APPLIED MICROBIOLOGY, Feb. 1973, p. 216-221

Vol. 25, No. 2
Printed in U.S.A.

Simple Method for Culturing Anaerobes

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Received for publication 3 August 1972

A simple, effective method is needed for growing obligate anaerobes in the clinical laboratory. This report describes a pre-reduced anaerobic bottle that can be taken to the bedside for direct inoculation, provides a flat agar surface for evaluation of number and morphology of colonies, and can be incubated in conventional bacteriological incubators. Each anaerobic culture set consisted of two bottles containing brain heart infusion agar and CO_2. Gentamicin sulfate (50 μg/ml) was added to one of these to inhibit facultative enteric bacilli. Comparison of the anaerobic bottles with an identical aerobic bottle which was also routinely inoculated permitted early identification of anaerobic colonies. Representative species of most anaerobic genera of proven pathogenicity for man have been isolated from this system during 10 months of routine use.

A more satisfactory method is needed for growing obligate anaerobes in the clinical laboratory, because only a minority of these exacting bacteria grow in anaerobic jars used in the conventional manner (12, 13). Pre-reduced anaerobically sterilized (PRAS) roll tubes as described by Hungate (5) and modified by Moore (7) and Hungate (6) increase the yield of anaerobes, but their use is complicated and time-consuming because it involves the simultaneous handling of rubber stoppers, gas outlets, and roll tubes during primary inoculation and transfer of cultures. A special apparatus of streaking prehardened roll tubes (7), a machine for rotating the tubes while the agar is hardening (7), and modified microscopes for photomicrography of colonies in PRAS tubes (2) have been devised to simplify this system. Instead, they emphasize and accentuate its basic complexity.

Glove boxes (1, 4, 9, 11) are other complicated devices for growing obligate anaerobes, and they take up too much space in the busy clinical laboratory when used as anaerobic incubators and for anaerobic storage of media. Moreover, these complex methods that yield more anaerobes in the experimental laboratory are no more effective than the anaerobic jar in the isolation of anaerobes from clinical specimens (J. E. Rosenblatt, A. M. Fallon, and S. M. Finegold, Abstr. Annu. Meeting Amer. Soc. Microbiol., p. 94, 1972).

This report describes a system that was designed to meet the clinical need for a simple, effective method for recovering obligate anaerobes. It is a pre-reduced anaerobic bottle that can be taken to the bedside for direct inoculation, provides a flat agar surface for gross or microscopic evaluation of colonial morphology, and can be incubated in conventional bacteriological incubators. Its efficiency was evaluated by testing it on a routine basis in the clinical laboratory by three criteria: (i) recovery of bacteria that did not grow aerobically but were observed in Gram stains; (ii) isolation of a wide range of pathogenic anaerobic bacteria; and (iii) comparison to a GasPak jar closed immediately after the inoculation of a single specimen and not opened for 48 hr. By all of these criteria, the anaerobic bottle described in this report has been a spectacular success.

MATERIALS AND METHODS

Preparation of the anaerobic culture bottles. Nonselective medium was prepared by dissolving 26 g of brain heart infusion (BHI) agar in 500 ml of boiling distilled water. Amounts of 5 ml were pipetted into 4-oz (ca. 120-ml) medicine bottles. The bottles were immediately stoppered with red rubber stoppers (catalogue no. 2330, Arthur H. Thomas Co., Philadelphia, Pa.) and evacuated to 20 μm of Hg with a vacuum pump; 7.0 ml of CO_2 was then added by syringe (final concentration, about 5%). The 100% CO_2 was obtained by connecting the syringe to a cylinder of the compressed gas through sterilized rubber tubing of appropriate diameter. The stoppers were covered with wrapping paper or foil, and the bottles were autoclaved for 15 min at 15 psi. The bottles were laid flat while cooling so that a thin layer of agar hardened on one side. A small quantity to clear, colorless water of condensation formed in the bottles after the agar hardened.

Selective medium was prepared exactly as the nonselective except that 50 μg of gentamicin sulfate per ml was added to the boiling water immediately after addition of the BHI agar. The concentration of gentamicin after autoclaving was sufficient to suppress the growth of almost all enteric facultative anaerobes.

As an aerobic control, a third bottle containing air but no gentamicin was prepared in an identical fashion. The aerobic bottle was closed with a black screw cap which was loosened after inoculation. Anaerobic growth was indicated by the appearance of colonies in the anaerobic bottles that did not grow in the aerobic bottles.

