Laboratories, Inc.). A citric acid-sodium bicarbonate tablet was added to the plate that was designated the control to provide CO₂ (14), and the plate was immediately incubated aerobically at 37°C. Corresponding cultures were incubated anaerobically in an anaerobe jar (BBL) containing an anaerobic indicator strip (BBL), and the jar was charged by the addition of a GasPak H₂-CO₂ generator (BBL). The anaerobe jar to be used for incubation at 22°C was left at room temperature, and the anaerobe jar to be used for 4°C incubation was allowed to stand at 22°C (room temperature) for 30 min for the activation of the catalyst before being incubated at 4°C. An alternate system for the development of an anaerobic atmosphere, Bio-Bag (Type A) (Marion Scientific), was also used. The inoculated Jembec plate was placed in the bag with the indicator and H₂-CO₂ generator, and the bag was sealed by using a commercial heat sealing device. The bags were placed at 22 and 4°C after activation of the catalyst for 30 min at 22°C. Controls were incubated for 48 h at 37°C before examination. Anaerobically incubated (22 and 4°C) plates were aerobically incubated at 37°C for 48 h before examination. A CO₂ generation tablet was added to provide CO₂ during aerobic incubation (analogous to the control plate). Morphology, Gram stain patterns, and oxidase reactions were determined after 48 h of aerobic incubation. Subcultures were made to ensure the purity of each culture before carbohydrate utilization was determined.

RESULTS AND DISCUSSION

The ability of *N. gonorrhoeae* to survive anaerobically was first tested by using laboratory strains. Anaerobic survival was both strain and temperature dependent (Table 1). All strains maintained viability better at 27°C than 37°C, and the strains that required arginine, hypoxanthine, and uracil (AHU auxotype) survived better than strains of other auxotypes. The strains

FIG. 1. Growth of *N. gonorrhoeae* laboratory strains after anaerobic incubation. *N. gonorrhoeae* CDC 005 (A) and CDC 221 (B) were inoculated onto GCI agar plates and streaked in a quadrant pattern. The plates were incubated in the anaerobic chamber at 27°C for 5 days, removed, and incubated aerobically for 24 h at 37°C to allow the organism to grow.

of the AHU auxotype grow more slowly than did strains of other auxotypes; their increased anaerobic survival may therefore be a function of a lower rate of metabolism. The increased survival at a lower temperature, 27°C, supports this conclusion. It should be noted that all of the strains tested showed a detectable viability after 48 h of anaerobic incubation at 27°C. This observation is of particular interest because it is well known that *N. gonorrhoeae* rapidly loses viability when incubated aerobically at room temperature.

The results presented in Table 1 document the survival of gonococcal strains that were incubated anaerobically on the surface of agar plates. We also incubated these strains anaerobically in minimal salts solution (9) or GC medium broth (Difco) supplemented with 1% IsoVitaleX (BBL). None of the strains incubated in either substance survived after 24 h of anaerobic incubation (data not shown). Thus, it appears that the presence of agar stabilizes the organism, perhaps preventing death by autolysis.

We performed the experiments which gave the results presented in Table 1 by diluting the *N. gonorrhoeae* cultures sufficiently to yield approximately 10^2 , 10^3 , and 10^4 colonies on the respective dilution plates. If more colonies were inoculated onto the plates an apparent protection was observed. This is demonstrated in Fig. 1, which shows cultures streaked in a quadrant pattern and incubated anaerobically at 27°C for 5 days and then allowed to grow aerobically for 24 h. The CDC 005 strain survived well in all

quadrants, as would be expected from the data in Table 1. The CDC 221 strain did not survive well in the quadrant with low initial numbers of cells but did survive well in the primary quadrant. Thus, a higher cell density in the initial inoculation on the plate appears to afford protection against loss of viability upon anaerobic incubation.

Because of our success in maintaining viability during anaerobic incubation of laboratory strains of *N. gonorrhoeae*, we turned our attention to clinical isolates. Urethral exudate specimens from 95 male patients and cervical exudate specimens from 34 female patients were obtained from the Monroe County Health Department Venereal Disease Clinic. The specimens were inoculated onto Jembec plates (15) containing Martin-Lewis agar and incubated in the presence of CO₂ under each of the following conditions: (i) 37°C, aerobic; (ii) 22°C, aerobic; (iii) 22°C, anaerobic; and (iv) 4°C, anaerobic. The plates incubated aerobically with CO₂ at 37°C were examined for typical gonococcal growth after 48 h and served as controls. After 48 h, plates incubated under conditions ii, iii, and iv were aerobically incubated at 37°C with CO₂ for 48 h. All strains were verified as *N. gonorrhoeae* on the basis of cell morphology, Gram stain reaction, oxidase reaction, and the production of acid from glucose but not maltose, sucrose, or lactose. Specimens in this series were not incubated anaerobically at 37°C because of the overgrowth of anaerobic organisms indigenous to the genitourinary tract.

