

Obtaining an anaerobic atmosphere for growth of strictly anaerobic bacteria in medical bacteriology

Evaluation of automated evacuation-replacement system

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Abstract

The performance of an automatic evacuation-replacement system – BACT-R (Sobioda, Montbonnot Saint Martin, France) – to create an anaerobic atmosphere in jars was evaluated for approximately fifty anaerobic strains. This system was compared to the growth of 51 strains in anaerobic cabinets and jars using anaerobic condition generation bags. The study showed that the anaerobic condition created by the evacuation-replacement system allows for the growth of isolated strictly anaerobic bacteria in medical bacteriology identical to that of the two compared systems. The jar opening tests showed the absence of the toxic effect of oxygen on 9 strains with the exception of one strain. On the other hand, the growth was slowed for six of the strains.

Keywords: Anaerobic condition, technique.

Introduction

While strictly anaerobic micro-organisms constitute a significant part of the commensal flora, they are implicated in a large number of generalised or localised infections, or of endogenous or exogenous origin. Their sensitivity to oxygen and their often slow growth make it difficult to bring them to light. In addition, anaerobic infections are frequently mixed infections, associating aerobic and strict anaerobic bacterial, requiring the use of selective environments. So, it is very important to optimise the culture growth conditions in order to obtain cultures as quickly and as reliably as possible.

The degree of sensitivity to oxygen of anaerobic bacteria varies from one species to another. Certain

species can tolerate contact with oxygen in the air for many hours (*Bacteroides fragilis* for example); others are killed after contact lasting only minutes (*Clostridium butyricum* for example). Different systems can be used to obtain an anaerobic atmosphere.

Anaerobic cabinets, biological safety cabinets blanketed with an oxygen-free gas, allow work to be performed under continuous anaerobic condition and is the ideal equipment for isolating anaerobic bacteria in biological samples. It is the only devices that allows the growth of micro-organisms that are extremely sensitive to oxygen. Nevertheless, these anaerobic cabinets are highly expensive to purchase and very cumbersome. Most frequently, discontinuous anaerobic condition

systems are used in medical biological laboratories.

The most common systems are jars or individual bags. Anaerobic condition is created thanks to the use of **mixtures of reagents that trap the oxygen and produce carbon dioxide**; for example GENbox anaer® or GENbag anaer® (bioMérieux, Marcy l'Étoile, France), Anaerogen® (Oxoid, Dardilly, France) or Anaerocult® (VWR, Fontenay-sous-Bois, France). These reagents are a good alternative, allowing good strict anaerobic growth isolated in medical bacteriology (1), (2) and are less expensive in terms of initial investment.

Anaerobic condition can also be obtained with the **evacuation-replacement technique**. This technique requires the use of jars that have an evacuation system, a vacuum source and an oxygen-free gas mixture. A number of automatic systems are available on the market: BACT-R (Sobioda, Montbonnot Saint-Martin, France), Anoxomat (Mart, Lichtenvoorde, Hollande), MACS-mics (AES Laboratoire, Bruz, France). Anoxomat was recently quoted, proving the interest in these types of systems (3). BACT-R, tested in our study, is a microprocessor-controlled programmable system that creates a vacuum of about 0.6 bar in a jar and then fills it with the blend of gases chosen. This operation is repeated six times to obtain an anaerobic condition quickly.

The study's objective was to validate the use of a BACT-R system by testing growth on about 50 strict anaerobic species that belong to the main types that can be responsible for anaerobic infections. The growth of these species was compared using four different types of anaerobic condition generation: the BACT-R system with two different gases, the anaerobic cabinet, and a chemical anaerobe generation system.

The rapid creation of an anaerobic atmosphere does not prevent the bacteria from being killed if the contact time with air is too long. For this reason, jar opening tests were included in this study in order to provide recommendations for the use of this system.

I. Materials and methods

A) Bacterial strains

Fifty-one species from the laboratory collection were included. These species originated from clinical samples or collections. They were divided as follows: 24 belonging to the Clostridium genus, 17 were Gram negative bacilli (*Bacteroides*, *Prevotella*, and *Fusobacterium* genus) and 10 des cocci or non-spore-forming Gram positive bacilli (Table I).