Method of inoculation. Liquid specimens were carefully injected into the bottle by syringe so that air was not introduced. Material was removed from swabs by immersion into a 4-ml tube of BHI broth containing 0.2% agar which was then aspirated into a syringe and injected in the same fashion as liquid material. Amounts of 1 ml of each specimen were inoculated into the selective, the nonselective, and the aerobic bottles. Swabs were transported to the laboratory immersed in deep tubes of Stuart's transport media.

To ensure isolated colonies, Gram stains were made immediately on smears of purulent and putrid materials. If microscopic examination showed that the bacterial inoculum would be heavy, a 1:100 dilution in BHI broth was used to inoculate the nonselective and aerobic bottles. The selective (gentamicin) bottle was always inoculated from undiluted liquid material or the original BHI broth.

The inoculated bottles were placed on the flat side (agar down) for 10 min. This step allowed the specimen to spread and inoculate the agar.

Culture methods. The agar was examined daily for colonies that did not grow in the aerobic bottle. As soon as these appeared (often in 24 hr), they were subcultured by opening the bottle and inoculating them into appropriate media for identification and onto agar plates containing 5% rabbit blood. Anaerobes were identified according to standard methods (10). Subcultures were incubated in Brewer jars with GasPak envelopes (disposable hydrogen plus carbon dioxide generator envelopes, BBL). The bottle was quickly stoppered and reevacuated so that additional anaerobic growth could develop. The other anaerobic bottle was left unopened to allow slower anaerobic growth to materialize with no exposure to air.

Comparison of culture results and Gram stains. Gram stains were made of smears of all specimens before they were inoculated into the anaerobic bottles. Correlations were then made between bacteria observed in Gram stain and culture results. The aim was to determine whether bacteria seen in the Gram stain, and not evident in aerobic culture, could be recovered in the anaerobic culture.

Comparison of the anaerobic bottles to the GasPak system. Sixty specimens of patient material obtained from abscesses, infected wounds, and purulent body fluids were cultured simultaneously in both systems. Media for incubation in GasPak jars included agar plates of BHI, BHI plus gentamicin sulfate (50 μg/ml), and 5% rabbit blood in blood agar base. Agar plates were freshly prepared every 3 days and stored at 4 C until the day of use. The method of inoculation was as described above and was identical for each system, except that the inoculum for the agar plates was two drops of the liquid material or the undiluted BHI broth streaked across the surface in the conventional manner. As soon as the plates from one specimen were inoculated, they were placed in a Brewer jar, the GasPak envelope was activated, and the jar was sealed. In no instance was the Brewer jar opened before 48 hr of incubation. Cultures were examined serially and not discarded before 7 days of incubation. All colony types developing on the agar plates and the anaerobic bottles were identified, and the results were tabulated for comparison.

RESULTS

Specimens were obtained from all services of a University Hospital with an average census of 300. All pus and body fluids except sputum and urine and all putrid material except feces were cultured anaerobically (7,500 specimens). Specimens obtained from the mouth, nasopharynx, cervix, and vagina were cultured anaerobically only on special request. During a 10-month period 2,242 anaerobes were isolated from 1,104 cultures (14.7% of 7,500). Representative species of many genera of medically important anaerobes were isolated by this technique (Table 1). *Bacteroides*, especially *B. fragilis* (437 isolates), was the most common organism and was frequently recovered in pure culture or as the only anaerobe present. *B. melaninogenicus* was the second most common *Bacteroides* species (224 isolates) and the third most common anaerobe. *Streptococcus* was the next most common genus, with *S. intermedius* (271 isolates) second only to *B. fragilis* in the total number of isolations. Many of the medically important clostridia (18 species) were identified during the period of this study with *Clostridium perfringens* most common (62 isolates). The anaerobic bottle was also very efficient in the isolation of the various members of the *Actinomyces* group (26 isolates).

Anaerobes often grew in the form of discrete colonies so that pure subcultures were easily obtained. At first, the growth in about one-third of the cultures was so heavy that subcultures of a sweep of the agar surface were required to obtain isolated cultures. Later on, it was found that dilution of specimens shown by Gram stain to contain heavy inocula substantially reduced the number of cultures with confluent growth. Gentamicin sulfate at a concentration of 50 μg/ml inhibited the facultative enteric rods more effectively than sodium azide (0.1%), which was used during the first few

TABLE 1. *Anaerobic bacteria isolated during 10 months of use of the anaerobic bottle*

