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Medium for Selective Isolation of *Fusobacterium nucleatum* from Human Periodontal Pockets

CLAY B. WALKER,* DIANE RATLIFF, DONNA MULLER, ROBERT MANDELL, AND SIGMUND S. SOCRANSKY

Forsyth Dental Center, Boston, Massachusetts 02115

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A selective medium, CVE agar, was developed for the isolation of *Fusobacterium nucleatum* from subgingival plaque of periodontally diseased patients. The medium contained 1.0% Trypticase (BBL Microbiology Systems), 0.5% yeast extract, 0.5% NaCl, 0.2% glucose, 0.02% L-tryptophan, 1.5% agar, and 5% defibrinated whole sheep blood. Erythromycin and crystal violet were added as the selective inhibitory agents at concentrations of 4 and 5 μg/ml, respectively. The medium permitted almost total recovery of *F. nucleatum* when compared with a nonselective medium and suppressed the recovery of most remaining species by 6 to 8 orders of magnitude. Microorganisms suppressed to a lesser degree included *Selenomonas sputigena*, *Actinobacillus actinomycetemcomitans*, *Eikenella corrodens*, and some strains of *Peptostreptococcus*. The distinct colonial morphology of *F. nucleatum* on CVE agar made differentiation relatively easy when contaminants were present. With this medium, *F. nucleatum* was enumerated from 278 subgingival plaque samples and accounted for <1.0 to >25% of the cultivatable microbiota.

Fusobacteria, in particular the species *Fusobacterium nucleatum*, are commonly isolated from human subgingival plaques associated with both healthy and periodontally diseased sites. The organisms have been found in subgingival sites associated with long-standing gingivitis (10, 27), in association with spirochetes in lesions of acute necrotizing ulcerative gingivitis (9), in periodontosis (13, 14), and in periodontitis (4, 6, 19, 20, 26, 28).

F. nucleatum strains isolated from human periodontitis lesions have been shown to accelerate alveolar bone loss when implanted as monocontaminants in gnotobiotic rats (A. Tanner, personal communication). In addition, *F. nucleatum* and *Treponema denticola* strains isolated from human subgingival plaque, when mixed together, were infective for mice. Infection could not be achieved with treponemes alone or when either component was replaced by nonoral strains (C. B. Walker, B. J. Veltri, B. Laughton, and T. D. Wilkins, Program Abstr. Intersci. Conf. Antimicrob. Agents Chemother. 17th, New York, N.Y., abstr. no. 171, 1977). Although animal studies have demonstrated the pathogenic potential of *F. nucleatum*, they do not clarify its role in the etiology of human periodontal diseases.

One approach to determining the role of an organism in an infectious disease is to determine its incidence and proportions in diseased and healthy sites. To facilitate such studies, rapid means of enumeration are required. Methods currently available for the detection and enumeration of groups of organisms from a large number of plaque samples include the use of direct microscopy, fluorescent-antibody techniques, and selective media. Direct microscopy may present misleading results, since other microorganisms in subgingival plaque, including *Capnocytophaga* and "fusiform-shaped" *Bacteroides* (26), have cellular morphologies similar to that of *F. nucleatum*. In our laboratory, fluorescent-antibody techniques have not been successful since the specific antibodies produced to *F. nucleatum* appear to be largely strain specific (unpublished data). Although a number of selective media are available for fusobacteria (2, 12, 16-18, 21), they either fail to sufficiently suppress the majority of contaminating subgingival microorganisms or else significantly suppress the fusobacteria.

The purpose of the present investigation was to develop a selective medium that would recover the majority of *F. nucleatum* present in subgingival plaque samples and suppress most of the other organisms.

