Genotypic Diversity and Virulence Traits of Streptococcus Sobrinus Isolated From Caries-Free Children and Children Suffering Severe Early Childhood Caries

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Objective: *To evaluate the genotypic diversity and some virulence traits of Streptococcus sobrinus (S. sobrinus) isolated from caries-free children and children suffering severe early childhood caries (SECC).*

Methods: *S. sobrinus isolated from stimulated whole saliva samples of 91 caries-free children and 87 SECC children were subcultured, identified by polymerase chain reaction and genotyped by arbitrarily primed polymerase chain reaction. Polysaccharide synthesis ability, acidogenicity, aciduricity and the adherence ability of these S. sobrinus isolates were measured.*

Results *The frequency of S. sobrinus detection was 18.39% (16/87) in SECC children, which was significantly higher than that (3.30%, 3/91) in caries-free children. One to three different genotypes of S. sobrinus were detected in each SECC child. Only one genotype was colonised in each caries-free child. In SECC children, the production of water-insoluble glucan (WIG) was positively correlated with the ability of S. sobrinus adhering to a glass surface.*

Conclusion: *The presence of S. sobrinus could be a risk factor for high caries activity in severe early childhood caries. The multi-genotypes could be related to different caries suceptibility. Water-insoluble glucan plays an important role in the adherence and accumulation of S. sobrinus on tooth surfaces.*

Key words: *Streptococcus sobrinus, genotypic diversity, virulence trait, cariogenic ability, severe early childhood caries*

Dental caries is an infectious and transmissible disease. Mutans Streptococci (MS) are generally considered to be the principal etiological agent of dental caries[1,2], of which *Streptococcus mutans* (*S. mutans*) and *Streptococcus sobrinus* (*S. sobrinus*) are closely associated with the development of early childhood caries (ECC). Although the prevalence was distinctly lower than *S. mutans* and always coexisted with *S. mutans*[3], S. sobrinus could be linked to dental caries, which showed high cariogenic potentials in experimental animals[4,5]. The prevalence of *S. sobrinus* is more closely associated with high caries activity than that of *S. mutans*[6-8]. In other studies, cases of children harboring both *S. mutans* and *S. sobrinus* had a significantly higher caries incidence than those with *S. mutans* or *S. sobrinus* alone[9-12].

The genetic diversity of *S. mutans* has been studied in some details. *S. mutans* has significant genetic diversity at a clonal level, even within the same mouth[5,13-15]. Caries-active subjects tend to carry more genotypes of *S. mutans* and multiple strains with stronger cariogenic ability, compared with caries-free subjects[16-21]. Although *S. sobrinus* is considered to be the most acido-

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genic bacteria among oral streptococci[5,22,23] and there is evidence that caries increment in children with both *S. mutans* and *S. sobrinus* is higher than in those with *S. mutans* alone[7,11,24,25], very little is known about the genotypic diversity of *S. sobrinus* and the cariogenic virulence traits of different genotypes.

The aim of the present study was to investigate the relationship between clonal diversity and some virulence traits of *S. sobrinus* isolated from caries-free children and children suffering severe early childhood caries (SECC), including acidogenicity, aciduricity, sucrose-dependent adherence on glass surfaces and extracellular polysaccharide synthesis ability.

Materials and methods

Subjects

A total of 178 children aged from 42 to 54 months were recruited from 14 urban kindergartens located within 10 kilometers from the Hospital of Stomatology, Peking University, Beijing, China. Eighty-seven children who had more than 5 decayed teeth were grouped into the experimental group (SECC group) and 91 caries-free children into the control group. Informed consents were obtained from their parents. Their general information of systemic medical histories was obtained from their parents and nurseries. All the children had no chronic diseases, and had not received any antibiotic therapy or fluoride therapy 2 weeks before saliva collection. The ethical approval of this study was obtained from the Human Research Ethics Committee of Peking University Medical Science Centre (IRB00001052-5132).

