DOI: 10.1111/j.1365-263X.2010.01047.x

INTERNATIONAL JOURNAL OF PAEDIATRIC DENTISTRY

Detection of oral streptococci with collagen-binding properties in saliva specimens from mothers and their children

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International Journal of Paediatric Dentistry 2010; 20: 254–260

Background. Approximately 10–20% of *Streptococcus mutans* strains have been reported to possess collagen-binding properties, whereas other species in the oral cavity with those properties remain to be elucidated.

Aim. To identify strains with collagen-binding properties and analyse their characteristics in comparison with *S. mutans*.

Design. A total of 110 expectorated saliva specimens were collected from 55 pairs of mothers and their children. Bacterial strains with collagen-binding properties were isolated and the species specified. In addition, strains with collagen-binding properties isolated from mother–child pairs were analysed using molecular biological approaches.

Results. The detection frequency of strains with collagen-binding properties was shown to be 40.9%, among which *S. salivarius* was the most frequently detected, followed by *S. mutans*. The collagen-binding activity of the *S. mutans* group was the highest, followed by *S. salivarius*. In addition, *S. mutans* and *S. salivarius* strains from 3 and 1 mother–child pairs, respectively, were shown to be the same clones.

Conclusions. Our results indicate that *S. mutans* and *S. salivarius* are major species with collagen-binding properties in the oral cavity, and that strains with such properties may be related to mother–child transmission.

Introduction

It has been estimated that about 500 bacterial species inhabit the human oral cavity, among which *Streptococcus mutans* is one of the most well known, as it is a pathogen related to dental caries[1,2]. Oral streptococci including *S. mutans* are considered to be major causative agents of bacteraemia and infective endocarditis[3]. Recent studies that analysed bacteraemia in humans have shown that not only invasive dental procedures, such as tooth extraction, but also conservative and preventive procedures, and even tooth brushing can cause bacteraemia[4–6]. In general, bacteraemia is considered to be transient in healthy individuals, whereas infective endocarditis can be induced in subjects with various types of cardiac disorders[7].

Bacterial binding to extracellular matrix proteins, such as collagen, fibronectin, and laminin, is considered to be an essential step in the pathogenesis of infective endocarditis[8]. A nationwide survey of infective endocarditis cases in Japan showed that the most common microorganism was Gram-positive cocci and the oral streptococci involved were major pathogenic species[9]. Thus, streptococcal species with collagen-binding properties in the oral cavity should be regarded as highly virulent strains for the onset of infective endocarditis. In our previous studies, approximately 10–20% of *S. mutans* strains were shown to possess collagen-binding properties due to the presence of the *cnm* gene encoding the 120-kDa collagen-binding adhesin (Cnm)[10–12]. However, except for *S. mutans*,

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the entire range of species with collagen-binding properties in the oral cavity remains to be elucidated. The purpose of the present study was to identify oral streptococcal strains with collagen-binding properties in saliva specimens obtained from mothers and their children. In addition, the features of the various strains for each species were investigated to consider the possibility of mother to child transmission.

Materials and methods

Subjects and clinical specimens

The protocols of the present study were approved by the Ethics Committee of Osaka University Graduate School of Dentistry. Prior to collection of the specimens, the subjects were informed of the contents of the study and gave approval for their participation. The subjects were mothers and their children who participated in oral hygiene instruction sessions held at Osaka University Dental Hospital from August 2008 to May 2009. A total of 110 expectorated saliva specimens were collected from 55 mother–child pairs (23 boys, 32 girls) (age ranges: mothers, 23–47 years; children, 3–11 years). Saliva specimens were obtained prior to giving detailed tooth brushing instructions, which enabled us to determine the natural bacterial profiles of each subject, as described previously[13]. Briefly, following mouth washing with water, non-stimulated expectorated whole saliva was collected from each subject in a sterile plastic tube and immediately placed on ice. The saliva specimens were then immediately transported to our laboratory to perform the following procedures.