Organism	Pure culture	Mixed with other anaerobes only	Mixed with aerobes only	Mixed with aerobes and anaerobes	Total anaerobes isolated	Sources of specimen[a]
Bacteroides fragilis	30	15	154	238	437	a,c,d,e,g,i,j,m,n,o,p,q,s,w
B. melaninogenicus	—	19	17	188	224	a,b,c,d,e,f,g,j,k,l,n,o,s,w,z
B. oralis	3	15	14	113	145	a,b,d,e,g,j,k,l,m,n,o,q,w
B. nodosus	3	15	5	89	112	a,b,d,e,f,g,k,o,w,q,t
B. variabilis	—	2	7	58	67	a,d,g,k,n,q,s
B. corrodens	—	10	7	57	74	a,b,c,d,g,j,k,n,o,t
Sphaerophorus necrophorus	2	1	—	14	17	a,b,d,f,k
Fusobacterium fusiforme	—	4	1	31	36	a,b,d,e,g,k,o
Clostridium perfringens	5	3	17	37	62	a,d,e,g,h,k,o,s,t,w
Clostridium species[b]	9	4	9	93	115	a,d,g,h,k,o,p,q,s,t,w
Streptococcus intermedius	10	31	45	185	271	a,c,d,f,g,j,k,n,o,r,t
S. micros	3	4	7	21	35	a,b,c,d,f,g,j,k,r,t
S. anaerobius	1	2	4	26	33	a,b,c,d,g,k,t
S. productus	—	3	1	21	25	a,d,g,k,t
S. parvulus	3	2	2	12	19	a,b,c,g,k,m,o,r
Anaerobic corynebacteria	11	6	32	38	87	a,c,d,e,g,k,n,o,t
Gaffkya anaerobia	4	5	30	44	83	a,c,d,e,g,k,n,o,t
Anaerobic *Lactobacillus*	2	7	3	59	71	a,b,d,g,j,k,n,o,q,s,t,v
Actinomyces israelii	1	—	—	1	2	g,a
Actinomyces species[c]	—	—	6	18	24	a,b,c,f,g,k
Eubacterium species	—	2	2	13	17	a,g,k,o
Veillonella	1	8	45	145	199	a,b,c,d,e,g,j,k,o,s,t,w
Miscellaneous[d]	4	9	11	63	87	a,c,d,g,i,k,n,r,w,t
Totals	92	167	419	1,564	2,242	

[a] (a) Intra-abdominal or pelvic infections; (b) mandibular abscess; (c) cellulitis of face or jaw; (d) surgical wound infections; (e) gall bladder or bile drainage; (f) brain abscess or meningitis; (g) respiratory secretions obtained by tracheal puncture, bronchial washings, or endotracheal tube; (h) gas gangrene of extremity; (i) joint fluid; (j) empyema; (k) skin infections or abscess (other than face or jaw); (l) mediastinal infections; (m) osteomyelitis; (n) urine or prostatic secretion; (o) peritonsillar abscess; (p) bowel prep; (q) kidney; (r) sinus; (s) lung; (t) eye.

[b] Eighteen well-defined species were isolated and identified.

[c] Includes *Actinomyces naeslundii*, *A. odontolyticus*, *A. eriksonii*, and unspeciated strains.

[d] *Vibrio sputorum*, *Propionobacterium*, *Fusobacterium* spp., *Peptococcus*, anaerobic *Sarcina*, *Catenabacterium* spp., *Streptococcus lanceolatus*, *Ramibacterium* spp., and *Bacteroides* spp.

weeks of this study. Because confluent growth is often due to enteric organisms, isolated colonies of anaerobes were usually available on the gentamicin bottle even when the companion bottle was overgrown with enteric bacilli. Occasional strains of *Bacteroides corrodens* and *Sphaerophorus necrophorus* have failed to grow on the gentamicin-containing agar, despite good growth in the bottle containing only BHI agar and CO_2.

In only one instance were bacteria seen in the Gram stain that could not be accounted for by the isolates obtained from the anaerobic bottles. One strain of *S. necrophorus* (*Bacteroides funduliformis*) seen on the Gram stain of purulent drainage from the ear and the cerebrospinal fluid obtained from a child with meningitis grew in thioglycolate broth but failed to grow either in the anaerobic bottles or on blood agar and BHI agar plates incubated in the GasPak jar. Seventeen other strains of *S. necrophorus* (Table 1) grew well in the anaerobic bottles.

