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Development and Evaluation of the Quick Anaero-system–A New Disposable Anaerobic Culture System

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Background : We developed a new disposable anaerobic culture system, namely, the Quick anaero-system, for easy culturing of obligate anaerobes.

Methods : Our system consists of 3 components: 1) new disposable anaerobic gas pack, 2) disposable culture-envelope and sealer, and 3) reusable stainless plate rack with mesh containing 10 g of palladium catalyst pellets. To evaluate the efficiency of our system, we used 12 anaerobic bacteria. We prepared 2 sets of ten-fold serial dilutions of the 12 anaerobes, and inoculated these samples on Luria-Bertani (LB) broth and LB blood agar plate (LB-BAP) (BD Diagnostic Systems, USA). Each set was incubated in the Quick anaero-system (DAS Tech, Korea) and BBL GasPak jar with BD GasPak EZ Anaerobe Container System (BD Diagnostic Systems) at 35-37℃ for 48 hr. The minimal inoculum size showing visible growth of 12 anaerobes when incubated in both the systems was compared.

Results : The minimal inoculum size showing visible growth for 2 out of the 12 anaerobes in the LB broth and 9 out of the 12 anaerobes on LB-BAP was lower for the Quick anaero-system than in the BD GasPak EZ Anaerobe Container System. The mean time (±SD) required to achieve absolute anaerobic conditions of the Quick anaero-system was 17 min and 56 sec (±3 min and 25 sec).

Conclusions : The Quick anaero-system is a simple and effective method of culturing obligate anaerobes, and its performance is superior to that of the BD GasPak EZ Anaerobe Container System. *(Korean J Lab Med 2010;30:133-7)*

Key Words : *Anaerobic bacteria, Culture techniques, Culture media*

INTRODUCTION

Obligate anaerobes cannot survive in the presence of oxygen because they are deficient in the enzymes superoxide dismutase and catalase that destroy the lethal superoxide radicals formed in the presence of oxygen. Therefore, obligate anaerobes are very susceptible to atmospheric oxygen. The cultivation of obligate anaerobes requires the rapid generation of an atmosphere with oxygen levels below 0.5% [1].

Several techniques can be used to rapidly generate anaerobic atmosphere for the cultivation of anaerobes [2-4]. Anaerobic chamber is a convenient tool for culturing anaerobic organisms in large scale studies. However, it is not commonly used in clinical microbiology laboratories because it is not cost effective.

It is relatively inexpensive to generate anaerobic conditions by chemical means as compared to using the expensive and bulky anaerobic chamber. Generally, several microbiological laboratories utilize a chemical compound to generate anaerobic conditions in an anaerobic jar. Many chemical systems produce hydrogen and carbon dioxide from

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tablets of sodium borohydride, sodium bicarbonate-citric acid, etc. In Korea, the most commonly used chemical systems in clinical microbiological laboratories are disposable anaerobic systems containing sodium borohydride. A variety of commercially available products contain sodium borohydride systems such as Anaerobic System (Difco Laboratories, Detroit, MI, USA), Genbox anaer system (bioMerieux, Marcy-l'Etoile, France), GasGendicator system (Adams Scientific Inc., West Warwick, RI, USA), BBL GasPak, BBL GasPakPlus, and BD GasPak EZ Anaerobe Container Systems (Becton Dickinson Microbiology Systems, Cockeysville, MD, USA) [5].

However, the existing disposable anaerobic gas systems using sodium borohydride have a disadvantage that they require more time to achieve an absolute anaerobic condition [5]. Therefore, sometimes, obligate anaerobes may not grow in such conditions. Among the several systems used for the cultivation of anaerobes, the anaerobic systems based on borohydride, including Anaerobic System (Difco Laboratories), GasGendicator (Adams Scientific Inc.), GasPak (BBL), GasPakPlus (BBL), and Genbox anaer (bioMerieux), showed a 10-20% failure rate, wherein the system failure was confirmed when O_2 concentration in the system was approximately 0.16% after 1 hr [5].

To overcome these problems, we developed the Quick anaero-system for convenient and efficient anaerobic culture. We evaluated the efficacy of the Quick anaero-system using 12 anaerobes cultured in commonly used media by routine aerobic manipulation methods.

MATERIALS AND METHODS

1. Bacterial strains

We used the following strains in our study: *Bacteroides fragilis* (ATCC 25285), *Bacteroides vulgatus* (KCTC 2639), *Bifidobacterium bifidum* (KCTC 3281), *Clostridium difficile* (KCTC 5009), *Clostridium septicum* (ATCC 12464), *Eubacterium limosum* (KCTC 3266), *Fusobacterium nucleatum subsp. polymorphum* (KCTC 2488), *Mobiluncus mulieris* (ATCC 35239), *Peptostreptococcus asaccharolyticus* (KCTC 3321), *Porphyromonas gingivalis* (ATCC 33277), *Propionibacterium acnes* (KCTC 3314), *Veillonella criceti* (ATCC 17747).

