

# Serotype specific polymerase chain reaction identifies a higher prevalence of *streptococcus mutans* serotype *k* and *e* in a random group of children with dental caries from the Southern region of India

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## Abstract

**Background:** The development of dental caries has been associated with the oral prevalence of *Streptococcus mutans*. Four serotypes of *S. mutans* have been reported, namely serotype *c*, *e*, *f*, and *k* that are classified based on the composition and linkages of cell wall polysaccharides, response to physiological reactions, sero-specificity and 16s rRNA homology. Although the oral prevalence of *S. mutans* serotype *c* in Indian subjects with or without caries is known, the prevalence of the other three serotypes, *e*, *f*, and *k* are not known. Hence in this study, we have investigated the occurrence of the *e*, *f*, and *k* serotypes in children with or without caries within the age group of 6-12 years. **Materials and Methods:** Genomic DNA isolated from whole saliva of caries active (CA) and caries free (CF) groups were first screened for the presence of *S. mutans* by strain specific polymerase chain reaction (PCR). Those samples that tested positive for the presence of *S. mutans* were further analyzed by serotype specific PCR to identify the prevalence of the serotypes. **Results:** Strain specific PCR indicated a higher prevalence of *S. mutans* in CA group (80%) relative to CF group (43%). Further analysis of the *S. mutans* positive samples in both groups indicated a higher prevalence of serotype *k* and *e*, followed by serotype *f* in CA group. **Conclusion:** The present data clearly establishes a novel *S. mutans* serotype prevalence hierarchy in children from this region, compared with those that have been reported elsewhere. Besides, the data are also clinically significant as the occurrence of serotype *k* has been associated with infective endocarditis.

**Keywords:** *Streptococcus mutans* serotypes *e*, *f* and *k* in dental caries, *Streptococcus mutans* serotype specific polymerase chain reaction, *Streptococcus mutans* serotypes in dental caries, *Streptococcus mutans* serotypes in India

## Introduction

The development of dental caries is a multistep process that often begins with the formation of plaque substance, a structurally and functionally defined biofilm with microbial communities organized in a polysaccharide matrix.<sup>[1]</sup> The formation of dental plaque is a normal event even in an otherwise healthy mouth with no caries, and the microbial

communities colonizing them primarily exist as symbionts, some of which essentially behave as commensals to prevent the pathogenic species from gaining access to the tooth surface.<sup>[2]</sup> In individuals with poorer oral hygiene the microbial population of the plaque shifts toward acidogenic and acid-tolerant species that consequently promotes active carious lesions.<sup>[3,4]</sup> The two most widely studied cariogenic bacteria are mutans streptococci (MS) and *Lactobacillus*, the levels of which have been found to correlate with the number of decayed teeth or decayed, missing or filled teeth (DMFT).<sup>[1,3,5]</sup> More specifically, the association of *Streptococcus mutans* of MS group with cariogenesis has been extensively characterized.<sup>[5]</sup> At the time of biofilm formation, the *S. mutans* adheres to the tooth surface with its dextran glucose rich polysaccharide capsule that promotes an acid-tolerant biofilm environment.<sup>[6,7]</sup> As *S. mutans* in biofilm begins to divide and form microcolonies, its metabolic process breaks down dietary sucrose to glucose and fructose. While fructose is fermented for energy, glucose is polymerized into an extracellular dextran, which cements *S. mutans* to tooth enamel to provide a matrix for dental plaque. In the absence of dietary sucrose, *S. mutans* depolymerizes the extracellular dextran polymer to glucose molecules again for carbon source, which results in the production of lactic acid within the biofilm that subsequently decalcifies enamel.<sup>[8]</sup> Decalcification of enamel produces white spots on the tooth surface and is often considered as a primary carious lesion.