MATERIALS AND METHODS

Bacterial strains. Sixty characterized strains representing the groups of microorganisms most frequently isolated from human periodontal pockets were

used as test organisms. These included a reference strain of *F. nucleatum* (ATCC 10953) and 15 strains of fusobacteria isolated from periodontal lesions and identified as *F. nucleatum* based on the criteria given by Holdeman and Moore (7). The *F. nucleatum* strains included both type I (formerly *F. nucleatum*) and type II (formerly *Fusobacterium polymorphum*) strains based on the classification of Baird-Parker (3). A nonoral strain, *Bacteroides fragilis* VPI 8708AP, with previously described minimal inhibitory concentrations (MICs) and growth characteristics, was used as a reference strain (C. B. Walker, Ph.D. thesis, Virginia Polytechnic Institute and State University, Blacksburg, 1977).

Cultivation. Before the study, stock cultures were preserved in Todd-Hewitt broth (Difco Laboratories, Detroit, Mich.) supplemented with 0.5% dimethyl sulfoxide and frozen in liquid N_2. During the study, all strains, except *Bacteroides asaccharolyticus* and *Bacteroides melaninogenicus*, were maintained on Trypticase-soy (T-soy) blood agar (BBL Microbiology Systems, Cockeysville, Md.) and transferred weekly. Strains of *B. asaccharolyticus* and *B. melaninogenicus* subspecies were maintained on T-soy blood agar supplemented with 5 μg of hemin (equine III, Sigma Chemical Co., St. Louis, Mo.) per ml and 0.5 μg of menadione (Sigma) per ml and transferred weekly. All strains were incubated at 37°C in an anaerobic chamber containing an atmosphere of 80% N_2, 10% CO_2, and 10% H_2.

Broth media. Broth media used for the cultivation of the bacteria were as follows: Mycoplasma broth base (BBL Microbiology Systems) supplemented with 0.3% glucose (MBB); Mycoplasma broth base supplemented with 0.3% glucose, 0.1% formate, 0.15% fumarate, and 0.1% KNO_3 (MBB-FF); and CS4 broth which consisted of 1.7% Trypticase (BBL Microbiology Systems), 0.3% yeast extract (Difco), 0.5% NaCl, 0.12% K_2HPO_4, 0.05% sodium thioglycolate, 0.05% Tween 80, and 0.2% glucose. All broth media were supplemented with 5 μg of hemin per ml and 0.5 μg of menadione per ml and were prepared prereduced (7).

Broth cultures. Broth inocula were prepared by transferring three to five colonies from a T-soy blood agar to a broth medium. Fusobacteria, *Selenomonas*, streptococci, oral campylobacters, and peptostreptococci were inoculated into MBB. Actinobacilli were inoculated into MBB to which filter-sterilized $NaHCO_3$ was added at the time of inoculation to give a final concentration in the medium of 2 mg/ml. *Capnocytophaga, B. asaccharolyticus, B. melaninogenicus, Bacteroides ureolyticus, Eikenella corrodens*, anaerobic vibrios, and the "corroding" *Bacteroides* were inoculated into MBB-FF. CS4 broth was used for the actinomyces. All broth cultures were inoculated by use of a VPI Anaerobic Culture System (Bellco Glass, Inc., Vineland, N.J.) while using an O_2-free mixture gas of 80% N_2, 10% CO_2, and 10% H_2. Cultures were incubated at 37°C for 24 to 48 h. Bacterial strains which did not grow to sufficient turbidity in broth media were grown on T-soy blood agar, scraped from the agar surface, and suspended in prereduced one-fourth-strength Ringer solution (1).

Agar media. T-soy blood agar plates (BBL Microbiology Systems) were purchased commercially and stored at 5°C until used. The composition of CVE agar was as follows (per liter): Trypticase (BBL Microbiology Systems), 10.0 g; yeast extract (Difco), 5.0 g; sodium chloride (J. T. Baker Chemical Co., Phillipsburg, N.J.), 5.0 g; glucose (Baker), 2.0 g; tryptophan (Sigma), 0.2 g; agar (Difco), 15.0 g; crystal violet (Baker), 0.005 g; erythromycin (Sigma), 0.004 g; and defibrinated sheep blood (BBL Microbiology Systems), 50.0 ml. The pH was adjusted to 7.0 to 7.2 before autoclaving at 121°C for 20 min. Crystal violet was added before autoclaving; erythromycin and defibrinated whole sheep blood were added aseptically to the medium after it had been autoclaved and cooled to 50 to 55°C. The agar plates were stored aerobically at 5°C and used within 10 days after preparation.

