Establishment and Distribution of Actinomyces viscosus and Actinomyces naeslundii in the Human Oral Cavity

RICHARD P. ELLEN
Faculty of Dentistry, University of Toronto, Toronto, Ontario, Canada MSG 1G6

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The intraoral establishment and proportional distribution of suspected periodontal pathogens Actinomyces viscosus and Actinomyces naeslundii have been implicated in the etiology of periodontal disease primarily because of their association with naturally transmissible and experimentally induced periodontal lesions in laboratory animals (13, 15–18, 21). However, little is known about their ecological relationship with the human host and their impact on the course of naturally occurring human periodontal diseases. Presumably, this lack of information reflects previously encountered difficulties in rapidly enumerating these species in clinical samples and in differentiating them from other facultatively anaerobic, gram-positive, pleomorphic rods and filaments that also colonize the mouth. Such identification problems were evident in a previous attempt to trace the establishment of Actinomyces species in the developing oral flora of newborn infants (20). The recent development of a partially selective, differential medium for detecting facultatively anaerobic Actinomyces colonies (6) has facilitated the processing of numerous clinical samples, making the present investigation feasible. This report describes investigations into the host age at which A. viscosus and A. naeslundii infect humans and the distribution of the two species in the mouth. Data are presented that indicate that the closely related species A. viscosus and A. naeslundii differ markedly in their patterns of human intraoral colonization.

MATERIALS AND METHODS

Populations under investigation. The 108 subjects for this investigation ranged in age from 3 months to 30 years. Thirty-six healthy infants were selected from the population at Edgewood Manor Day Nursery and the Family Practice Unit of St. Michael's Hospital, Toronto, Canada. Fifty-seven children from 3 to 16 years of age were sampled at the Dental EDentistry clinics, University of Toronto. The adult population included 15 subjects, 20 to 30 years of age, who were selected from the staff and students of the Faculty of Dentistry and were found to be relatively free of clinical signs of periodontal disease.

Sample collection and cultural conditions. Samples of whole saliva were collected from all the subjects. Unstimulated whole saliva was collected in sterile vials from the children and adults. Infant saliva samples were collected by absorbing the saliva bathing their mucosal surfaces with calcium alginate swabs (Calgiswabs, Wilson Diagnostics, Inc., Glenwood, Ill.). Pooled smooth-surface plaque samples were obtained from all dentulous subjects by swabbing the facial and lingual surfaces of their maxillary anterior teeth. Additional swab samples were collected from the dorsum of the tongue and from the buccal mucosa of the adult subjects. The calcium alginate swabs were partially dissolved in 2.0 ml of modified Ringer solution with hexametaphosphate (23) by vibrating the sample tubes on a Vortex mixer for 20 s.

Tenfold serial dilutions of each sample were prepared in 0.05% yeast extract broth (Difco), and appropriate dilutions were spread in duplicate on plates of tryptic soy agar (Difco) containing 5.0%...
sheep blood and Columbia CNA agar (Difco) containing 20.0 μg of 1CdSO₄ · 8H₂O per ml (CNAC-20) (6). Undiluted swab samples of plaque and infant saliva were also inoculated directly onto CNAC-20. For the whole saliva samples, the lowest dilution plated on CNAC-20 was 10⁻³. The blood plates were incubated at 35°C for 4 days in an atmosphere of 95% N₂ and 5% CO₂. The CNAC-20 plates were incubated for 2 days in 90% air and 10% CO₂, to encourage the growth of Actinomyces which could grow aerobically (6).

Collection and analysis of data. The number of colony-forming units (CFU) per milliliter of sample was determined by calculating the average colony count on duplicate plates of dilutions yielding between 20 and 200 colonies. The number of facultatively anaerobic Actinomyces was determined by counting only the large, white, smooth, opaque CNAC-20 colonies previously shown to contain bacteria resembling either A. viscosus or A. naeslundii (6). Questionable colony types were isolated, and pure cultures thereof were identified by cultural methods (6, 14). Catalase activity to differentiate between catalase-positive A. viscosus-like and catalase-negative A. naeslundii-like CNAC-20 isolates was tested by applying 3.0% H₂O₂ directly to the colonies. The proportions of catalase-positive and catalase-negative isolates in the samples were determined on the same plates used for the colony count. If no catalase-positive colonies were detected, plates from successively lower dilutions were chosen.

