Prevalence of Five Biofilm-Related Oral *Streptococci* Species from Plaque

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Objective: *To examine the prevalence of five oral streptococci species of severe early childhood caries (S-ECC) and caries-free (CF) groups.* ***Study design:*** *Supra gingival plaque samples were obtained from 198 Thai children with ages ranging from one to six years old. Eighty-seven subjects had no caries (dmft=0), and 111 had S-ECC. After DNA extraction,* S. mutans, S. sobrinus, S. sanguinis, S. oralis*, and* S. gordonii *were identified by standard PCR using species-specific primers. Statistical analysis determined the differences among prevalence rates of each species using Pearson chi-square test. The relationship among dmft score, age, sex and caries status within each group was analyzed by logistical regression ($p \leq 0.05$).* ***Results:*** *Sex was not correlated with any of the species detected in both groups (mean age =3.09, mean±SD of dmft=11.04±7.89).* S. mutans *was found at greatest prevalence in both groups followed by* S. oralis. S. gordonii *was detected at a high prevalence, but* S. sobrinus *and* S. sanguinis *were lower in S-ECC when compared with those from the CF group.* ***Conclusion:*** *S. mutans was associated significantly with S-ECC ($p \leq 0.05$). Caries prevalence was highest (56.5%) in subjects infected by* S. mutans *alone.* S. sanguinis *prevalence was higher in the CF group, but not statiscally different. Infection with MS did not show higher caries prevalence.*

Keywords: *DNA, PCR, oral streptococci, plaque, mutans streptococci*
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INTRODUCTION

Severe early childhood caries (S-ECC) has been a leading chronic disease in the world including Thailand.[1,2] It results not only in local pain and health problems but also reduces general growth and development, loss of self-esteem and might lead to psychological problems.[3,4] Children who experience early childhood caries are at higher risk for developing new carious lesions in both primary and permanent dentitions when compared to children without disease. In the oral cavity, more than 800 species of microorganisms live in a complex community called biofilm or dental plaque.[5] During caries development, bacteria play a major role in destroying tooth structures. The development of ECC usually starts from the labial aspects of the upper maxillary incisors and then spreads quickly. Heavy plaque accumulation is an important sign of this type of caries.[6] *Mutans streptococci* or MS (*Streptococcus mutans* and *Streptococcus sobrinus*) have been the main focus in caries research for more than a decade.[7,8] They are commonly isolated from plaque and saliva taken from subjects with or without dental caries.[9-17] The rise of a wide variety of modern molecular techniques provides an in depth evaluation and new discoveries especially in microbiology have been reported continuously over the past ten years.[18-22] Recent studies have demonstrated that *S. mutans*, *Candida albicans*, and *lactobacilli* species were isolated more frequently from ECC infected dentin while *Streptococcus oralis*, *Streptococcus sanguinis* and *Streptococcus gordonii* were found at a higher prevalence from caries-free children.[21] Interestingly, MS are encountered less frequently at the advancing front of dentin caries, whereas *lactobacilli*, *prevotellae* and *bifidobacterium* are more prevalent.[21,22] On the other hand, numerous studies have found high amounts of *S. mutans* in caries-free (CF) children.[21-25] It is more evident that the counteractions between these bacteria might modulate the outcome of clinically present cavities. An interspecies relationship of oral microorganisms in dental plaque has also become more evident. The competition between interspecies involves several mechanisms including the excretion of

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inhibiting substances against competitors. In the early phase of biofilm formation, oral streptococci are the pioneer bacteria to colonize tooth surfaces.[5] *S. gordonii, S. sanguinis, S. oralis* and *S. mitis* biovar 1 are the main early colonizers.[5] Recent reports have suggested an association between *S. sanguinis* and healthy oral conditions.[25] Several studies have pointed out that it antagonizes *S. mutans*.[26] The earlier colonization of *S. sanguinis* might affect or interrupt the colonization of *S. mutans*.[26,27] Most oral streptococci possess glucosyltransferase (GTF) enzymes that are considered to be an obligatory factor in biofilm formation on tooth surfaces.[13] Since both commensal and cariogenic streptococci have GTF enzymes, the proposed detection would be based upon these genes to determine the bacterial composition of dental plaque. Studies that have analyzed samples from Thai children, either from plaque or saliva, were by means of culture-based techniques excluding not-yet-cultivated species. Although those studies demonstrated an association between MS and childhood caries, other oral streptococci species and their relationship have not yet been reported. Molecular methods for bacterial identification and enumeration now are performed routinely to obtain more precise results. PCR is suitable for the specific detection and identification of this bacteria.[13,24]

