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New Medium for Isolation of *Actinomyces viscosus* and *Actinomyces naeslundii* from Dental Plaque

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Metronidazole (10 μg/ml) and cadmium sulfate (20 μg/ml) were added to a gelatin-based medium to select for microaerophilic *Actinomyces* species from dental plaque samples. The new medium (GMC), when incubated anaerobically, allowed 98% recovery of seven pure cultures of *Actinomyces viscosus* and 73% recovery of eight pure cultures of *Actinomyces naeslundii*, while suppressing 76% of the total count of other orgnisms in dental plaque samples. In 203 plaque samples, recoveries of *A. viscosus* and *A. naeslundii* on GMC and another selective medium for oral *Actinomyces* (CNAC-20) were compared. Recovery of *A. viscosus* was comparable on the two media. Recovery of *A. naeslundii* was significantly higher on GMC than CNAC-20 ($P < 0.001$), and GMC allowed a more characteristic cell morphology of both organisms. GMC medium appears to be useful for the isolation and presumptive identification of *A. viscosus* and *A. naeslundii* from dental plaque.

Gram-positive pleomorphic rods belonging to the genus *Actinomyces* are frequent isolates from human dental plaque. These organisms become prominent as plaque develops (S. A. Syed, W. J. Loesche, and H. Loe, J. Dent. Res. **54**:A72, abstr. no. 109, 1975) and are the dominant organisms found in marginal dental plaque associated with experimental and naturally occurring gingivitis (13; W. J. Loesche and S. A. Syed, J. Dent. Res. **54**:A71, abstr. no. 108, 1975). Gram-positive pleomorphic rods also appear to be the dominant morphological types seen in subgingival plaque removed from clinically healthy gingiva (16). The association of elevated levels of the more oxygen-tolerant *Actinomyces* species, such as *Actinomyces viscosus* and *Actinomyces naeslundii*, with human periodontal disease (21) and root surface caries (18) has emphasized the need for improved methods of studying the ecology of these organisms.

The culturing of large numbers of clinical samples is most practical with the use of a combination of selective and nonselective media. The development of CNAC-20 medium (5), which uses cadmium sulfate as the selective agent, has greatly facilitated the study of oral *Actinomyces*. The importance of Gram-stain morphology, i.e., branching filaments, in the presumptive identification of *Actinomycetes* has somewhat limited the use of the CNAC-20 medium, since *A. viscosus* and *A. naeslundii* often appear as coccobacillary forms on that medium. We have observed that CNAC-20 permits classical *Actinomyces* cell morphology when incubated anaerobically, but overgrowth of plaque anaerobes is then a common occurrence. Efforts were therefore directed toward development of a medium which would allow growth and characteristic Gram-stain morphology of microaerophilic *Actinomyces* isolates, while limiting the growth of anaerobic organisms found in plaque. In the present investigation, the antimicrobial agent metronidazole (Flagyl), which inhibits many anaerobic species in vitro (19), and cadmium sulfate were combined in an effort to develop a medium specific for the microaerophilic *Actinomyces* species.

MATERIALS AND METHODS

Experimental media. Metronidazole and either kanamycin sulfate or cadmium sulfate ($3CdSO_4 \cdot 8H_2O$), in various concentrations, were added to the enriched gelatin agar of Syed (17). Kanamycin and cadmium sulfate were autoclaved with the autoclavable constituents of the medium. Metronidazole was added to the cooled medium after filter sterilization. Metronidazole was prepared by pulverizing tablets of the prescription preparation (Flagyl), and all weights refer to the weight of the active ingredient after compensation for inert fillers, e.g., 10 mg of metronidazole = 22.4 mg of Flagyl powder.

A nonselective medium (MM10; 14) and the CNAC-20 selective medium for *A. viscosus* and *A. naeslundii* (5) were prepared as originally described. The experimental media and MM10 were incubated in an anaerobic glove box with 85% nitrogen, 5% CO_2, and 10% hydrogen (1), and CNAC-20 was incubated in a candle

jar as recommended by Ellen and Balcerzak-Raczkowski (5).

Investigation with pure cultures. The following recent isolates from human dental plaque were tested on the various media: *A. viscosus* strains 21, GA, 55-2, 27-8, and 60-4; *A. naeslundii* strains 55-7, 57-3, 59-6, 57, and M12; and *Lactobacillus casei* 51L.