B) Anaerobic Cabinet

The anaerobic cabinet used was a biological safety cabinet with rigid plates, temperature regulated to 37 °C (Don Whitley Scientific, AES Laboratoire, Bruz, France). The gas used was a blend of $H_2 : CO_2 : N_2 = 10 : 10 : 80$ v/v. The anaerobic conditions were verified continuously due to the presence of a resazurin solution in the presence of cysteine. This solution, colourless in the absence of oxygen, turns pink if the anaerobic condition fails.

C) Anaerobic condition generation bag

The anaerobe generation systems used were GENbags (bioMérieux, Marcy-l'Étoile, France). These bags allow the anaerobic condition to be generated quickly, without the addition of water or a catalyser. Their chemical composition (activated carbon, sodium ascorbate, and other organic and mineral components) absorb oxygen and liberate carbon dioxide.

D) BACT-R

The BACT-R system allows for an anaerobic condition to be established after six evacuation-replacement cycles. The manufacturer's recommended gas mixture is $H_2 : CO_2 : N_2 = 5 : 5 : 90$ v/v. The 5 per cent hydrogen content gas blanket to be maintained in the laboratory. For reasons of the study, the equipment was adapted to

Table I. Bacterial strains used to evaluate the BACT-R system

Genera and bacterial species	Number of isolates tested
Spore-forming Gram positive bacilli	
<i>Clostridium perfringens</i>	3
<i>Clostridium butyricum</i>	5
<i>Clostridium difficile</i>	10
<i>Clostridium sporogenes</i>	2
<i>Clostridium bifermentans</i>	1
<i>Clostridium ramosum</i>	1
<i>Clostridium paraputrificum</i>	2
Gram negative bacilli	
<i>Bacteroides fragilis</i>	4
<i>Bacteroides ovatus</i>	1
<i>Bacteroides vulgatus</i>	1
<i>Bacteroides caccae</i>	1
<i>Bacteroides thetaiotaomicron</i>	1
<i>Bacteroides distasonis</i>	1
<i>Bacteroides stercoris</i>	1
<i>Prevotella bivia</i>	1
<i>Prevotella melaninogenica</i>	1
<i>Prevotella intermedia</i>	1
<i>Fusobacterium varium</i>	1
<i>Fusobacterium nucleatum</i>	1
<i>Fusobacterium necrophorum</i>	2
Gram positive cocci	
<i>Peptostreptococcus anaerobias</i>	1
<i>Finegoldia magna</i>	2
<i>Peptostreptococcus asaccharolyticus</i>	1
<i>Micromonas micros</i>	1
Non-spore-forming Gram positive bacilli	
<i>Propionibacterium acnés</i>	2
<i>Eubacterium lentum</i>	1
<i>Bifidobacterium longum</i>	1
<i>Bifidobacterium bifidum</i>	1

deliver both this gas mixture and the gas used for the anaerobic cabinet (10 per cent H₂). The jars were equipped with a palladium catalyst. The catalyst was regenerated after each use (1 h 30 at 180 °C) to eliminate the water of condensation. Note that the catalyst regeneration can be performed at 37 °C, also, but for a period of 12 hours (i.e. overnight).

E) Environment

The bacterial strains, stored at -80 °C in brain heart infusion broth additivated with 15 per cent glycerol, were inoculated on freshly prepared Columbia blood agar (5 per cent). For the bacterial enumeration, the inoculation was performed using TGYH (tryptone, glucose, hemin and yeast extract) brain heart infusion broth.

F) Method

1) Validation of bacterial strain growth using the BACT-R system

After verifying the purity of the microbial cultures, a colony of each strain was inoculated in TGYH broth and incubated for 24 to 48 hours according to the bacterial species. Dilutions of 100 in 100 were made and each dilution was inoculated on four Columbia blood agar using the WASP spiral automatic plater (Don Whitley Scientific, AES Laboratoire, Bruz, France). The blood agar plates were then placed as quickly as possible under anaerobic condition using the four different systems

- the anaerobic cabinet,
- the GENbag system,
- the BACT-R system using the manufacturer's recommended gas for laboratories (5 per cent H₂),
- the BACT-R system using the anaerobic cabinet gas (10 per cent H₂).

