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Molecular analysis of age-related changes of *Streptococcus anginosus* group and *Streptococcus mitis* in saliva

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The purpose of this study was to survey the prevalence of streptococcal species, especially *Streptococcus anginosus* (which has been reported to be associated with cancer in the upper digestive tract), *Streptococcus constellatus*, and *Streptococcus intermedius* in the saliva of different age groups. A sequence analysis of 16S rDNA was performed and DNA quantified using real-time polymerase chain reaction. The *S. anginosus* level increased with age, whereas the levels of *S. constellatus* and *S. intermedius* did not change. *Streptococcus mitis* was the predominant species in the saliva of all the age groups but, unlike the *S. anginosus*, the proportion of *S. mitis* in the salivary bacteria decreased with age. The increase in *S. anginosus* with age should be carefully monitored because of its association with diseases, including cancer.

Key words: real-time polymerase chain reaction; saliva; *Streptococcus anginosus*; *Streptococcus mitis*

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Streptococcus anginosus is a currently recognized species of the '*Streptococcus milleri*' group, the name used for heterogeneous oral streptococcal strains associated with purulent infections (14). The *S. milleri* group comprises at least three different species: *S. anginosus*, *Streptococcus constellatus*, and *Streptococcus intermedius* (14). Awareness of the clinical importance of *S. anginosus* has gradually increased because several studies have reported a close association between *S. anginosus* infection and cancer in the upper digestive tract (3, 7, 8, 12). In spite of this clinical significance, information on the prevalence of *S. anginosus* in the oral cavity is limited because most studies on this subject were performed before the current classification criteria of the *S. anginosus* group was established (11). Recent analyses using real-time polymerase chain reaction (PCR) found extremely low levels of *S. anginosus* in the saliva (2, 10). However, these studies did not consider the variety of *S. anginosus* strains. Nor was age considered, although microbial infection in the oral cavity appears to change with age (5). It was reported that the streptococcal salivary colony-forming units were higher in adults than in children, and that the isolation frequency and proportion of streptococcal species change with age (11). The incidence of cancer is highest in people in their sixties (1). The oral cavity may act as a reservoir of *S. anginosus*, a potential pathogen, and saliva is the most probable carrier. The emphasis of this study was the molecular analysis of subsets of the salivary microbiota in different age groups, focusing on *S. anginosus*.

Material and methods

Saliva samples

Saliva samples were obtained from systemically healthy volunteers aged 25–70 years. These samples were centrifuged, then frozen and stored at −80°C until use. Written informed consent was obtained from all the volunteers.

Bacterial strains

S. anginosus ATCC 33397, *S. intermedius* ATCC 27335, *S. constellatus* ATCC 27823, *Streptococcus mutans* LM 7, *Streptococcus sobrinus* AHT, *Streptococcus sanguinis* ATCC 10556, *Streptococcus gordonii*

Table 1. S. anginosus, S. constellatus, and *S. intermedius* strains and accession numbers for the DDBJ/EMBL/GenBank nucleotide sequence databases from which DNA sequence data were used for designing specific primers

Species/strains	Accession numbers
S. anginosus strain ATCC33397	AF352808
S. anginosus genotype VA8466	AF306838
S. anginosus strain 920	AF145246
S. anginosus strain 1007	AF145245
S. anginosus strain 414	AF145243
S. anginosus strain 21	AF145242
S. anginosus strain 1204	AF145240
S. anginosus strain 367	AF145239
S. anginosus strain GTC822	AB006121
S. anginosus strain GTC821	AB006120
S. constellatus strain 1259	AY277942
S. constellatus strain 919	AY277941
S. constellatus strain 1192	AY277940
S. constellatus strain 15	AY277939
S. constellatus strain 857	AY277938
S. constellatus strain 1	AY277937
S. constellatus strain ATCC27823	AF104676
S. constellatus strain 206	AF104677
S. constellatus strain VAMC3868	AF169356
S. constellatus strain VAMC5464	AF169353
S. intermedius strain 488	AF104673
S. intermedius strain 125	AF104672
S. intermedius strain 535	AF104674
S. intermedius strain ATCC27335	AF104671
S. intermedius strain B33	AJ491836

ATCC 10558, *Streptococcus mitis* ATCC 6249, and *Streptococcus salivarius* ATCC 9759 were cultured.