The purpose of this study was to investigate the prevalence of five oral streptococci species between S-ECC and CF subjects. The hypothesis to be tested was that the prevalence of *S. oralis, S. sanguinis* and *S. gordonii* from S-ECC plaque is lower than from CF plaque, but the prevalence of *S. mutans* and *S. sobrinus* is higher. In addition, the study analyzed the association between these bacteria and caries status, age and sex of subjects within each group.

MATERIAL AND METHOD

The study protocol was approved by the Human Institutional Review Board of Mahidol University (MU-IRB 2009/287.2611). Subjects were selected from patients who came for dental treatment at the Pediatric Dental Clinic, Faculty of Dentistry, Mahidol University, Bangkok, Thailand. Consent was obtained from parents of all subjects. A total of 198 children were selected. Eighty-seven of these subjects were CF having no caries or existing restorations (dmft=0). Additional bite wing radiographs were obtained when posterior contacts were tight. One hundred eleven were diagnosed having S-ECC as defined by the American Academy of Pediatric Dentistry,[28] as the presence of one or more decayed (noncavitated or cavitated lesions), missing (due to caries), or filled tooth surfaces in any primary tooth in a child 71 months of age or younger. In children younger than three years of age, any sign of smooth-surface caries is indicative of S-ECC. From ages three through five, one or more cavitated, missing (due to caries), or filled smooth surfaces in primary maxillary anterior teeth or a decayed, missing, or filled score of ≥4 (age 3), ≥5 (age 4) or ≥6 (age 5) surfaces constitutes SECC. Scores for decayed, missing, and filled teeth were recorded. Plaque samples were collected at the first time of their visits. Subject selection and sample collection were conducted by three pediatric dentists who already calibrated and obtained consensus based on the given guidelines (kappa =0.92). Subjects who had professional prophylaxis and fluoride application within three months or were taking any kind of antibiotics were excluded.

Pooled plaque samples were collected by sterile gracey curette starting from buccogingival surfaces and the accessible proximal surfaces of the molars and canines. The collected plaque samples were released from the curette by agitation in 1.0 ml of TE buffer. All sample solutions were calibrated in an opaque McFarland tube containing a cell concentration equal to 10^8 and immediately transported in ice to the Oral Biology Laboratory, Faculty of Dentistry, Mahidol University and stored at -20ºC until the extraction process.

The reference strains used in this study were *S. mutans* (GS5), *S. sobrinus* (ATCC 33478), *S. oralis* (ATCC 10557), and *S. gordonii* (ATCC10558). The strains were purchased from the American Type Culture Collection. These organisms were routinely cultured in brain heart infusion broth (BHI, Difco Laboratories, Detroit, MI) and Mitis-Salivarius (MS) agar (Difco).

DNA Extraction

Extraction was based on the enzymatic reaction as recommended by the manufacturers using commercial kit (NucleoSpin Genomic Purification Kit, McCheary-Nagel, Germany) with an additional step for gram-positive bacteria lysis.[18] In brief, pellet cells were resuspended in 180 µl of T1. Then 10 µl mixture solution of 2 µl mutanolysin (Sigma Aldrich, USA) was added in10 µl lysozyme (10 mg/ml) in all samples and incubated at 56ºC for three hours. Next, 200 µl B3 buffer was added and further incubated at 70ºC for ten minutes. After, 210 µl ethanol was added in a spin column and centrifuged at 11,000x rpm or the maximum speed of the centrifuge machine. Then 500 µl of BW buffer was added and centrifuged. Next, 600 µl B5 buffer was added and centrifuged. After, 50 µl BE buffer (pre-incubated at 70ºC for ten minutes) was added and incubated at room temperature for one minute before final centrifuge.