TABLE 2. Anaerobic survival of clinical isolates

No. and sex of patients	No. of positive specimens ^a			
	Aerobic, 37°C, zero time	Aerobic, 22°C, 48 h	Anaerobic, 22°C, 48 h	Anaerobic, 4°C, 48 h
Male				
22	12	10	12 ^b	12
59	37	ND ^c	37 ^b	ND
14	7	ND	7 ^d	ND
Female				
34	11	ND	11	ND

^a One specimen was taken from each patient. See the text for a detailed explanation of incubation conditions.

^b Cultures were incubated anaerobically in a GasPak system.

^c ND, Not determined.

^d Cultures were incubated anaerobically in a BioBag system.

The results obtained for clinical isolates of *N. gonorrhoeae* are summarized in Table 2. All specimens that were positive for *N. gonorrhoeae* after immediate incubation at 37°C were also positive if they were incubated anaerobically for 48 h at either 22 or 4°C before aerobic incubation. Corresponding specimens incubated aerobically for 48 h at 22°C before being incubated at 37°C showed either a decrease in organism number or a complete absence of *N. gonorrhoeae* colonies (Fig. 2).

Clinical specimens in this study were initially incubated anaerobically at 37°C in GasPak jars for 48 h before being aerobically incubated.

After the initial anaerobic incubation, we occasionally noted that in addition to the growth of anaerobic gram-negative bacilli, some specimens exhibited a slight growth of organisms that proved to be gonococci. A study in which strict anaerobic conditions were used was undertaken to investigate this growth, which was thought to have occurred because of residual oxygen in the medium. Specimens of male urethral exudates were immediately placed into anaerobic transport tubes (AnaCult; BBL) and then inoculated onto prereduced Martin-Lewis agar plates. Plates were incubated in the anaerobic chamber at 37°C for 48 h. Corresponding controls were incubated aerobically at 37°C with CO₂. Of 31 specimens, 21 were positive for gonococci after aerobic incubation. Of these 21 positive specimens, 3 showed small, discrete colonies after incubation under strict anaerobic conditions. Gram-stained smears of these colonies showed normal-appearing, gram-negative diplococci, similar in size and shape to aerobically grown *N. gonorrhoeae* and having none of the bizarre forms noted by Ferguson (5). Attempts to subculture these isolates anaerobically were not successful. Subcultures removed from the chamber and incubated aerobically grew as well as the matching controls. The organisms were identified as *N. gonorrhoeae* because they were gram-negative diplococci, oxidase positive, and able to produce acid from glucose but not maltose, sucrose, or lactose. Auxotyping was carried out (9, 19) on the anaerobically grown isolates and their matching aerobic control isolates, and identical results were obtained. Auxotypes of the three isolates were zero auxotype (wt), Arg⁻, and Val⁻ Pro⁻. Attempts to reinitiate

FIG. 2. Growth of *N. gonorrhoeae* clinical isolates after being incubated under various conditions. A single urethral specimen was inoculated onto four Jembec plates and incubated under the following conditions: 37°C, aerobic (A); 22°C, aerobic (B); 22°C, anaerobic (C); and 4°C, anaerobic (D) (see the text). Incubations under the conditions described for B, C, and D were followed by 48 h of aerobic incubation at 37°C to allow the organism to grow.

anaerobic growth of these organisms after aerobic subculturing by the addition of the electron acceptors KNO_3 or fumaric acid (or both) to the medium were not successful.

In summary, we determined that both laboratory strains and clinical isolates of *N. gonorrhoeae* survived when incubated anaerobically on agar. The survival rates of laboratory strains were higher when lower temperatures were maintained. In fact, clinical isolates were observed to survive well at 4°C, a temperature that rapidly results in a loss of viability of aerobically incubated gonococci.

Anaerobic incubation could potentially be very useful for delaying the initiation of growth of clinical specimens. It would be very convenient for a clinical laboratory which is not staffed on weekends or holidays to be able to wait until Monday to grow specimens received on Friday. In addition, viable colonies would always be available for subculturing so that test results could be confirmed.

We also used anaerobic incubation in transporting gonococcal strains to other laboratories. Agar slant tubes, with a resazurin butt to indicate the maintenance of anaerobiosis, were prepared as described above. An *N. gonorrhoeae* strain was heavily inoculated onto the slant in the anaerobic chamber and the tube was sealed. Such a tube could be sent in a standard culture mailing tube through first-class mail regardless of season, since temperature does not appear to affect the anaerobic survival of heavy inocula. This eliminates the expense and preparation necessary to transport cultures in a frozen state by special carrier; in addition, the loss of viability that often occurs with lyophilized cultures is avoided.

Thus, anaerobic conditions may be extremely important in the transport of clinical and research strains. It must be noted, however, that the anaerobic incubation conditions did not result in an *N. gonorrhoeae* isolation rate greater than that of standard techniques.

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