2. The Quick anaero-system

The Quick anaero-system—a new disposable anaerobic culture system—consists of 3 components (registered with the Korean Intellectual Property Office; registration no. 10-0791977, no. 20-0436738) (Fig. 1).

1) Disposable anaerobic gas pack

To achieve absolute anaerobic conditions, we have developed a new disposable anaerobic gas pack (DAS Tech, Gwangju, Korea). The gas pack is based on the following chemical principle: silica (SiO_2) and sodium borohydride ($NaBH_4$) tablets react with tap water to generate a volatile hydride (SiH_4). In contrast to other kits based on the borohydride system, we used SiO_2 for rapid hydride generation.

The generation of the hydride was verified by inductively coupled plasma-mass spectrometry (ICP-MS; Thermo Electron Corporation, Waltham, MA, USA) by using the Spectroflame system (SPECTRO A.I. GmbH, Kleve, Germany). The hydrogen from the volatile hydride reacts easily with oxygen in the presence of a palladium catalyst and forms water vapor. The disposable anaerobic gas pack consists of 2 parts. One part generates hydrogen; this part contains 2 sodium borohydride tablets and silica. The second part generates carbon dioxide; this part contains 1 citric acid tablet and 1 sodium bicarbonate tablet. Both parts have an attached narrow-tipped plastic tube into which 10 mL of tap water is poured when it is used.

2) Closed anaerobic culture unit

The unit is composed of a disposable culture envelope, a reusable rack, and a sealer (DAS Tech).

3) Catalyst unit

We used 10 g of 0.5% palladium-coated alumina pellets as catalyst (Heesung Engelhard, Seoul, Korea); the pellets were placed on a stainless mesh that was attached below

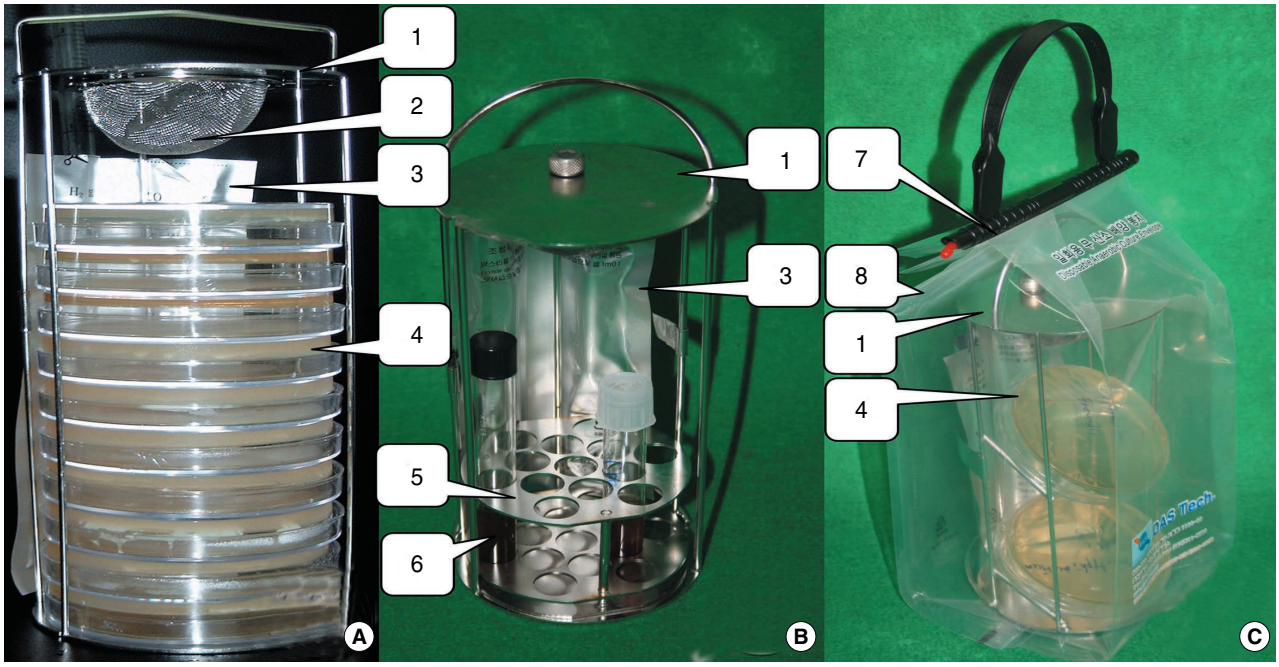

Fig. 1. Features of the Quick anaero-system: Stacked media (A), tube (B), and fully assembled Quick anaero-system with 2 media on which colony formation can be observed through disposable culture-envelope (C). The components of the Quick anaero-system and consumables are indicated by numbers as follows: (1) reusable stainless plate rack with mesh containing palladium catalyst pellet; (2) palladium catalyst pellet; (3) a new disposable anaerobic gas pack; (4) culture media plate; (5) reusable stainless tube rack; (6) tube; (7) sealer; and (8) disposable culture-envelope.

the roof of the reusable rack.