The frequency of A. viscosus and A. naeslundii isolation was calculated and expressed as the percentage of subjects in each age group whose samples yielded detectable CNAC-20 Actinomyces isolates. The infant group was composed of 15 predentates and 21 infants with teeth. The group of preschool-aged children (3 to 4 years) included 9 subjects. Among the school-aged children, 10 were 5 to 6 years of age; 14 were 7 to 8 years; 11 were 9 to 10 years; 7 were 11 to 12 years; and 6 were 13 to 16 years of age.

For each sample, the proportions of A. viscosus and A. naeslundii (CNAC-20 plate counts) among the total CFU recovered (blood plates) were calculated. An estimate of the efficiency of CNAC-20 in recovering Actinomyces from the samples was obtained by comparing the number of Actinomyces CFU detected on CNAC-20 with the number of CFU recovered on blood agar that could be identified subsequently as A. viscosus or A. naeslundii. This estimate was made on five of the clinical samples. Every colony on blood agar plates containing 50 to 100 colonies was isolated. Pure cultures of the isolates were Gram stained. Gram-positive rods, pleomorphic rods, filaments, and cocccobacilli were submitted to several cultural tests (6, 14) to determine their resemblance to A. viscosus and A. naeslundii.

An index was calculated to determine the relative preferences of A. viscosus and A. naeslundii for colonizing specific intraoral surfaces. The index consisted of the ratio of catalase-positive to catalase-negative CFU in samples from only those subjects found to be infected with both catalase-positive and catalase-negative Actinomyces. Thus, for any intraoral site, a mean ratio significantly greater than 1.0 indicated a predominance of catalase-positive CFU among the facultatively anaerobic Actinomyces isolated; a mean ratio of less than 1.0 indicated a predominance of catalase-negative isolates.

RESULTS

Establishment of facultatively anaerobic Actinomyces in the human mouth. The frequency of isolation of bacteria resembling A. viscosus and A. naeslundii is illustrated in Fig. 1. In general, the frequency of facultatively anaerobic Actinomyces isolation increased with age, similar to findings for anaerobic Actinomyces reported by others (20, 22). Catalase-negative Actinomyces were detected in 40% of the predentate infants and 82% of the infants with teeth. The saliva samples of all other subjects contained catalase-negative isolates. Although the plaque samples from most subjects also contained catalase-negative CNAC-20 CFU, there were some samples in which they were not detected. In contrast, the establishment of catalase-positive Actinomyces in the oral cavity was delayed. They were not detected in the samples from predentate infants. The frequency of their detection in saliva and plaque samples increased slowly, reaching an isolation frequency greater than 50% by age 7.

Neither saliva nor plaque samples from any of the age groups yielded a 100% detection frequency for catalase-positive Actinomyces.

Intraoral distribution of facultatively anaerobic Actinomyces. Data reflecting the proportions of Actinomyces-like isolates in the total cultivated flora should be considered accu-
rate only to the limits of the methods used to enumerate them. We have found previously that some stock strains of both A. viscosus and A. naeslundii either fail to grow on CNAC-20 or lose the ability to grow on it after extended laboratory culture (6; unpublished observations). However, pure cultures of stock and freshly isolated strains that can grow on CNAC-20 yield equivalent numbers of CFU on CNAC-20 and nonselective media (6). In the samples from this study chosen to estimate the efficiency of CNAC-20 in recovering facultatively anaerobic Actinomyces, the number of Actinomyces CFU on CNAC-20 represented 70.0 ± 12.0% (mean ± standard error; range, 36.8 to 90.4) of the predominant CFU recovered on tryptic soy blood agar that could be identified subsequently as A. viscosus or A. naeslundii.