Isolation of strains with collagen-binding properties from saliva specimens

Identification of the strains with collagen-binding properties in the saliva specimens was carried out using a method described previously[12], with some modifications. Briefly, type I collagen in 0.25 M acetic acid (Sigma-Aldrich Inc., St Louis, MO, USA) was coated onto 96-well Tissue Culture plates (Beckton Dickinson, Franklin Lakes, NJ, USA) overnight at 4 °C, then the plates were washed three times with PBS and blocked for 1.5 h with 5% BSA in PBS at 37 °C, and washed again with PBS containing 0.01% Tween 20. The saliva specimens cultured overnight in 10 mL of brain heart infusion (BHI) broth (Difco Laboratories, Detroit, MI, USA) were centrifuged and dissolved with 1 mL of PBS, after which 200-μL samples were added to each well of the plates. After 3 h of incubation at 37 °C, non-adherent cells were removed by washing three times with PBS. Finally, adherent cells were removed by vigorous manipulation using repeated pipetting, which were added to the BHI broth. The overnight-grown cultures were inoculated onto Mitis-salivarius (MS) agar (Difco) plates or MS agar plates, containing bacitracin (0.2 U/mL; Sigma Chemical Co.) and 15% (w/v) sucrose (MSB agar plates). One colony each was picked up randomly from the MS agar (SN700 series strains) and MSB agar (SN800 series strains) plates, and examined.

Measurement of collagen-binding activities

Collagen-binding activities were determined using a method described previously[12], with some modifications. Briefly, type I collagen was coated onto 96-well plates, then the plates were washed and blocked for 1.5 h at 37 °C, as described above. Next, the wells were washed again with PBS containing 0.01% Tween 20. Cells from overnight cultures of the tested strains were grown in BHI broth, as noted above, then collected by centrifugation and added to the wells, after which the bacterial numbers were adjusted with PBS. After 3 h of incubation at 37 °C, adherent cells were washed three times with PBS, then fixed with 200 μL of 25% formaldehyde at room temperature for 30 min. After another three washes with PBS, the adherent cells were stained with 200 μL of 0.05% crystal violet (Wako Pure Chemical Industry, Osaka, Japan) in water for 1 min and washed three times with PBS, then the dye was dissolved by adding 7% acetic acid (200 μL) before determining the A_{595} value. The collagen-binding activities of the strains are expressed as a percentage as compared

to the binding property of *S. mutans* strain TW871, which was defined as 100%.

Specification of bacterial species

Genomic DNA was extracted from each strain using a conventional method, then 16S rRNA (approximately 1500 bp) was amplified by PCR using AmpliTaq Gold polymerase (Applied Biosystems, Foster City, CA, USA) with the primers 8UA (5′-AGA GTT TGA TCC TGG CTC AG-3′) and 1540R (5′-AAG GAG GTG ATC CAG CC-3′), as described previously[13]. Amplified fragments from the strains were cloned into a pGEM-T Easy vector (Promega, Madison, WI, USA) and their nucleotide alignments were determined using a dye-terminator reaction with a DNA sequencing system (ABI PRISM 310 Genetic Analyzer; Applied Biosystems) and BigDye terminator cycle sequencing kit. In order to obtain the complete sequence, the internal primers 16S-IN1 (5′-TCG TTG CGG GAC TTA ACC CAA CAT C-3′) and 16S-IN2 (5′-GAG CAA CGC CGC GTG AGT GAA GAA G-3′) were utilized. Data analysis was performed using GeneWorks software (IntelliGenetics, Mountain View, CA, USA). The 16S rRNA sequences obtained were compared with those available in the GenBank, EMBL, and DDBJ databases using the gapped BLASTN 2.0.5 program obtained from the National Center for Biotechnology Information server (http://www.ncbi.nlm.nih.gov/BLAST/).