DNA extraction

Genomic DNA was isolated from saliva and bacteria by a standard phenol–chloroform method. DNA content was determined spectrophotometrically.

PCR cloning and sequence analysis

Equal amounts of DNA extracted from the saliva of 10 people were mixed for each age group (25–49-year-old group, 50–69-year-old group and 70-year-old group) and used as templates. For amplification of a portion of the 16S rDNA gene of many oral bacteria from saliva, PCR was performed with primers Ust1X and Ust2E (Tables 1 and 2), and PCR cloning and sequence analysis of 16S rDNA were performed as described previously (4). A species was determined when its sequence had greater than 90% homology to bacteria.

Alignment and primers

16S rDNA sequences of 10 strains of *S. anginosus*, 10 strains of *S. constellatus*, 5 strains of *S. intermedius* (Table 1) and other streptococcal species were aligned by Clustal W (13) to design specific primers for amplification of 16S rDNA of *S. anginosus*, *S. constellatus*, and *S. intermedius*. Primers Ust1 and Ust2 modified from Ust1X and Ust2E were used for amplification of the 16S rDNA gene of many oral bacteria (Table 2).

Quantitative real-time PCR

Real-time PCR was performed on the ABI Prism Sequence detection System 7700 (Applied Biosystems, Foster City, CA) using SYBR green chemistry. The reaction mixture in a total volume of 25 µl contained SYBR Green Core Regent (Applied Biosystems), 3 mM $MgCl_2$, 200 nM of each primer, and 5 µl of DNA solution. The reaction was started with an incubation of 2 min at 50°C, followed by 10 min at 95°C, then 50 cycles of 15 s at 95°C and 1 min at 68°C.

Statistical analysis

Differences in the levels of *S. anginosus*, *S. constellatus*, and *S. intermedius* DNA in the three age groups were statistically analyzed using the Mann–Whitney *U*-test.

Results

Distribution of *Streptococcus* in saliva

The diversity of the bacterial flora in saliva was examined in three age groups. Table 3 describes species that had more than 90% similarity to partial sequences of 16S rDNA obtained for clones of salivary DNA. In all, 119 of 192 clones were identified as *Streptococcus*. *Streptococcus* accounted for 93% of identified strains in 25–49-year-olds, 45% in 50–69-year-olds, and 58% in 70-year-olds. *S. mitis* was the most frequently detected species in all age groups, and the proportion decreased with increasing in age. *S. mitis* accounted for 76% of *Streptococcus* in subjects aged 25–49 years, 46% in 50–69-year-olds, and 43% in 70-year-olds. Diversity of bacterial species increased as age increased. *S. anginosus* was not detected in any age group. *S. constellatus* was the only species detected among the *S. anginosus* group.

Table 2. Primers used in this study

Primer	Purpose	Bacterial specificity	Sequence	Position[a]
F13	Real-time PCR	*S. anginosus*	CTAATACATGCAAGTAGG	48
F6	Real-time PCR	*S. anginosus*	CAAGTAGGACGCACAGTT	58
F8	Real-time PCR	*S. anginosus*	CAAGTAGGACGCACAGTC	58
R3	Real-time PCR	*S. anginosus*	CAAGCATCTAACATGTGTTAC	186
ConF2	Real-time PCR	*S. constellatus* (*S. intermedius*)	CACCGTAGTTTACTACACCGTATT	78
ConR4	Real-time PCR	*S. constellatus* (*S. intermedius*)	CTACCATGCAGTAAATGTTC	181
Ust1	Real-time PCR	Oral bacteria	GAACGGGTGAGTAACGCGTAGGT	106
Ust2	Real-time PCR	Oral bacteria	CACTCACGCGGCGTTGCTCGGTC	387
Ust1X	PCR cloning	Oral bacteria	GCTCTAGAGAACGGGTGAGTAACGCGTAGGT	106
Ust2E	PCR cloning	Oral bacteria	GGAATTCCACTCACGCGGCGTTGCTCGGTC	387

[a]The 5′ position in *Escherichia coli* 16S rDNA numbering convention.

