Serotype distribution of *Streptococcus mutans* a pathogen of dental caries in cardiovascular specimens from Japanese patients

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The involvement of oral bacteria in the pathogenesis of cardiovascular disease has been studied, with *Streptococcus mutans*, a pathogen of dental caries, detected in cardiovascular lesions at a high frequency. However, no information is available regarding the properties of *S. mutans* detected in those lesions. Heart valve specimens were collected from 52 patients and atheromatous plaque specimens from 50 patients, all of whom underwent cardiovascular operations, and dental plaque specimens were taken from 41 of those subjects prior to surgery. Furthermore, saliva samples were taken from 73 sets of healthy mothers ($n=73$) and their healthy children ($n=78$). Bacterial DNA was extracted from all specimens, then analysed by PCR with *S. mutans*-specific and serotype-specific primer sets. The detection rates of *S. mutans* in the heart valve and atheromatous plaque specimens were 63 and 64 %, respectively. Non-*c* serotypes were identified with a significantly higher frequency in both cardiovascular and dental plaque samples from the subjects who underwent surgery as compared to serotype *c*, which was detected in 70–75 % of the samples from the healthy subjects. The serotype distribution in cardiovascular patients was significantly different from that in healthy subjects, suggesting that *S. mutans* serotype may be related to cardiovascular disease.

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INTRODUCTION

Streptococcus mutans, a major pathogen of dental caries, has been detected in heart valves and isolated from the blood of patients with infective endocarditis (IE), suggesting its close relationship with IE (Vose *et al.*, 1987; Ullman *et al.*, 1988; Gauduchon *et al.*, 2001; Nomura *et al.*, 2006). *S. mutans* is classified into four serotypes (*c/e/f/k*) based on the chemical composition of its cell surface serotype-specific rhamnose–glucose polymers (RGPs), which form a backbone of rhamnose polymers with side chains of glucose polymers (Hamada & Slade, 1980; Nakano *et al.*, 2004a). Serotype *c* is reported to be the most prevalent in oral isolates at approximately 70–80 %, followed by *e*, *f* and *k* (Hamada *et al.*, 1980; Hirasawa & Takada, 2003; Shibata

et al., 2003; Nakano *et al.*, 2004b). In our previous study, four *S. mutans* strains isolated from the blood of patients with bacteraemia after tooth extraction or IE were characterized; however, none of those strains were classified as serotype *c* (Fujiwara *et al.*, 2001).

The serotype-specific RGPs of *S. mutans* were demonstrated to play important roles in streptococcal adherence to human monocytic and fibroblastic cells, and speculated to be its most efficient cytokine-stimulating components (Engels-Deutsch *et al.*, 2003). In addition, the hydrophilic nature of RGPs is known to be associated with the resistance to phagocytosis by human polymorphonuclear leukocytes (Tsuda *et al.*, 2000). Further, RGPs were shown to possess properties allowing them to bind directly to human platelets and trigger their aggregation in a dose-dependent manner (Chia *et al.*, 2004). In our previous study, serotype *k* strains featured a drastic reduction in the amount of

Abbreviations: IE, infective endocarditis; RGP, rhamnose–glucose polymer.

glucose side chains and were shown to be less susceptible to phagocytosis by human polymorphonuclear leukocytes than the parent strain (Nakano *et al.*, 2004a). However, the virulence of each serotype of *S. mutans* in blood remains to be elucidated.

Recently, the association of cardiovascular diseases and periodontitis has received attention because of results showing that periodontopathic bacterial DNA could be detected in the diseased cardiovascular tissues (Scannapieco *et al.*, 2003). We previously reported that not only periodontopathic bacterial species, but also several streptococcal species, were detected in cardiovascular specimens, such as heart valve and atheromatous plaque, and *S. mutans* was the most frequently detected (Nakano *et al.*, 2006a). In the present study, detection of *S. mutans* in additional specimens, as well as serotype determination of *S. mutans*-positive specimens, was carried out.

METHODS

Specimens from subjects undergoing a cardiovascular operation.

