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Proficiencies of Three Anaerobic Culture Systems for Recovering Periodontal Pathogenic Bacteria

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Anaerobic culture is employed routinely in the primary isolation of periodontal pathogenic bacteria. However, little or no data exist on the relative abilities of the Coy anaerobic chamber (Coy Laboratory Products, Grass Lake, Mich.), the GasPak (Becton Dickinson Microbiology Systems, Cockeysville, Md.), and the AnaeroPack (Mitsubishi Gas Chemical America, Inc., New York, N.Y.) systems to grow important periodontal species, including *Porphyromonas gingivalis*, *Prevotella intermedia/nigrescens*, *Bacteroides forsythus*, *Eubacterium* species, *Campylobacter* species, *Fusobacterium* species, and *Peptostreptococcus micros*. A total of 78 specimens from advanced periodontitis lesions were collected anaerobically, plated on enriched blood agar medium, and incubated at 35°C for 5 to 7 days in each anaerobic culture system. The three culture systems were equally efficient in isolating *Porphyromonas gingivalis* and *Prevotella intermedia/nigrescens*. The Coy anaerobic chamber yielded the highest proportional recoveries of *Campylobacter* ($P = 0.0001$; nonparametric analysis of variance) and *Eubacterium* ($P = 0.009$). The Coy anaerobic chamber and the GasPak system demonstrated higher proportional recoveries of *Bacteroides forsythus* ($P = 0.0006$) and *Peptostreptococcus micros* ($P = 0.0001$) than the AnaeroPack system. The AnaeroPack system was most efficient in growing *Fusobacterium* species ($P = 0.0001$). Overall, the Coy anaerobic chamber and the GasPak system showed the highest proportional recoveries of putative periodontal pathogens, but the recoveries by the various anaerobic test systems varied considerably from sample to sample.

Microbial diagnosis of oral infections is performed by using culture, direct microscopic examination, immunoserological identification, and nucleic acid-based methods (18). Clinical oral microbiology laboratories employ one or a combination of these methods, depending on the pathogens to be identified. Rarely does one detection method prove optimal for all situations.

Periodontal infections involve mainly anaerobic bacteria, including *Porphyromonas gingivalis*, *Bacteroides forsythus*, *Prevotella intermedia/nigrescens*, *Peptostreptococcus micros*, *Actinobacillus actinomycetemcomitans*, *Fusobacterium* species, *Eubacterium* species, and *Campylobacter* species (7, 13). Culture constitutes the conventional methodology for identifying periodontal pathogens (9). Nonselective culture comprises the most effective method to elucidate all major pathogenic components of the periodontal microbiota, to identify the presence of unusual periodontal pathogens, and to determine the antimicrobial susceptibility of periodontal pathogens (9, 14, 15). Selective culture is routinely used to recover periodontal *A. actinomycetemcomitans* (14).

The anaerobic chamber provides a convenient culture system for large-scale studies of strictly anaerobic and/or facultatively anaerobic bacteria and is widely used in oral microbiology laboratories (17). Chemically generated anaerobic systems such as the BBL GasPak system (Becton Dickinson Microbiology Systems, Cockeysville, Md.) and the AnaeroPack system (Mitsubishi Gas Chemical America, Inc., New York, N.Y.) may represent attractive alternatives to anaerobic chamber systems, especially for smaller laboratories. The GasPak system generates an anaerobic environment by means of a carbon dioxide and hydrogen generator, water, and a palladium catalyst (1, 17). The AnaeroPack system employs one or two chemical sachets that, after contact with oxygen, generate the anaerobic environment (3, 19). Jar systems constitute the most popular anaerobic culture methodology in the clinical laboratory (6). The efficiency of available anaerobic culture systems has been studied for medical bacteria (3, 6), but to the best of our knowledge, no study has compared the abilities of current anaerobic systems to support the growth of periodontopathic species. Therefore, the present study was performed to determine the relative recoveries of important periodontal bacteria in the Coy anaerobic chamber (Coy Laboratory Products, Grass Lake, Mich.), the GasPak, and the AnaeroPack culture systems.