Table 3. Bacterial species inferred from PCR cloning analysis of the saliva of healthy people in the three age groups

Species	No. of clones			
	25–49 years	50–69 years	70 years	Total
S. mitis	32	13	21	66
S. salivarius	2	0	7	9
S. infantis	3	3	6	12
S. sanguinis	1	4	2	7
S. parasanguinis	0	4	4	8
S. australis	0	2	5	7
S. cristatus	1	1	2	4
S. constellatus	0	1	0	1
S. anginosus	0	0	0	0
Unidentified *Streptococcus*	3	0	2	5
Other bacterium	3	34	36	73

Primer design

An alignment of *S. anginosus*, *S. intermedius*, *S. constellatus*, and other *Streptococcus* revealed a variety of *S. anginosus* strains in 16S rDNA (Fig. 1). In previous studies, a variable region was used as primer to detect *S. anginosus* (2, 3, 10). Three different forward primers were designed to determine if this variable region is suitable as an *S. anginosus*-specific primer (Fig. 1, Table 2). F6 and F8 primers are highly specific but include the variable region used for detecting *S. anginosus* in former studies (2, 3, 10). F13 primer is less specific but does not include this variable region. In order to maintain specificity to *S. anginosus*, these three forward primers were used with a specific reverse primer, R3 (Fig. 1, Table 2). The alignment also revealed a strong similarity between *S. intermedius* and *S. constellatus*, making it difficult to design specific primers to distinguish *S. constellatus* from *S. intermedius*. Therefore, a primer set (ConF2, ConR4) that detected both *S. constellatus* and *S. intermedius* was designed (Table 2).

Quantification system of *S. anginosus*, *S. constellatus*, and *S. intermedius*

Real-time PCR was used to analyze DNA extracted from *S. anginosus*, *S. constellatus*, *S. intermedius*, *S. mitis*, *S. gordonii*, *S. salivarius*, *S. mutans*, *S. sanguinis*, *and S. sobrinus* in order to examine quantification systems with new primer sets of *S. anginosus* and *S. constellatus* (*S. intermedius*). When 10^1–10^6 fg DNA from *S. anginosus* was added in serial dilutions to the reaction mixture for real-time PCR systems of *S. anginosus*, detection and quantification were linear over the range of the DNA concentration examined. The real-time PCR system of *S. constellatus* (*S. intermedius*) was examined in the same way, and a similar result was obtained. When 10^4 fg of DNA from other species were assayed, the calculated values derived from a standard curve were lower than the detectable level, 10^1 fg. It was therefore concluded that each system was specific for each species. Three *S. anginosus* forward primers were then examined to determine whether they were suitable for the *S. anginosus* quantification system. *S. anginosus* DNA in saliva samples was quantified, but the quantified values derived from each system were different. Figure 2 depicts quantified values of *S. anginosus* in the saliva from the 50–69-year-old group. The system using F6 and F8 primers often exhibited completely different values from one another (6 of 18 samples showed more than 10 times difference), and they were not proportional. The system using the F13 primer usually had the highest values, which were comparatively close to the second highest values. As these results suggested that F6 and F8 primers are too specific to quantify various types of *S. anginosus* present in the oral cavity, F13 was chosen as the primer to quantify *S. anginosus* in the present study.