Characterization of S. mutans *strains isolated from mother–child pairs*

First, conventional biochemical analysis findings, such as sugar fermentation profiles for 1% mannitol, sorbitol, raffinose, and melibiose as well as negative results for dextran agglutination were confirmed, as described previously[14]. Serotype determination was carried out using an immunodiffusion method with rabbit antisera specific for serotypes *c*, *e*, *f*, and *k*, as well as PCR with serotype-specific sets of primers, as described previously[15,16]. Then, genomic DNA was extracted from each strain, and detection of *cnm* genes encoding Cnm was carried out using the primer sets cnm-3F (5′-GAC AAA GAA ATG AAA GAT GT-3′) and cnm-1730R (5′-GCA AAG ACT CTT GTC CCT GC-3′), with TaKaRa Ex *Taq* polymerase (TAKARA BIO, Inc., Otsu, Shiga, Japan), as described previously[12].

Evaluation of mother to child transmission

In order to compare fingerprinting patterns, random amplified polymorphic DNA (RAPD) analysis was performed using Ready-To-Go RAPD analysis beads and primers (primer 1; 5′-GGT GCG GGA A-3′, primer 6; 5′-CCC GTC AGC A-3′) (GE Healthcare Bio-Sciences KK, Tokyo, Japan), according to the manufacturer's instructions. The amplicons were separated by electrophoresis on 1.5% agarose gels to compare the fingerprinting patterns of each strain.

Statistical analyses

Statistical analyses were performed using the StatView 5.0 computational software package (SAS Institute Inc., Cary, NC, USA). Fisher's exact probability test was utilized to compare the detection frequency of the species in the mothers with that of those in their children. As for the collagen-binding activities of each species, intergroup differences of various factors were estimated using a statistical analysis of variance (ANOVA) for factorial models. Fisher's protected least-significant difference (PLSD) test was used to compare individual groups. A P value of <0.05 was considered significant.

Results

Oral streptococcal strains with collagen-binding properties were present in the saliva specimens of 19 mothers (34.5%) and 23 children (41.8%), of which nine *S. mutans* strains and 36 non-*S. mutans* strains had collagen-binding properties. Both *S. mutans* and non-*S. mutans* strains were isolated from one mother and two children. The bacterial colonies on MSB agar plates were confirmed as *S. mutans* according to the biochemical properties, whereas those of non-*S. mutans* strains on MS agar plates were specified based on the nucleotide alignment of 16S rRNA. The most frequently detected species was *Streptococcus*

salivarius, followed by *S. mutans*, *Streptococcus mitis*, and *Streptococcus parasanguinis* (Table 1). *S. mutans* was more frequently detected in the mothers, whereas the detection rate of *S. salivarius* in the children was shown to be higher than that in the mothers, though those differences were not statistically significant. On the other hand, the detection rate of *S. salivarius* in the children was significantly higher than that of any other species isolated in the present study ($P < 0.01$). As for the collagen-binding activities of each species, *S. mutans* was the highest, followed by *S. salivarius* and *S. mitis*, and the activities of *S. mutans* and *S. salivarius* were significantly higher than that of *S. mitis* ($P < 0.001$ and $P < 0.01$, respectively) (Fig. 1).

S. mutans strains with collagen-binding properties were isolated from nine subjects (Table 2) and PCR analyses showed that all possessed the *cnm* gene. Of those, six were isolated from mothers (subjects 15M, 16M, 20M, 23M, 25M and 46M) and three from children (subjects 20C, 23C and 25C). In addition, *S. mutans* strains without collagen-binding activities were isolated from the two children of subjects 15M and 46M, whereas none was isolated from the child of 16M. The serotypes of the three mother–child pairs with *S. mutans* strains with collagen-binding properties (subjects 20M–20C, 23M–23C and 25M–25C) were totally consistent. In addition, the collagen-binding rates of the strains from the mothers were quite similar to those from their children. Furthermore, RAPD analysis showed that the fingerprinting patterns of these mother–child pair strains were consistent, while those of the 15M–15C and 46M–46C pairs were totally different

Table 1. Bacterial species with collagen-binding properties identified in saliva specimens.

