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Predominant Cultivable Flora Isolated from Human Root Surface Caries Plaque

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Plaque samples were obtained from tooth surfaces exhibiting typical lesions of root surface caries and were immediately cultured by a continuous anaerobic procedure. The bacterial composition of root caries flora was determined on individual samples. Representative isolates from each specimen were characterized by morphological and physiological criteria. In addition, fluorescent antibody reagents were used to confirm the identification of *Streptococcus mutans* and *Actinomyces viscosus*. The plaque samples could be divided into two groups on the basis of the presence or absence of *S. mutans* in the plaque. In group I plaques, *S. mutans* comprised 30% of the total cultivable flora. *S. sanguis* was either not found or was present in very low number. In group II plaques, *S. mutans* was not detected, and *S. sanguis* formed 48% of the total plaque flora. *A. viscosus* was the dominant organism in all plaque samples, accounting for 47% of the group I isolates and 41% of the group II isolates.

Root surface caries is a soft progressive lesion that is found on root surfaces which have lost connective tissue attachment and are exposed to the oral environment. Various terms, such as cemental caries, cervical caries, radicular caries, and erosion or senile caries, have been used in the literature to describe this form of decay. The root surface has a lower inorganic composition than the enamel surface so that the dissolution phenomenon involved in this type of caries may be quite distinct from the events which occur in enamel caries. The incidence of root caries is not well documented, often being ignored in clinical studies, but when reported has been less than enamel caries (5). Clinically, these lesions are difficult to restore and are prone to recurrent caries after restoration.

Jordan and Sumney (9) have evaluated the significance of the problem of root caries in humans. They commented on the necessity of determining the role of bacteria in the development of root surface caries and speculated that a unique bacterial flora is involved in this infection. In a subsequent study (12) these investigators qualitatively sampled the carious lesions (soft dentine) in the root surfaces of extracted human teeth and reported the presence of *Streptococcus mutans*, *Streptococcus sanguis*, filamentous organisms of the actinomycetes type, and enterococci. They also found aerobic gram-positive rods in the advancing front of the lesion which were identified as belonging to the genus *Arthrobacter*.

The present study was undertaken to determine the qualitative and quantitative composition of the plaque flora associated with human root surface caries lesions using anaerobic techniques (1) and a medium (14) which appear to permit optimal recovery of the plaque flora. The results of this investigation are described in this paper.

MATERIALS AND METHODS

Patient selection. Patients from the Operative and Periodontal Clinics of The University of Michigan Dental School were screened by full mouth radiographs. Approximately one in forty patients exhibited some root caries, with the final diagnosis being made by careful clinical examination. These patients gave a broad representation of the general population, varying in age (range 23 to 70 years), economic status, and cultural background. Both sexes (12 males and 3 females) were included in the study. These patients had previously undergone periodontal treatment which, in many cases, included periodontal surgery.

Sample collection and culturing procedure. Plaque samples were taken from buccocervical lesions prior to restorative treatment. Plaque associated with the root caries lesion was removed by a sterile orthodontic wire (1 cm in length) which was held in a hemostat. The wire containing the plaque material was dropped in a test tube that contained a prereduced transport medium, reduced transport fluid (RTF) (13). After collection, each sample was immedi-

ately brought to the bacteriological laboratory and dispersed for 10 s by sonic treatment under a stream of oxygen-free gas (13). The samples were introduced into an anaerobic chamber (Coy Manufacturing Co., Ann Arbor, Mich.) and serially diluted in RTF. Appropriate dilutions were plated on MM10 sucrose blood agar, a nonselective medium which appears to give maximal recovery of bacteria from dental plaque and permits reliable identification of extracellular polysaccharide producing organisms such as *S. sanguis*, *S. mutans*, and *S. salivarius* (14).

A duplicate set of plates for each sample was prepared and incubated in candle jars at 37 C for 1 week. At the end of the incubation period, total colony-forming units (CFU) for *S. mutans* and *S. sanguis* were determined with a stereomicroscope. The remaining colonies exhibited a limited number of colony morphologies, i.e., five to eight. At least 40 to 50 of these colonies per sample were Gram stained and grouped according to single-cell morphology and Gram-staining characteristics into one of the following genera: *Actinomyces*, *Veillonella*, and *Streptococcus*.