Fifty-two heart valve and fifty atheromatous plaque samples were obtained from subjects with cardiovascular diseases who visited Osaka Rosai Hospital. Of these, 41 subjects were referred to the Department of Dentistry and Oral Surgery for a dental examination prior to cardiovascular surgery, and from whom dental plaque specimens were obtained. All of the specimens were collected from December 2004 to August 2006 according to a protocol approved by the ethics committee of Osaka Rosai Hospital. The heart valve tissue specimens were excised during a valve replacement procedure, following diagnosis of aortic regurgitation, aortic stenosis, mitral regurgitation, mitral stenosis or tricuspid regurgitation, while the atheromatous plaque specimens were collected during treatment for a thoracic or abdominal aortic aneurysm. The specimens were aseptically cut into small pieces, and bacterial DNA was extracted as described previously (Nakano *et al.*, 2006a).

Specimens from mothers and their children. Seventy-three pairs of mothers (73 subjects) and their children (78 subjects) who came

to the Pedodontics Clinic of Osaka University Dental Hospital, Osaka, Japan, from June 2005 to August 2006 were analysed. None of the children or mothers had systemic disorders. Collection of clinical specimens was carried out in accordance with a protocol approved by the ethics committee of Osaka University Dental Hospital. Whole saliva samples were taken and bacterial DNA was extracted according to previously described methods (Hoshino *et al.*, 2004).

S. mutans detection and serotype determination.

S. mutans detection was carried out using primers targeting the *gtfD* gene (Hoshino *et al.*, 2004) (Table 1). The serotype of each sample was determined by serotype *c*-, *e*- or *f*-specific sets of primers (Shibata *et al.*, 2003), as well as a serotype *k*-specific set of primers generated in our previous study (Nakano *et al.*, 2004b). *S. mutans* MT8148 (*c*), NN2001 (*c*), NN2002 (*e*), NN2003 (*f*) and FT1 (*k*) were used as reference strains for *S. mutans* and each serotype (Nakano *et al.*, 2004b). Samples with a positive reaction to the *S. mutans*-specific set of primers but not with any of the serotype-specific sets of primers were confirmed using an additional *S. mutans*-specific set of primers based on the *gtfB* sequence (Oho *et al.*, 2000). In addition, sequence determination of the amplified fragments was carried out to confirm that they contained the target nucleotide alignment. The sensitivity of all the PCR methods was evaluated using diluted DNA extracted from the reference strains. In order to confirm that *S. mutans* DNA was present in the serotype undetermined specimens, a broad-range PCR assay targeting 16S rRNA and sequencing analysis was carried out, as described elsewhere (Rovero *et al.*, 2005; Nakano *et al.*, 2006a). Briefly, PCR products amplified by broad-range 16S rRNA primers [536f (5'-CAG CAG CCG CGG TAA TAC-3') and 1050r (5'-CAC GAG CTG ACG ACA-3')] were cloned into a pGEM-T Easy vector (Promega), after which ten clones were randomly chosen and their sequences determined. To identify the bacterial species, the sequences 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/>). Identification at the species level was determined when the sequence similarity was greater than 99 % of that of the GenBank prototype strain sequence.

Statistical analysis. Fisher's exact probability test was used for statistical analysis of the comparative frequency of each serotype. A *P* value of <0.05 was considered significant.

Table 1. PCR primers used for *S. mutans* detection and serotype determination

Primer	Purpose	Sequence (5'-3')	Expected size (bp)	Reference
MKD-F	<i>S. mutans</i> detection	GGC ACC ACA ACA TTG GGA AGC TCA GTT	433	Hoshino <i>et al.</i> (2004)
MKD-R		GGA ATG CCG ATC AGT CAA CAG GAT		
<i>gtfB</i> -F	<i>S. mutans</i> detection	ACT ACA CTT TCG GGT GGC TTG G	517	Oho <i>et al.</i> (2000)
<i>gtfB</i> -R		CAG TAT AAG CGC CAG TTT CAT C		
SC-F	Serotype <i>c</i> determination	CGG AGT GCT TTT TAC AAG TGC TGG	727	Shibata <i>et al.</i> (2003)
SC-R		AAC CAC GGC CAG CAA ACC CTT TAT		
SE-F	Serotype <i>e</i> determination	CCT GCT TTT CAA GTA CCT TTC GCC	517	Shibata <i>et al.</i> (2003)
SE-R		CTG CTT GCC AAG CCC TAC TAG AAA		
SF-F	Serotype <i>f</i> determination	CCC ACA ATT GGC TTC AAG AGG AGA	316	Shibata <i>et al.</i> (2003)
SF-R		TGC GAA ACC ATA AGC ATA GCG AGG		
CEFK-F	Serotype <i>k</i> determination	ATT CCC GCC GTT GGA CCA TTC C	294	Nakano <i>et al.</i> (2004b)
K-R		CCA ATG TGA TTC ATC CCA TAC C		

