Natural History of *Streptococcus sanguinis* in the Oral Cavity of Infants: Evidence for a Discrete Window of Infectivity

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Received 20 December 1999/Returned for modification 8 March 2000/Accepted 24 April 2000

The heterogeneous group of oral bacteria within the *sanguinis* (sanguis) streptococci comprise members of the indigenous biota of the human oral cavity. While the association of *Streptococcus sanguinis* with bacterial endocarditis is well described in the literature, *S. sanguinis* is thought to play a benign, if not a beneficial, role in the oral cavity. Little is known, however, about the natural history of *S. sanguinis* and its specific relationship with other oral bacteria. As part of a longitudinal study concerning the transmission and acquisition of oral bacteria within mother-infant pairs, we examined the initial acquisition of *S. sanguinis* and described its colonization relative to tooth emergence and its proportions in plaque and saliva as a function of other biological events, including subsequent colonization with mutans streptococci. A second cohort of infants was recruited to define the taxonomic affiliation of *S. sanguinis*. We found that the colonization of the *S. sanguinis* occurs during a discrete “window of infectivity” at a median age of 9 months in the infants. Its colonization is tooth dependent and correlated to the time of tooth emergence; its proportions in saliva increase as new teeth emerge. In addition, early colonization of *S. sanguinis* and its elevated levels in the oral cavity were correlated to a significant delay in the colonization of mutans streptococci. Underpinning this apparent antagonism between *S. sanguinis* and mutans streptococci is the observation that after mutans streptococci colonize the infant, the levels of *S. sanguinis* decrease. Children who do not harbor detectable levels of mutans streptococci have significantly higher levels of *S. sanguinis* in their saliva than do children colonized with mutans streptococci. Collectively, these findings suggest that the colonization of *S. sanguinis* may influence the subsequent colonization of mutans streptococci, and this in turn may suggest several ecological approaches toward controlling dental caries.
presumed to be \(S.\) \textit{sanguinis} (median of seven isolates per infant) were selected based on the same criteria as the natural history cohort and then further characterized based on the criteria given below. Written informed consent was obtained from all subjects as approved by the Institutional Review Board of the University of Alabama at Birmingham and the Jefferson County Health Department.

Sample procurement and bacteriology. Bacterial samples were obtained from saliva and plaque collected from both cohorts. Unstimulated saliva samples were collected with a sterile cotton swab from the sublingual area of the mouth until saturated. Samples from the teeth (plaque) were taken with a sterile toothpick; the toothpick was placed in each approximal site and then passed alone the ridge into the next approximal site of both upper and lower teeth. Swab and toothpick samples were placed into separate 1.0-ml reduced transport fluid vials (29) and then processed as previously described (8). Approximate dilutions of saliva and plaque samples were plated onto MM10-sucrose agar (20). After 3 days of anaerobic incubation (5% \(N_2\), 10% \(CO_2\), and 5% \(H_2\)), colonies presumed to be \(S.\) \textit{sanguinis} were selected from MM10-sucrose agar based on their firm, adherent, star-shaped colony morphology (20, 28). Discrete colonies were then isolated from subcultures and placed in the appropriate medium for the detection of hydrolysis of arginine and lack of fermentation of raffinose. (Mannitol fermentation differentiates mutants streptococci from \(S.\) \textit{sanguinis},) This characterization is consistent with the original classification of Carls-son and coinvestigators as group I:B of \(S.\) \textit{sanguinis} (4, 7). At these isolates were not saved after the biochemical tests were done, and as a new criteria became available (15), species delineation of \(S.\) \textit{sanguinis} (15, 24, 35), the same screening procedures were performed on isolates from the taxonomy cohort (characteristic morphology on MM10-sucrose medium, hydrolysis of arginine, and failure to ferment mannitol), but unlike isolates from the natural history cohort, species were not further delineated by more detailed biochemical and genetic bio-

ization. These additional tests included an extended panel of biochemical assays described by White and Beighton (35) and Kilian and coworkers (15), i.e., fermentation of amygdalin, inulin, melibiose, raffinose, and sorbitol; hydrolysis of arginine and esculin; and production of \(H_2O_2\). Enzymatic reactions, including \(\alpha\)-d-fucosidase, \(\alpha\)-L-fucosidase, \(\beta\)-d-glucosidase, \(\alpha\)-galactosidase, and \(\alpha\)-L-acetylgalactosaminidase, as described by White et al. (36), supplemented the battery of tests. All 291 isolates were subjected to these tests. As continue the prototype strain of \(S.\) \textit{sanguinis} (ATCC 10556), along with protot-

ypic strains of the \textit{Streptococcus} species \(S.\) \textit{gordonii}, \(S.\) \textit{parasanguinis}, \(S.\) \textit{oralis}, and \(S.\) \textit{mitis}, were subjected to the same battery of tests.