In addition, representative isolates were subcultured in an enriched tryptone-yeast extract broth, and their purity after growth was determined by Gram-staining characteristics. The ingredients present in 1 liter of medium were: tryptone (Difco), 10 g; yeast extract (Difco), 5 g; mineral salt solutions used in MM10 sucrose blood agar (14), 75 ml each; dithiothreitol, 0.2 g; sodium carbonate, 0.4 g; menadione, 0.5 mg. The last three compounds were sterilized by membrane filtration (0.22-μm pore size Millipore filter) and added to the cold sterile medium.

Biochemical studies. The ability of these isolates to ferment various carbohydrates was determined by adding membrane filter-sterilized carbohydrates to enriched tryptone-yeast extract broth to give a final concentration of 1% (wt/vol) test compound. All sugar media were tubed in 5-ml portions in screw-cap test tubes (15 by 100 mm) and then incubated inside the anaerobic glove box for 48 h before they were used for fermentation studies. The final pH of the uninoculated sugar media varied between 7.1 and 7.3. All test isolates were grown in enriched tryptone-yeast extract broth, which served as an inoculum for the different sugar media. All inoculated broths were incubated in the anaerobic glove box for 7 days, after which time the pH was measured using a Beckman pH meter and a glass combination electrode. Tests for nitrate reduction, esculin hydrolysis, catalase production, and gelatin liquefaction were performed by criteria recommended by the Anaerobe Laboratory (15).

Fatty acid analysis. Analysis of short-chain fatty acids was done on enriched tryptone-yeast extract glucose broth cultures by gas-liquid chromatography. A Varian gas chromatograph (model 2740) equipped with hydrogen flame ionization detectors was used. The gas-liquid chromatography column (6 feet by 1/4 inch; ca. 185 by 0.4 cm) was made of stainless steel and packed with 10% carbowax and 3% H_3PO_4 (Varian Aerograph). It was used both for free fatty acids and methylated fatty acid analyses. The fatty acid peaks were identified on the basis of relative retention times which were determined by analyzing known fatty acids under identical conditions. The presence of these acids as contaminants in the uninoculated broth was also determined.

Fluorescent antibody test. Representative isolates identified culturally as *Actinomyces viscosus* and *S. mutans* were stained with fluorescent antibody (FA) reagents. The actinomycetes used to prepare antisera for the FA test were: *A. viscosus* strain 21 (isolated in our lab from human dental plaque), *A. israelii* strain ATCC12102, *A. naeslundii* strain I (12), *A. odontolyticus* strain ATCC17929, *A. bovis* strain ATCC13682, *Arachnia propionica* strain ATCC14157, and *A. ericksonii* strain ATCC15423. *Rothia denticariosa* strain ATCC17931 was used for the preparation of FA conjugate. The following strains of *S. mutans* were used for the preparation of antisera: AHT, BHT, GS5, OIHI, and LM7. The details concerning antisera preparation and fluorescein labeling of antibodies are found elsewhere (5).

RESULTS

Two sets of plates were prepared for each sample, one set being incubated in the anaerobic glove box and the duplicate set in the candle jar under microaerophilic conditions. When the total CFU on the plates grown under these conditions were compared, the recoveries from most of the samples were higher on the anaerobic plates. The recovery of *S. mutans* was lower, and *S. sanguis* did not grow well on the plates incubated in the candle jar. Because of this, anaerobic plates were used for enumeration of total CFU and differential counts and the isolation and characterization of the organisms.

Colony morphology of all isolates was used to qualitatively evaluate the flora. Approximately 50 isolates from each of the 21 samples were Gram stained. This information was used to separate the isolates into the following categories: *S. mutans*, *S. sanguis*, *Actinomyces* species, *Veillonella* species, and other streptococcal species. Subsequently the *Actinomyces* isolates were found to be mainly *Actinomyces viscosus*.