The bacterial enumeration was performed after 24 and 48 hours of incubation and expressed in log₁₀ UFC/mL and the sizes of the colonies were compared.

2) Jar opening tests

In order to optimise the use of the BACT-R system, comparative bacterial enumeration was performed after anaerobic incubation (1) without opening the jar, and voluntarily opening the jar in two steps (after 1 hour and after 5 hours of incubation) (2) for 5 minutes each time, and (3) for 30 minutes each time. This was done to mimic the conditions encountered in a medical bacteriology laboratory. The gas used was the manufacturer's recommended gas. The bacterial enumeration and observation of the colonies was performed after 24 and 48 hours of incubation and compared to the growth of the same strains incubated for 24 and 48 hours in the anaerobic cabinet.

These tests were performed on the bacterial stains most sensitive to the toxicity of oxygen: *Clostridium butyricum* (2 strains), *Clostridium difficile* (3 strains), *Fusobacterium necrophorum* (2 strains), *Fusobacterium nucleatum* (1 strains), *Micromonas micros* (1 strains).

Table II. Bacterial enumeration as a function of the systems used for the anaerobic condition, expressed in log₁₀ UFC/mL (mean ± standard deviation) after 48 hours incubation

Bacterial strains	Anaerobic Cabinet	GENbag	BACT-R	
	H ₂ :CO ₂ :N ₂ 10:10:80		H ₂ :CO ₂ :N ₂ 5:5:90	H ₂ :CO ₂ :N ₂ 10:10:80
<i>Clostridium</i>	7.3 ± 1.0	7.2 ± 1.2	7.3 ± 1.0	7.4 ± 1.2
<i>C perfringens</i>	8.2 ± 0.2	8.2 ± 0.3	8.0 ± 0.1	8.6 ± 0.9
<i>C difficile</i>	7.2 ± 0.6	6.9 ± 0.6	7.2 ± 0.5	7.1 ± 0.6
<i>C butyricum</i>	6.2 ± 0.6	6.0 ± 0.7	6.2 ± 0.6	6.1 ± 0.4
Autres <i>Clostridium</i> ^a	7.6 ± 1.1	7.7 ± 1.3	7.5 ± 1.1	7.6 ± 1.4
Gram negative bacilli	8.8 ± 1.4	8.8 ± 1.3	8.8 ± 1.5	8.8 ± 1.3
<i>Bacteroides fragilis</i>	8.8 ± 0.7	8.7 ± 0.7	8.8 ± 0.7	8.8 ± 0.7
Autres <i>Bacteroides</i> ^a	9.5 ± 0.3	9.5 ± 0.3	9.6 ± 0.3	9.5 ± 0.3
<i>Prevotella sp</i> ^a	7.8 ± 2.8	8.0 ± 2.3	7.7 ± 2.3	8.0 ± 2.4
<i>Fusobacterium sp</i> ^a	8.1 ± 1.9	8.1 ± 2.0	8.1 ± 1.9	8.2 ± 1.9
Gram positive cocci^a	7.5 ± 0.8	7.4 ± 0.9	7.4 ± 0.9	7.4 ± 0.9
Non-spore-forming Gram positive bacilli^a	8.1 ± 0.3	8.0 ± 0.3	8.1 ± 0.4	8.1 ± 0.4

^a see the list of bacteria specials (Table I).