Among the children and adults, the average percentage of the salivary flora recovered on blood agar that could be accounted for by CNAC-20 Actinomyces isolates ranged from 5.2 ± 1.0 to 11.0 ± 3.0% (Tables 1 and 3). No trends of increasing or decreasing proportions with age were noted. Catalase-negative isolates predominated among CNAC-20 CFU recovered from saliva of subjects in all age groups. The mean percentage of catalase-negative isolates among salivary Actinomyces isolates was greater than 90% at all ages younger than the teenage and adult groups. Under the conditions used, the adult saliva samples contained an average of $8.9 \times 10^6$ catalase-negative and $2.9 \times 10^6$ catalase-positive Actinomyces CFU/ml.

The mean proportions of the cultivated dental plaque flora recovered as Actinomyces CFU on CNAC-20 were quite low in the children of all age groups, ranging from 0.6 ± 2.0 to 3.0 ± 1.7% (Table 1). Among these, catalase-negative isolates predominated in the plaques from the young children. The relative proportions of catalase-positive isolates generally increased with age. Catalase-positive isolates predominated in plaque samples from the teenage and adult groups (Tables 1 and 3).

Facultatively anaerobic Actinomyces accounted for 7.0 and 4.5% of the cultivated flora from the adult tongue and buccal mucosal samples, respectively. The frequency of isolation and the relative proportions of catalase-positive and catalase-negative Actinomyces isolates differed in samples from the two sites (Table 3). Catalase-negative CFU were detected in 100% of both tongue and buccal mucosa samples. In contrast, catalase-positive CFU were isolated from only 40% of the tongue and 38% of the cheek samples. Tongue samples contained an average of greater than fourfold more catalase-negative than catalase-positive isolates. Their average relative proportions in the buccal mucosa samples were almost equal.

Differences in the preferences of catalase-positive and catalase-negative Actinomyces for colonizing various sites in the human mouth were suggested by the mean relative proportions described above. These preferences became more evident when the ratio of their proportions was calculated for samples from only those subjects from whom both catalase-positive and
tive Actinomyces were isolated (Tables 2 and 3). The mean ratios for the saliva samples differed markedly from those of the plaque samples at all ages. With one exception (saliva samples from the teenage group), the ratio indicated an overall predominance of catalase-negative isolates in saliva samples and catalase-positive isolates in plaque. Differences in the ratio were also observed between the samples obtained from the tongue and buccal mucosa in adults (Table 3). The ratio indicated a predominance of catalase-negative isolates in the tongue samples, similar to that detected in saliva. In contrast, catalase-positive isolates predominated in buccal mucosa samples from those subjects with detectable levels of both catalase-positive and -negative CFU.

**DISCUSSION**

Information regarding the host age at which oral pathogens colonize, their distribution in the mouth, and the conditions necessary for successful implantation should suggest some means to prevent their establishment, and thereby lower the pathogenic potential of the flora. Data presented herein indicate that the suspected periodontal pathogens A. viscosus and A. naeslundii differ in both the host age at which they establish in the human mouth and their preferences for colonizing various intraoral sites. Catalase-negative isolates resembling A. naeslundii were found to colonize most infants, including 40% of the predentates, and to maintain predominance among facultatively anaerobic Actinomyces in saliva and on the tongue. These data agree with earlier findings that the proportions of bacterial species in saliva generally reflect the proportions on the tongue dorsum (9, 12, 19). In contrast, the colonization of bacteria resembling catalase-positive A. viscosus was delayed until after teeth had erupted, and even then their rise to predominance over catalase-negative Actinomyces in plaque was delayed for several years. Although the medium used to detect A. viscosus and A. naeslundii could only recover approximately 70% of their CFU cultivable on blood agar, it seems unlikely that undetected A. viscosus CFU could have comprised a significant proportion of the flora, considering the fact that infant saliva samples and plaque samples were plated undiluted.