S. mutans could be used as the basis for the division of the plaque samples into two groups. The frequency of occurrence of various bacterial species and their proportions in the two groups were determined (Table 1). All 13 samples in group I had *S. mutans* and *A. viscosus*. *S. sanguis* was found in three samples. *Veillonella* species (gram-negative anaerobic nonfermentative cocci) were found in five samples, whereas other streptococcal species (e.g., *S. miteor* and *S. faecalis*) were present in ten samples. The serological study showed that *S. mutans* serotypes *c*, *d*, and *e* (2) were present. More than one serotype could be detected in individual

TABLE 1. *Proportion of various bacterial species in human root surface caries plaque*

Organism	Percentage		Range (%)		Frequency[a]	
	Group I[b]	Group II[b]	Group I	Group II	Group I	Group II
S. mutans	30	0	4-57	0	13/13	0/8
S. sanguis	1	48	0-5	2-72	3/13	8/8
A. viscosus	47	41	4-80	20-80	13/13	8/8
Veillonella species	4	2	0-20	0-8	5/13	2/8
Streptococcal species	18	9	0-57	0-46	10/13	4/8

[a] Number of samples positive/total number of samples examined.

[b] Total viable counts for group I and II were 49×10^6, with a range of 1×10^6 to 130×10^6, and 47×10^6, with a range of 1×10^6 to 126×10^6, respectively. Group I, n = 13; group II, n = 8, where n = number of samples examined in each group.

plaque specimens. The second group of root caries plaque did not have detectable colonies of *S. mutans*, but *S. sanguis* and *A. viscosus* were found in all eight samples. *Veillonella* and streptococcal species were present in two and four samples, respectively. In group I plaque samples, *S. mutans* and *A. viscosus* were the dominant bacterial species and accounted for 30 and 47% of the total CFU. *S. sanguis* was present in low numbers even in those samples in which it was found, i.e., 4 to 5% of the total CFU. In group II plaque samples, *S. sanguis* and *A. viscosus* were the dominant bacterial species, accounting for 48 and 41% of the total isolates (Table 1).

Sugar fermentation and other physiological tests were done with representative isolates of the various groups other than *S. mutans* and *S. sanguis*. Sixty-three *Actinomyces* isolates were uniformly positive for catalase production, nitrate reduction, and glucose fermentation (Table 2). The fermentation products acetate, lactate, and succinate (molar ratio 1:2:1) were also identical. The following tests were negative for these isolates: indole production and gelatin liquefaction. This biochemical profile is compatible with the description of *A. viscosus* (6). These isolates varied with respect to other tests which could be used for the division of the organisms into subgroups. Twenty isolates were identified as *Veillonella* on the basis of their being anaerobic, failure to ferment sugars, ability to reduce nitrate, and production of acetate and propionate from lactate. Sixty-four streptococcal isolates were acidogenic and indole and catalase negative. The majority of these isolates failed to produce acid from mannitol, which differentiates them from *S. mutans*, and were considered to be *S. mitis*. However, some isolates were capable of fermenting mannitol and because they did not form a distinctive *S. mutans* colony on MM10 sucrose blood agar were considered to be enterococci.

TABLE 2. *Characteristics of A. viscosus and streptococcal species from human root surface caries plaque*[a]

Characteristic	No. of isolates positive	
	A. viscosus	*Streptococcus* sp.
Acid produced from:		
Glucose	63	64
Fructose	61	64
Sucrose	59	64
Lactose	43	64
Mannitol	2	15
Additional tests:		
Esculin hydrolysis	46	39
Nitrate reduction	62	ND[b]
Catalase	63	0
Indole	0	0
Gelatin liquefaction	0	ND
Glucose fermentation products		
Acetate, lactate, succinate[c]	63	ND

[a] Sixty-three isolates of *A. viscosus* and 64 isolates of streptococcal species were tested.

[b] ND, Not done.

[c] Molar ratio, 1:2:1.