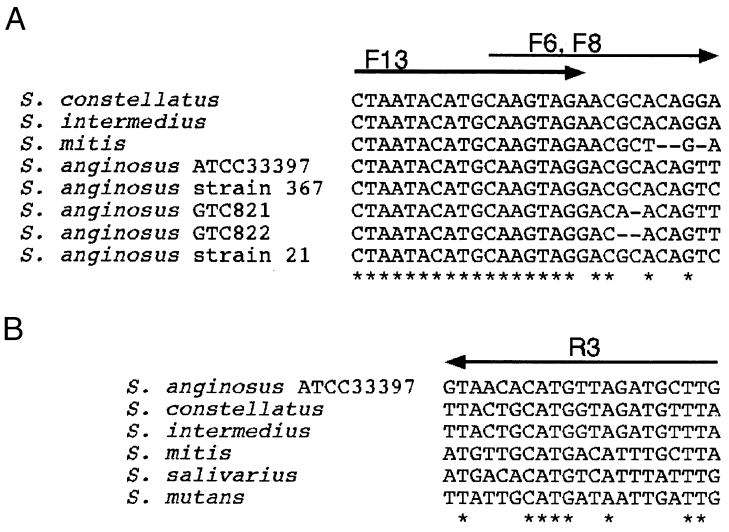

Fig. 1. Alignments of 16SrDNA of streptococcal species and *S. anginosus* strains. Arrows indicate primers. Stars represent identical nucleotides.

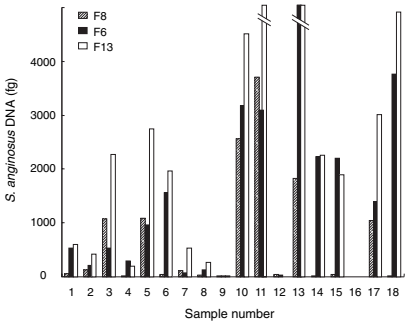

Fig. 2. Quantified values of *S. anginosus* DNA in 50–69-year-olds obtained using three different systems of real-time PCR. Ten ng of salivary DNA was used.

Quantification of *S. anginosus* and *S. constellatus* in saliva

The quantities of *S. anginosus*, *S. constellatus* (*S. intermedius*), and oral bacteria in saliva samples from 65 healthy people aged 25–70 years were determined, and the proportions of *S. anginosus* and *S. constellatus* (*S. intermedius*) in oral bacteria were calculated (Table 4). Results demonstrated that the average proportion of *S. anginosus* in oral bacteria increased with age: 0.38% at 25–49 years of age, 1.12% at 50–69 years, and 2.02% at 70 years. Statistical analysis indicated that the level was significantly higher at age 70 than at ages 25–49. Additionally, the oral bacteria in the saliva of 25% of 50–69-year-olds contained more than 2% *S. anginosus,* whereas the highest

Table 4. Average proportion of *S. anginosus* or *S. constellatus* (*S. intermedius*) to oral bacteria in the three age groups

	Average age	Sample no.	*S. anginosus* (%)	*S. constellatus* (*S. intermedius*) (%)
25–49 years	33.2	17	0.38 ± 0.32	0.06 ± 0.04
50–69 years	58.3	18	1.12 ± 1.74	0.11 ± 0.26
70 years	70	30	2.02 ± 3.49	0.07 ± 0.09

level found in the 25–49-year-old group was only 1.7% (data not shown). In contrast, the average ratio of *S. constellatus* (*S. intermedius*) to oral bacteria was about 10–40 times lower than that of *S. anginosus*, with no significant difference between age groups.

Discussion

Previous studies demonstrated very low levels of *S. anginosus* in saliva (2). In the present study, the distribution of streptococcal species in the saliva of healthy people was determined by clonal analysis of 16S rDNA sequence. It was demonstrated that *S. anginosus* as well as other *S. milleri* group species, *S. constellatus* and *S. intermedius* were minor species in all three age groups. However, quantification of *S. anginosus* DNA demonstrated that most people possess *S. anginosus* to some extent in their saliva. These results were obtained with the use of our new primer.

The average level of *S. anginosus* in the oral bacteria increased with age, whereas the average level of *S. constellatus* (*S. intermedius*) did not change and the proportion of *S. mitis* in the oral bacteria decreased. *S. mitis* was the predominant oral streptococcal species in infants, both in its prevalence and in its proportion of the oral streptococci. It may thus be the major component of the initially colonizing streptococcal microbiota of infants (9). The trend toward a decreasing proportion of *S. mitis* might start from an early age. Viridans group streptococci, including *S. mitis,* are known to induce inflammation of renal tissue (15). However, at least in the oral cavity, a prevalence of *S. mitis* may indicate young healthy microflora. In contrast, *S. anginosus*, which increased with increasing age in inverse proportion to *S. mitis*, should be carefully monitored due to its association with various kinds of infectious diseases such as endocarditis and cancer in the upper digestive tract.

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