3. Comparison of the efficiencies of Quick anaero-system and BD GasPak EZ Anaerobe Container System in BBL GasPak jar

We isolated 12 anaerobes for this study. The samples were retrieved from frozen stock culture by using a sterilized wire loop, streaked on a brain heart infusion (BHI) agar plate (Becton Dickinson Microbiology Systems) or Luria-Bertani blood agar plate (LB-BAP; Becton Dickinson Microbiology Systems), and incubated under anaerobic conditions at 37℃ for 48 hr. Next, the cells were resuspended in the fresh BHI broth or LB broth and incubated under the same conditions. The density of the organisms in the suspension was adjusted to that of 3 McFarland standards (about 10^9 colony forming units [CFU]/mL) by adding sterile normal saline. Subsequently, bacterial suspension was serially diluted in 10-fold steps up to the 9th dilution (about 10^0 CFU/mL) with LB broth in 96-well microplates. In addition, an aliquot of diluted bacterial suspension from each dilution well was inoculated on the LB-BAP. Duplicate dilution sets were prepared. One set of bacterial dilution was incubated in the Quick-anaero system and the other set was incubated in the BBL GasPak jar with BD GasPak EZ Anaerobe Container System (Becton Dickinson Microbiology Systems). Both systems were incubated at 35-37℃ for 48 hr. The minimal inoculum size that showed visible bacterial growth for all 12 anaerobes in both the systems was recorded. The experiment was repeated 2 times. Finally, the mean values of the test results were calculated for both systems and compared.

4. Determination of the lag time required by the Quick anaero-system to reach an oxygen-free state

The lag time required by the Quick anaero-system with 10 g of palladium catalyst pellets and disposable anaerobic gas pack to reach an oxygen-free state was determined by repeatedly measuring the oxygen concentration 99 times

using the PICK-O_2 sensor (International Technologies Dr. Gambert GmbH, Wismar, Germany).

RESULTS

1. Comparison of the efficiencies of the Quick anaero-system and BD GasPak EZ Anaerobe Container System in BBL GasPak jar

The results of comparison of the minimal inoculum size (CFU/mL) showing visible growth of all 12 anaerobes when incubated in the Quick anaero-system and BD GasPak EZ Anaerobe Container System are shown in Table 1. The minimal inoculum size that showed visible bacterial growth in the LB broth was in the range of 10^0-10^5 for the Quick anaero-system and 10^0-10^6 for the BD GasPak EZ Anaerobe Container System. The dilutions inoculated on LB-BAP were 10^2-10^6 for the Quick anaero-system and 10^0-10^9 for the BD GasPak EZ Anaerobe Container System. The comparison of the minimal inoculum size showing visible growth on the LB broth revealed 2 out of 12 anaerobes grew better when incubated in the Quick anaero-system as compared to when incubated in the BD GasPak EZ Anaerobe Container System. In contrast, 9 out of 12 anaerobes cultured on LB-BAP showed better growth when incubated in the Quick anaero-system than in the BD GasPak EZ Anaerobe Container System. Although all 12 anaerobes grew in both LB broth and on LB-BAP, most bacteria showed better growth in the LB broth than on LB-BAP.

Table 1. The results of comparison of the minimal inoculum size showing visible growth of 12 anaerobes when incubated in the Quick anaero-system and BD GasPak EZ Anaerobe Container System

Bacterial species	Minimal inoculum size (CFU/mL) showing visible bacterial growth			
	LB broth		LB blood agar plate	
	QAS	BD GasPak	QAS	BD GasPak
Bacteroides fragilis	10^1	10^1	10^2	10^7
Bacteroides vulgatus	10^5	10^6	10^4	10^9
Bifidobacterium bifidum	10^4	10^6	10^4	10^7
Clostridium difficile	10^0	10^0	10^3	10^5
Clostridium septicum	10^0	10^0	10^6	10^7
Eubacterium limosum	10^0	10^0	10^3	10^5
Fusobacterium nucleatum subsp. polymorphum	10^0	10^0	10^2	10^4
Mobiluncus mulieris	10^0	10^0	10^2	10^0
Peptostreptococcus asaccharolyticus	10^0	10^0	10^4	10^4
Porphyromonas gingivalis	10^0	10^0	10^4	10^4
Propionibacterium acnes	10^0	10^0	10^4	10^5
Veillonella criceti	10^0	10^0	10^4	10^5

Abbreviations: LB, Luria-Bertani; QAS, Quick anaero-system; BD GasPak, BD GasPak EZ Anaerobe Container System.