RESULTS AND DISCUSSION

The minimum amounts of genomic DNA required for *S. mutans* detection and serotype determination by PCR methods used in the present study were evaluated using serial dilution of known concentrations of genomic DNA extracted from the reference strains (Fig. 1). The PCR results with *gtfD*- and *gtfB*-based primer sets showed that the minimum amount of template DNA for *S. mutans* detection was 1–10 pg, which was also the detection limit for the template DNA for serotype *c/elfk*.

The detection rates of *S. mutans* in heart valve and atheromatous plaque specimens were 63.4 and 64.0 %, respectively (Table 2). In the heart valve specimens, 60.6 % of the *S. mutans*-positive samples were shown to be a single serotype, with serotype *e* the most prevalent followed by *c*, *f* and *k*. Specimens with multiple serotypes detected were also identified at a frequency of 15.2 %. As for the atheromatous plaque specimens, 31.2 and 59.4 % of the *S. mutans*-positive samples were shown to be single and multiple serotypes, respectively. In addition, there were eight heart valves and three atheromatous plaque specimens that did not react to any of the serotype-specific sets of primers. Those specimens were also confirmed to have a positive reaction toward the *gtfB*-based primers. In addition, broad-range PCR and sequencing analyses showed that *S. mutans* was identified in at least one of ten clones analysed.

Fig. 1. Sensitivity of the PCR methods for *S. mutans* detection and serotype determination. (a) A gel showing the detection limit using the *S. mutans*-specific sets of primers. The *gtfD*- and *gtfB*-based primer set were evaluated by genomic DNA from NN2001 (serotype *c*). (b) A gel showing the detection limit using the serotype-specific sets of primers. The serotype *c*-, *e*-, *f*-, and *k*-specific sets of primers were analysed by genomic DNA from NN2001 (*c*), NN2002 (*e*), NN2003 (*f*) and FT1 (*k*), respectively. Lanes: 1, 100 pg; 2, 10 pg; 3, 1 pg; 4, 0.1 pg; M, molecular size marker (100 bp DNA ladder).

The rates of *S. mutans*-positive specimens in dental plaque samples from subjects who underwent cardiovascular surgery, and in saliva samples from the mothers and their children ranged from 88.4 to 95.1 % (Table 3). However, the serotype distribution in the healthy mothers and children was quite different from that in the cardiovascular surgery subjects. In the subjects who underwent surgery, the proportion of specimens containing multiple serotypes was greater than that of specimens containing a single serotype. In addition, a non-*c* serotype was detected in cardiovascular surgery subjects more often than in the healthy children and their mothers. As for the serotype distribution in the mothers and children, specimens with a single serotype were detected more often than in the cardiovascular surgery subjects, with the detection of serotype *c* much higher than any of the other serotypes. In addition, we were unable to classify two (5.1 %) specimens from the cardiovascular surgery subjects as any known serotype, whereas all of those from mothers and children were classified. Table 4 summarizes the serotype distribution frequency of *S. mutans*-positive specimens. The frequency of serotype *c* in the cardiovascular specimens, as well as in the oral specimens from the subjects who underwent cardiovascular operations, was significantly lower than that of the oral specimens from the healthy mothers and children ($P < 0.05$). On the other hand, the rates for serotypes *e* and *k*, as well as untypable specimens, among the cardiovascular specimens were significantly higher than among the oral specimens from

Table 2. Detection and serotype distribution of *S. mutans* in cardiovascular specimens