The following stock strains, obtained from either M. A. Gerenczer (West Virginia University) or the American Type Culture Collection (ATCC), were tested: *A. viscosus* strains G26 and 472; *A. naeslundii* 61A; *Actinomyces israelii* strains 237 and 304; *Arachnia propionica* 427; *Actinomyces odontolyticus* ATCC 17929; *Rothnia dentocariosa* ATCC 17931; and *Bacterionema matruchotii* ATCC 14265.

All stock strains were periodically Gram stained and tested for aerobic growth, gelatinase and catalase activity, indole production, nitrate reduction, esculin hydrolysis, and acid production from lactose and mannitol. Cultures were grown for 48 h in a basal anaerobic broth in the anaerobic chamber, diluted in reduced transport fluid (14), and plated in triplicate on nonselective MM10, test media, and, where indicated, CNAC-20 agar.

Investigations with plaque samples. Plaque samples from about the gingival margin of an interdental papilla were removed from 203 single sites in 73 patients with gingivitis by means of a sterile Morse scaler tip held in a hemostat. After plaque removal, the scaler tip was placed in reduced transport fluid and taken immediately into the anaerobic chamber for dilution and plating on MM10, experimental media, and CNAC-20. After 7 days of incubation, representative colonies on the gelatin-metronidazole-cadmium medium (GMC) or CNAC-20 medium were Gram stained and tested for catalase and gelatinase activity, as well as the additional biochemical tests described above for pure cultures. Total counts of all colony-forming units on GMC and CNAC-20 were determined for 115 of 203 samples.

RESULTS

Media development. The gelatin medium, with various concentrations of metronidazole, kanamycin, or cadmium, was evaluated for the recovery of three strains of *A. viscosus* and for the recovery of plaque organisms other than *A. viscosus* or *A. naeslundii*. The total count of all organisms on the test medium was expressed relative to the total count on MM10 medium (Table 1).

The test media were evaluated in three series (Table 1). Metronidazole alone allowed good recovery of pure cultures of *A. viscosus*, but only moderate inhibition of plaque organisms other than the microaerophilic *Actinomyces*. The catalase-positive and gelatinase-negative colonies of *A. viscosus* on the metronidazole medium were white, smooth, convex, and approximately 1 to 3 mm in diameter. These colonies contained Gram-positive pleomorphic branching rods when stained after 5 days of growth. Many of the non-*Actinomyces* colonies were facultative gram-positive cocci.

In the second series, kanamycin sulfate was added to the metronidazole medium to inhibit these gram-positive cocci. Various concentrations of both additives resulted in no growth of any organism, apparently due to a synergistic action between the antimicrobial agents (Table 1).

The successful use of cadmium sulfate to inhibit gram-positive cocci (5) led to the addition of this substance to the metronidazole medium (Table 1). The medium containing cadmium sulfate (20 μg/ml) and metronidazole (10 μg/ml) allowed greater than 95% recovery of the pure cultures of *A. viscosus*, while inhibiting more than 75% of the other plaque organisms. Increasing the concentration of either component resulted in a decreased recovery of *A. viscosus* with no improvement in selectivity. On the basis of these data, the gelatin-based metronidazole (10 μg/ml) and cadmium sulfate (20 μg/ml) medium (GMC) appeared promising as a selective and differential medium for *A. viscosus*.

Investigation of pure cultures. Pure cultures of seven strains of *A. viscosus* were serially diluted and plated on the GMC and MM10 media. Recovery on GMC and MM10 was essentially identical, as was the recovery of five strains plated on CNAC-20 medium. All strains produced a similar white, smooth, convex colony 1.0 to 3.0 mm in diameter. Pure cultures of *A. naes-*

TABLE 1. *GMC media development*[a]

Additives (μg/ml)	Metronidazole (μg/ml)	Recovery of *A. viscosus*[b] (%)	Recovery of other organisms[c] (%)
None	10	100	47
None	16	89	51
Kanamycin			
20	16	0	0
10	16	0	0
5	10	0	0
5	5	0	0
Cadmium sulfate			
20	10	99	24
20	12	73	28
20	14	75	26
20	16	56	14
25	10	78	21

[a] All media based on gelatin agar of Syed (17).

[b] Recovery of three pure cultures relative to recovery on MM10 medium.