MICs. The MICs of erythromycin and crystal violet were determined by an agar dilution technique using the basal medium. Inocula were grown as described above, the turbidity was adjusted approximately equal to a no. 1 McFarland standard (1), and broth cultures were inoculated aerobically onto the agar surface by use of a Steers replicator (22). As soon as the inocula were dry, the agar plates were placed in an anaerobic chamber and incubated at 37°C for 48 h. The MIC was defined as the lowest concentration that gave no growth.

Recovery or suppression rate of medium. Pure cultures grown overnight in broth were sonicated for 5 s at an amplitude of 6 μm with an ultrasonic disintegrator (MSE, Inc., Westlake, Ohio) equipped with a 0.375-in. (ca. 0.95-cm) titanium probe. The broth culture was continuously flushed with a stream of O_2-free N_2 while being sonicated. Tenfold dilutions were then made in one-fourth-strength Ringer solution under a stream of O_2-free N_2 with a VPI Anaerobic Culture System. Triplicate T-soy blood agar and CVE agar plates were spread with 0.1 ml of inoculum at each dilution. The agar media were incubated at 37°C for 5 days in an atmosphere of 80% N_2, 10% CO_2, and 10% H_2.

Clinical samples. Subgingival plaque samples were taken from the base of the periodontal pocket from patients having advanced periodontal disease as described by Newman and Socransky (13). The samples were immediately placed in prereduced one-fourth-strength Ringer solution and sonicated for 10 s to disperse the plaque, and then 10-fold dilutions were made. Samples were plated onto CVE agar for enumeration of *F. nucleatum* and onto T-soy blood agar for total viable counts. All plates were incubated for 5 days at 37°C in an anaerobic chamber as previously described.

RESULTS

Selective agents. The MICs of erythromycin and crystal violet were determined for 13 strains of *F. nucleatum* isolated from human periodontal pockets. A total of 45 strains of other bacteria commonly encountered in such lesions were also tested. The *F. nucleatum* strains all had an MIC of 8 μg/ml or higher for erythromycin. The MICs of crystal violet were 32 μg/ml and higher, with the exception of one strain (Table 1). No differ-

TABLE 1. *MICs of erythromycin and crystal violet for F. nucleatum isolated from human periodontal pockets*

F. nucleatum strain	MIC (μg/ml)	
	Erythromycin	Crystal violet
Type I		
FDC 277	64	32
FDC 282	16	32
FDC 306	16	32
FDC 364	32	64
FDC 2097	32	32
Type II		
FDC 26	>64	128
FDC 101	16	32
FDC 262	32	32
FDC 304	16	8
FDC 317	>64	64
FDC 318	16	32
FDC 397	8	32
FDC 398	16	32

ences were found in the MICs obtained for type I and type II strains.

The oral campylobacter and some of the *Actinobacillus actinomycetemcomitans* strains exhibited relatively high resistance to both of these agents. However, most bacterial strains other than *Fusobacterium*, *Streptococcus*, and *Peptostreptococcus* were sensitive to low concentrations of both erythromycin and crystal violet. The streptococcal and peptostreptococcal species were often relatively resistant to one agent, but were inhibited by low concentrations of the other (Table 2).

Medium. Different concentrations of erythromycin and crystal violet were tested in the basal medium to determine the optimal recovery of six pure cultures of *F. nucleatum*. The strains used and the concentrations tested are given in Table 3. Concentrations of 4 μg of erythromycin per ml and 5 μg of crystal violet per ml gave a recovery comparable to the basal medium and T-soy blood agar. Higher concentrations of either of these agents resulted in a decrease in recovery.

