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Improved Isolation of Anaerobic Bacteria From the Gingival Crevice Area of Man

D. F. GORDON,[1] M. STUTMAN,[2] AND W. J. LOESCHE

Department of Bacteriology, Forsyth Dental Center, Boston University School of Graduate Dentistry, Boston, Massachusetts, and Department of Oral Biology, University of Michigan School of Dentistry, Ann Arbor, Michigan 48104

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A roll tube technique (Hungate method) was employed in an attempt to cultivate a maximal portion of the organisms in the gingival crevice area of man. This technique achieves an anaerobic state by flushing the local environment with oxygen-free gas. Once collected, the crevicular debris was immediately placed into sterile oxygen-free test tubes which were flushed out by the oxygen-free gas. In this manner, the samples were weighed, dispersed, diluted, and cultured in roll tubes and plates. The medium for control (Brewer Jar technique) and Hungate techniques was Heart Infusion Agar fortified with 10% defibrinated horse blood. When the Hungate technique was used, the recovery of viable bacteria, as a percentage of the direct microscopic count, was significantly greater than plates incubated aerobically or utilizing the Brewer Anaerobic technique. Cultural counts by using the Hungate method averaged 41.3% for six samples when 90% nitrogen and 10% hydrogen were used, 70.4% for eight samples when 85% nitrogen, 10% hydrogen, and 5% carbon dioxide were used, and 63.4% for eight samples when 100% carbon dioxide was the gaseous atmosphere. At no time were cultural counts, by using anaerobic plates (Brewer Jar), more than 24% of the direct microscopic count. This suggests that exclusion of oxygen and the presence of carbon dioxide maximized recovery of gingival crevice bacteria.

The gingival crevice area of man appears to harbor an anaerobic microflora. Thus, when bacteriological samples were removed, dispersed, diluted, and plated on nutritionally complex media, twice as many organisms were recovered when the high dilution plates were incubated in an anaerobic atmosphere than when incubated in air (2, 3, 9). Most of the aerobic isolates were facultative bacteria, as they would grow also in an anaerobic environment (3). When the viable counts were compared with the total microscopic counts, only 20 to 25% of the organisms present were cultivated (9). The inability to cultivate all of the gingival crevice bacteria was attributed to the presence of nonviable bacteria, the absence of a medium that would be nutritionally adequate for all of the indigenous gingival crevice organisms, or to the failure to disperse completely the clumps of bacteria present in the sample. Recently Aranki et al. (1) were able to markedly improve the recoveries of anaerobes from gingival crevice samples by performing the various bacteriological procedures in an anaerobic glove box in an oxygen-free atmosphere. Their work suggested that many of the gingival organisms were killed by oxygen during the manipulative procedures prior to anaerobic incubation.

The purpose of the present investigation was to develop a means of increasing the recovery of viable organisms from the human gingival crevice by achieving continuous anaerobiosis in all phases of the experimental procedure. The technique developed by Hungate (4) was used for this purpose.

MATERIALS AND METHODS

Complete anaerobiosis. Atmospheric oxygen was eliminated or greatly diluted in the vicinity of the bacteriological sample by means of an oxygen-free gas (OFG). Commercial gas mixtures containing (i) 90% nitrogen and 10% hydrogen; (ii) 85% nitrogen, 10% hydrogen, and 5% carbon dioxide, or (iii) 100% carbon dioxide were made oxygen-free by passing the gas through a Pyrex tube containing hot copper turnings (4, 8). The OFG was passed through neoprene tubing fitted at the end with a stainless-steel

[1] Present address: Department of Allied Health, Quinnipiac College, Hamden, Conn. 06518.

[2] Present address: 6218 MacDonald Avenue, Hampstead, Quebec, Canada.

needle. This was used to either displace or prevent air from entering the sample, diluent, and media during collection, preparation, and inoculation.

Preparation of diluent and medium. The diluent contained 0.067 M phosphate buffer (*p*H 7.2), 0.1% peptone (Difco), 0.001% sodium thioglycolate, and 0.001% resazurin. The medium contained 4% Heart Infusion Agar (Difco), 10% defibrinated horse blood, 0.001% sodium thioglycolate, 0.001% resazurin, and menadione (0.5 μg/ml). The blood and menadione were flushed with OFG and added aseptically to the autoclaved medium.

