Evaluation of two anaerobic systems for isolation of anaerobes

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Abstract

Many systems are available for the isolation of anaerobic bacteria from clinical specimens. The jar system is the oldest and more adapted while the pouches are not popular with many investigators. The anaerobic chambers are expensive to maintain and technically inflexible. This study evaluated the efficacy of the Oxoid anaerobic jar and the GENbag pouches as anaerobic incubation systems. Anaerobic cultures were set up for 145 middle ear exudates and incubation was in the anaerobic jar, GENbag or a combination of both. The effect of specimen transport system and time lapse before culturing on the performance of the anaerobic systems were evaluated. Ten genera of anaerobic bacteria were isolated with both systems (P>0.05). *Peptostreptococcus* and *Prevotella* were isolated more frequently in Oxoid jar than in GENbag (P<0.05) but both systems were not discriminatory for *Clostridium*, *Propionibacterium* and *Veillonella*. The use of GENbag as a backup to Oxoid jar increased isolation rate from 56.6% to 90.3% (P>0.05). Type of transport media or vehicle did not affect the recovery of anaerobes adversely as did delay in processing of specimen. A careful application of a number of variables may improve isolation of anaerobes from clinical specimens.

Introduction

Culture remains the gold standard for the identification and speciation of anaerobic bacteria. Three factors usually will determine the level of success achieved in isolating anaerobic bacteria from clinical specimens. These are the methods used in collecting the appropriate specimens, transport and handling of specimens and the efficiency of the incubation system.[1-3] After proper selection and collection of specimens, success in isolating anaerobes depends on the ability to maintain the oxygen tension within the specimen or culture at a relatively low level. The anaerobic jars were the first system introduced for incubating anaerobic cultures with anaerobiosis generated through the evacuation-replacement technique or the use of gas generating envelopes (BBL GasPak envelopes; Becton Dickinson Microbiology Systems, Cockeysville, Md.). In other to limit exposure time of specimens and cultures to oxygen especially during processing the anaerobic chambers were introduced. The demand for cost effectiveness and elasticity have necessitated the introduction of new systems amongst which are the pouches that exhibit considerable flexibility. The pouch system and anaerobic jars or containers depend mostly on chemical anaerobe-atmosphere generating systems (Biobag type A, Marion Scientific, Kansas City, Mo; AnaeroPack systems, Mitsubishi Gas Chemical America, Inc., New York, N.Y.; BBL GasPak envelopes, Becton Dickinson Microbiology Systems, Cockeysville, Md.; GENbag system, bioMerieux, sa, 69280 Marcy-1 Etiole, France; Anaerobic Pouch System Catalyst-Free, Difco Laboratories, Detroit, Mich.) for anaerobiosis.

A deluge of chemicals, reagents, media and equipment are commercially available for anaerobic study. These attest to the growing clinical significance of anaerobic bacteria, therefore the continuous assessment of these facilities to validate their functionality and scope is required. Earlier studies have shown that chemical anaerobic atmosphere-generating systems (AnaeroPack, Biobag type A, and the BBL GasPak system) compared favourably with the anaerobic chambers.[1,2] With most anaerobes, the AnaeroPack sachet was comparatively more effective than the anaerobic chamber and other systems.[2] The better performance of the AnaeroPack system was associated with the higher CO_2 concentration generated (18% *vs* 4-10 % in the GasPak system and 10% in chambers). In contrast, the Coy anaerobic chamber and the GasPak system were more efficient than the AnaeroPack system in recovering *Bacteroides forsythus* and *Peptostreptococcus micros* from periodontal specimens, though all three systems were equally efficient in isolating *Porphyromonas* and *Prevotella*.[3]

Some of the earlier studies comparing non-chamber incubation system processed specimen either partly or wholly in the atmosphere of the anaerobic chamber,[1,2] this may not be the case in Centre that do not have anaerobic chamber; and the impact of this on the recovery of different groups of anaerobic bacteria is worth investigating. Considering the non-portability of the anaerobic chamber, specimens will equally need to be transported to the laboratory for processing, irrespective of the final incubation system adopted. The efficiency of the transport system in sustaining anaerobes in specimens and the permissible time lag for optimal recovery of anaerobes are essential.