Strains of S. sobrinus

Stimulated whole saliva was collected when the children were asked to chew 1 g paraffin (Orion Diagnostica,) and spit saliva continously into a 15 ml sterilized centrifuge tube for five minutes and diluted in sterilized PBS. The aliquots were inoculated on Trypticase Yeast-Extract Cystine Sucrose Bacitracin (LAB) agar for *S. sobrinus* isolation. The DNA extraction of isolates was identified as *S. sobrinus* by polymerase chain reaction (PCR) with primers for gtf I as described previously[26], and genotyped by arbitrarily primed PCR (AP-PCR) with the random primer OPA-13 (5'-CAG CAC CCA C-3'). One strain – representative of each genotype identified from each group – was selected for virulence traits. All clinical isolates were stored in BHI (brain heart infusion) with 50 % glycerol at -70° C for further analysis.

Virulence traits analysis

S. mutans UA159 and *S. sobrinus* OMZ176 were used as reference strains. *S. sobrinus* strains and reference strains were inoculated onto BHI agar plates, and incubated at 37°C in mixing air containing 5% CO_2 for 3 days. After incubation, *S. sobrinus* strains were inoculated into 1 ml BHI broth for 1 day, and a 100 μl aliquot of each culture was inoculated into 100 ml fresh BHI broth for 20 hours. Cells were harvested by centrifugation, washed three times with sterile saline, and re-suspended in sterile saline at OD_{570nm} = 0.8-1.0 using a UV Spectrophotometer (ELx 808, BioTek). In virulence traits analysis, each strain was tested three times by using separately grown cultures. The experimental data of each culture were recorded and all values were averaged.

Acidogenicity and aciduricity

Aliquots of 100 μl of 20 hours cultures were inoculated into 1 ml BHI broth with a final concentration of 5% (w/v) glucose at pH 3.5, 4.0, 4.5, 5.0, 5.5, 6.0, 6.5 and 7.0, and grown at the same conditions for 48 hours. Cultures were then centrifuged at 4°C (3,000 rpm) for 15min and the supernatant was collected for final pH measurement with a standardized pH meter (Model HI3111, Hanna). The pellets were washed once with sterile saline, re-suspended with 1ml sterile saline, and measured at OD_{570nm}. The readings were defined as aciduricity of the strains. Sterile saline was used as a control.

Extracellular polysaccharide synthesis

Aliquots of 100 μl of 20 hours cultures were transferred to 1ml fresh BHI broth with 1.0% sucrose and grew at the same conditions for 20 hours. Cultures were then centrifuged at 4°C (3,000 rpm) for 15 min and the supernatant was collected for the measurement of water-soluble glucan (WSG). The pellet was washed twice with phosphate buffered saline (PBS, pH = 8.0) and supernatants were combined for WSG extraction. Then the pellet was dissolved in 0.5N NaOH for 30 min at room temperature and used for water-insoluble glucan (WIG) extraction. The NaOH incubation was repeated twice and the supernatants were collected by centrifugation and combined with previous portions for WIG extraction. Three volumes of ethanol were added to culture supernatants and stored at 4°C overnight to precipitate the polysaccharides. The precipitation was collected by centrifugation and dissolved in the same volume of deionized water for WSG and 0.5N NaOH for WIG. Total amounts of WSG

and WIG were measured by the phenol sulphuric acid method[27] and expressed as glucose equivalent (μg/ml).

Adherence analysis

Sucrose-dependent adherence of growing cells of *S. obrinus* was determined according to previous published procedures[28,29] with slight modification. Aliquots of 100 μl of 20 hours cultures were transferred to 1 ml fresh BHI broth containing 1% sucrose and grew under the same conditions at a 30-degree angle for 20 hours in a glass test tube (10 by 75 mm). After incubation, the tubes were vigorously mixed with a vortex mixer for 1 min, then detached cells were transferred to a second tube. One-millilitre sterile saline were added to the first tube and agitated for 1 min: the released cells were transferred to a third tube. All test tubes were vortexed, then serially diluted, plated on BHI plates, and incubated at 37°C for 2 days before colonies were counted. The percentage adherence was determined by dividing the colony-forming unit (CFU) of adherent cells by the total counts of CFU.