There were 24 positive cultures obtained from the 60 specimens cultured simultaneously in the anaerobic bottles and on agar plates of BHI, BHI plus gentamicin, and 5% rabbit blood incubated in GasPak jars. As shown in Table 2, 64 strains were isolated from the anaerobic bottle and 56 from the GasPak system. There were three negative cultures in the GasPak system that showed organisms on the Gram stain and produced growth in the anaerobic bottles. These included *B. fragilis*, *B. melaninogenicus*, and *Streptococcus intermedius* from an abscess, *Clostridium* species from an abdominal fluid, and *B. oralis*, *Gaff-*

kya anaerobia, and *Clostridium capitovale* from an abdominal drain. The GasPak system also failed to isolate from an infected abdominal incision a *B. fragilis* strain that grew heavily on the anaerobic bottles in mixed culture with a *C. perfringens*. The only significant isolate that failed to grow in the anaerobic bottle was an *Actinomyces israelii* strain that was not seen on Gram stain and produced two colonies on the blood agar plate incubated in the GasPak jar. This organism, which was isolated from material excised from a late abdominal wound infection, grew well on the anaerobic bottles on subculture. One strain each of anaerobic *Lactobacillus*, anaerobic *Corynebacterium*, and *Veillonella* that grew in the anaerobic bottles failed to grow in the GasPak system but were not considered failures in Table 2 because they were present in mixed culture with heavier growth of more important anaerobes. One strain each of *Gaffkya anaerobia* and anaerobic *Corynebacterium* that grew in the GasPak system failed to grow on the anaerobic bottles and were not considered failures for the same reason.

TABLE 2. *Comparison of anaerobic bottles with agar plates incubated in GasPak jars*[a]

Bacterium	No. of isolates		
	Bottles	Plates	Combined
Bacteroides fragilis	15	13	15
B. melaninogenicus	6	6	6
B. variabilis	6	6	6
B. nodosus	5	5	5
B. oralis	4	3	4
Fusobacterium fusiforme	5	5	5
Streptococcus intermedius	3	2	3
S. parvulus	2	2	2
Gaffkya anaerobia	2	2	3
Anaerobic corynebacteria	3	2	4
Clostridium perfringens	2	2	2
Clostridium species	6	4	6
Veillonella	2	1	2
Anaerobic *Lactobacillus*	3	2	3
Actinomyces israelii	0	1[b]	1
Total isolates	64	56	67
No. of positive cultures	23	21	24
Failures[c]	1	4	—

[a] Results of 24 positive cultures.
[b] Two colonies of *A. israelii*.
[c] Number of cultures in which significant anaerobes were not recovered.

DISCUSSION

The anaerobic bottle described in this report is designed to meet the need of clinical laboratories for a simple effective method of culturing clinically significant anaerobic bacteria. It is not intended to replace the glove box and PRAS roll tube in those laboratories engaged in sophisticated research in anaerobic bacteriology. The efficiency of this anaerobic system was tested by two primary criteria: (i) recovery of bacteria that did not grow aerobically but were observed in Gram strains and (ii) comparison with the GasPak jar closed immediately after the inoculation of a single specimen and not opened for 48 hr. Correlation of culture results with Gram stains is a more realistic standard of efficiency for clinical specimens than a comparison with other anaerobic culture systems, because it tests the capacity of a system to recover all significant organisms instead of testing its relative efficiency with respect to other methods. By this criterion of recovering representatives of all bacteria seen on Gram stain, the anaerobic bottle has been a spectacular success. The only failure was one strain of *Sphaerophorus necrophorus* that also failed to grow in the GasPak system. Seventeen other strains of *S. necrophorus* were isolated during the 10-month period of this study. The wide range of anaerobic bacteria shown in Table 1 shows that representative species of nearly all anaerobes of proven pathogenicity have been recovered.

Comparison with the GasPak system also provided interesting results (Table 2). Although the GasPak jar used in this unconventional and impractical manner (entire jar for a single specimen) was more efficient than anticipated, the anaerobic bottles were clearly superior. Only one significant organism, *Actinomyces israelii*, failed to grow in the anaerobic bottles. This was not seen in the Gram stain and formed only two colonies on the blood agar plate. The GasPak system, however, failed to isolate significant anaerobes from 4 of the 24 positive cultures and produced only 56 isolates compared to 64 anaerobes identified from the anaerobic bottles.

The frequent successful isolation of the exacting, gram-negative, nonsporeforming rods (*Bacteroides* spp.), even without accompanying growth of aerobic bacteria to lower the E_h (Table 1), underscores the effectiveness of the anaerobic bottle. This simple method depends on pre-reduction by evacuation of oxygen before inoculation of the specimen. Daily inspection of the agar surface for colonies is achieved without exposure to air. The presence of gentamicin sulfate (50 μg/ml) in one bottle greatly

reduces the number of facultative, gram-negative rods and simplifies management of the cultures. Experienced technologists adapt readily to the use of the anaerobic bottles and are quickly able to differentiate colony types and to select single colonies for subcultures.