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MATERIALS AND METHODS

Microbial sampling and processing. The study material consisted of microbiological samples from deep periodontal pockets submitted by extramural dentists to the Oral Microbiology Testing Laboratory at the University of Southern California School of Dentistry. Samples originated from 45 females and 33 males, aged 25 to 77 years, with advanced periodontitis. Forty-six patients were diagnosed with adult periodontitis, 16 were diagnosed with rapidly progressive periodontitis, 14 were diagnosed with refractory periodontitis, and 2 were diagnosed with postlocalized juvenile periodontitis.

Each individual contributed microbial samples from three deep (5-mm or more) periodontal pockets. Sample sites were isolated with cotton rolls, supragingival plaque was removed, and one sterile paper point was inserted to the depth of each periodontal pocket sampled and retained therein for 10 s. The three paper points were then transferred to a 2-ml screw-cap glass vial containing VMGA III transport medium (5% Bacto Gelatin, 0.05% Thione E Peptone [Becton Dickinson Microbiology Systems, Cockeysville, Md.), 0.2% washed Bacto Agar, 0.05% thioglycolic acid, 0.05% L-cysteine-HCl, 1.0% Na glycero-

TABLE 1. Proportional recoveries of suspected periodontal pathogens by three anaerobic culture systems

Bacterium	% of pathogens recovered						P[a]	Proficiency[b]
	Coy chamber		GasPak		AnaeroPack			
	Mean (SD)	Range	Mean (SD)	Range	Mean (SD)	Range		
Porphyromonas gingivalis	3.69 (7.28)	0–39.53	4.11 (8.29)	0–38.01	3.29 (6.85)	0–28.95	0.81	
Prevotella intermedia	4.25 (7.82)	0–39.61	3.65 (7.14)	0–38.27	3.62 (6.34)	0–27.36	0.12	
Bacteroides forsythus	1.09 (1.81)	0–8.84	1.67 (3.40)	0–22.22	0.99 (2.44)	0–14.89	0.0006	C or G > A
Campylobacter sp.	2.47 (5.02)	0–38.89	0.56 (1.42)	0–7.41	0.60 (1.74)	0–12.63	0.001	C > A or G
Eubacterium sp.	1.00 (1.95)	0–10.25	0.67 (1.16)	0–4.70	0.72 (1.37)	0–5.66	0.009	C > A or G
Fusobacterium sp.	2.84 (4.25)	0–26.88	2.94 (3.84)	0–25.21	3.98 (4.07)	0–17.98	0.0001	A > C or G
Peptostreptococcus micros	5.30 (7.49)	0–37.25	6.19 (8.10)	0–39.06	3.47 (4.72)	0–20.61	0.0001	C or G > A

[a] By nonparametric analysis of variance.
[b] C, Coy chamber; G, GasPak system; A, AnaeroPack system.

phosphate, 0.0005% phenylmercuric acetate, 0.0003% methylene blue, 0.024% $CaCl_2 \cdot 6H_2O$, 0.042% KCl, 0.1% NaCl, 0.01% $MgSO_4 \cdot 7H_2O$) (11). The samples were processed within 1 to 3 days of sampling. This time period reflects the usual delay in transporting samples to the microbiology laboratory. Möller (11) showed that VMGA III transport medium was able to sustain the viability of oral anaerobes for at least 3 days.

Samples were processed in room atmosphere and immediately incubated in the test anaerobic culture systems. Microorganisms were mechanically dispersed from the paper points with a Vortex mixer at the maximal setting for 45 s. The bacterial suspension was then serially diluted in 10-fold steps in VMG I anaerobic dispersion solution (0.25% tryptose, 0.25% Thione E Peptone, 0.5% NaCl) (11). By using a sterile bent glass rod, 0.1-ml aliquots from 10^3 to 10^5 dilutions were inoculated onto three sets of freshly prepared plates containing 4.3% brucella agar (BBL Microbiology Systems, Cockeysville, Md.), 0.3% Bacto Agar, 5% defibrinated sheep blood, 0.2% hemolyzed sheep erythrocytes, 0.0005% hemin, and 0.00005% menadione. After incubation at 35°C for 5 to 7 days, total viable counts and the percentage of each test bacterium were determined in each of the three anaerobic culture systems. The identification methods of Slots (15) and commercial micromethod systems were employed for presumptive bacterial identification.

Anaerobic systems. (i) Coy anaerobic chamber. The Coy anaerobic chamber consists of a flexible glove box filled with 85% N_2–10% H_2–5% CO_2 and heated palladium catalyst pellets. Anaerobiosis of the chamber was monitored by using a BBL disposable anaerobic indicator strip (Becton Dickinson).