Table III. Biological enumeration of the species without and with jar opening during incubation, expressed in log₁₀ UFC/mL

Strains bacterial	24 hours incubation				48 hours incubation			
	Anaerobic Cabinet	BACT-R			Anaerobic Cabinet	BACT-R		
		Without opening	Opening 2 x 5 min ^a	Opening 2 x 30 min ^a		Without opening	Opening 2 x 5 min ^a	Opening 2 x 30 min ^a
	8.4	8.3	n/e ^b	n/e	8.4	8.3	8.3	8.3
	6.8	n/e	n/e	n/e	6.8	6.8	6.8	6.7
	8.9	8.7	n/e	n/e	8.9	8.7	8.7	8.8
	8.2	8.2	8.1	n/e	8.2	8.2	8.2	8.1
	6.5	6.1	n/e	n/e	6.5	6.2	6.2	5.5
	8.0	7.8	7.9	n/e	8.0	7.8	7.9	7.8
	6.3	6.2	6.3	6.3	6.4	6.4	6.4	6.3
	5.0	5.0	4.9	4.9	5.0	5.0	4.9	4.9
	No culture present				5.4	5.5	5.6	5.6

^a 1st opening at H +1 after starting incubation, 2nd opening at H +5 after starting incubation.

^b n/e : not enumerable, punctiform colonies.

Results and discussion

The comparison of the biological enumeration of the 51 species in the different anaerobic atmospheres did not show any significant variation after 48 hours of incubation (Table II). Therefore it can be said that the BACT-R system offers a similar growth to the other anaerobe generation systems, be the continuous (anaerobic cabinet) or discontinuous (GENbag) (average variation of 0.1 to 0.2 log₁₀ UFC/mL). Only the *C. difficile* species cultivated a little less well with the GENbag (6.9 ± 0.6 compared to 7.2 or 7.1 ± 0.6 with the other techniques).

The growth of species highly sensitive to oxygen, such as *Fusobacterium sp* or *C. butyricum* showed extremely similar results between the BACT-R system and the other anaerobic condition generation techniques. For the species currently isolated in medical bacteriology, the gas

recommended by the manufacturer yields results similar to the gas traditionally used in anaerobic cabinets (richer in hydrogen and CO₂) (Table II). Only two strains showed greater growth by a half log in the presence of more hydrogen-rich gas (one strain of *C. perfringens*, and one strain of *C. sporogenesi*).

The discontinuous anaerobic condition generation method requires the laboratories to leave jars open in time to observe the isolation. In order to measure the consequence of opening the jars on the anaerobic bacteria growth, bacterial enumeration tests were performed after opening the jars in two occasions and during the incubation time. The opening time was 5 minutes (minimum time) and 30 minutes.

These tests were performed on species that are highly sensitive to oxygen (2 species of *F. necrophorum*, 1 species of *F. nucleatum*, 2 species of *C. butyricum*'), on 3 species of *C. difficile*, micro-organism used regularly for research in medical bacteriology laboratories, non-sporing in the selective isolation environment used and therefore more sensitive to the toxic action of oxygen and on a slow-growth species *Micromonas micros* (Table III).

The strains of *C. butyricum* and *M. micros* have a similar growth regardless of the jar opening. Only the species of *Fusobacterium* and *C. difficile* showed growth retardation compared to the anaerobic cabinet or unopened jar. This growth retardation was important both due to the opening of the jar and the period it was opened. Nevertheless, the biological enumeration at 48 hours was identical with the exception of one species of *C. difficile* where the growth was less than 0.7 log₁₀ UFC/mL.

Conclusion

This study showed that the BACT-R system allows creating an anaerobic condition where the quality is identical to the two atmospheres compared, the anaerobic cabinet and the anaerobic condition generation bags.

This automated evacuation-replacement procedure, much less expensive than an anaerobic cabinet, with the advantage of being very easy to use. This system remains, however, a discontinuous anaerobe generation system, but the anaerobic condition is created faster than when using bags (3).

The jar opening tests show that if the anaerobic condition is quickly recreated, its effect on bacterial growth is inconsequential, causing only a delay in growth. Nonetheless, it must be kept in consideration that the oxygen in the air is toxic for the anaerobic bacteria and therefore if the jar may be opened on multiple occasions for adding plates or observing the cultures, the opening time must be kept as short as possible.

In the BACT-R system, there are integral controls

associated with the evacuation-replacement cycles that allow, from when the atmosphere is created, to be in control of the culture conditions. Finally, this system, with the same gas, allows establishment of a microaerophilic atmosphere with only three evacuation-replacement cycles and a capnophile atmosphere with a blanket of carbon dioxide.

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