Nested and standard PCR

The sequences of all primers used in this study are listed in Table 1. Each PCR mixture (25 µl) consisted of 10 mM Tris-HCl buffer (pH 8.3), 1.5 mM MgCl2, 50 mM KCl, 200 µM each of dNTP, 1 µM oligonucleotide primers and 2 units of *Taq* DNA Polymerase (*iTaq* DNA Polymerase with dNTP, Geneaid Biotech Ltd, Taiwan). For universal primers and nested PCR for *S. mutans*, thermal cycler (GeneAmp PCR System 9600 PCR Machine, PerkinElmer, CA, USA) was set for 30 cycles; each cycle consisted of a denaturation step at 95ºC for 15 minutes, extra denaturation at 95ºC for 15 minutes, annealing at 51ºC for one minute and extension at 72ºC for two minutes, with final extension at 72ºC for ten minutes.[23] For *S. sanguinis,* the sequence consisted of 30 cycles. Each cycle consisted of a denaturation strep at 98ºC for 30 seconds, extra denaturation at 98ºC for ten second,

Table 1. Primers used in this study

Primer name		Nucleotide sequence (5' to 3')	Amplicon (bp)	Annealing Temp (C)	Ref.
Universal 16SrRNA	F	5'-AGAGTTTGATCMTGG CTCAG-3'	1505	51	Sato *et al*, 2003 [23]
	R	5'-TACGGYTACCTTGTTACGACTT-3'			
S. mutans (nestedPCR)	Sm1F	5'-GGTCAGGAAAGTCTGGAGTAAAAGGCTT-3'	282	51	Sato *et al*, 2003 [23]
	Sm2R	5'-GCG GTA GCT CCG GCA CTA AGC C-3'			
S. sobrinus (nestedPCR)	SobF	5'-CGCACTTGCTCCAGTGTTACTAA-3'	546	51	Sato *et al* , 2003 [23]
	SobR	5'-GCC TTT AAC TTC AGA CTT AC-3'			
S. sanguinis gtfP	MKP-F	5'-GGATAGTGGCTCAGGGCAGCCAGTT-3'	313	69	Hoshino *et al*, 2004 [30]
	MKP-R	5'-GAACAGTTGCTGGAC TTGCTTGTC-3'			
S. oralis gtfR	MKR-F	5'-TCCCGGTCAGCAAACTCCAGCC-3'	374	66	Oho *et al*, 2000 [29]
	MKR-R	5'-GCAACCTTTGGATTTGCAAC-3'			
S. gordonii gtfG	MKG-F	5'-CTATGCGGATGATGCTAATCAAGTG-3'	440	70	Oho *et al*, 2000 , [29]
	MKG-R	5'-GGAGTCGCTATAATCTTGTCAGAAA-3'			

Table 2. The prevalence of five oral streptococci species in three age groups in caries-free and caries-active groups

Organisms	CF (%)	S-ECC (%)
S. oralis	1-3.11= 29.72 4-5.11= 44.45 ≥6 =40	1-3.11= 31.57 4-5.11= 41.07 ≥6 = 66.67
S. gordonii	1-3.11= 10.81 4-5.11= 13.34 ≥6 = 0	1-3.11= 0 4-5.11= 5.35 ≥6 = 41.66
S. sanguinis	1-3.11= 25 4-5.11= 14.89 ≥6 = 10	1-3.11= 5.26 4-5.11= 10.17 ≥6 = 27.77
S. mutans	1-3.11=38.9 4-5.11=37 ≥6 =52.2	1-3.11=60 4-5.11=57.1 ≥6 =52.2
S. sobrinus	1-3.11=5.6 4-5.11=7.4 ≥6 =20	1-3.11=10 4-5.11=7.1 ≥6 =17.4

Age group 1-3.11-year-old children
4-5.11-year-old children
≥ 6-year-old children

Table 3. Caries prevalence in children infected with *S. mutans* alone, *S. sobrinus* alone, *S. sanguinis* alone, *S. mutans*+ *S. sobrinus*, and *S. mutans* + *S. sanguinis*