In addition, the DNA coding for 16S rRNA (rDNA) loci of 20 randomly chosen isolates of the 291 isolates placed within one of the four \(S.\) \textit{sangui-

nis} biovars (5) were selected via PCR using custom-designed primers. The primers

(5'-GGCTCGACGAGCAGACCCTGCG-3' and 5'-AGCCGCGGTTG

CTCGTCAAGG-3'), produced a single amplicon of approximately 360 bp. This number of nucleotides proved sufficient to determine each strain’s phylogenetic affiliation (described below). At least two amplicons from different reactions were used to determine the phylogenetic affiliation of each strain. All amplicons were sequenced (Qiagen Quick PCR Purification kit; Qiagen, Chatsworth, Calif.). The similarity (\(S\)) index (21) ranged from 0.73 to 1.00, with a mean of 0.94. Interesting, the strain with the lowest \(S\) (0.73) still showed its highest similarity with the 16S rRNA locus of \(S.\) \textit{gordonii}.

RESULTS

Taxonomic characterization of \(S.\) \textit{sanguinis}. In the original natural history study, we selected and biochemically defined \(S.\) \textit{sanguinis} at the time using a simple identification scheme based on the existing literature (7). Subsequent to that study, emended taxonomic definitions became available, making it necessary to reconfirm our original characterization of \(S.\) \textit{sanguinis}. In addition, and independent from taxonomic realign-

ments, the species name was changed to “\(S.\) \textit{sanguinis}.” Because only a few isolates of strains presumed to be \(S.\) \textit{sanguinis} were saved from the natural history cohort, we enrolled a second group of infants (taxonomy cohort) for the purpose of con-

firming that the isolates that we described in the natural history cohort were, indeed, \(S.\) \textit{sanguinis}. Isolates selected by the same approach as for the original natural history cohort (i.e., selection based upon colony morphology, cleavage of arginine, and production of \(H_2O_2\)) were characterized using the biochemical tests described by Kilian and coworkers and White and Beight-

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onized with S. sanguinis. Wilcoxon sign-rank test). Over all the samples from infants colonized with S. sanguinis, the average level of S. sanguinis was 2.0 × 10^5 CFU/ml of unstimulated saliva. This comprised 0.1% of the total cultivable bacteria in saliva. In plaque, S. sanguinis comprised 10% (±18.3%) of the total cultivable bacteria from dentate infants. As new teeth emerged in the infants, the levels of S. sanguinis in saliva increased (r = 0.89; P = 0.0001; Pearson correlation analysis). There were no racial or gender differences in any of the measured aspects of initial acquisition of S. sanguinis.

Relationship between colonization by S. sanguinis and mutans streptococci. Because colonization by S. sanguinis not only precedes that by mutans streptococci but, like that by mutans streptococci, is dependent on the presence of teeth, we wondered whether colonization with S. sanguinis influenced subsequent colonization with mutans streptococci. In addition, S. sanguinis is thought to be an antagonist of mutans streptococci. To address this query, we employed Cox regression analysis (stepwise selection) using the time of oral colonization of mutans streptococci as the dependent variable and time of infection during a discrete window period is analogous to what was experienced at 2 and 3 years of age, but failed to show a significant correlation, perhaps due to the low prevalence of caries in this population of children at 2 and 3 years of age (10 and 23%, respectively).

To further examine the possible antagonism between S. sanguinis and mutans streptococci, we averaged the levels of S. sanguinis in saliva for periods before and after colonization with mutans streptococci (Fig. 3). On average, the pre- and post-mutans streptococci saliva samples were comprised of 6.8 and 4.8 samples, respectively, from 37 infants. (Eight infants did not harbor detectable levels of mutans streptococci.) We then compared pre-mutans streptococci levels (4.5 × 10^5 CFU/ml) to post-mutans streptococci levels (1.9 × 10^5 CFU/ml) and found that the S. sanguinis levels were significantly greater (P = 0.05, paired t test) in saliva before the initial colonization by mutans streptococci than after. As expected, pre- and post-mutans streptococci levels of S. sanguinis within each individual were positively correlated with each other (r = 0.43; P = 0.01). Interestingly, the eight infants who appeared to be free of mutans streptococci exhibited higher average levels of S. sanguinis in their saliva (5.7 × 10^5 CFU/ml) than did the 37 mutans streptococci-infected infants (4.6 × 10^5 CFU/ml); this difference was also statistically significant (P = 0.03, Wilcoxon rank sum test).