Data analysis

Software SPSS 13.0 was used to analyse the data in the present study. The relationship between clonal diversity and caries index was tested by Spearman's rank correlation analysis. To analyse the acidogenicity, aciduricity, percentage of adherent cells, and extracellular polysaccharide synthesis of *S. sobrinus* strains, the data distribution was analysed first for continuous data. If the data showed a normal distribution, the student *t* test was used. Otherwise, the Mann-Whitney U nonparametric test was used. In the SECC group, associations between extracellular polysaccharide synthesis and adherence were tested by Pearson rank correlation analysis. Statistical significance was defined as $P \leq 0.05$.

Results

Identification and genotyping

Merging the two groups, the frequency of *S. sobrinus* detection was 10.67% (19/178) in children aged from 42 to 54 months in our study. The frequency of *S. sobrinus* detection was 18.39% (16/87) in SECC children, which was significantly higher than 3.30% (3/91) in caries-free children (chi-square test, $P < 0.01$).

Fifty-three *S. sobrinus* strains were identified by PCR with primers for gtf I and classified into 23 distinct genotypes by AP-PCR (Table 1). In the SECC group, *S. sobrinus* were detected with one to three different genotypes in each saliva sample. Only one genotype was detected in each caries-free child. One genotype (H) was shared by three children: C60, C61 and C15.

Since only three genotypes of *S. sobrinus* were detected in 91 caries-free children, the differences of cariogenic virulence traits between the caries-free group and the SECC group were not analysed statistically. The values of the same genotype H were averaged and analysed as one strain. The cariogenic virulence traits of *S. sobrinus* from SECC children were compared with reference strains *S. mutans* UA159 and *S. sobrinus* OMZ176.

Table 1 The number of genotypes of *S. sobrinus* among 19 subjects

Subject	Isolates (n = 53)	Genotypes (n = 23)
SECC group		
C2	2	2 (A,B)
C15	1	1 (H1*)
C16	2	1 (C)
C19	2	2 (D,E)
C20	3	1 (F)
C23	1	1 (G)
C30	3	1 (I)
C33	4	3 (J,K,L)
C36	2	2 (M,N)
C39	7	2 (O, P)
C49	3	1 (Q)
C57	7	1 (R)
C60	1	1 (H2*)
C61	2	1 (H3*)
C62	6	1 (S)
C74	3	1 (T)
Caries-free group		
F83	2	1 (U)
F111	1	1 (V)
F116	1	1 (W)

*: The genotype H shared in three children C15, C60 and C61.

Table 2 Final pH values of *S. sobrinus* isolated from SECC group and caries-free group at different initial pH values ($\bar{\chi} \pm s$).

Initial pH	Caries-free group (n = 3)	SECC group (n = 20)	OMZ176	P value	UA159	P value
7.0	4.07 ± 0.08	4.07 ± 0.06	4.00 ± 0.01	0.00*	4.06 ± 0.01	0.27Φ
6.5	4.04 ± 0.07	4.04 ± 0.06	4.01 ± 0.02	0.04*	4.04 ± 0.01	1.00*
6.0	4.02 ± 0.06	4.01 ± 0.06	4.04 ± 0.01	0.13*	4.03 ± 0.01	0.39*
5.5	4.00 ± 0.03	4.00 ± 0.07	4.07 ± 0.01	0.00*	4.06 ± 0.00	0.00*
5.0	4.02 ± 0.05	4.05 ± 0.18	4.06 ± 0.03	0.91Φ	4.10 ± 0.03	0.29Φ
4.5	4.25 ± 0.16	4.07 ± 0.21	4.09 ± 0.00	0.78*	4.10 ± 0.01	0.62*
4.0	3.93 ± 0.07	3.94 ± 0.07	4.00 ± 0.00	0.00Φ	4.05 ± 0.00	0.00*
3.5	3.48 ± 0.03	3.49 ± 0.02	3.50 ± 0.00	0.02Φ	3.57 ± 0.01	0.00*

*Student T-test

ΦMann-Whitney U test

Table 3 Bacterial growth (OD_{570nm} of *S. sobrinus* isolated from SECC group and caries-free group at different initial pH values ($\bar{\chi} \pm s$).