	SECC subjects n (% of all caries-active)	CF subjects	Total	Caries prevalence (%)
Positive for *S. mutans*	26(55.3•)	34	60	43.3
Positive for *S. sobrinus*	6 (12.8)	15	21	29.6
Positive for *S. sanguinis*	7(14.9)	18	25	28
Positive for *S. mutans* + *S. sobrinus*	6(12.8)	15	21	29.6
Positive for *S. mutans* + *S. sanguinis*	7(14.9)	18	25	28

• a Pearson Chi-square test showing significant differences in the prevalence of *S. mutans* and caries status *(p<0.005)* as detected by PCR between S-ECC group and CF group.
b Fisher's exact test. No statistically significant differences in the prevalence of *S. sobrinus* and caries status were found between S-ECC group and CF group.
c Fisher's exact test. No statistically significant differences in the prevalence of *S. sanguinis* and caries status were found between S-ECC group and CF group.

annealing at 69 °C for one minute and extension at 70°C for one minute, with final extension at 72°C for five minutes. *S. mutans* GS-5 and *S. sobrinus* 6715 DNA was used as the positive control and 5 µl of water as the negative control. Positive and negative controls were included in each PCR set and in all sample processes. A 100-bp DNA Ladder (100 bp DNA Ladder, Geneaid Biotech Ltd, Taiwan) was used as a molecular size marker. PCR products were checked by electrophoresis on 1.0% agarose gel (Broad Separation Range for DNA/RNA agarose, Fisher Scientific, UK) in 1XTris–borate EDTA buffer (100 mM Tris, 90 mM borate; 1 mM EDTA, pH 8.4). Gels were stained with ethidium bromide. Image results were captured with a digital imaging system (Molecular Imager ®Gel docTM Systems, Bio-Rad Laboratories Inc., CA, USA).

Statistical Analysis

All data were recorded and then transferred to SPSS 14.0 software (Microsoft Corporation, CA, USA) for analysis. Pearson Chi-square test for statistical analysis of the detec-

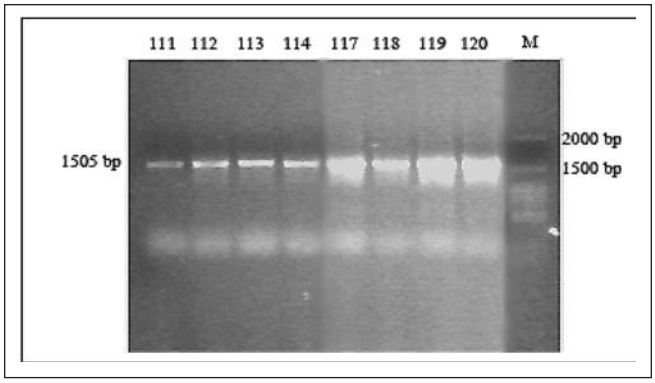

Figure 1. The PCR was amplified using 16SrRNA primers. All samples showed the same 1505-bp amplicons on 1% agarose gel.

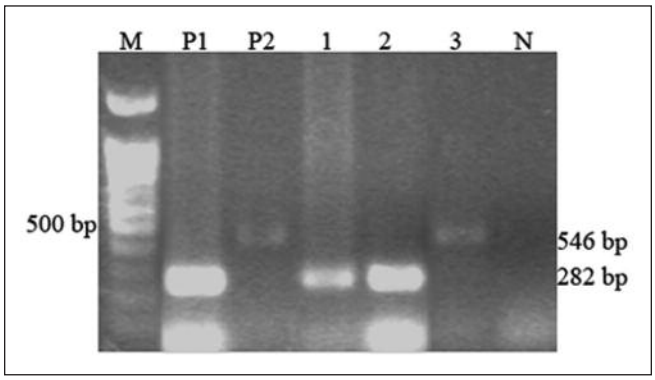

Figure 2. Detection of *S. mutans* and *S. sobrinus* in dental plaque samples by PCR. Lane M; 100 bp DNA marker; lanes P1, P2, and N are chromosomal DNA from *S. mutans* (GS-5), *S. sobrinus* (ATCC 33478), and negative control (distilled water), respectively; lanes 1, 2, and 3 show PCR products from plaque samples from different children.

tion rate between each species between two groups was used. The relationship between dmft score, age, sex and caries status within each group was analyzed by logistical regression. Values of $p \leq 0.05$ were accepted as a significantly different.