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Strains of MS are classified into nine distinct serotypes – *Streptococcus cricetus* (serotype a), *Streptococcus rattus* (serotype b), *S. mutans* (serotypes c, e, f and k), *Streptococcus sobrinus* (serotypes d and g), *Streptococcus downei* (serotype h), *Streptococcus ferus* (serotype c) and *Streptococcus macacae* (serotype c) based on the composition and linkages of cell wall polysaccharides, physiological reactions, serological specificity and 16s rRNA homology.<sup>[9,10]</sup> More recently, primers designed to amplify specific variant regions of rhamnose-glucose polymer (*rgp*) operon, which encodes for enzymes that catalyze the formation of serotype specific *rgps* in the cell wall are used to classify the four serotypes of *S. mutans*: c, e, f and k.<sup>[11,12]</sup> While variations downstream of *rgpF* gene region are used to type c, e and f, variations within the *rgpF* is used to type serotype k.<sup>[11,12]</sup> Recent studies have indicated an association between cariogenic *S. mutans* serotypes and infective endocarditis (IE), an inflammatory condition of endothelial cells of the heart. Indeed *S. mutans* serotypes have been found in the heart valves and blood samples of patients with IE.<sup>[13]</sup> These findings highlight the clinical significance of elucidating the prevalence of *S. mutans* serotypes in a population. Investigation on the prevalence of *S. mutans* serotype c in children from India with or without caries identified its wider prevalence in both noncaries and caries group, as has been reported elsewhere.<sup>[14]</sup> However, the prevalence of the other three serotypes: e, f and k in Indian subjects with or without caries remains yet to be identified. In this study, we sought to investigate, (1) the prevalence of *S. mutans* in school going children from a defined location within the Southern region of India, (2) the prevalence of the three *S. mutans* serotypes e, f and k in *S. mutans* positive subjects by serotype specific polymerase chain reaction (PCR) technique, and (3) the association of the occurrence of serotypes with caries.

## Materials and Methods

### Subject selection and sample size

School children in the age group of 6-12 years hailing from the coastal region of state of Tamil Nadu, South India, were selected by random sampling and screened for dental caries. Children with DMFT (permanent dentition)/dmft (deciduous dentition) index >5 or <1 but otherwise clinically healthy were included in the study, after obtaining informed consent from their respective parents. Children with recent or long term antibiotic therapy were excluded from the study. The DMFT/dmft index of 5 was used as a cut-off to define caries activity in the community addressed in this study, but not that of the general population. Sixty children with DMFT/dmft index >5 and 39 children with DMFT/dmft index <1 were selected for inclusion in the study. The sample size was calculated based on standard normal distribution with reference to the prevalence of *S. mutans* and its serotypes e, f and k in Japanese population, as the three serotypes have been simultaneously studied only in these samples by serotype specific PCR. The study protocol was approved by the Institutional Ethics Committee.

### Collection of saliva, DNA extraction, serotype specific polymerase chain reaction and data analysis

After obtaining demographic details, 2 ml of unstimulated saliva was collected from each child in a 15 ml sterile tube (cat #546021, Tarsons, Kolkata, India), which was stored at – 20°C until transported to the laboratory. At the time of DNA extraction, the saliva samples stored in the freezer were thawed and bacterial cells were pelleted by spinning them at 3000 rpm for 10 min at room temperature. Pelleted cells were resuspended in 100 µl of 1X phosphate buffered saline, pH 7.2 containing 20 µl of fresh lysozyme (5 mg/ml, cat #L6876, Sigma-Aldrich, St. Louis, MO, USA), and the cell suspension was incubated at 4°C for 15 min. An equal volume of cell lysis buffer containing 0.1% sodium dodecyl sulfate, 25 mM EDTA, 75 µg/100 µl proteinase-K (cat # P2308, Sigma-Aldrich, St. Louis, MO, USA) and 200 mM Tris-Cl at pH 8 (cat #T6664, Sigma-Aldrich, St. Louis, MO, USA) was then added to the cell suspension and incubated at 55°C for 30 min. Following lysis, 2.5 volume of cold ethanol was added to the cell lysates to precipitate genomic DNA. The tubes were then centrifuged at 12,000 rpm for 15 min at room temperature to pellet the genomic DNA precipitate. Subsequently, the pellet was washed twice with 0.25 ml of 70% ethanol and then dissolved in 25 µl of double deionized water (cat #W4502, Sigma-Aldrich, St. Louis, MO, USA). PCR amplification was performed on 25 ng of DNA samples with strain or serotype specific primers as described elsewhere.<sup>[10]</sup> The amplification conditions were similar to those published earlier,<sup>[10]</sup> except that 25 cycles or 35 cycles of amplification were employed for strain specific or serotype specific PCR, respectively. A negative control reaction without DNA was run to confirm the specificity of amplification in experimental samples. Chromosomal DNA of *S. mutans* serotypes e, f, and k (kind gift of Kazuhiko Nakano, Department of Pediatric Dentistry, Osaka University Graduate School of Dentistry, Japan) were used as a positive control for amplification reactions. Statistical significance of the data was evaluated by Chi-square test.