	Heart valve (<i>n</i> =52)	Atheromatous plaque (<i>n</i> =50)
Age (range)	67.8 ± 8.7 (49–87)	70.8 ± 7.2 (56–82)
Gender (male : female)	36 : 16	37 : 13
Positive for <i>S. mutans</i>	33/52 (63.4 %)*	32/50 (64.0 %)*
Single serotype	20/33 (60.6 %) [†]	10/32 (31.2 %) [†]
<i>c</i>	7	5
<i>e</i>	11	4
<i>f</i>	1	1
<i>k</i>	1	0
Multiple serotype	5/33 (15.2 %) [†]	19/32 (59.4 %) [†]
<i>c</i> and <i>e</i>	3	10
<i>c</i> and <i>k</i>	0	3
<i>e</i> and <i>f</i>	0	1
<i>e</i> and <i>k</i>	2	2
<i>c</i> , <i>e</i> and <i>k</i>	0	3
Serotype undetermined	8/33 (24.2 %) [†]	3/32 (9.4 %) [†]

*The percentage is expressed in comparison to all (100 %) of the heart valve and atheromatous plaque specimens.

[†]The percentage is expressed in comparison to all (100 %) of the *S. mutans*-positive specimens.

Table 3. Detection and serotype distribution of *S. mutans* in the oral cavity

	Cardiovascular surgery patient (n=41)	Healthy mother (n=73)	Healthy child (n=78)
Age (range)	66.1 ± 7.8 (49–80)	37.7 ± 4.2 (28–50)	7.2 ± 2.4 (2–12)
Gender (male : female)	30 : 11	0 : 73	40 : 38
Specimens	Dental plaque	Saliva	Saliva
Positive for <i>S. mutans</i>	39/41 (95.1 %)*	68/73 (93.2 %)*	69/78 (88.4 %)*
Single serotype	16/39 (41.0 %) [†]	51/68 (75.0 %) [†]	54/69 (78.3 %) [†]
<i>c</i>	2	43	50
<i>e</i>	14	8	4
<i>f</i>	0	0	0
<i>k</i>	0	0	0
Multiple serotype	21/39 (53.8 %) [†]	17/68 (25.0 %) [†]	15/69 (21.7 %) [†]
<i>c</i> and <i>e</i>	14	14	9
<i>c</i> and <i>f</i>	0	3	2
<i>c</i> and <i>k</i>	2	0	2
<i>e</i> and <i>f</i>	0	0	2
<i>c</i> , <i>e</i> and <i>f</i>	1	0	0
<i>c</i> , <i>e</i> and <i>k</i>	4	0	0
Serotype undetermined	2/39 (5.1 %) [†]	0/68 (0 %) [†]	0/69 (0 %) [†]

*The percentage is expressed in comparison to all (100 %) of the dental plaque and saliva specimens.

[†]The percentage is expressed in comparison to all (100 %) of the *S. mutans*-positive specimens.

the healthy mothers and children ($P < 0.05$). We acknowledge that the control group was not an ideal match for the cardiovascular subjects. However, no difference in *S. mutans* serotype distribution with gender was observed among the children analysed in this study. Also, the serotype distribution patterns of the children and mothers were not significantly different. We surmise, therefore, that more elderly males (matching the cardiovascular test subjects) are also likely to harbour a similar serotype range as the mothers and children employed in this study.

To the best of our knowledge, this is the first report of *S. mutans* serotype distribution in cardiovascular specimens. We previously reported that four blood isolates of *S. mutans* from subjects with bacteraemia after tooth extraction or IE belonged to serotypes *e*, *f* or *k* (Fujiwara *et al.*, 2001; Nakano *et al.*, 2004a). It is generally known that 70 to 80 % of *S. mutans* oral strains belong to serotype *c* (Hamada *et al.*, 1980; Hirasawa & Takada, 2003; Shibata *et al.*, 2003; Nakano *et al.*, 2004b), and the saliva samples from the healthy mothers and children were consistent

Table 4. Serotype distribution of *S. mutans*-positive specimens

A significant difference ($P < 0.05$) was found as compared to the oral specimens from the healthy mothers and healthy children by Fisher's exact probability test.

	Cardiovascular		Oral		
	Heart valve (n=33)	Atheromatous plaque (n=32)	Cardiovascular surgery patients (n=39)	Healthy mothers (n=68)	Healthy children (n=69)
<i>c</i>	10 (30.3 %)* [†]	21 (65.6 %)* [†]	23 (59.0 %)* [†]	60 (88.2 %)	63 (91.3 %)
<i>e</i>	16 (48.5 %) [†]	20 (62.5 %)* [†]	33 (84.6 %)* [†]	22 (32.4 %)	15 (21.7 %)
<i>f</i>	1 (3.0 %)	2 (6.3 %)	1 (2.6 %)	3 (4.4 %)	4 (5.8 %)
<i>k</i>	3 (9.1 %)*	8 (25.0 %)* [†]	4 (10.3 %)*	0 (0 %)	2 (2.9 %)
Unserotypable	8 (24.2 %)* [†]	3 (9.4 %)* [†]	2 (5.1 %)	0 (0 %)	0 (0 %)

*Healthy mothers.