(ii) BBL GasPak system. The GasPak system includes a 2.5-liter jar with palladium catalyst pellets and a GasPak anaerobic envelope. Pellets were heated in a 125°C oven for 2 h before each use. Prior to the incubation of the blood agar plates, the GasPak anaerobic envelope was activated by adding 10 ml of water to the envelope. The final CO_2 concentration was 4 to 10% (3). The anaerobic conditions were monitored, after 60 min of incubation, by using the BBL disposable anaerobic indicator strip.

(iii) AnaeroPack system. The AnaeroPack system includes a rectangular container (9.5 by 6.75 by 3.25 in.; 2.5 liters) and one AnaeroPack sachet. The sachet was opened and placed into the container along with inoculated blood agar plates and a BBL disposable anaerobic indicator strip. After 60 min of incubation, the oxygen concentration was less than 1% and the CO_2 concentration was approximately 18% (3).

To ensure quality, lids for the jars and containers of the BBL GasPak and the AnaeroPack systems were inspected and sealed as described in the manufacturer's instructions. Also, catalyst pellets for the Coy anaerobic chamber and the GasPak system were reactivated before each use.

Statistical analysis. Differences in the bacterial colony counts (proportional recovery) of the study organisms were analyzed by a nonparametric analysis of variance. It was not uncommon for a test species not to grow in one or more of the anaerobic culturing systems. This led to a distribution that could not be analyzed by standard parametric methods. Hence, a nonparametric repeated-measure analysis of variance was used (5). A P value of 0.05 was considered significant for the quantitative nonparametric analysis of variance. A second analysis examined the ability of three anaerobic systems to detect the test species. Data were dichotomized on the basis of detection or no detection of the species. The McNemar chi-square test (10) was employed to determine statistical differences in detection rate between any two culture systems. Each anaerobic culture system was compared to the two other culture systems examined, leading to a total of three tests per bacterium. To control for repeated testing for each test species, a significance level of 0.017 was used for the McNemar chi-square tests.

RESULTS

The Coy chamber, the GasPak system, and the AnaeroPack system yielded, on average, approximately 10^7 viable counts per sample. No marked difference in total colony counts was observed between the culture systems tested.

When quantitative nonparametric analysis of variance was used, the three culture systems showed similar proportional recoveries of *Porphyromonas gingivalis* and *Prevotella intermedia* (Table 1). However, the Coy anaerobic chamber yielded the highest proportional recovery of *Campylobacter* species ($P = 0.0001$) and *Eubacterium* species ($P = 0.009$) (Table 1). The AnaeroPack culture system demonstrated the highest proportional recovery of *Fusobacterium* species ($P = 0.0001$). The AnaeroPack system showed the lowest proportional recoveries of *B. forsythus* ($P = 0.0006$) and *Peptostreptococcus micros* ($P = 0.0001$) (Table 1).

Table 2 describes the number of samples that showed a test species in one culture system and not in another. The Coy anaerobic chamber system exhibited a higher recovery rate of *Campylobacter* species than the GasPak ($P = 0.0004$) and AnaeroPack ($P = 0.001$) systems. The GasPak system tended to be more efficient than the AnaeroPack system in recovering *Peptostreptococcus micros* ($P = 0.03$).

DISCUSSION

This study compared the proficiencies of three anaerobic culture systems for recovering periodontopathic bacteria. The microorganisms isolated from the 78 test specimens were representative of those from periodontitis lesions in the United States (12).

The three anaerobic culture systems seemed equally efficient in recovering *Porphyromonas gingivalis* and *Prevotella intermedia/nigrescens*. However, the Coy anaerobic chamber system and the GasPak system were more efficient than the AnaeroPack system in growing *Campylobacter* species, *Eubacterium* species, *B. forsythus*, and *Peptostreptococcus micros*. The AnaeroPack system was more efficient in growing *Fusobacterium* species. Overall, the Coy anaerobic chamber and the GasPak systems demonstrated slightly higher proportional recoveries of periodontal anaerobes.