## Results

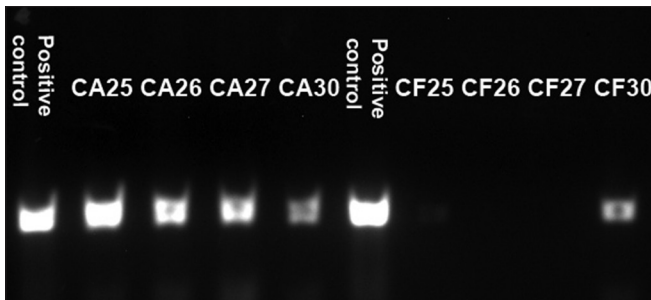
### Selection of caries active and caries free children and determination of overall prevalence of *Streptococcus mutans*

In order to determine the prevalence of *S. mutans* serotypes e, f and k in children of age group 6-12 years, the oral cavity of 99 school children of afore mentioned age group were screened by a single examiner to select the caries active (CA) and caries free (CF) groups. The intra examiner reliability was assessed by screening twice on 25 children at an interval of 2 weeks and the data was assessed by reliability test and kappa value was found to be >0.91. Those children with clinically identifiable DMFT/dmft index >5 were considered as CA, while those with DMFT/dmft index <1 were considered as CF.<sup>[15]</sup> DMFT/dmft was chosen instead of DMFS as DMFT/dmft, (1) is a World Health Organization standardized criteria for clinical registration of dental caries,<sup>[16]</sup> (2) provides

a rapid way of screening a large number of subjects in field trips,<sup>[16]</sup> and (3) is commonly used in epidemiological studies in school children worldwide.<sup>[16]</sup> Hence, based on the above criterion 60 and 39 children were selected for inclusion in CA and CF groups respectively. Subsequently, unstimulated saliva samples were obtained from both groups of children and screened to first identify the prevalence of *S. mutans* by strain specific PCR amplification method. This method uses primers that anneal and amplify a segment of *GTFB* gene specifically from *S. mutans* and hence may be used to detect its overall prevalence.<sup>[10-12]</sup> Gel analysis of the PCR amplified samples showed *S. mutans* specific band in 80% (48/60) of CA and 43% (17/39) of CF groups, which indicated a statistically higher prevalence of *S. mutans* in the CA group ( $P = 0.0001$ ). The PCR amplicons were also confirmed for specificity of amplification of *S. mutans GTFB* gene by direct sequencing. Some of the samples that were analyzed showed a less intense amplification relative to others [Figure 1, compare lanes among lanes in CA and CF groups], but nevertheless were included in the study as it only reflected differential count of *S. mutans* population among the tested samples.

**Determination of prevalence of *Streptococcus* serotypes *e, f* and *k* in *Streptococcus* positive caries active and caries free samples by serotype specific polymerase chain reaction**

Next, we sought to identify the prevalence of the three *S. mutans* serotypes *e, f* and *k* in samples that had tested positive for the presence of *S. mutans* to investigate the possible association of serotype prevalence and dental caries. This category included 48 samples from CA group and 17 samples from CF group. Serotype specific PCR was performed on the above samples with primers that specifically amplify either one of the three serotypes along with respective positive controls, as described earlier.<sup>[10]</sup> Gel analysis of the PCR amplification reaction showed serotype specific DNA band with respective set of primers for *e, f* or *k* in both CA and CF groups, which were confirmed by their relative size of migration with reference to respective positive controls [Figure 2] and direct sequencing. The number of samples that tested positive for either one of