In Nigeria the anaerobic jar is in common use while the pouch systems are not very popular with many investigators who are sceptic about their efficiency. In our Centres and many other Institutions the glove boxes are not available. Recently, we acquired some GENbag pouches and instituted this study to compare their effectiveness as alternative incubation system to the anaerobic jar we are much familiar with. This study in addition, considered the contributing role of choice of method for processing specimens: by direct inoculation at patient's bedside or clinician consulting room; effectiveness of transport medium; and time lapse before cultures were set up.

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Materials and Methods

Incubation systems

The BBL GasPak system included the GasPak jar (2.5 litres) with a catalyst chamber containing new palladium catalysts and the GasPak Anaerobe envelope. Catalysts were activated in a hot air oven at 140°C for 2 h before use. Anaerobic conditions in jars were obtained with the GasPak envelope and were monitored with a disposable BBL Dry Anaerobic Indicator Strip (Becton Dickinson).

The GENbag system consisted of the GENbag pouch and GENbag anaer sachet. The sachet generates 18% CO_2 on contact with oxygen and does not require the addition of water. One GENbag anaer sachet was used for a GENbag that contains not more than 3 plates. Anaerobic conditions were monitored with BBL Dry Anaerobic Indicator Strip.

Specimen collection

Exudates of ear effusion from 145 cases of chronic otitis media (COM) were collected through tympanocentesis by aspiration of the effusion directly from the middle ear after proper disinfection of the external ear canal. The meatus was swabbed with warm sterile water and coated with tincture of iodine. Tympanocentesis was done using an 18 gauge medicut which consisted of an 18 gauge needle covered by plastic canula attached to 2mL syringe. Pooled specimens <2 mL were not included in this study as this volume will not satisfy the experimental requirements and may imply prior therapy either antibiotic or unorthodox. In some cases, repeated sampling was done to obtain more specimens. Both ears were sampled for cases of bilateral infections. Patients for this study were from the ENT clinics of the Lagos State University Teaching Hospital, Ikeja. Diagnosis and specimen collection were done by a consultant otologist. Exclusion criteria were antibiotic history 2 months to presentation, and or absence of or inadequate exudates.

Inoculation pattern

Immediately after collection anaerobic agar plates (Shaedler anaerobe agar [Oxoid], Wilkins-Chalgren anaerobe agar [Oxoid], kanamycin blood agar 75 ug/mL supplemented with vitamin K_1 and L-cysteine hydrochloride) were inoculated directly with 0.2 mL of ear exudates either at the patient's bedside or the otologist consulting room. The remaining specimen was distributed into transporting media as follows: 0.4 mL in Portagerm vial (bioMerieux); six alginate swabs each absorbed with 0.2 mL and a set of 3 swabs transported in Amies charcoal medium (Oxoid) and Portagerm tubes (bioMerieux). Smears were prepared directly from specimens and Gram stained to serve as quality checks for culture results.

Experimental design

A set of the three inoculated anaerobic plates were placed inside a GENbag pouch system (bioMerieux sa, 69280 Marcy-1 Etiole, France) for temporary incubation for between 2-4h. This was the median time spent in transit from specimen collection to processing. Incubation was within the prevailing room temperatures (28°C to 32°C) and anaerobiosis was achieved with the paper sachet anaerobic gas generator (GENbag anaer). A set of plates in the pouch system was thereafter transferred into Oxoid anaerobic jar (10 plates per jar) and incubated under 10% CO_2 and 10% H_2 generated from the Oxoid GasPak anaerobe kit for 3-7 days at 37°C. The remaining set of plates in the pouch system was incubated further at 37°C for 3-7 days maintaining anaerobic conditions with fresh GENbag anaer sachet. Specimens in Portagerm vials and the Alginate swab specimens were processed for anaerobic incubation both in the GENbag system and Oxoid anaerobic jar. Specimens were withdrawn and cultured on selective anaerobic agar plates 2h, 6h and 9h after specimen collection. Anaerobic conditions were monitored with BBL Dry Anaerobic Indicator Strip. Isolates were Gram stained and identified further by conventional methods and the API system.