Initial pH	Caries-free group (n = 3)	SECC group (n = 20)	OMZ176	P value*	UA159	P value*
7.0	0.82 ± 0.06	0.87 ± 0.11	0.76 ± 0.03	0.00	0.81 ± 0.11	0.02
6.5	0.86 ± 0.10	0.86 ± 0.06	0.76 ± 0.02	0.00	0.71 ± 0.07	0.00
6.0	0.75 ± 0.01	0.76 ± 0.06	0.75 ± 0.02	0.00	0.70 ± 0.02	0.00
5.5	0.69 ± 0.04	0.71 ± 0.07	0.65 ± 0.04	0.00	0.57 ± 0.05	0.00
5.0	0.51 ± 0.11	0.58 ± 0.08	0.54 ± 0.06	0.02	0.54 ± 0.02	0.02
4.5	0.38 ± 0.09	0.42 ± 0.08	0.37 ± 0.10	0.01	0.38 ± 0.06	0.00
4.0	0.30 ± 0.09	0.27 ± 0.06	0.34 ± 0.02	0.00	0.33 ± 0.01	0.00
3.5	0.24 ± 0.04	0.23 ± 0.04	0.30 ± 0.01	0.00	0.30 ± 0.01	0.00

*Student T-test

Acidogenicity and aciduricity

The final pH values of the caries-free group and the SECC group were similar from initial pH 3.5 to 7.0 (Table 2). Compared to reference strains OMZ176 and UA159, the final pH values of the SECC group at initial pH 6.5 and 7.0 were significantly higher than those of OMZ176 ($P < 0.01$), but similar to those of UA159 ($P > 0.05$). From initial pH 3.5 to 6.0, the final pH values were lower than those of OMZ176 and UA159, though not all the differences were statistically significant.

For the aciduricity measurement (Table 3), there was no bacterial growth at initial pH 3.5 to 4.0, and the OD_{570nm} were similar to the baseline. From initial pH 4.5 to 7.0, the bacterial growth of the SECC group was significantly higher than that of the reference strains OMZ176 and UA159 ($P < 0.05$).

Extracellular polysaccharide synthesis and adherence to glass surfaces

S. sobrinus clinical isolates were able to synthesise extracellular WSG and WIG using sucrose as the substrate (Table 4).

In the SECC group, the difference of WIG synthesis ranged from 34.90 μg/ml to 125.92 μg/ml (mean

Table 4 WIG synthesis, WSG synthesis, percentages of cells adhering to glass surfaces in the presence of sucrose of *S. sobrinus* isolated from SECC group and caries-free group ($\overline{\chi} \pm s$)

Virulence factors	Group of children	
	SECC group (n=20)	Caries-free group (n=3)
% of adherence	60.15 ± 23.71	74.32 ± 26.10
WIG synthesis μg/ml	64.48* ± 22.06	65.67 ± 14.98
WSG synthesis μg/ml	34.57* ± 12.22	37.93 ± 19.00

*Paired sample t test, t = 5.01, $P < 0.01$.

64.48 ± 22.06 μg/ml) and the difference of WSG synthesis ranged from 17.44 μg/ml to 67.46 μg/ml (mean 34.57 ± 12.22). The mean value of the WIG synthesis was significantly higher than that of the WSG synthesis (paired sample t test; $P < 0.01$). There was a close correlation between the WIG synthesis and percentages of growing cells adhering to glass surfaces in the presence of sucrose in the genotypes isolated from the SECC children (Pearson; r = 0.50; $P = 0.02$). But the level of WSG synthesis was not correlated to the percentages of growing cells adhering to glass surfaces in the SECC subjects (Pearson; r = 0.02; $P = 0.93$).