[†]Healthy children.

with that (Table 3). On the contrary, the detection rate of serotype *c* in the cardiovascular subjects was significantly lower than that in the healthy subjects (Tables 2 and 3), which led us to speculate that the serotype distribution of *S. mutans* in cardiovascular subjects is significantly different from that in healthy subjects, and that non-*c* serotypes of *S. mutans* might be possible risk factors for cardiovascular diseases. Further, the number of the subjects with multiple serotypes was higher in the cardiovascular subjects than the healthy subjects, in whom a single serotype *c* was predominant. In addition, most of the *S. mutans*-positive specimens showed a positive reaction for at least one of the serotype-specific sets of primers; however, eight (24.2 %) heart valve specimens, three (9.4 %) atheromatous plaque specimens and two (5.1 %) dental plaque specimens did not react to any of the serotype-specific sets of primers (Tables 2 and 3). All of these specimens were also confirmed to have a positive reaction to the additional *S. mutans*-specific sets of primers designed based on the nucleotide alignment of the other gene. Also, the nucleotide alignment of those amplicons was shown to be the targeted sequence. Since the sensitivity of all of the PCR methods used in this study was shown to be at nearly the same level (Fig. 1) and the broad-range PCR method also detected the 16S rRNA alignment of *S. mutans*, we concluded that at least one additional unknown serotype was present in the cardiovascular specimens.

Serotype *k* *S. mutans* was first isolated from the blood of IE subjects and characterized by lack of the glucose side chain of the serotype-specific RGPs (Fujiwara *et al.*, 2001), which resulted in a low antigenicity and a low susceptibility to phagocytosis by human polymorphonuclear leukocytes (Nakano *et al.*, 2004b). Serotype *k* *S. mutans* strains have also been detected in the oral cavities of healthy children with a prevalence of 2 % (Nakano *et al.*, 2004b). In the present study, there were several *S. mutans*-positive cardiovascular specimens that could not be classified into any of the known serotypes by PCR. We recently analysed the serotypes of 100 *S. mutans* oral isolates from 100 children, as well as 2500 from 50 children, and all of the strains belonged to the *c*, *e*, *f* or *k* serotypes (Nakano *et al.*, 2004a), which indicated that the prevalence of a new serotype in the oral cavity would be extremely low, even though it likely exists. If a new serotype of *S. mutans* with a variation of RGPs is found to be selectively present in cardiovascular subjects, then the variation of serotype-specific RGPs may be related to the pathogenesis of *S. mutans* with cardiovascular diseases.

The reason why non-*c* serotype *S. mutans* was detected more frequently in the cardiovascular specimens than in those from the oral cavity remains to be elucidated. Since serotype-specific RGPs have been shown to be involved with virulence in blood (Tsuda *et al.*, 2000; Engels-Deutsch *et al.*, 2003; Chia *et al.*, 2004; Nakano *et al.*, 2004a), it is possible that an alteration in chemical composition of the RGPs in each serotype is directly related to virulence. Another possible hypothesis is that non-*c* serotype strains

possess common properties other than serotype-specific RGPs, which contribute to their virulence in blood. We previously demonstrated that inactivation of the *pac* gene encoding the major surface antigen protein antigen *c* (PAC) of *S. mutans* resulted in lowered susceptibility to phagocytosis by human polymorphonuclear leukocytes *in vitro* and a longer duration of bacteraemia in a rat model (Nakano *et al.*, 2006b). In that study, analysis of clinical oral isolates of *S. mutans* without PAC expression showed them to be predominantly detected among the non-*c* serotypes. In addition, genes encoding collagen-binding adhesins of *S. mutans* were frequently identified in non-*c* serotype strains (Sato *et al.*, 2004). Although the clinical significance of *S. mutans* detection in cardiovascular specimens remains to be elucidated, subjects with non-*c* serotype may be at risk for cardiovascular diseases.

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