If oral bacteria are transmitted between humans primarily via saliva, then the relative ease with which specific bacteria are transmitted would likely be influenced in part by their relative numbers in saliva. In addition, differences in their preferred intraoral sites of colonization would be expected to affect the probability of implantation, dependent upon the availability of a specific local environment conducive to colonization at the time of transmission. For example, Streptococcus salivarius, which colonizes the tongue and is present in high numbers in adult saliva, has been shown by Carlsson and co-workers to establish in the mouths of infants preferentially, and thereby may affect the colonization of other intraoral flora, including catalase-negative Actinomyces. However, the incidence of these bacteria in the mouths of infants may be influenced by the age at which they establish.

**TABLE 2. Ratio of catalase-positive to catalase-negative Actinomyces-like isolates in samples from children**

<table>
<thead>
<tr>
<th>Subject age (years)</th>
<th>No. of subjects</th>
<th>Mean ratio (catalase-positive/negative CFU)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Saliva</td>
</tr>
<tr>
<td>3-4</td>
<td>24/9</td>
<td>0.23</td>
</tr>
<tr>
<td>5-6</td>
<td>4/10</td>
<td>0.10</td>
</tr>
<tr>
<td>7-8</td>
<td>12/14</td>
<td>0.19</td>
</tr>
<tr>
<td>9-10</td>
<td>7/11</td>
<td>0.14</td>
</tr>
<tr>
<td>11-12</td>
<td>4/7</td>
<td>0.12</td>
</tr>
<tr>
<td>13-16</td>
<td>4/6</td>
<td>1.32</td>
</tr>
</tbody>
</table>

* Only children from whom both catalase-positive and catalase-negative Actinomyces were isolated.
* Number of children sampled.

**TABLE 3. Recovery of facultatively anaerobic Actinomyces from various intraoral sites of adults**

<table>
<thead>
<tr>
<th>Site sampled</th>
<th>Mean % of cultivated flora</th>
<th>Mean % of cultivated Actinomyces</th>
<th>Mean ratio* (catalase-positive/negative CFU)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Catalase positive</td>
<td>Catalase negative</td>
<td>Catalase positive</td>
</tr>
<tr>
<td>Saliva</td>
<td>1.5 ± 0.6 (0.1-6.4)</td>
<td>4.3 ± 0.8 (0.6-12.9)</td>
<td>23.9 ± 7.1</td>
</tr>
<tr>
<td>Smooth-surface</td>
<td>4.4 ± 1.4 (0.1-19.5)</td>
<td>2.9 ± 1.3 (0.1-16.9)</td>
<td>58.3 ± 11.2</td>
</tr>
<tr>
<td>dental plaque</td>
<td>(0.9-4.9)</td>
<td>(0.8-12.8)</td>
<td>15.7 ± 7.8</td>
</tr>
<tr>
<td>Tongue</td>
<td>2.9 ± 1.1 (0.1-13.5)</td>
<td>1.6 ± 0.7 (0.2-10.4)</td>
<td>51.2 ± 9.5</td>
</tr>
</tbody>
</table>

* Includes only subjects from whom both catalase-positive and catalase-negative Actinomyces were detected.
* Mean ± standard error of mean (range).
* Standard error same as for catalase positive.
of neonates (3). Thus, the large surface area of the tongue, which is a prime ecological niche for S. salivarius (10, 11, 19), provides an environment suitable for colonization by the relatively high numbers of S. salivarius cells transmitted via adult saliva. In contrast, the establishment of Streptococcus mutans and Streptococcus sanguis, which preferentially colonize teeth, is delayed until after tooth eruption (1, 4, 5). In the case of S. mutans, adult salivary numbers are relatively low compared with those of other oral streptococci, and oral colonization is often delayed until after the first year of age (1, 4, 5, 22). In addition to its favored, and perhaps essential, ecological niche being unavailable until teeth erupt, the frequency with which infants’ teeth are exposed to high enough S. mutans doses to favor implantation may be rare.