When 100% carbon dioxide was employed, 0.5% sodium bicarbonate (final concentration in medium) was autoclaved separately, equilibrated with CO_2, and added to the diluent and medium so as to neutralize the acidity created by the dissolved carbon dioxide. The diluent and medium were prepared in 100-ml amounts in 250-ml Erlenmeyer flasks fitted with gauze stoppers. Immediately after autoclaving, these solutions were flushed with OFG until the resazurin was colorless and sterile neoprene stoppers were inserted into the flasks. If it was necessary to add any ingredient after autoclaving, these were also flushed with the OFG. After addition of blood to the medium, a change in the resazurin indicator could not be visualized.

The diluent was tubed in 9-ml amounts and portions of the medium were tubed in 4-ml amounts and maintained at 48 C until inoculated. Both the diluent and medium were placed in sterile test tubes (16 by 150 mm) and flushed with OFG. The remaining medium was poured into petri plates which were used in culturing the gingival debris under conventional aerobic and anaerobic techniques.

Collection of sample. Periodontally normal individuals (i.e., no periodontal pockets greater than 3 mm and no evidence of gingivitis or bone loss) ranging in age from 21 to 33 yr served as subjects. These volunteers had not eaten for at least 2 hr prior to sampling. They were seated adjacent to the OFG source so that the sample could be collected under a stream of OFG. Samples were removed from sulci which were less than 3 mm in depth and showed no clinical signs of inflammation. Crevicular debris was placed immediately on a piece of sterile aluminum foil contained in a preweighed tube. The tube was flushed with OFG, stoppered, and quickly weighed to obtain the wet weight of the crevicular debris.

Dispersion, dilution, and plating of sample. A 9-kc Raytheon sonic oscillator was used to disperse the gingival crevice debris. The oscillator chamber was fitted with a neoprene stopper containing two open glass tubes, one of which was connected to the OFG and the other served as a vent. Reduced diluent (15 ml) was placed in the chamber. The small piece of aluminum foil containing the crevicular debris was then carefully removed from the tube under coverage of the OFG and placed in the oscillator. The sample was dispersed for 30 sec under a continuous flow of OFG. Samples were removed from the oscillator and serially diluted. It was possible to execute this procedure without oxidation of the resazurin indicator in the diluent.

Samples (0.1 ml) of the appropriate dilution were plated and incubated as follows. (i) *Roll tubes, complete anaerobiosis* (*4*). Tubes were prepared in triplicate by inoculating the reduced blood-agar medium maintained at 48 C. The tubes were stoppered and rolled gently in cold water to obtain an even, thin film of blood-agar around the inside of the tube. (ii) *Conventional anaerobic technique.* Three blood-agar petri plates were inoculated in room atmosphere, and the sample was spread on the surface by means of a sterile glass rod. These plates were incubated in Brewer Jars containing 85% nitrogen, 10% hydrogen, and 5% carbon dioxide. In this procedure, residual oxygen in the presence of hydrogen is converted to water by means of a heat-activated platinum catalyst contained in the lid of the Brewer Jar. (iii) *Conventional aerobic techniques.* Three blood-agar plates treated as above except that they were incubated aerobically.

All tubes and plates were incubated at 35 C for 5 days. Total microscopic counts were obtained by placing the dispersed material from the sonic oscillator in a Petroff-Hauser counting chamber and counting the bacteria by using a dark-field microscope. Clumps of bacteria were occasionally observed and were counted as a single microscopic unit. The statistical significance of the data was determined by the Student *t* test.

RESULTS

All samples were collected, dispersed, and diluted under conditions of complete anaerobiosis, i.e., resazurin remained colorless. Samples of the final dilution were then used to inoculate roll tubes or were used to inoculate plates in the conventional manner. The results obtained when the OFG was 90% nitrogen and 10% hydrogen are shown in Table 1. The mean total microscopic count was $24.7 \pm 8.7 \times 10^{10}$ per gram (wet weight) of crevicular debris. Under aerobic conditions, 14.8% of these organisms were cultured (Table 4). When conventional anaerobic incubation was employed, the recoveries improved to 22.2%. When oxygen was completely excluded, 41.3% of the bacteria proved to be viable.

Carbon dioxide appears to be essential for the growth of certain oral anaerobes, i.e., *Bacteroides melaninogenicus* and *Fusobacterium nucleatum* (Loesche, *unpublished results*). These organisms, readily identified by distinct colonial appearance, were not observed in the role tube isolates. Thus, the omission of CO_2 from the OFG would be expected to give lower recoveries than might otherwise be achieved by the inclusion of this gas. The experiment was repeated with the addition of 5% CO_2 in the OFG. Eight samples which contained an average of $26.9 \pm 11.7 \times 10^{10}$ bacteria per gram (wet weight) were each treated as before. The aerobic count was $2.2 \pm 1.5 \times 10^{10}$/g (wet weight); the conventional anaerobic count was $5.4 \pm 2.9 \times 10^{10}$/g (wet weight); whereas the