	Detection rates (%)		
	Mothers (*N* = 55)	**Children (*N* = 55)**	**Total subjects (*N* = 110)**
Positive subjects*	19 (34.5%)	23 (41.8%)	42 (38.2%)
MSB plates			
S. mutans	6 (10.9%)	3 (5.5%)**	9 (8.2%)***
MS plates			
S. salivarius	9 (16.4%)	17 (30.9%)	26 (23.6%)
S. mitis	3 (5.5%)	4 (7.3%)**	7 (6.4%)***
S. parasanguinis	2 (3.6%)	1 (1.8%)**	3 (2.7%)***

*There was one mother and two children positive for both *S. mutans* and *S. salivarius*.
,*There were significant differences in the detection frequencies of *S. salivarius* and others between the children** and all subjects***($P < 0.01$).

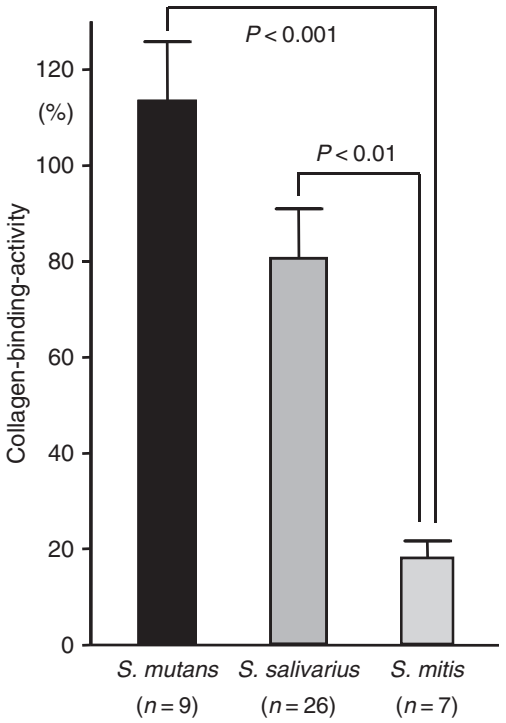

Fig. 1. Collagen-binding properties of strains isolated from saliva specimens. The collagen-binding activity of each strain was calculated based on a comparison to that of *S. mutans* TW871, which was considered to be 100%. There were statistically significant differences detected by Fisher's PLSD test.

Table 2. Subjects with *S. mutans* strains with collagen-binding properties.

Subject	Age	Gender	Strain	Serotype	Collagen-binding activity* (%)
15M	40	F	SN815M	*f*	113.8 ± 2.4
16M	40	F	SN816M	*c*	113.8 ± 1.2
20M	32	F	SN820M	*f*	156.5 ± 4.8
20C	9	F	SN820C	*f*	164.4 ± 8.6
23M	33	F	SN823M	*e*	127.7 ± 5.9
23C	7	F	SN823C	*e*	137.2 ± 1.3
25M	NR	F	SN825M	*c*	74.0 ± 1.9
25C	4	F	SN825C	*c*	86.4 ± 6.4
46M	37	F	SN846M	*f*	37.3 ± 0.3

NR, not recorded.
*The collagen-binding activity of each strain is expressed as compared to that of strain TW871, which was considered to be 100%.