Representative *A. viscosus* isolates from group I and II plaques were stained with FA conjugates prepared against different *Actinomyces* species (Table 3). Of 52 isolates, 42 gave a positive test with the *A. viscosus* antisera. Five isolates gave reactions with FA conjugates of *A. naeslundii* and could not be typed by FA conjugates prepared against other actinomycetes. The remaining five isolates could not be typed by the prepared antisera. This could indicate that these organisms represented a serotype for which typing serum was not available. Thus over 80% of the isolates appeared by serological tests to be *A. viscosus*.

TABLE 3. *Fluorescent antibody reactions of A. viscosus isolates from root surface caries plaque stained with different Actinomyces species antisera*[a]

Organism	Strain no.	FA titer	No. of isolates positive[b]
A. viscosus	21	1:8	42/52
A. israelii	12102	1:8	0/52
A. naeslundii	I	1:2	5/52
A. odontolyticus	17929	1:2	0/52
A. bovis	13682	Undiluted	0/52
A. eriksonii	15423	Undiluted	0/52
Arachnia propionica	14157	Undiluted	0/52
Rothia denticariosa	17931	1:4	0/52

[a] Five isolates could not be typed.

[b] Number of isolates positive/total number of isolates examined.

DISCUSSION

The root surface must be exposed to the oral environment before the clinical manifestation of the carious lesion. This is substantiated by the fact that the majority of the patients had previously undergone periodontal surgery and later developed root caries. Absence of active enamel caries in these patients and differences in the chemical composition of enamel and cementum raised the question of whether there is a specific bacterial flora involved in this lesion which might be different from that of enamel caries. To study these aspects, plaque samples were removed from the diseased site, viz., root surface caries lesion, and the composition of the bacterial flora was determined.

The data obtained strongly suggest that the dominant bacterial species found in these plaques was *A. viscosus*. This organism has been shown by various investigators (7, 8) to produce root caries and periodontal disease in experimental animal model systems. Its presence in high numbers would seem to implicate this organism as a prime suspect in human root surface caries.

The presence of *S. mutans* in high numbers in certain plaque samples (group I) suggests that this organism might also be important in producing root caries lesions in man. As with *A. viscosus*, this organism has been reported to produce root caries lesions in gnotobiotic rats (3) and has been isolated from root surface lesions in man (7, 12).

The significance of a high proportion of *S. sanguis* in the group II samples is uncertain at this time. This organism is usually considered as a member of the normal flora of the oral cavity and is one of the first organisms to colonize the tooth. To our knowledge it has not been considered as a pathogen in any dental infection. The inverse relationship between *S. mutans* and *S. sanguis* noted in coronal caries (10) is seen in these samples.

Jordan and Hammond (7) cultured the softened dentine of root caries lesions in extracted teeth and isolated *A. viscosus* and *A. naeslundii*. They did not culture the surface plaque, nor were they able to quantitate the flora present in the carious dentine. They showed that *A. viscosus* and *A. naeslundii* strains isolated from such lesions could produce periodontal disease and root surface caries in gnotobiotic rats. They also commented on the occurrence of streptococci, including *S. mutans*, in the root caries lesions of these extracted teeth.

Sumney and Jordan (12) studied the qualitative composition of the bacterial flora present in the deeper carious dentine of extracted human teeth with root caries lesions. They did not culture the surface plaque which was discarded before the dentine was cultured. They reported the presence of *S. sanguis*, *S. mutans*, *Actinomyces* species, enterococci, staphylococci, etc., in the lesion. They found aerobic gram-positive diphtheroids from the advancing front of the lesion which they characterized as *Arthrobacter*.

Although *A. naeslundii* has been reported to be involved in cemental caries and periodontal disease in animal model system (7, 11), it could not be isolated as a predominant organism from root caries plaque in our studies. Failure to find this organism in root caries plaque does not preclude the possibility of its role in root caries infection in humans. It is possible that *A. naeslundii* is not prominent in the carious plaque but is confined to softened dentine. However, five isolates of actinomycetes reacted with *A. naeslundii* antisera (Table 3), suggesting that the organism could be present in some root surface caries lesions. Further studies are required to determine its prevalence in human root caries infection.

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