[c] Recovery of organisms other than those resembling *A. viscosus* from three plaque samples relative to recovery on MM10.

lundii showed some variability in recovery on GMC and CNAC-20 media (Table 2). The mean recovery of eight strains tested on GMC was 73%, and the mean recovery of five strains tested on CNAC-20 medium was 76%. Colony morphology of *A. naeslundii* on the GMC medium was similar to that of *A. viscosus,* but the colonies were 0.5 to 1.5 mm in diameter. Gram stains made from colonies on GMC showed *A. naeslundii* to have a thinner, more pleomorphic cell morphology than *A. viscosus.* All *A. naeslundii* colonies were catalase negative when exposed to a drop of 30% H_2O_2.

A. israelii, A. odontolyticus, R. dentocariosa, and *L. casei* were not recoverable on GMC. Recovery of *A. propionica* on GMC was good, but the rough irregular colonies were easily distinguished from *A. viscosus. B. matruchotii* ATCC 14265 grew on GMC, but the rough raised colonies did not resemble the colony morphology of *A. viscosus.*

Investigation of plaque samples. Since the GMC medium appeared to allow the quantitative recovery and differentiation of pure cultures of *A. viscosus* and *A. naeslundii,* it was evaluated for its usefulness in the presumptive identification and quantitation of these organisms in plaque samples. Plaque samples from 203 single sites were diluted and plated on GMC, CNAC-20, and MM10 media. After incubation, GMC plates were refrigerated for 1 h and gelatinase-positive colonies were circled (17). Colonies resembling *A. viscosus* or *A. naeslundii* were Gram stained and tested for catalase activity.

Characteristic colonies that were gram-positive, pleomorphic rods, gelatinase negative, and catalase positive were given a presumptive identification as *A. viscosus.* Similar organisms that were catalase negative were given a presumptive identification of *A. naeslundii.* Colonies were identified on CNAC-20 according to the colony morphology described by Ellen and Balcerzak-Raczkowski (5). Of 43 representative colonies picked from the GMC medium and subjected to more extensive biochemical evaluation, only 2 were found to be incompatible with the presumptive identification.

The mean recovery of *A. viscosus* from 203 dental plaque samples was 2.45×10^5 on the GMC medium and 1.34×10^5 on the CNAC-20 medium (Table 3). The GMC recovery represented 8.7% and the CNAC-20 recovery 8.1% of the mean total colonies isolated on the nonselective medium. The mean recovery of *A. naeslundii* was 2.59×10^5 on the GMC medium and 8.71×10^4 on the CNAC-20 medium (Table 3). The recovery of *A. naeslundii* on the two media was significantly different by the pairwise *t* test ($P < 0.001$). The GMC recovery of *A. naeslundii* represented 9.2% of the mean total count on the nonselective medium, compared to a 5.3% recovery on the CNAC-20 medium.

Recovery from dental plaque of organisms resembling *A. viscosus* was greater on GMC than on CNAC-20 in 34% of the samples, equal on the two media in 47% of the samples, and greater on CNAC-20 in 19% of the samples (Ta-

TABLE 2. *Recovery of pure cultures of A. naeslundii*

A. naeslundii strains	Recovery (%)[a]	
	GMC[b]	CNAC-20[c]
55-7	100	NT[d]
57-3	76	NT
59-6	84	NT
63	22	100
57	54	56
M12	100	100
61A	47	43
12104	100	81
All strains	73	76

[a] Mean of three samples as percentage of growth on the nonselective medium MM10.
[b] Gelatin medium of Syed (17) with metronidazole (10 μg/ml) and cadmium sulfate (20 μg/ml).
[c] Columbia CNA agar (Difco) with cadmium sulfate (20 μg/ml) as described by Ellen and Balcerzak-Raczkowski (5).
[d] NT, Not tested on CNAC-20 medium.

TABLE 3. *Effect of GMC and CNAC-20 on recovery of A. viscosus, A. naeslundii, and other organisms from dental plaque*[a]

Organism	No. of plaque samples cultured	GMC		CNAC-20		Significance[b]
		Mean colony-forming units	Recovery (%)[c]	Mean colony-forming units	Recovery (%)[c]	
A. viscosus	203	2.45×10^5	8.7	1.34×10^5	8.1	$P = 0.06$
A. naeslundii	203	2.59×10^5	9.2	8.71×10^4	5.3	$P < 0.001$
All other organisms	115	2.29×10^6		1.52×10^6		$P < 0.001$

[a] GMC: Gelatin agar (17) with metronidazole (10 μg/ml) and cadmium sulfate (20 μg/ml). CNAC-20: Columbia CNA agar with cadmium sulfate (20 μg/ml) (5).
[b] Difference between media by pairwise *t* test.
[c] Recovery on selective medium as percentage of total colonies on the nonselective medium MM10.

ble 4). Overall, the recovery of *A. viscosus* on GMC was greater than or equal to the recovery on CNAC-20 in 81% of the samples. Recovery of organisms resembling *A. naeslundii* was significantly greater on GMC than CNAC-20 ($P < 0.001$, Wilcoxon rank sum test).