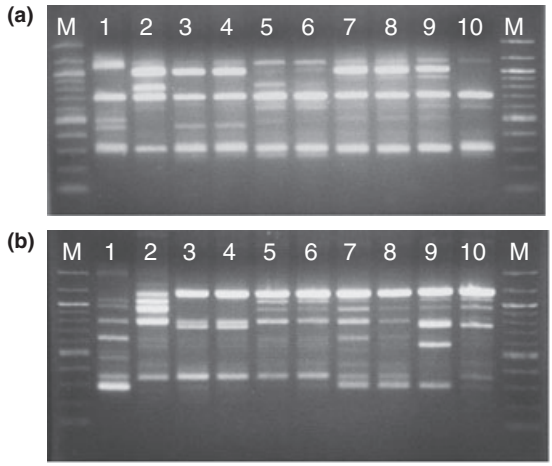

Fig. 2. Evaluation of mother–child transmission of *S. mutans* was performed using an RAPD method with Primer 1 (a) and Primer 6 (b). The SN800 series of strains were grown on MSB agar and analysed. M, 100-bp ladder. Lanes: 1, SN815M; 2, SN815C; 3, SN820M; 4, SN820C; 5, SN823M; 6, SN823C; 7, SN825M; 8, SN825C; 9, SN846M; 10, SN846C. M and C indicate mother and child, respectively.

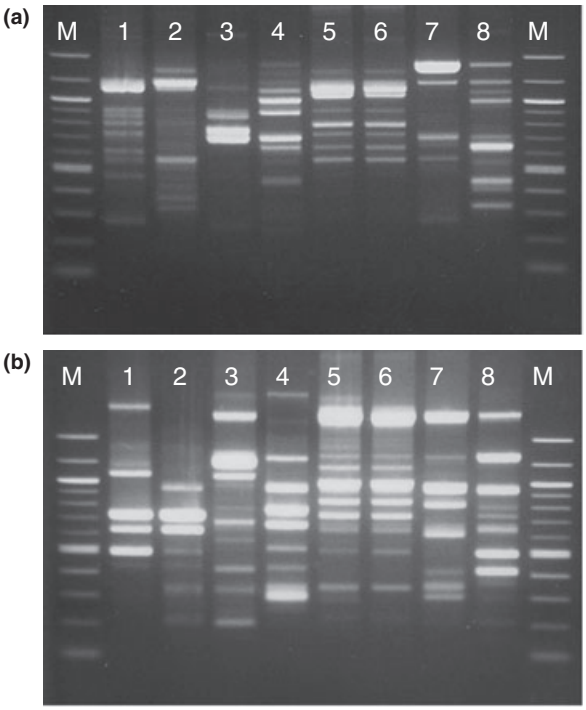

Fig. 3. Evaluation of mother–child transmission of *S. salivarius* was performed using an RAPD method with Primer 1 (a) and Primer 6 (b). The SN700 series of strains were grown on MS agar and analysed. M, 100-bp ladder. Lanes: 1, SN724M; 2, SN724C; 3, SN744M; 4, SN744C; 5, SN749M; 6, SN749C; 7, SN754M; 8, SN754C. M and C indicate mother and child, respectively.

(Fig. 2). As for *S. salivarius*, there were four mother–child pairs (24M–24C, 44M–44C, 49M–49C, and 54M–54C) who possessed species with collagen-binding properties. However, only the 49M–49C pair showed similar fingerprinting patterns, while those of the remaining three pairs were different (Fig. 3).

Discussion

The collagen-binding property of the bacterial strains present in the oral cavity is considered to be one of the most important factors for their virulence for infective endocarditis[17]. Previously, we focused on the collagen-binding properties of *S. mutans* species[11,12], while there are few reports of those properties in other oral streptococci. Thus, the methods used for specification of strains with collagen-binding properties in the obtained saliva specimens were newly constructed for the present study. In order to specify all oral bacterial strains with collagen-binding properties, saliva specimens rather than isolated strains were used for the collagen-binding assays. Strains with those properties were considered to be present at the bottoms of the wells, which were removed and then streaked onto selection agar plates for determination of oral streptococci. Although saliva specimens were used in the present study, it would also be interesting to evaluate dental plaque specimens, which will be carried out in subsequent studies.

To the best of our knowledge, this is the first study to investigate the presence of bacterial species with collagen-binding properties in saliva specimens. Notably, approximately one-third of all our subjects possessed bacterial species with those properties. A total of 9 *S. mutans* strains and 36 non-*S. mutans* strains showed collagen-binding properties, among which *S. salivarius* and *S. mutans* were frequently identified. However, the detection rate of *S. mutans* was lower in the children and that of *S. salivarius* higher in the children, as compared with the mothers. These findings indicate that the distribution of species with collagen-binding properties might be different between children and their mothers. The collagen-binding properties of *S. mutans* were previously reported to be associated with root

caries, which generally occur in adults[18]. Thus, it is speculated that *S. mutans* strains with collagen-binding properties are predominantly present in adults, rather than children.