Relationship between sanguinis streptococcus colonization and caries. We compared the time to initial colonization with S. sanguinis and its levels in both saliva and plaque to caries experience at 2 and 3 years of age, but failed to show a significant correlation, perhaps due to the low prevalence of caries in this population of children at 2 and 3 years of age (10 and 23%, respectively).

TABLE 1. Age of infants at initial detection of S. sanguinis in either plaque or saliva samples

<table>
<thead>
<tr>
<th>Sample</th>
<th>Mean ± SD</th>
<th>Median</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saliva</td>
<td>13.9 ± 6.0</td>
<td>12.2</td>
<td>4.0–29.5</td>
</tr>
<tr>
<td>Plaque</td>
<td>10.6 ± 5.2</td>
<td>9.1</td>
<td>5.2–36.5</td>
</tr>
<tr>
<td>Either</td>
<td>9.7 ± 3.1</td>
<td>9.0</td>
<td>3.9–20.9</td>
</tr>
</tbody>
</table>

DISCUSSION

Within this population of urban infants from Birmingham, Ala., initial colonization by S. sanguinis occurs during a discrete window of infectivity, around 9 months of age. Acquisition during a discrete window period is analogous to what was
observed for the acquisition of mutans streptococci, which occurred around a median age of 26 months (8). Also similar to mutans streptococci, colonization by S. sanguinis follows and is significantly correlated to the emergence of primary teeth. Unlike colonization by mutants streptococci (8), however, all of the infants eventually acquired S. sanguinis, albeit some 12% of the infants did so later than the median window period shown in Fig. 1. The earlier colonization with S. sanguinis than with mutans streptococci may reflect the greater affinity for attachment of S. sanguinis to tooth surfaces than mutants streptococci (34).

The present study confirms and extends the pioneering work of Carlson and coworkers (7), who first showed that the colonization of both S. sanguinis and mutants streptococci is dependent upon the presence of teeth. Extrapolation from the original data of Carlson (7) shows close agreement with data from the present study as evident when comparing the cumulative probability of infection curves (Fig. 4). Also in agreement with Carlson’s group is the strong correlation between time to colonization of S. sanguinis and the time of emergence of the primary dentition (Fig. 1). That colonization with S. sanguinis is dependent upon the presence of teeth is further supported in the present study, which shows that detection of S. sanguinis in plaque precedes its detection in saliva by more than 4 months. In another study in Boston area infants, Smith and coworkers (26) reported that 7 of 14 (50%) infants were colonized by S. sanguinis by 12 months of age. This observation agrees well with our observation that S. sanguinis was detected at a median age of 12 months in unstimulated saliva.

The notion of a time-dependent window for acquisition of members of the oral biota requires an understanding of the limitations in defining such a window. Populations may differ in time to acquisition based upon environmental and developmental exposures, such as sucrose consumption and enamel hypoplasia, to name only two possible determinants of early colonization. The method of detection (e.g., cultural, PCR, or DNA probes) dictates time to infection, but methods can vary widely. Moreover, a possibly more correct designation for a window period would be window of colonization rather than infectivity, because our methods indicate colonization only after sufficient levels are present for detection by cultural methods. More sensitive methods (e.g., PCR and DNA probes) would likely detect S. sanguinis, S. mutans, or other oral indig

The time of initial detection of oral bacteria is also dependent upon the design of the study, that is, longitudinal versus cross-sectional. Study results may differ based on this factor alone. We prefer longitudinal surveys, as sustained colonization can be distinguished from transient infections, but longitudinal studies are expensive and loss to follow-up is often a problem, especially with the mobility inherent in urban populations.

As shown in the present study, the levels of S. sanguinis increase with the age of the infant. After the mutants streptococci colonize the oral cavity, however, the levels of S. sanguinis decrease. We attribute this initial increase in S. sanguinis prior to the colonization of mutants streptococci to the availability of new colonization sites as primary teeth emerge. Other investigators have reported an increase in total streptococci correlated with an increase in the number of teeth present (31), but the same investigators did not show that this increase was correlated to increases in S. sanguinis. These conflicting data may be due to the fact that Tappuni and Challacombe (31) reported a low isolation frequency of only 5.4% for S. sanguinis from dentate infants aged 1 to 3.4 years. In contrast, we show that 100% of dentate infants were colonized by S. sanguinis prior to 18 months of age. These authors also isolated S. sanguinis from predentate infants (1.7%), as did we (2%), but our subsequent samples failed to document persistent colonization prior to tooth emergence.