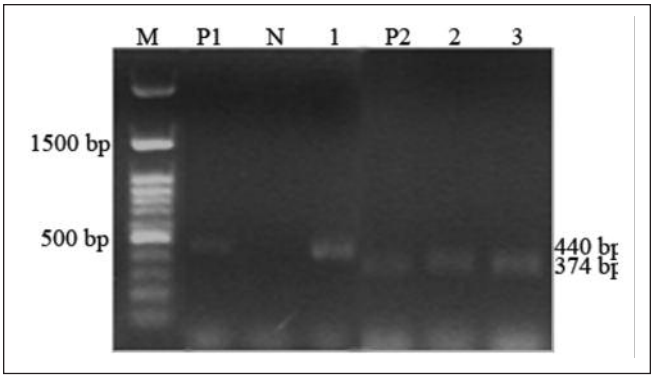

Figure 3: Detection of *S. gordonii* and *S. oralis* in plaque samples by PCR. Lane M; 100 bp DNA marker; lanes P1, N, and P2 are chromosomal DNA from *S. gordonii* (ATCC 10558), negative control (distilled water), and *S. oralis* (ATCC 10557), respectively; lanes 1, 2, and 3 show PCR products from plaque samples from different children in CF subjects.

RESULTS

Study population demographics and dental caries status: Mean age was 3.09 (range from 1.08 to 6.11 year). Mean±SD of dmft were 11.04±7.89. The mean ± SD of dmft of the study population were 11.04±7.89. Each PCR reaction was performed separately. Sex was not correlated significantly with any of the species detected in both groups. *S. mutans* was found highest in both groups followed by *S. oralis*. *S. gordonii* was detected more frequently in S-ECC but for *S. sobrinus* and *S. sanguinis* were lower when compared with that from the CF group. The ratio of *S. mutans*/*S. sobrinus* was higher in S-ECC than in CF (3.79: 1.86). The prevalence of *S. mutans* in the S-ECC group was significantly different from that of the CF group ($p \leq 0.05$).

DISCUSSION

We obtained similar associations between *S. mutans* and caries, when compared with previous studies.[9,10,12] The prevalence rates of *S. mutans* and *S. sobrinus*, found in this study

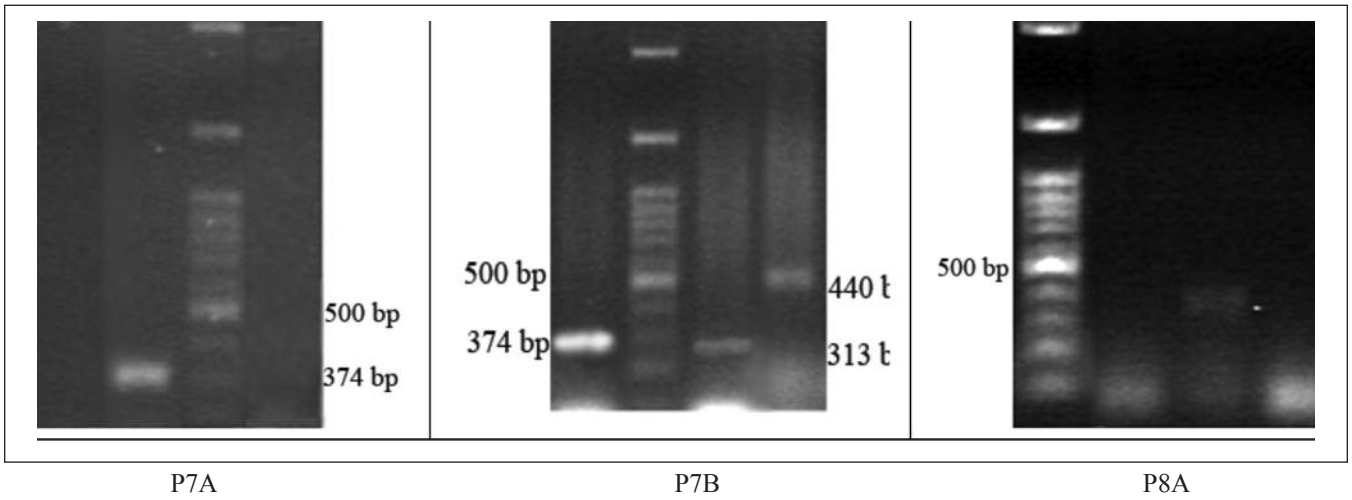

Figure 4: Detection of three oral *streptococci* species in plaque samples from S-ECC subjects. The PCR was amplified using *S. sanguinis* MKP-specific primers, *S. oralis* MKR-specific primers, and *S. gordonii* MKG-specific primers, representing 313-bp, 374-bp, and 440-bp amplicons, respectively. Lane M; 100 bp DNA marker.

