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Anaerobic Bag Culture Method

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In a new method of anaerobic culture, a transparent, gas-impermeable bag is used and the anaerobic environment is established with copper sulfate-saturated steel wool. An Alka-Seltzer tablet generates carbon dioxide. The agar plate surface can be inspected through the bag at any time without interrupting the anaerobic atmosphere or disturbing other specimens. Methylene blue indicator strips are completely reduced by 4 h after the bag is set up and have remained reduced for as long as 3 weeks. Growth of 16 different stock culture anaerobes was generally equivalent by the bag and GasPak jar methods. Yield and growth of anaerobic isolates also were equivalent with 7 of 10 clinical specimens; from the other 3 specimens, 13 isolates were recovered, 5 by both the bag and jar methods and the rest by one method or the other. No consistent differences were found between the anaerobic bag and GasPak jar methods in the yield of anaerobes from clinical specimens. Early growth (24 h of incubation) of anaerobes from one specimen was detected with the bag method.

Currently used methods for culturing anaerobes include the glove box, roll tubes, and the anaerobic jar. Although the yield of anaerobic isolates from properly collected and transported clinical specimens has been approximately equal from these systems (6), each one has certain disadvantages. Because the glove box is expensive and requires considerable space, it is often not suitable for the average clinical laboratory. The roll tube method requires a special apparatus and prereduced media, making this system also somewhat expensive and technically difficult. Anaerobic jars usually contain multiple plates from a number of different specimens and generally are not opened until after 48 h of incubation, so individual plates cannot be inspected without opening the jar and exposing all the plates to oxygen at the same time. Proponents of the roll tube and glove box systems point out that the ability to inspect and work with an individual roll tube or plate without exposing other specimens to oxygen is a significant advantage. The recognition of growth in these cultures after only 24 h of incubation can result in more rapid reporting of results to clinicians.

We developed and studied a method of anaerobic culture that allows use of conventional plating media in an individual unit that can be inspected and processed without exposing other plates to oxygen. Individual plates are sealed within a transparent, gas-impermeable bag containing steel wool saturated with copper sulfate as the method of establishing an anaerobic atmosphere (2, 5). This report describes that system and our experience in comparing the growth of stock cultures and clinical isolates in the anaerobic bag and in the conventional GasPak anaerobic jar.

MATERIALS AND METHODS

The transparent gas-impermeable bag is available commercially (Anaerobag, Cedanco Co., Framingham, Mass.). Each bag is 30.5 by 51 cm and can be cut into four smaller bags of equal size; the edges are heat sealed by using a bar-type heat sealer (National Scientific Co., catalogue no. A5015). The undersurface of the agar plate lid is swabbed with a few drops of an antifogging solution (Keep Klear, Valspar Corp., Lyons Ill.) to prevent accumulation of moisture that would impair visibility. A 5-g portion of grade 0 steel wool is placed in an appropriate-sized container (plastic cassette tape holders have been used by us) and is saturated by pouring 50 ml of an acidified copper sulfate solution (2) over it (this solution should be freshly prepared each week); the excess copper sulfate solution is drained off. The inoculated agar plate is placed in the bag first and then, as rapidly as possible, the steel wool container with one-quarter of an Alka-Seltzer tablet in a small piece of aluminum foil beside it and a methylene blue indicator strip (GasPak) are placed in the bag, a small drop of water is placed on the Alka-Seltzer tablet, and the open end of the bag is sealed (Fig. 1). It may be useful to include a desiccant in the bag (we have used Drierite enclosed

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FIG. 1. *Sealed bag after 4 h of incubation at 37 C. Moisture condenses over steel wool at room temperature but agar surface is visible through the bag. Methylene blue strip is completely reduced (white).*

in a small plastic bag) and to aspirate air from the open end of the bag (with rubber tubing connected to a vacuum source) for a few seconds just before sealing the bag. However, we did not routinely use these measures and have no evidence that they are necessary. Measurements of oxygen and carbon dioxide concentrations in the sealed bag at various intervals during incubation at 37 C were made by mass spectroscopy (Medspec medical mass spectrometer, model MS-8).

Standard inocula of 16 different stock culture isolates were prepared by initial subculturing to a brucella blood agar plate and subsequent subculturing of isolated colonies after 72 h of incubation to thioglycolate broth. A loopful (0.01 ml) of the 24-h thioglycolate broth culture was streaked on each plate used in the study. The anaerobic bag and GasPak jar (using GasPak H_2-CO_2 generator envelopes) were set up simultaneously and incubated for 48 h. The anaerobic bag also was inspected after 24 h of incubation for notation of growth and colony size. Comparison of colony size and numbers was done by one observer only. Colony size was evaluated as tiny, small, medium, or large; number of colonies present was evaluated on a scale of 1+ to 4+, depending on the number of colonies in the orginal inoculating site and in the first, second, and third streak areas.