Total counts of other colonies on GMC were significantly greater than or equal to the total counts on CNAC-20 in 73% of the samples. This suggests that CNAC-20 medium incubated under microaerophilic conditions is more selective than the GMC medium incubated under anaerobic conditions. However, *A. viscosus* and *A. naeslundii* together accounted for approximately 18% of the total count on GMC and approximately 13% of the total count on CNAC-20. Thus the absolute and relative recoveries of these microaerophilic *Actinomyces* were higher from plaques cultured on the GMC medium (Tables 3 and 4).

DISCUSSION

The association of *A. viscosus* and *A. naeslundii* with periodontal disease and root surface caries has emphasized the need for improved methods of studying the ecology of these organisms in dental plaque. The GMC medium combines selective and diagnostic factors which make it useful in a presumptive diagnostic scheme.

The observation that *A. viscosus* exhibited classical Gram-stain morphology when incubated anaerobically but not in a candle jar suggested that microaerophilic conditions should not be used as the means of selecting against the great number of anaerobes in dental plaque. Metronidazole was therefore chosen as the primary selective agent in a medium designed to be incubated anaerobically while favoring the isolation of the microaerophilic organisms *A. viscosus* and *A. naeslundii*.

TABLE 4. *Comparison of GMC and CNAC-20 in their ability to recover A. viscosus and A. naeslundii from dental plaque*

Organism	No. of plaque samples	Ability to recover[a]		
		GMC > CNAC-20	GMC = CNAC-20	CNAC-20 > GMC
A. viscosus	203	69	95	39[b]
A. naeslundii	203	89	89	25[c]
All other organisms	115	84	0	31[c]

[a] Number of samples in which recovery on one medium was greater than (or equal to) the recovery on the other. GMC: Gelatin agar (17) with metronidazole (10 μg/ml) and cadmium sulfate (20 μg/ml); CNAC-20: Columbia CNA agar with cadmium sulfate (20 μg/ml) (5).

[b] No significant difference between recoveries on GMC and CNAC-20; $P = 0.06$, Wilcoxon rank sum test.

[c] Difference between recoveries on GMC and CNAC-20, $P < 0.001$, Wilcoxon rank sum test.

Chow et al. (3) reported that 66% of 358 clinical isolates of anaerobes were inhibited by 6.25 μg of metronidazole per ml. Tally et al. (19) found that 95% of 54 strains of various anaerobic bacteria were inhibited by 6.25 μg/ml. However, metronidazole has been shown to be relatively ineffective in vitro against most microaerophilic *Actinomyces*, with 12 of 15 *A. viscosus* strains resistant to >125 μg/ml (12). The inability of metronidazole to inhibit aerobic organisms has recently been explained. The antibacterial activity of metronidazole depends on reduction of the parent compound to a more active form, which appears to produce deoxyribonucleic acid mutations (7). Production of the active form requires a highly negative redox potential, which may not be present in aerobes or facultative anaerobes (7, 8).

The use of metronidazole alone as a selective agent allowed the growth of a large number of facultative gram-positive cocci. For this reason, kanamycin sulfate and cadmium sulfate were tried in conjunction with metronidazole. A gelatin medium was used as the base for these selective media to allow differentiation of catalase-positive, gelatinase-positive organisms, e.g., *Propionibacteria*, which might otherwise be confused with *A. viscosus*. Kanamycin proved to be synergistic with metronidazole, as has been reported for metronidazole and other aminoglycosides (20), and was dropped from further study. Cadmium has been shown to inhibit the growth of many gram-positive organisms at ≤10 μg/ml (2) and was the selective agent in the CNAC-20 medium. The combination of metronidazole and cadmium sulfate effectively suppressed anaerobes and facultative gram-positive cocci while allowing good recovery of the microaerophilic *A. viscosus* and *A. naeslundii* strains.

The GMC medium, when incubated anaerobically, appears to be useful in the study of dental plaque samples. Organisms resembling *A. viscosus* and *A. naeslundii* are readily identified as the only gram-positive pleomorphic rods on GMC which produce white, smooth, convex colonies and are gelatinase negative.

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