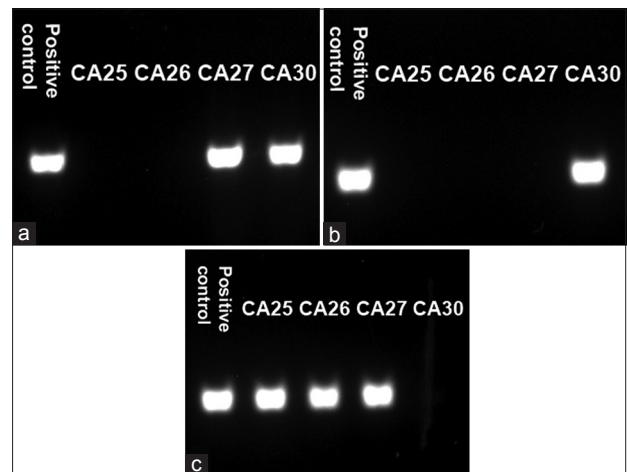
**Figure 1:** A representative gel image of polymerase chain reaction based detection of *Streptococcus mutans* in caries active and caries free sample groups is shown. Note that the intensity of *S. mutans* specific band varies among samples, which is a reflection of variation in the *S. mutans* population in each of the sample

the serotype *e, f* or *k* in CA group was 7, 3 and 13 respectively, while the same in CF group was 1, 2 and 4 respectively. The occurrence of *k* serotype was highest in both CA and CF groups, which was followed by *e* and *f* serotypes in CA group. The occurrence of these two serotypes, that is, *e* and *f* in CF group were too low to be analyzed for its order of prevalence. Statistical analysis of the above data showed a nonsignificant trend toward positive association between caries and higher prevalence of either of the serotypes, especially that of *k* and *e* [Table 1]. There was also a nonsignificant trend towards positive association between caries and cumulative percent

**Table 1: Percent distribution of *S. mutans* and its serotypes, *e, f* and *k* in CA and CF groups**

Variables	Children with DMFT/dmft index >5 [CA group] (n=60) (%)	Children with DMFT/dmft index <1 [CF group] (n=39) (%)
Age (range)	6-12 years	6-12 years
<i>S. mutans</i> positive	48/60 (80)*	17/39 (43.5)*
Single serotypes	23/48 (47.9)#	7/17 (41.2)#
<i>e</i>	7 (14.5)	1 (5.9)
<i>f</i>	3 (6.3)	2 (11.8)
<i>k</i>	13 (27.1)	4 (23.5)
Multiple serotypes	7/48 (14.5)	5/17 (29.4)
<i>e</i> and <i>f</i>	0	0
<i>e</i> and <i>k</i>	4	1
<i>f</i> and <i>k</i>	2	2
<i>e, f</i> and <i>k</i>	1	2
Undetermined serotype	18 (37.5)	5 (29.4)

\*The percentage is expressed relative to all (100%) of the CA and CF samples; #The percentage is expressed relative to all (100%) of *S. mutans* positive CA and CF samples. *S. mutans*: *Streptococcus mutans*; CA: Caries active; CF: Caries free; DMFT/dmft: Decayed, missing or filled teeth



**Figure 2:** A representative gel image of serotype specific polymerase chain reaction of caries active and caries free samples that had tested positive for *Streptococcus mutans* is shown (a) Serotype *e*, (b) Serotype *f*, and (c) serotype *k*

prevalence of single serotypes (*e*, *f* or *k*) [Table 1]. However, no such trend was observable with independent or cumulative percent prevalence of multiserotypes (*e* and *k*, *f* and *k* or *e*, *f* and *k*) [Table 1]. About 37.5% of CA and 29.4% of CF samples were not typeable for any of the three serotypes and hence were classified as undetermined serotype [Table 1].

## Discussion

Serotypes of *S. mutans* have been implicated in the development of dental caries.<sup>[10,11]</sup> In this study, we have analyzed the saliva samples of children with caries (CA) or without caries (CF) to investigate the prevalence of the three *S. mutans* serotypes: *e*, *f* and *k*. Both group of samples were initially screened for the prevalence of *S. mutans* by a strain specific PCR amplification protocol, which showed *S. mutans* presence in 80% ( $n = 48/60$ ) and 43% (17/39) of saliva samples from CA and CF groups respectively. The prevalence status was found to be statistically significant ( $P = 0.0001$ ) in agreement with those published earlier.<sup>[5]</sup> Subsequent serotype specific PCR of those saliva samples that had tested positive for *S. mutans* showed a high prevalence of serotype *k* in both CA

and CF groups [Table 1], which were followed by *e* and *f*. Most of the studies, however, have reported the occurrence of serotype *e* with highest prevalence that ranged from 2% to 30% followed by serotypes: *f* and *k* in the range of 1-25% and around 5% respectively [Table 2].<sup>[17-28]</sup> Since the findings of our study are in contrary to those that have been observed earlier, we speculate that this could be due to a shift in *S. mutans* serotype in subjects from this region. Such serotype shifts have been reported elsewhere in other strains such as *Streptococcus agalactiae* from Group B *Streptococcus* for example, where in *S. agalactiae* serotype *la* was found to occur consistently with a higher prevalence in North American population relative to those from other geographic regions ( $P < 0.005$ ).<sup>[29]</sup>