Recovery of pure cultures of *F. nucleatum*. The recovery rates of 12 pure cultures of *F. nucleatum*, representing both type I and type II, were determined on CVE agar as compared with T-soy blood agar (Table 4). Recovery rates ranged from 79 to 167%. The two distinct colony types were readily apparent and could be used to distinguish the two types of *F. nucleatum*. On CVE agar, type I formed a 2-mm transparent, smooth, round blue colony having an entire edge with a darker blue center. Type II formed a 1-

TABLE 2. *MICs of erythromycin and crystal violet for bacteria most commonly encountered in human periodontal pockets*

Bacteria	MIC (μg/ml)	
	Erythromycin	Crystal violet
Gram-positive organisms		
Actinomyces israelii	≤0.06	1.0
Actinomyces naeslundii	≤0.06	1.0
Actinomyces odontolyticus	0.125	4.0
Actinomyces viscosus	≤0.06	1.0
Streptococcus species	0.125–0.5	8–16
Peptostreptococcus species	8–32	1–4
Gram-negative organisms		
Anaerobic *Vibrio*	0.5–1	1–4
Bacteroides asaccharolyticus	0.5	1.0
Bacteroides fragilis	8.0	2.0
Bacteroides melaninogenicus subsp. *intermedius*	1.0	1.0
Bacteroides melaninogenicus subsp. *melaninogenicus*	0.25	1.0
Bacteroides ureolyticus	0.125	4.0
Capnocytophaga gingivalis	1.0	1.0
Capnocytophaga ochracea	1.0	1–2
Capnocytophaga sputigena	2.0	2.0
Corroding *Bacteroides*	1–4	8–16
Eikenella corrodens	2.0	1–128
Selenomonas sputigena	4.0	4.0
Actinobacillus actinomycetemcomitans	1–32	32–>128
Oral *Campylobacter*	8.0	8–64

TABLE 3. *Effect of different concentrations of crystal violet and erythromycin in CVE agar on the recovery of pure cultures of F. nucleatum*

Strain	% Recovery[a] at following concn (μg/ml) of crystal violet and erythromycin, respectively:					
	0, 0	5, 4	10, 4	5, 8	10, 8	20, 16
Type I						
FDC 277	102	97	0.4	0.05	0.1	0.01
FDC 282	104	105	11	7	7	0.5
FDC 364	99	99	99	97	72	40
Type II						
FDC 304	100	96	20	5	6	0.01
FDC 397	110	108	62	0.6	0.1	0.01
FDC 398	91	89	82	85	83	71

[a] Colony-forming units on T-soy blood agar considered as 100%.

to 2-mm transparent, round or irregular blue colony with a speckled appearance within the colony. There were no apparent differences between the recovery of either type I or type II on CVE agar. Total viable counts of both types on CVE agar were comparable to those obtained on T-soy blood agar.

TABLE 4. *Recovery of F. nucleatum strains on CVE agar compared with T-soy blood agar*

F. nucleatum strain	CFU[a] recovered on CVE agar[b]	CFU recovered on T-soy blood agar[b]	% Recovery
Type I			
FDC 277	5.9×10^8	5.8×10^8	101
FDC 282	11.0×10^9	7.3×10^9	137
FDC 314	1.6×10^9	1.7×10^9	94
FDC 364	2.0×10^9	2.1×10^9	95
Type II			
FDC 26	3.1×10^9	3.0×10^9	103
FDC 101	4.0×10^9	4.1×10^9	98
FDC 262	6.6×10^8	7.0×10^8	94
FDC 317	2.0×10^9	1.2×10^9	167
FDC 318	6.9×10^8	6.9×10^8	100
FDC 397	5.2×10^9	3.6×10^9	144
FDC 398	2.3×10^9	2.4×10^9	96
ATCC 10953	1.9×10^9	2.4×10^9	79

[a] CFU, Colony-forming units.
[b] Mean of triplicate plates.