Despite occasional reports (14; Rosenblatt et al., Abstr. Annu. Meeting Amer. Soc. Microbiol., p. 94, 1972) to the contrary, other simple methods used in clinical anaerobic bacteriology are not effective in most laboratories. Blood agar plates incubated in evacuated jars provide no method for anaerobic storage of media, cannot be examined daily without exposure to air, and, when used in the conventional manner, support the growth of only a minority of exacting anaerobes (12, 13). Highly reduced liquid or semisolid media are not quantitative; facultative organisms frequently overgrow the anaerobes, and some anaerobes are inhibited by oxidized reducing agents. Further, serial subcultures are needed to detect growth and to obtain isolated colonies, so that bacterial identification takes too long.

Most attempts to improve anaerobic culture methods aim at lowering and rigidly controlling the E_h. Even momentary exposure of the specimen to atmospheric oxygen must be avoided to get the best anaerobic culture results (4, 12, 13). Inhibitory products are also formed during the storage of media in the presence of oxygen (3, 8). During the past 10 years the two major improvements in the cultivation of anaerobes are the PRAS roll tubes and the glove box. The combined use of these techniques could prevent exposure of the specimen and the media to atmospheric oxygen. The glove box alone, no matter how rigidly controlled, will not recover anaerobes exposed to air while sitting on the ward and the laboratory bench. The use of the PRAS roll tube as the sole technique in anaerobic culture work is tremendously complex, as it involves special machinery and special skills. The preference of most laboratories for agar plates incubated in evacuated jars comments pointedly on the complexity, expense, and general unacceptability of the glove box and the PRAS roll tube. These methods have been simplified recently (1, 4, 6, 7), but not enough to make them acceptable to most routine laboratories.

The anaerobic bottle described in this report can be inoculated at the bedside. Subcultures may be made to agar plates incubated in GasPak jars or to additional anaerobic bottles. Ideally, oxygen and oxidative products of media should be excluded from the specimen at each step in management from the time of collection to final identification. Subcultures of the heavy inocula obtained from colonies on the anaerobic bottles, however, invariably produced growth on agar plates incubated in GasPak jars.

By inoculating a third bottle containing air, presumptive anaerobic culture results may often be reported within 24 to 48 hr by comparing colonies on the agar surfaces of aerated and anaerobic bottles. Routine, daily examination of the agar surfaces for colonies appearing only in the anaerobic bottles is achieved without exposure to air. The PRAS roll tube could not be used in the same manner as our anaerobic bottle, because the flat agar surface in the bottle is necessary for inoculation without a platinum loop. As the specimen spreads out over the flat surface, it often inoculates the surface with *isolated* colonies (Fig. 1) that can be obtained for pure subcultures. The roll tube, on the other hand, must be inoculated with a loop because of the curved surface. The flat agar surface of the anaerobic bottle is also better for direct evaluation of the colonial

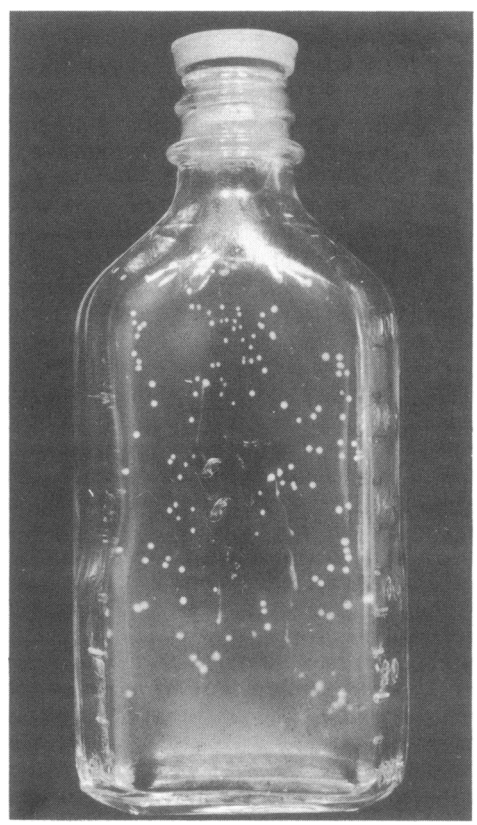

FIG. 1. *Colonies of Bacteroides fragilis after 5 days of growth on the pre-reduced anaerobic bottle.*

morphology, the free-standing bottle is more convenient for bedside use and cultivation in standard bacteriological incubators, and the flat agar layer is much easier to prepare. For these reasons, the bottles described in this report would seem to offer the clinical laboratory an effective but much simpler method for routine diagnosis of anaerobic infections.

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