To some extent, differences in findings among the various studies may arise from the definition of the species comprising the sanguinis complex. As a result of recent reexamination of the oral streptococci, the group of oral bacteria formerly classified as S. sanguinis have now been divided into at least two species, S. sanguinis and S. gordonii, each species having three to four biootypes or biotypes (15, 35). This reclassification is not without controversy, however. The three authoritative reviews involved with reexamination of the classification of viridans streptococci lack agreement, particularly with regard to the grouping of S. sanguinis (10, 15, 35). This ambiguity is further heightened by the fact that the genetic determinants of the four biovars of the newly designated S. sanguinis vary to the extent that they may constitute separate species (10, 22). Our data confirm this lack of genetic homogeneity in that the 16S rDNA sequences varied as much within biovars as between biovars and there was no apparent overlap or clustering within biovars and their 16S rDNA loci (Y. Pan, Y. Li, and P. W. Caulfield, unpublished data). In fact, Willcox (37) argued for at least nine species within the “S. sanguinis group” based on biochemical and genetic data. Nonetheless, the isolates we selected based primarily on colony morphology bore the highest phylogenetic affiliation with the S. sanguinis prototype strain, ATCC 10556. This affiliation may be somewhat misleading, however, because only a few sequences from the 16S rDNA locus of S. sanguinis are present in the GenBank database. It can be argued that the genetic data support splitting the sanguinis streptococci into at least two genetic groups based on 16S rDNA locus diversity alone. Clearly, the sanguinis group of streptococci that we describe here is well defined in terms of their discrete ecological characteristics (i.e., colonization site and time of acquisition) as well as their phylo-
genetic affiliation. We and others (23) suggest that similarity indices based only on genetic data (e.g., DNA-DNA hybridization and rRNA) using more or less arbitrary cutoff points for defining a species may be inappropriate when applied to an ecologically defined population of bacteria.

Perhaps the most interesting finding of this study, and of possible clinical relevance, is the relationship between levels and time of colonization of S. sanguinis and subsequent colonization of mutans streptococci. Serial samples of saliva, which contained higher levels of S. sanguinis, were associated with a 6-month delay in the colonization of mutans streptococci. Consistent with this finding is our observation that early colonization of S. sanguinis in an infant results in the later colonization of mutans streptococci. The notion that delayed colonization of mutans streptococci may lead to less caries has been demonstrated by Köhler and coworkers (16). Whether the early introduction of S. sanguinis into the oral cavity of infants or increasing its relative proportions by artificial means could affect subsequent mutans streptococci colonization or caries has yet to be demonstrated. The concept of effector or protective mechanisms to displace another organism has precedence, however (25). Efforts to artificially implant oral streptococci in the oral cavity to serve as an effector strain have been proposed by others (30).

Another interesting observation was that the colonization of mutans streptococci might have adversely influenced the S. sanguinis levels, since they were shown to drop significantly following colonization of the mutans streptococci. This finding is consistent with the notion that S. sanguinis and the mutans streptococci antagonize or compete with each other in the oral cavity. Further supporting this possible antagonism is the observation that the eight infants who did not acquire mutans streptococci during the window period had significantly higher mean levels of S. sanguinis than mutans streptococcus-infected infants (5.7 × 10^5 CFU/ml versus 4.6 × 10^6 CFU/ml; P = 0.03). Taken as a whole, the data here indicate that S. sanguinis and the mutans streptococci compete for colonization of the infant and that one affects the colonization of the other.

From these data, we speculate that early colonization of S. sanguinis could delay the colonization of the mutans streptococci. This, in turn, could result in a reduction in dental caries, as delayed colonization of mutans streptococci has been associated with lower caries scores (16). Repeating these studies in a more caries-active population may bear out this relationship. If such a relationship exists, one might also predict lower caries levels or time to initial infection. Further manipulation of this relationship may involve the early introduction of S. sanguinis into the mouths of infants, serving as an effector strain, possibly reducing the risk for future caries. What remains, and is currently under investigation, is determining the source of S. sanguinis to the infant. If the mother is the primary source of S. sanguinis, as has been shown in the case of mutans streptococci (17), then measures that foster the transmission of S. sanguinis to her infants may be a viable means of protecting the infant from mutans streptococci infection and caries. Caution is warranted here, however, because the sanguinis streptococci have been implicated with life-threatening diseases, including bacterial endocarditis.

ACKNOWLEDGMENTS

Support for this study came from USPHS research grants RFP 5-83-3R, RO3 DE10224, and P50 DE11147 from the National Institute of Dental and Craniofacial Research, National Institutes of Health, Bethesda, MD 20892.

We thank Zhenmei Lu and Winnie Lee for their technical assistance in the area of clinical microbiology.

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Editor: E. I. Tuomanen