Suppression of pure cultures of other bacteria. To determine the suppression rate of CVE agar for other bacteria, we compared total viable counts recovered on CVE agar with those recovered on T-soy blood agar for 35 strains, representing 20 species of bacteria other than fusobacteria (Table 5). Counts on CVE agar for most strains were suppressed by a factor of 10^6 to 10^8 when compared with the total viable counts obtained on T-soy blood agar. However, *E. corrodens*, *Selenomonas sputigena*, and certain strains of *A. actinomycetemcomitans* were not suppressed to this extent. There were individual strains of peptostreptococci that were suppressed only 1 or 2 logs. However, there were also members of this genus that did not grow on CVE agar. This variability was strain specific and was not related to any particular species of *Peptostreptococcus*.

Recovery of *F. nucleatum* from periodontally diseased sites. *F. nucleatum* was enumerated from 278 samples of subgingival plaque from 22 patients having advanced periodontal disease. The patients ranged in age from 13 to 63 years, had periodontal pockets of 6 mm or greater, and had not received antibiotics or periodontal treatment in the 12 months preceding

TABLE 5. *Suppression of non-fusobacteria on CVE agar compared with T-soy blood agar*

Bacteria	CFU[a] recovered on CVE agar[b]	CFU recovered on T-soy blood agar[b]	Suppression rate of CVE agar
Gram-positive organisms			
Actinomyces israelii	$<1.0 \times 10^2$	6.3×10^9	10^7
Actinomyces naeslundii	$<1.0 \times 10^2$	1.2×10^9	10^8
Actinomyces odontolyticus	$<1.0 \times 10^2$	1.1×10^{10}	10^8
Actinomyces viscosus	$<1.0 \times 10^2$	1.9×10^{10}	10^8
Propionibacterium acnes	$<1.0 \times 10^2$	1.0×10^{10}	10^8
Streptococcus species	$<10^2$–10^4	1.0×10^{10}	10^6–10^8
Peptostreptococcus species	$<10^2$–10^9	1.0×10^{10}	10^1–10^8
Gram-negative organisms			
Bacteroides asaccharolyticus	$<1.0 \times 10^2$	1.8×10^{10}	10^8
Bacteroides melaninogenicus subspecies	$<1.0 \times 10^2$	3.2×10^{10}	10^8
Bacteroides fragilis	$<1.0 \times 10^2$	2.3×10^{10}	10^8
Capnocytophaga gingivalis	$<1.0 \times 10^2$	6.1×10^9	10^7
Capnocytophaga ochracea	$<1.0 \times 10^2$	4.7×10^9	10^7
Capnocytophaga sputigena	$<1.0 \times 10^2$	2.1×10^{10}	10^8
Eikenella corrodens	3.0×10^7	1.4×10^{10}	10^3
Oral *Campylobacter*	1.7×10^7	2.8×10^{10}	10^3
Selenomonas sputigena	4.7×10^6	2.1×10^{10}	10^4
Actinobacillus actinomycetemcomitans	10^4–10^8	1.0×10^{10}	10^2–10^6

[a] CFU, Colony-forming units.
[b] Mean of triplicate plates.

the study. Total viable counts were determined on T-soy blood agar incubated anaerobically. *F. nucleatum* accounted for <1.0 to >25% of the cultivated microbiota (Table 6).