Evidently, members of the genus Actinomyces also differ in their ease of implantation in humans. A. naeslundii, which colonizes the tongue and is present in relatively high numbers in saliva, is readily transmitted to the infant and may establish in the predentate mouth. In contrast, oral colonization of A. viscosus is delayed. This may be explained in part by its preference for colonizing teeth and its relatively low salivary numbers available for transmission. It is probable that other age-related phenomena also influence the ability of specific bacteria to colonize. In studies to determine the relationship between the age of rats and their susceptibility to the implantation of A. viscosus Ny-1, Brecher and van Houte have found that the organism established more readily in older than in younger rats (Int. Assoc. Dent. Res., abstr. 462, 1976). It is of interest that the infecting strain was recovered in higher proportions of the flora from the rats’ teeth and buccal mucosa than from their tongues (S. Brecher and J. van Houte, personal communication). Thus, the most heavily colonized rat sites were the same as those yielding the highest relative proportions of A. viscosus in the present investigation of humans.

Factors influencing the dissimilarities in A. viscosus and A. naeslundii patterns of intraoral colonization have not yet been elucidated. It is doubtful that differences in their nutrient requirements have a profound effect, since taxonomic studies have revealed few dissimilarities in the two species except for distinct differences in their catalase-like activities (14; E. D. Fillery, G. H. Bowden, and J. M. Hardie, Int. Assoc. Dent. Res., abstr. L-218, 1975). Moreover, reported similarities in their deoxyribonucleic acid base ratios (A. L. Cokendall, T. W. Lee, and A. T. Brown, Int. As-

soc. Dent. Res. abstr. 74, 1974) and cross-reactions between some antigens (8) suggest that what are now termed A. viscosus and A. naeslundii may eventually be classified as catalase activity variants of one species. Since several members of the dental plaque flora are known to produce hydrogen peroxide, it is possible that the increased proportions of A. viscosus recovered from plaque accumulations may have resulted from the induction of catalase activity among populations of Actinomyces cells in response to high local hydrogen peroxide concentrations. However, the findings in this study that catalase-negative Actinomyces predominated in plaque collected from young children and the findings of Socransky and co-workers that catalase-positive A. viscosus is one of the first species to recolonize cleaned adult teeth (S. S. Socransky, A. D. Manganiello, D. Propas, V. Oram, and J. van Houte, J. Periodontal. Res., in press) do not support this contention. If catalase-like activity is of any selective advantage for A. viscosus cells, it does not appear to be a basic ecological determinant affecting their initial colonization.

A more likely explanation for differences in the proportional distribution of A. viscosus and A. naeslundii may involve the selectivity with which they adhere to various oral surfaces. Specificity in the extent to which bacteria can attach to surfaces has been substantiated as a major ecological pressure regulating the indigenous microbial flora of the mouth and has been implicated among factors influencing the tissue tropisms of overt pathogens (7, 10). The fact that A. viscosus colonizes cleaned teeth more readily than A. naeslundii (Socransky et al., in press), whereas the number of A. viscosus cells in saliva available for attachment is severalfold fewer, suggests that A. viscosus cells have a greater affinity for teeth than cells of A. naeslundii. Such differences in affinity for teeth and salivary cell concentrations have been used to predict the probability of colonization by oral streptococci and lactobacilli on smooth surfaces of teeth (24).

A. viscosus and A. naeslundii may also differ in the degree to which they can adhere to accumulated plaque deposits. Differences in the ability of some A. viscosus and A. naeslundii strains to aggregate selectively with glucose-grown strains of plaque streptococci have been reported (R. P. Ellen and I. B. Balcerzak-Raczkowski, J. Periodontal Res., in press). In addition, Bourgeau and McBride have shown recently that A. viscosus cells bind readily to sucrose-grown S. mutans and S. sanguis cells and to glucans elaborated by these streptococci (2). The ability of A. naeslundii to aggregate
under similar conditions has not been reported. The full impact of adherence-related phenomena and interbacterial affinities on the establishment and proportional distribution of oral Actinomyces species is not yet clear. However, it is evident that A. viscosus and A. naeslundii, which are similar enough physiologically to be considered members of the same species, differ greatly in the pattern in which they naturally infect the human host.

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LITERATURE CITED