Recoveries of anaerobes from 10 clinical specimens by the two methods were compared. All specimens were collected by members of the Section of Infectious Disease from infected patients using techniques that avoided contamination with normal flora and were placed in anaerobic transport vials (1). In the laboratory, the specimen was aspirated from the transport vial with an 18-gauge needle and syringe. All plates utilized in the study were inoculated simultaneously with two drops from the 18-gauge needle per plate. A brucella blood agar plate and a brucella agar plate containing 5% lysed sheep blood and kanamycin and vancomycin were inoculated for each specimen and each method. Plates were examined after 48 h of incubation for comparison of anaerobic growth. The anaerobic bag was also examined after 24 h to determine early growth in that system. Plates having no anaerobic growth were reincubated for 3 weeks and reexamined at that time. Isolation and identification methods were those described by Sutter et al. (7).

RESULTS

Oxygen and carbon dioxide measurements. Oxygen concentrations (mean of two values) measured in the anaerobic bag were 7.3% at 30 min of incubation, 3.7% at 60 min, 0.7% at 90 min, and 0.14% at 189 min. In the GasPak jar, the values (measured by J. Finegold using an oxygen sensor [Beckman 39553] and analyzer [Beckman Fieldlab]) (mean of two) at 37 C were 1.7% at 30 min, 0.3% at 60 min, 0.2% at 90 min, and 0.17% at 180 min.

Carbon dioxide concentration in the anaerobic bag was 6.6% (mean of four values) at 90 min.

The methylene blue indicator strips in the bags were partially reduced (pale blue color) at 180 min and were completely reduced by 4 h. In four anaerobic bags incubated for 3 weeks, the indicators remained reduced for the entire period.

Comparison of stock cultures. The results with single stock-culture isolates of 16 different anaerobes are shown in Table 1. In only one instance was an organism (*Fusobacterium varium*) judged to have formed larger colonies in the jar method. Quantitative growth was somewhat greater in the jar method with two

organisms (*Peptostreptococcus micros* and *Propionibacterium acnes*). Because of the subjective and inexact nature of these measurements, these small differences were not considered significant, and overall growth of stock anaerobes was thought to be equivalent by the two methods.

TABLE 1. *Recovery and colony size of stock-culture anaerobes by anaerobic bag and GasPak jar methods (48-h incubation)*

Organism[a]	Quantitative recovery[b]		Colony size[c]	
	Bag	Jar	Bag	Jar
Bacteroides fragilis	2+	2+	Tiny	Tiny
B. melaninogenicus	4+	4+	Medium	Medium
Fusobacterium mortiferum	3+	3+	Medium	Medium
F. necrophorum	3+	3+	Large	Large
F. varium	4+	4+	Medium	Large
Peptostreptococcus anaerobius	4+	4+	Small	Small
Peptococcus magnus	4+	4+	Tiny	Tiny
Peptostreptococcus micros	1+	2+	Tiny	Tiny
Veillonella alcalescens	4+	4+	Small	Small
V. parvula	4+	4+	Small	Small
Clostridium innocuum	4+	4+	Medium	Medium
C. ramosum	4+	4+	Medium	Medium
Actinomyces visocsus	4+	4+	Medium	Medium
Eubacterium lentum	4+	4+	Small	Small
Lactobacillus plantarum	4+	4+	Medium	Medium
Propionibacterium acnes	2+	3+	Tiny	Tiny

[a] Single isolate of each organism.
[b] Quantitation based on number of colonies present in original inoculum site and first, second, and third streak areas.
[c] Colony size determined by gross visual inspection.

Comparison of clinical specimens. Recoveries of anaerobes were compared from the following 10 clinical specimens: respiratory secretions (percutaneous tracheal aspiration), five; pus from area of osteomyelitis, two; and pus from subphrenic abscess, appendicitis wound, and empyema, one each. Recoveries of specific organisms and their growth were identical from seven of the ten specimens (Table 2). Recoveries were nonidentical from three specimens, including five isolates recovered by both methods from the same specimens; growth of these five isolates was equivalent. Eight other isolates were recovered by one method but not the other; three of these (*Actinomyces* spp., *Bacteroides* spp., and *P. micros*) were isolated by the jar method but not by the anaerobic bag method. Five isolates were recovered by the anaerobic bag method but not by the jar method. A *Bacteroides* sp. was isolated by using the bag method but from a different specimen than that from which a similarly identified isolate was recovered by the jar method. However, species of those organisms recovered from clinical specimens by only one method were isolated from plates incubated by the other method in another part of the study, i.e., from other clinical specimens or stock cultures.