Discussion

Generally, it is said that dissimilarities of subjects and techniques may have contributed to the difference in findings. The culture technique is the best way to obtain the bacterial colonies in order to detect their genotypic diversity and virulence traits. In the present study, the prevalence of *S. sobrinus* in 42- to 54-month-old children was 10.67%, 18.39% in SECC children and 3.30% in caries-free children respectively, which was similar with previous studies that examined preschool children using culture technique[6,30-32], and lower than those studies using the PCR detection technique[11,33]. It was suggested the presence of *S. sobrinus* was a risk factor for high caries activity in severe early childhood caries.

In the present study, one to three different genotypes of *S. sobrinus* were detected in each SECC child, while only one genotype was detected in each caries-free child. This suggested that SECC children harbored more genotypes of *S. sobrinus* than caries-free children, which was similar with earlier studies[18,34-36]. Though many studies have reported the relationship between clonal diversity and caries activity of *S. mutans*, very little is known about the genotypic diversity and cariogenic virulence traits of *S. sobrinus*.

Acidogenicity is one of the major cariogenic virulence factors of the mutans streptococcus (MS) group. In particular, acid production at low pH is thought to be an important ecological determinant in dental caries[23]. However, Köhler et al[37], Mattos-Graner et al[16] and Napimoga et al[17] reported that no correlation between acid production of MS and caries status was found when they compared MS acid production both at pH 5.5 or neutral pH. In the present study, the final pH values of the two groups were found to be similar from the initial pH level 3.5 to 7.0, though only three *S. sobrinus* isolates were identified from caries-free subjects, which was too small to be analysed statistically. At a lower pH level of 3.5 to 6.0, the acid production of *S. sobrinus* isolates in the SECC group was higher than that of the reference strains OMZ176 and UA159. Aciduricity is another cariogenic virulence factor of MS group. The high acid tolerance meant that the bacteria can grow and carry out glycolysis at a low pH value, and can drive the environment to an even lower pH value[5,22,23]. In this study, all the *S. sobrinus* isolates survived at pH levels as low as 4.5 and were capable of producing acid in this acidic condition, which confirmed that *S. sobrinus* isolates had a strong acidogenicity and aciduricity at low pH.

S. sobrinus clinical isolates in the present study were able to synthesise extracellular WSG and WIG using sucrose as the substrate. WSG serves as an energy store to extend acid production when the carbohydrate source is limited. WIG is the major matrix component that promotes dental plaque formation, which in turn enhances MS accumulation on tooth surfaces. In our study, a significant positive association was observed between the level of WIG synthesis and the proportion of adherent cells in the presence of sucrose in SECC subjects, but not the level of WSG synthesis. Similar effects of WSG and WIG in caries-free children could not be analysed because of the limitation of the amount

of strains. These findings were similar with earlier reports about *S. mutans* clinical isolates from caries-active and caries-free subjects[17,19,20]. This suggested that WIG would be the major matrix enhancing bacteria accumulation on tooth surfaces, and the isolates from subjects with high caries activity were more capable of colonising and accumulating on teeth surface and consequently induced caries.

In the present study, the number *S. Sobrinus* strains for the caries free children (n = 4) is too low compared to the SECC children (n = 49), even though an almost equal number of SECC children (n = 87) and caries children (n = 91) were recruited. It is suggested that more than 1,000 caries free children might be suitable in order to get matched *S. Sobrinus* strains for the caries free children.

In the present study, one genotype of *S. sobrinus* was shared by three SECC children: C60, C61 and C15. There might be potential horizontal transmission between C60 and C61 for they were from the same kindergarten. Since C15 was not found to be related to C60 and C61, C15 sharing one genotype of *S. sobrinus* with C60 and C61 could be considered as an overlap phenomenon of the *S. sobrinus* genotype in individuals. Kozai[38] reported similar phenomenon.

This study revealed that genetic polymorphism of *S. sobrinus* was related to caries susceptibility. WIG played an important role in the adherence and accumulation of *S. sobrinus* on tooth surfaces in SECC children.

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