2. Determination of the lag time required by the Quick anaero-system to reach an oxygen-free state

The mean lag time (±SD) required for reaching an oxygen-free state was 17 min 56 sec (±3 min 25 sec). The minimum and maximum lag time required for reaching the oxygen-free state were 14 min 7 sec and 33 min 48 sec, respectively.

3. Deduction of the chemical formula and determining the efficiency of palladium catalyst in the Quick anaero-system

The chemical reaction for the generation of the volatile hydride was deduced as follows: $BH_4^- + 15H^+ + 3SiO_2 \rightarrow H_3BO_4 + 3SiH_4\uparrow + 2H_2O$. We postulated that the volatile SiH_4 gas generated in this reaction reacts with oxygen in the air to yield water as follows: $SiH_4 + O_2 \rightarrow Si + 2H_2O$.

DISCUSSION

It is difficult for a clinical microbiologist to set up an anaerobic culture because it needs complicated culture media and rapid methods to reduce the lag time for achieving oxygen-free state.

We developed the Quick anaero-system and evaluated its efficiency based on the growth of 12 strains of obligate anaerobes on commonly used LB broth or LB-BAP after aerobic manipulation.

The lag time to achieve an oxygen-free environment in the anaerobic jar may have an important effect on the viability of highly sensitive anaerobes. The mean time to reach an O_2 concentration of 0.5% was 72-370 min for several

anaerobic systems utilizing sodium borohydride [5]. Long time lag of the conventional anaerobic systems may exert a harmful effect on the more sensitive anaerobes. However, the mean time (±SD) required to achieve absolute anaerobic conditions in the Quick anaero-system was 17 min 56 sec (±3 min 25 sec). This short time lag may ensure the viability of obligate anaerobes in the initial phase of incubation during anaerobic culture. The rapidity of this system may be mainly attributable to adoption of effective disposable anaerobic gas pack and palladium catalyst. The new disposable anaerobic gas pack in the Quick anaero-system afforded maximum generation of volatile hydride. The hydrogen reacted quickly and efficiently with oxygen in the presence of the palladium catalyst to form water vapor. The palladium catalyst effectively catalyzed the reaction and facilitated the formation of an oxygen-free environment in the Quick anaero-system.

The comparison of the culture results obtained in this study indicates that the performance of the Quick anaero-system is superior to the BD GasPak EZ Anaerobe Container System. The good results of the Quick anaero-system may be attributed to the short time lag required to achieve an oxygen-free state. The difference between the performances of the 2 systems was observed more clearly on LB-BAP; the difference may be attributed to the tendency that the growth of anaerobes on LB-BAP was poorer than that in LB broth. Unlike the other 11 anaerobes, *M. mulieris* growth on LB-BAP was better in BD GasPak EZ Anaerobe Container System than in the Quick anaero-system. However, the reason for this difference is not clear. A mild degree of variability was observed between the results according to the batch of the test, species of anaerobes, and the media or anaerobic system used. Therefore, we used the mean values of the test results for comparing the results of the 2 systems. Further studies with a large number of obligate anaerobes of various species are required to clarify all the differences and the underlying mechanisms.

In summary, the Quick anaero-system is a simple and effective method for culturing obligate anaerobes, and the performance of this system is superior to that of the BD GasPak EZ Anaerobe Container System. The Quick anaero-system is a powerful method that requires only a small amount of initial inoculum.

REFERENCES

1. Loesche WJ. Oxygen sensitivity of various anaerobic bacteria. Appl Microbiol 1969;18:723-7.
2. Citron DM. Specimen collection and transport, anaerobic culture techniques, and identification of anaerobes. Rev Infect Dis 1984;6(S1):S51-8.
3. Bennett GN, Hickford JG, Zhou H. Convenient anaerobic techniques, science from the supermarket shelf. Anaerobe 2006;12:49-51.
4. Strobel HJ. Basic laboratory culture methods for anaerobic bacteria. Methods Mol Biol 2009;581:247-61.
5. Imhof A and Heinzer I. Continuous monitoring of oxygen concentrations in several systems for cultivation of anaerobic bacteria. J Clin Microbiol 1996;34:1646-8.