Statistical analysis

The various data were analysed with the Pearson Chi square test at P=0.05. Data analysed included: recovery rate for anaerobes in aspirate specimen incubated in GENbag only and GENbag serving as a transit for Oxoid jar incubation; the effect of the transportation method and time lapse on the final recovery of anaerobes in the clinical specimen (vials, tubes and swab specimens). The z score test was used to analysed the difference in efficiency of GENbag pouch and the Oxoid jar in recovering anaerobes in transported specimens.

Results and Discussion

The present study recorded 56.6% (82 of 145 specimens) success in recovering anaerobes in middle ear aspirates cultured at the point of collection and incubated in GENbag anaer system. When cultures were transferred from the GENbag system after 2-4 h into the Oxoid jar system for further incubation, success rate rose to 90.3% (131 of 145 specimens) (Table 1). Though this increase in recovery was not statistically significant (P>0.05), it is of immense clinical importance if patients' health is the overriding factor. This may give credence to the use of the GENBag system as a backup incuba-

Table 1. Predominant anaerobic bacteria genera recovered from specimens.

Genera	Frequency of Recovery							
	Asp		Portvial		AlgAmies		AlgPortube	
	Ge	GeOx	Ge	Ox	Ge	Ox	Ge	Ox
Bacteroides	15	18	11	18	10	16	10	15
Fusobacterium	12	20	13	19	5	10	7	14
Porphyromonas	8	16	9	15	5	8	3	12
Prevotella+	18	25	15	28	9	19	8	16
Clostridium	8	8	7	8	7	8	6	7
Peptococcus	5	6	7	6	3	5	6	4
Peptoniphilus	2	5	4	9	3	5	2	4
Peptostreptococcus+	11	28	10	18	9	24	10	20
Propionibacterium	2	3	1	2	0	1	1	1
Veillonella	1	2	1	1	0	1	0	1
Total	82	131	78	124	51	97	53	94
Percentage	(56.6)	(90.3)	(53.8)	(85.5)	(35.2)	(66.9)	(36.6)	(64.8)
Mean	8.2	13.1	7.8	12.4	5.1	9.7	5.3	9.4
Standard deviation	(5.5)	(9.0)	(4.5)	(8.2)	(2.5)	(7.3)	(3.0)	(6.5)

Asp, aspirate; Ge, GENbag; GeOx, GENbag + Oxoid jar incubation; Ox, Oxoid jar; P>0.05 for differences in recovery of anaerobes collectively comparing GENbag with Oxoid jar +; P<0.05 for differences in isolation comparing GENbag and Oxoid jar.

tion system especially where strict anaerobic conditions are required. Leke *et al.*[4] used the GENbag anaer device to maintain anaerobic conditions in fresh stool samples from point of collection to processing for the isolation of bifidobacteria. The oxygen requirement of *Isoptericola hypogeus* sp. nov., a facultative anaerobe was established by absence and presence of growth in cultures incubated in GENbag anaer and GENbag microaer respectively.[5] In a similar study, it was established that *Bacteroides massiliensis* sp. nov is a strict anaerobe by growth in GENbag anaer and absence of growth in GENbag microaer systems.[6] These findings validate the GENbag anaer device as an efficient anaerobic incubation system. The GENbag anaer generates 15-18% CO_2 within the first 45-60 min of incubation (bioMerieux) and this makes it comparable to the AnaeroPack sachet that generates about the same anoxic conditions.[2]