Review of these limited results does not suggest that there is any substantial difference in the total yield or growth of anaerobes from clinical specimens cultured in these two systems. There was one instance in which growth of anaerobes from a clinical specimen was observed by inspecting the plate in the anaerobic bag after 24 h of incubation. Because corresponding aerobic cultures were negative, the

TABLE 2. *Recovery of anaerobes from 10 clinical specimens by anaerobic bag and GasPak jar methods*[a]

Identical recoveries from seven specimens[b]	Nonidentical recoveries from three specimens		
	Organisms recovered by both methods[b, c]	Organisms recovered by one method only	
		Bag	Jar
Bacteriodes fragilis subsp. *fragilis*	*B. melaninogenicus* subsp. *intermedius*	*B. melaninogenicus* subsp. *intermedius*	*Actinomyces* sp.
B. fragilis subsp. *thetaiotaomicron*	*B. oralis*	*Bacteroides* sp.[d]	*Bacteroides* sp.
B. melaninogenicus subsp. *melaninogenicus*	*Bacteriodes* sp.	*Clostridium ramosum*	*Peptostreptococcus micros*
B. melaninogenicus subsp. *intermedius*	*Fusobacterium necrophorum*	*P. acnes*	
Fusobacterium nucleatum	*F nucleatum*	*Veillonella parvula*	
Peptococcus variabilis			
P. magnus			
P. previotii			
Propionibacterium acnes			
Eubacterium sp.			

[a] Incubation period, 48 h; negative plates reincubated for 3 weeks.
[b] No significant differences in recovery or colony size noted.
[c] Recovery by both methods from the same specimen.
[d] Recovered by one method only, from a different specimen than other *Bacteroides* sp.

recognition of definite anaerobic growth could have been made at that early time.

DISCUSSION

This study demonstrates that use of the anaerobic bag is a simple and efficient method for culturing clinically significant anaerobes. Measurements of carbon dioxide and oxygen concentrations indicate that appropriate levels of these gases are achieved in this system. Comparisons of growth of various stock cultures and isolates from 10 clinical specimens indicate that similar results are obtained in both the anaerobic bag and the GasPak jar.

Practical advantages of the anaerobic bag method include the use of conventional plated media and the ability to examine plates within the bag at any time without disturbing the anaerobic atmosphere. Plates may be examined earlier than the usual 48 h for jars and compared with growth on aerobic plates. Likewise, plates in the bag having no growth or colonies identical to those on aerobic cultures may be reincubated or discarded, at the discretion of the bacteriologist, after rapid visual examination of the sealed bag.

All components of the bag method are inexpensive and easily obtainable. The bags themselves are reusable, being limited only by a progressive decrease in size each time the sealed end is cut off to open the bag. The estimated cost of setting up one bag (excluding the cost of the heat sealer and media) is $0.10. The number of bags used will depend upon the number of selective plates required. Usually one selective and one nonselective plate will suffice but some laboratories may use additional selective plates for certain types of specimens. The anaerobic bag is probably most efficiently used for primary culture only. Subcultures from the original plates can be incubated in jars (or thioglycolate broth) because there is no necessity to examine individual plates at frequent intervals. The anaerobic bag would seem to be most useful for those laboratories not already using a glove box or the roll tube method and that process more than one or two specimens daily.

Other workers have described self-contained anaerobic culture units (other than roll tubes) that can be inspected at any time without interrupting the anaerobic atmosphere or disturbing other specimens. Matthews and Karnauchow (4) used a transparent polyethylene bag sealed with cellulose tape with a pyrogallic acid-alkali mixture to establish the anaerobic atmosphere. However, they did not compare their method with any other method or evaluate it with clinical specimens. Davis et al. (3) described a pre-reduced anaerobic bottle that compared well with the GasPak jar in the recovery of anaerobes from clinical specimens; however, this bottle has the technical disadvantages of roll tubes in that colonies growing on agar inside the bottle must be inspected from the outside and may be difficult to recognize; manipulations using loops or pipettes must be performed through the narrow neck of the bottle. The anaerobic bag method described herein has the advantages of the above systems coupled with the convenient use of plating media and demonstrated efficacy in the growth of anaerobes from stock cultures and clinical specimens.

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