We identified *S. mutans*, *S. salivarius*, *S. mitis* and *S. parasanguinis* strains with collagen-binding properties in saliva specimens obtained from the present mothers and children, among which the *S. mutans* and *S. salivarius* strains showed significantly greater levels of collagen-binding properties than the *S. mitis* strains. Bacteraemia is generally transient, even though the associated oral bacteria invade the bloodstream, whereas the duration of bacteraemia occasionally causes infective endocarditis in subjects with certain kinds of heart diseases[17]. *S. mutans* strains with collagen-binding properties were found in 10.9% of the mothers and 5.5% of the children, whereas 16.4% and 30.9%, respectively, were found to possess *S. salivarius* strains with those properties. Both species are considered to be possible pathogens for infective endocarditis, though their isolation frequency is lower as compared to other oral streptococcal species such as *Streptococcus sanguinis*[9,17]. Although the present results were obtained from subjects without systemic disorders, we intend to clarify these findings in subjects at high risk for infective endocarditis in subsequent studies.

S. mutans strains with collagen-binding properties were isolated from three of the children as well as their mothers, and serological and molecular biological analyses showed that the strains from the mother–child pairs were the same clones. Thus, we concluded that those strains had been transmitted from the mothers to their children. We previously hypothesized that strains with *cnm* genes are highly transmissible from mothers to children was presented in our previous study[12], which may also be supported by the present findings. On the other hand, we previously found that the rate of mother–child transmission in children under the age of 10 was approximately 70% and that the rate of transmitted strains from mothers was significantly higher in girls than boys[19]. It is of interest that all three of the present children determined to possess transmitted strains were girls, while another subject whose *S. salivarius* strain was determined to be transmitted from the mother was also a girl. These findings indicate that oral streptococcal strains with collagen-binding properties are transmissible, especially between mothers and their daughters, though that should be confirmed with larger scale studies in the future.

Several cell surface proteins of *S. mutans* related to collagen binding have been reported, among which the approximately 120-kDa Cnm protein should be regarded as a major factor[10,12]. Collagen-binding adhesins with a high homology to Cnm have been reported in studies of *Staphylococcus aureus* (Cna) and *Streptococcus equi* (Cne)[20,21]. Furthermore, Southern hybridization of *cnm* using strains with collagen-binding properties revealed positive bands for all of the *S. mutans* strains examined, while no bands were identified for the other strains, even under less stringent conditions (data not shown). Thus, it is reasonable to speculate that the collagen-binding adhesin homologous to Cnm is not present in species generally found in the oral cavity, other than *S. mutans*.

In summary, we determined oral streptococcal species with collagen-binding properties in saliva specimens from mothers and their children. *S. mutans* and *S. salivarius* were frequently isolated, and their collagen-binding activities were high as compared to other species identified in the specimens. In addition, strains with collagen-binding properties had a possible association with mother to child transmission. Due to the limited number of participants in the present study, only limited conclusion can be stated, though we hope that they initiate similar studies by other research groups worldwide.

What this paper adds

- *S. mutans* and *S. salivarius* are major species with collagen-binding properties found in saliva specimens.
- *S. mutans* strains with collagen-binding properties may be transmissible from mother to child.

What this paper is important for paediatric dentists

- *S. mutans* strains with collagen-binding properties are considered to be associated with risks for development of caries and onset of infective endocarditis in individuals with underlying heart diseases.

Acknowledgments

This study was supported by a Grant-in-Aid for Scientific Research (A) 19209063 from the Japan Society for Promotion of Science, as well as Grants-in-Aid for Young Scientists (A) 21689052, (B) 21792066, and (B) 21792067 from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

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