On the face value the Oxoid jar system yielded more anaerobic isolates than the GENbag system but the difference was not significant (P>0.05). The seemingly better performance of the Oxoid system was with *Peptostreptococcus* and *Prevotella* spp. Both systems were not discriminatory with respect to *Clostridium*, *Propionibacterium* and *Veillonella* (Table 1). Previous studies have shown that the performance of an anaerobic system may be organism dependent. For instance, the Coy anaerobic chamber was most efficient in the recovery of *Campylobacter* and *Eubacterium*, the Coy anaerobic chamber and the GasPak system were more promising with *B. forsythus* and *P. micros* than the AnaeroPack system. In contrast, the AnaeroPack system was most efficient in growing *Fusobacterium* spp.[3]

If cost will constitute constrain to the use of a pouch system, we evaluated the use of transport facility in conveying anaerobic specimens. Aspirates were either transported direct in Portagerm vials or alginate swab samples were conveyed in Amies medium and Portagerm tube and processed after different time intervals. The results indicate no significant difference in the efficiency of the transport media irrespective of the incubation system adopted. However, loss in recovery of anaerobes occurred when there was a delay of 9 h before specimens were cultured (Table 2). Previous studies have shown varied survival time of anaerobes in transport media.[3,4,7] Amies charcoal transport medium was able to preserve Gram negative anaerobic bacilli (*Bacteroides* and *Prevotella*) and *Clostridium* at room temperature for 7 days optimally.[7] This study however used clinical isolates, but Portagerm Amies + agar rectal swabs have been shown to preserved anaerobes for upward of 48 h.[4] Doan *et al.*[3] employed VMGA III transport medium (Becton Dickinson Microbiology Systems, Cockeysville, Md.) to transfer periodontal specimens and reported recovery of anaerobes even after 3 days. Choice of transport medium may be a matter of interest or pooled experience from different investigators.

Procurement and maintenance are baseline reasons for the absence of anaerobic chambers in many Centre (we do not have one). Where anaerobic culture is intended, the jar system and now the pouches become cost-effective alternatives. Usually, with inability to maintain sufficient anaerobiosis from specimen collection to processing, success rate has been low. The present study has shown that the combination of the pouch and the jar systems will improve the recovery of anaerobes from clinical specimens. Where cost may be a limiting factor, a careful selection of transport medium and processing within the first 2-6 h will be adequate for isolation of anaerobes from clinical specimens.

Table 2. Analysis of data on the impact of timing on the recovery of anaerobic bacteria in ear aspirates transported in different medium.

Time differences	X^2 calculated	P	Remark
Portvial + GENbag			
2 h ; 6 h	1.396	0.40<P< 0.50	P>0.05*
2 h ; 9 h	8.044	0.01<P< 0.25	P<0.05°
Portvial + Oxoid jar			
2 h ; 6 h	0.851	0.60<P< 0.70	P>0.05*
2 h ; 9 h	4.500	0.10<P< 0.20	P>0.05*
AlgAmies + GENbag			
2 h ; 6 h	5.152	0.05< P < 0.10	P>0.05*
2 h ; 9 h	18.357	P< 0.0005	P<0.0005°
AlgAmies + Oxoid jar			
2 h ; 6 h	8.004	0.01< P < 0.025	P<0.05°
2 h ; 9 h	12.188	0.0005< P < 0.005	P<0.005°°
AlgPortube + GENbag			
2 h ; 6 h	8.098	0.01<P<0.025	P<0.05°
2 h ; 9 h	14.300	0.0005 <P<0.005	P<0.005°°
AlgPortube + Oxoid jar			
2 h ; 6 h	4.319	0.10<P<0.20	P>0.05*
2 h ; 9 h	12.807	0.0005<P<0.005	P<0.005°°

Portvial, Portagerm vial; Oxoid jar, BBL Oxoid jar system with BBL GasPak envelope; AlgAmies, Alginate swab transported in Amies charcoal medium; AlgPortube, Alginate swab transported in Portagerm tube; 2h, 6h, 2h, 9h, evaluation between the first and the last; GENbag pouches used the GENbag anaer sachet for CO_2 generation. *No significant difference was observed in recovery of anaerobe from aspirates irrespective of incubation method adopted; ° degree of significance in storage period of specimen in transport medium before processing.

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