More recent case controlled studies have identified multiserotypes in up to 53.8% ( $n = 21/39$ ) of subjects.<sup>[10]</sup> However, we observed multiserotypes only in 14.5% ( $n = 7/48$ ) and 29.4% ( $n = 5/17$ ) of CA and CF samples respectively. This contradictory observation may be due to the fact that up to 37.5% of CA and 29.4% of CF samples were nontypeable and hence may have contributed toward the lower prevalence of multiserotypes. In addition, inclusion of serotype *c* analysis and discerning of

**Table 2: Distribution of *S. mutans* serotypes *e*, *f* and *k* in the oral cavity of individuals from different population, and techniques used to identify them**

Population	Method	Number of subjects	e %	f %	k %	Reference
Denmark	Immunofluorescence and biochemical tests	114	7	6	-	[17]
North America (Illinois)	Biochemical tests	194	5	-	-	[18]
Japan	FITC	83	1.60	-	-	[19]
Saudi Arabia	Biochemical tests	196	7.1	-	-	[20]
North America	Biochemical tests					[20]
Great Lakes		194	0.5			
Orlando		30	0			
San Diego		24	4.2			
Hawaiian		50	2			
Japan	Cell diffusion	22	18.18	-	-	[21]
Columbia	Culture medium	142	14.1	25.4	-	[22]
Maine, South America	Culture medium		21.1	10.6		
Maine, South America	FA methods		23.8	5.3		
Iceland	Cell diffusion	75	25.30	-	-	[23]
Finland	Immunodiffusion	16	10	-	-	[24]
Vietnam	Immunodiffusion	16	31	-	-	[24]
Finland	Immunodiffusion	34	26	3	-	[25]
Japan	DNA finger printing	76	12.50	-	-	[26]
Japan	PCR	432	13.30	1.90	-	[11]
Japan	Immunodiffusion	100 (2002)	17	3	2	[9]
		50 (2003)	16	4	2	
Japan	PCR	410	7.00	1.00	-	[27]
Japan	PCR	73 (mothers)	32.4	4.4	0	[10]
		78 (children)	21.7	5.8	2.9	
Thailand	Immunodiffusion, PCR	50	22.80	4.40	2.80	[28]

PCR: Polymerase chain reaction; *S. mutans*: *Streptococcus mutans*; FITC: Fluorescein IsoThioCyanate; FA: Fluorescent antibody

undetermined serotypes may have significantly increased the probability of multisero-type identification. It is important to note that most of the studies that reported *S. mutans* serotypes prevalence employed biochemical, immunodiffusion or immunofluorescence techniques to determine them. *S. mutans* serotype specific PCR is a relatively recent technical advent, which was invented and used successfully by Shibata *et al.* and Nakano *et al.*,<sup>[11-13]</sup> to identify the prevalence in children including those with IE. This technique was employed in the present study as it: (1) Is highly sensitive and is believed to be more effective in detecting the presence of target molecule (of a strain or serotype) relative to immunostaining<sup>[30]</sup> and is capable of detecting *S. mutans* and its serotypes from 1 to 10 pg of template DNA,<sup>[10]</sup> and (2) provides a platform to screen large number of samples rapidly. Based on these facts, we believe that the inclusion of other detection techniques such as biochemical, immunodiffusion or immunofluorescence methods in this study may not have contributed to an increase in the significance of percent prevalence of either single or multiple serotypes. Hence, in the present scenario, the identification of a nonsignificant trend towards positive association between caries and prevalence of independent serotypes, especially that of *k* and *e*, may be expected to reach a significant trend when a larger sample size is analyzed.

## Conclusion

This study brings forth two observations: (1) A novel prevalence hierarchy of the three determined *S. mutans* serotypes: *k*, *e* and *f* in the saliva of school children from this region, and (2) a nonsignificant positive association between caries and serotypes *k* and *e*.

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