TABLE 1. *Recovery of bacteria from gingival crevice debris*

Sample no.	Wet weight of sample (mg)	Microscopic count [$\times 10^{10}$/g(wet wt)]	Viable counts [$\times 10^{10}$/g(wet wt)]		
			Aerobic	Anaerobic (Brewer Jar)	Anaerobic (roll tubes), 90% N_2, 10% H_2
1	1.0	40.5	3.1	9.1	16.8
2	2.0	28.5	1.5	4.1	8.1
3	1.5	21.0	1.7	4.7	8.6
4	2.4	19.3	$<10^{10}$	2.0	4.0
5	2.3	22.1	$<10^{10}$	1.0	5.1
6	2.0	16.5	6.3	9.7	15.4
SD[a]	1.9 ± 0.5	24.7 ± 8.7	3.15 ± 2.2	5.1 ± 3.6	9.6 ± 5.3

[a] Standard deviation. Differences between aerobic and anaerobic (Brewer Jar), $P = 0.18$; between aerobic and anaerobic (roll tubes), $P = 0.03$; and between anaerobic (Brewer Jar) and anaerobic (roll tubes), $P = 0.06$.

TABLE 2. *Recovery of bacteria from gingival crevice debris*

Sample no.	Wet weight of sample (mg)	Total microscopic count [$\times 10^{10}$/g (wet wt)]	Viable counts [$\times 10^{10}$/g (wet wt)]		
			Aerobic	Anaerobic (Brewer Jar)	Anaerobic (roll tubes), 85% N_2, 10% H_2, 5% CO_2
1	2.8	10.7	1.0	2.6	9.8
2	2.2	16.3	1.2	3.3	10.4
3	0.5	30.0	5.1	9.0	14.6
4	1.8	35.0	$<10^{10}$	4.0	16.6
5	1.8	20.7	1.2	2.4	21.7
6	1.6	20.6	1.5	5.1	15.9
7	1.0	37.1	3.1	9.6	31.2
8	1.4	45.0	2.6	7.3	22.8
SD[a]	1.6 ± 0.7	26.9 ± 11.7	2.2 ± 1.5	5.4 ± 2.9	17.9 ± 7.1

[a] Standard deviation. Differences were significant between aerobic and anaerobic (Brewer Jar), $P = 0.01$; between aerobic and anaerobic (roll tubes), $P = <0.01$; and between anaerobic (Brewer Jar) and anaerobic (roll tubes), $P = 0.01$.

roll tube count was 17.9 ± 7.1 × 10^{10}/g (wet weight; Table 2). All differences were highly significant, i.e., $P \leq 0.01$. The roll tube recovery was 70.4% of the microscopic count and included colonies of *B. melaninogenicus* which appeared in five of the samples. Viable counts of this organism alone averaged 5.4% of the microscopic count. Aerobic and conventional anaerobic counts represented 8.7 and 20.6%, respectively, of the total count

High recoveries of bacteria from the rumen (5) and from the mouse cecum (10) were achieved with 100% CO_2 as the OFG. Accordingly, the experiment was repeated by using 100% CO_2. Eight samples containing 15.5 ± 3.9 × 10^{10} bacteria per gram (wet weight) were dispersed, diluted, and plated as before. The recoveries obtained were as follows: aerobic, 2.0 ± 0.4 × 10^{10}/g (wet weight); conventional anaerobic, 3.7 ± 0.9 × 10^{10}/g (wet weight); roll tube, 9.8 ± 2.8 × 10^{10}/g (wet weight; Table 3).

All differences were highly significant, i.e., $P < 0.01$. The per cent recoveries with 100% CO_2 were aerobic, 12.3%; conventional anaerobic, 24.0%; and roll tube, 63.4% (Table 4).

DISCUSSION

Anaerobic incubation yielded significantly higher recoveries than aerobic incubation. Of the two anaerobic procedures tested, the roll tube method, when the incubating atmosphere contained CO_2, permitted significantly higher recoveries than the anaerobic jar method (Table 4). The only differences in sample manipulation between these methods in the present experiment had to do with the plating of the diluted sample.