Other authors have reported similar results. Downes et al. (4) evaluated the Anaerobe Systems (San Jose, Calif.) anaerobic chamber, the Anaerobic Pouch System Catalyst-Free (Difco Laboratories, Detroit, Mich.), and the Bio-Bag Environmental Chamber Type A (Marion Scientific, Division of Marion Laboratories, Inc., Kansas City, Mo.) for the cultivation of anaerobic bacteria. They concluded that the anaerobic chamber was more efficient than the pouch systems in recovering fastidious anaerobes. Cox et al. (2) compared the proficiencies of the Bactron IV anaerobic chamber (Sheldon Manufacturing, Cornelius, Oreg.), the GasPak, and the AnaeroPack jar

TABLE 2. Effectiveness of three anaerobic culture systems in detecting the presence of suspected periodontal pathogens

Test species	No. of samples showing test species		χ^{2b}	P^c
	Coy chamber+/GasPak−[a]	GasPak+/Coy chamber−		
Porphyromonas gingivalis	7	6	0.04	0.845
Prevotella intermedia	8	5	0.35	0.556
Bacteroides forsythus	11	14	0.18	0.671
Campylobacter sp.	33	3	12.5	0.0004
Eubacterium sp.	16	7	1.76	0.185
Fusobacterium sp.	1	3	0.5	0.48
Peptostreptococcus micros	2	6	1	0.317
	Coy chamber+/AnaeroPack−	AnaeroPack+/Coy chamber−		
Porphyromonas gingivalis	11	8	0.24	0.626
Prevotella intermedia	5	7	0.17	0.683
Bacteroides forsythus	17	11	0.64	0.423
Campylobacter sp.	31	4	10.41	0.001
Eubacterium sp.	18	10	1.14	0.285
Fusobacterium sp.	1	5	1.33	0.248
Peptostreptococcus micros	15	7	1.45	0.228
	GasPak+/AnaeroPack−	AnaeroPack+/GasPak−		
Porphyromonas gingivalis	8	6	0.14	0.705
Prevotella intermedia	5	10	0.83	0.361
Bacteroides forsythus	13	4	2.38	0.123
Campylobacter sp.	12	15	0.17	0.683
Eubacterium sp.	11	12	0.02	0.883
Fusobacterium sp.	1	3	0.5	0.48
Peptostreptococcus micros	14	2	4.5	0.034

[a] +, growth; −, no growth.
[b] By the McNemar chi-square test.
[c] *P* of 0.017 denotes significant difference.

systems. By measuring the bacterial colony sizes, they concluded that the anaerobic chamber showed a better recovery of anaerobic bacteria than the anaerobic jar systems tested.

Subgingival periodontopathic organisms differ in oxygen sensitivity. Loesche (8) demonstrated that fastidious oral anaerobic species are incapable of growing at partial oxygen levels of greater than 0.5%. Even if a short exposure to oxygen may not kill oral anaerobic bacteria, anaerobic culture systems aim to achieve anaerobiosis as soon as possible after sample processing. The higher proportional recovery of *Eubacterium* species in the Coy anaerobic chamber in our study may in part be due to the longer exposure time to oxygen of samples in the anaerobic jar systems. The reason for the higher proportional recovery of *Campylobacter* species, which may grow in the presence of low concentrations of oxygen, is not clear. Cox et al. (2) indicated that clinical samples processed within the anaerobic chamber showed better recovery than those processed in air.

Delaney and Onderdonk (3) and Van Horn et al. (19) reported a high proficiency of the AnaeroPack system for growing clinically significant anaerobes. However, these two studies included laboratory bacterial strains, whereas we examined the primary recovery of periodontopathic species in samples from periodontal lesions. It is well known that laboratory adaptation of anaerobic species facilitates bacterial subculture (17). Differences in microbiological findings may also be due to differences in sample processing and culture media (16).

The Coy anaerobic chamber can process high volumes of bacterial plates and exhibits good recovery for most subgingival anaerobic organisms but can be expensive to purchase and maintain, costing in excess of $10,000. The BBL GasPak system is limited to processing a few bacterial plates at a time but costs only approximately $400 per jar and $2.00 per anaerobic atmosphere-generating envelope. The Coy anaerobic chamber also requires a relatively large space, while the GasPak jar is small enough to fit a medium-size incubator. For oral microbiology laboratories that process a limited number of anaerobic samples, the GasPak anaerobic culture system seems to offer a convenient and effective method for recovering periodontal pathogens.

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