showed similar results to those previously reported. Noticeably, most of the studies with subjects residing in tropical areas produced similar results.[9,10,12] However, it was quite different from those of Okada *et al* [11] In that study, the prevalence of *S. mutans* and *S. sobrinus* in three- to-five-year-old Japanese preschool children was quite high. However, a majority of studies compared the difference between prevalence of MS and ethnicity suggesting that there was no correlation between ethnicity and prevalence of MS and lactobacillus species. Since most studies were conducted using samples obtained from immigrants or minorities, diet and/or residence areas where subjects permanently lived in might have affected the microbiota prevalence more than the ethnicity. Moreover, when subjects were infected by both *S. mutans* and *S. sobrinus,* the caries prevalence was the same as subjects infected by *S. sobrinus* alone. There might be a high possibility that *S. sobrinus* was not found without the presence of MS, and this situation was observed in *S. sanguinis* as well. Some previous reports mentioned that caries prevalence was higher when subjects were infected by MS.[11,14] However, consensus has not been reached on the results of this issue. The total amount of *S. sanguinis* was found at a higher prevalence than *S. sobrinus* in both groups, and was detected highest in the CF group aged one-to-three-years and eleven month old.

S. sanguinis colonizes the oral cavity of healthy subjects at a higher rate when compared with that from S-ECC. Although the result was not statistically powered to test for the correlation, possibly from the small sample size, it revealed similar conclusion to previously reports.[25] Few studies suggested that *S. sanguinis* might be transmitted horizontally like *S. mutans*. Further studies are suggested to provide more information on this issue. When compared among age groups, this study revealed that when the subject age is increased, *S. sanguinis* prevalence also increased in S-ECC but decreased in CF subjects. The possible reasons for an increasing rate in S-ECC are that *S. sanguinis* can survive in a wide range of pH even in low pH as found in subjects with caries. The second reason is that some strains of *S. sanguinis* are capable of producing acid rapidly. Although it might be a prominent species that colonizes early on tooth surfaces, it may prepare an environment that is suitable for the overgrowth of more aciduric species such as MS. On the other hand, in subjects without caries, *S. sanguinis* decreased, which might be from an inhibition or replacement by other bacteria which are non cariogenic bacteria.[26] For *S. gordonii* and *S. oralis,* higher total prevalence rates were obtained S-ECC

Several reports showed that some strains of *S. mitis* biovar 1 and *S. oralis* may play an important role in caries development by modifying the environment in dental plaque to become favorable for the succession of aciduric species. Further studies are needed to find an association between plaque index and *S. gordonii* prevalence clinically. From *in vitro* studies, several articles reported that *S. gordonii* is one of the early colonizers in plaque formation. It competes with *S. sanguinis* and *S. mutans* for oxygen. In dual bacteria biofilm formation, *S. mutans* was inhibited and even disappeared in cultures when co-cultured with *S. oralis*.[26] The real biofilm community is certainly more complicated than the laboratory setting.

CONCLUSION

This study demonstrated that in Thai children, sex and age are not associated with the detection rate of any of the streptococci mentioned in both groups. Only the prevalence of *S. mutan*s in the S-ECC group was significantly higher than in the CF group. The carious lesions and nondisruption from any methods of plaque controls might provide nondisturbing reservoir sites for the biofilm involved with streptococci species such as *S. gordonii* and *S. oralis*. The amount of *S. mutan*s alone was not indicative of caries incidence. These findings have suggested that an association between *S. mutan*s, *S. sobrinus* and *S. sanguinis* might affect caries outcomes more than previously thought. Further studies are required on this issue.

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