TABLE 3. *Recovery of bacteria from gingival crevice debris*

Sample no.	Wet weight of sample (mg)	Total Microscopic count [× 10^{10}/g (wet wt)]	Viable counts [× 10^{10}/g (wet wt)]		
			Aerobic	Anaerobic (Brewer Jar)	Anaerobic (roll tubes), 100% CO_2
1	1.1	8.1	<1.0	2.4	5.5
2	1.8	16.6	2.1	3.9	12.7
3	1.3	17.3	1.9	3.8	8.6
4	1.8	20.8	1.8	4.1	13.3
5	2.0	15.7	2.0	3.4	9.7
6	1.9	11.8	1.4	2.4	7.0
7	2.2	17.7	2.4	4.2	12.2
8	2.1	15.7	2.4	5.0	9.2
SD[a]	1.8 ± 0.4	15.5 ± 3.9	2.0 ± 0.4	3.7 ± 0.9	9.8 ± 2.8

[a] Standard deviation. Differences were significant between aerobic and anaerobic (Brewer Jar), $P = <0.01$; between aerobic and anaerobic (roll tubes), $P = < 0.01$; and between anaerobic (Brewer Jar) and anaerobic (roll tubes), $P = <0.01$.

TABLE 4. *Effect of oxygen exposure on percentage recovery[a] of bacteria from gingival crevice debris*

Air exposure	Percentage recovery with gas atmosphere of		
	90% N_2 and 10% H_2	100 % CO_2	85% N_2, 10% H_2, and 5% CO_2
During plating and incubation (aerobic)	14.8 ± 15.6[b]	12.3 ± 2.1	8.7 ± 3.9
During plating (Brewer Jar)	22.2 ± 19.2	24.0 ± 4.2	20.6 ± 6.9
No exposure (roll tube)	41.3 ± 26.9	63.4 ± 8.1	70.4 ± 20.7

[a] Per cent recovery = viable count divided by microscopic count × 100.

[b] ±, standard deviation. Differences were significant between aerobic and Brewer Jar in an atmosphere of 100% CO_2, $P = <0.01$; between aerobic and Brewer Jar in an atmosphere of 85% N_2, 10% H_2, and 5% CO_2, $P = <0.01$; between Brewer Jar and roll tube in an atmosphere of 100% CO_2, $P = <0.01$; between Brewer Jar and roll tube in an atmosphere of 85% N_2, 10% H_2, and 5% CO_2, $P = <0.01$; and between atmospheres of 90% N_2 and 10% H_2 and 100% CO_2 with roll tube, $P = 0.02$.

In the roll tube method, the sample was added to agar medium maintained at 48 C in the absence of atmospheric oxygen. The colonies developed within and on the surface of the agar medium. In the anaerobic jar method, the diluted samples were exposed to atmospheric oxygen during the brief period in which they were spread over the agar surface. The inferior recovery of the anaerobic jar method would seem to be due to the exposure of the bacteria or medium, or both, to oxygen. In different experiments, the time between plating and removal of air from the anaerobic jar varied between 20 and 45 min. This brief exposure to air apparently is sufficient to kill many strict anaerobes (6). Similar findings were reported recently by McMinn and Crawford (7). They found that *Propionibacterium propionicum* would not survive beyond 30 min in aerobic transport broth.

The higher counts obtained with the roll tube method raised the question as to whether these isolates are more oxygen-sensitive members of known oral species or whether they are to date undescribed species. Spears and Freter (10) reported that 10 roll tube isolates, on subculture, would not grow in anaerobic jars. Several rumen species, isolated and maintained in roll tubes, would not grow as surface colonies in the presence of oxygen levels greater than 0.7%. However, several oral species, isolated and maintained in Brewer Jars, grew reliably in the presence of 4% oxygen (6). It would seem that some anaerobic bacteria are uniquely sensitive to oxygen and are different from others that can survive short exposures to air and grow in the presence of low levels of oxygen. Thus, many of the roll tube isolates upon further investigation may prove to be species not presently known to reside in the oral cavity.

The most difficult problem in maintaining strict anaerobiosis was during the sample collection. The subject was seated in the laboratory and the

sample was transferred from crevice to an oxygen-free test tube in less than 5 sec. In previous count studies of human gingival debris (2, 3, 9), material was collected aerobically and subjected to atmospheric conditions for an unspecified time before anaerobic incubation. The present investigation in terms of total microscopic count and viable recoveries in anaerobic jars compared quite well with the results of these previous studies. However, the present recovery from roll tube was about three times higher. This would suggest that the failure to grow more gingival organisms in these earlier studies was due to a killing effect of atmospheric oxygen during the manipulation of the sample prior to anaerobic incubation (1).

The roll tube procedure is a relatively simple technique for obtaining an anaerobic environment suitable for the isolation of strict anaerobes which colonize in large numbers the mucous membranes of mammals. The necessary equipment is inexpensive and requires little bench space. The technique itself is simple, and, given its marked superiority in isolating bacteria from a wide range of specimens, should be a routine procedure in various laboratories.

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