DISCUSSION

A number of selective media for the isolation of oral fusobacteria have been reported (2, 12, 16–18, 21). In most of these, crystal violet or a similar dye, such as ethyl violet, gentian violet, or brilliant green, was used to achieve selective bacterial inhibition. Fusobacteria as a group are relatively resistant to these dyes. Several other groups of oral organisms, such as *A. actinomycetemcomitans*, *E. corrodens*, oral *Campylobacter*, the corroding *Bacteroides*, and some of the streptococci, also demonstrate a similar resistance. In several of the selective media, a second selective agent, usually streptomycin (2, 12, 16) or vancomycin (12), was incorporated. However, a number of oral microorganisms, other than fusobacteria, are relatively resistant to both antibiotics. This is particularly true for many of the anaerobic bacteria which are often predominant in periodontal lesions. De Araujo and Gibbons have shown that streptomycin in the medium of Omata and Disraely (16) had no effect under anaerobic conditions on either the number of fusobacteria or the number of other organisms recovered (5). In our laboratory, vancomycin has been found to be ineffective against a wide range of oral organisms, including *A. actinomycetemcomitans*, oral *Campylobacter*, anaerobic vibrios, corroding *Bacteroides*, *B. asaccharolyticus*, *B. melaninogenicus*, *E. corrodens*, and *Capnocytophaga* (T. Niebloom, personal communication).

In routine antibiotic susceptibility testing, *F. nucleatum* was relatively resistant to erythromycin, and a number of other oral bacteria were inhibited by low concentrations of this antibiotic. This has been reported for strains of *F. nucleatum* from nonoral sites as well (8, 11, 23–25). It appeared that erythromycin and crystal violet together might be effective for the selective recovery of *F. nucleatum*.

TABLE 6. *Incidence of F. nucleatum in subgingival plaque from human periodontal pockets*

Incidence (%)	No. of samples	% of samples
<1.0	174	63
1–5	34	12
5–10	34	12
10–15	12	4
15–20	8	3
20–25	5	2
>25	11	4

The basal medium, supplemented with 5% whole sheep blood, was nutritionally adequate for pure cultures of *F. nucleatum* since total viable counts obtained were similar to the counts on T-soy blood agar. The medium was formulated with Trypticase and glucose contents lower than those in most media, which did not affect the growth of *F. nucleatum*, but may have been less than optimal for more fastidious organisms. Tryptophan has been previously reported to be stimulatory for oral fusobacteria (17). Although Trypticase is relatively rich in tryptophan, the addition of 0.02% L-tryptophan increased the total viable counts of *F. nucleatum* recovered on CVE agar. Yeast extract was added since it has been shown to be useful in obtaining maximum growth of fusobacteria (15). Sodium chloride was added to make the medium isotonic so that whole blood could be added. The addition of whole blood to the medium facilitated the recovery of *F. nucleatum* from subgingival plaque samples and may have enhanced the recovery of a number of anaerobic bacteria other than fusobacteria. However, the inclusion of erythromycin and crystal violet at concentrations of 4 and 5 μg/ml, respectively, was sufficient to suppress the majority of these organisms. The agents also slowed the growth rate of *F. nucleatum*, but did not have an apparent effect on total viable counts recovered, providing the medium was incubated anaerobically for 4 to 5 days. An increase in the concentration of either erythromycin or crystal violet resulted in a decrease in the recovery of *F. nucleatum*.

Although contamination by non-fusobacteria does occur on this medium, it is not a significant problem. Based on the suppression rates obtained with pure cultures, the major contaminants expected would be *S. sputigena*, *E. corrodens*, *A. actinomycetemcomitans*, and, occasionally, certain strains of the genus *Peptostreptococcus*. All of the organisms form small colonies on CVE medium that are distinctly different from the two colony types formed by *F. nucleatum*. A contaminant that was expected due to its similarity to the fusobacteria was *Leptotrichia buccalis*. This organism produces a 4- to 5-mm rough blue colony on CVE agar that is readily distinguishable from *F. nucleatum* colonies. In the sites that we sampled, both the incidence and the recovery of *L. buccalis* were very low.

There are undoubtedly other organisms that will grow on this medium which were not tested in pure culture. However, over the course of a year, *F. nucleatum* has been enumerated in 278 subgingival plaque samples; in all samples the contaminants were easily differentiated from *F. nucleatum*. Spot checks of colonies considered to be *F. nucleatum* consistently revealed orga-

nisms with the morphological and physiological characteristics of this species.

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