Original Article

Polymerase chain reaction-based identification of various serotypes of *Streptococcus mutans* in adults with and without dental caries

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Abstract

Background: Dental caries is a multistep process which initiates the development of plaque, defined as a structured biofilm containing microbial communities. Teeth provide unique surfaces for bacterial colonization. Serotypes of *Streptococcus mutans* implicate the development of dental caries.

Aim: The aim of the study was to determine the prevalence and association of serotypes of *S. mutans* in groups with and without dental caries.

Materials and Methods: One hundred and fifty adults aged between 18 and 35 years were included in the study. Supragingival plaque samples were collected, followed by deoxyribonucleic acid extraction. Polymerase chain reaction was performed to identify *S. mutans* and its serotypes. Proportions of *S. mutans* and its serotypes were correlated with caries-active (CA) and caries-free (CF) groups.

Results: CA group showed 66.7% positivity for *S. mutans* and CF group showed only 42.7% of positivity. Serotype C showed a higher proportion followed by E, F, and K in the CA group, whereas in the CF group, higher proportion was observed with K followed by C, E, and F. 70.8% cases showed single serotype in the CA group and 83.3% in CF group. Multiple serotypes were seen in 29.2% in the CA group and 16.7% in the CF group.

Conclusions: The study clearly established variation in proportions of *S. mutans* and its serotypes between CA and CF groups. Positive correlation was observed in the CA group for *S. mutans* and its serotypes.

Keywords: Polymerase chain reaction; serotypes; *Streptococcus mutans*

INTRODUCTION

Dental caries is a multistep process which initiates by the development of plaque, defined as a structured biofilm containing microbial communities.[1] Worldwide, prevalence of dental caries is as high as 100% among adults.[2] According to a recent systematic review and meta-analysis, the overall prevalence of dental caries in the Indian population between the age group of 3 and 75 years was 54.16%, which was in agreement with the National Oral Health Survey 2004.[3] In rural adolescents, the prevalence of dental caries is as high as 72%.[4] Risk factors for dental caries are microbiological and dietary factors.[5]

Clarke (1924) separated an organism from carious lesions naming it *Streptococcus mutans*, as he assumed

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the oval shaped cells he detected were mutant forms of Streptococci. Nevertheless, it was only in the late 1950's that *S. mutans* received a greater consideration from the scientific community, and by the mid 1960s, it was accepted as a principal etiological agent in dental caries.[2] In the following two decades, investigators began to unearth the pathophysiology of *S. mutans*. During this period, the paramount techniques for studying *S. mutans in vitro* and *in vivo* were evolved.[6]

Although colony-forming units microbial method using mitis-salivarius-bacitracin agar and monoclonal antibodies provided a valuable evidence of *S. mutans* as an early initiator of decay, molecular techniques such as polymerase chain reaction (PCR) detect live/dead bacteria. In addition, molecular methodologies can determine not only the basis of cariogenic bacteria, but also the potential virulence each strain attained or is likely to be transmitted.

It was recognized that the serotypes of *S. mutans* (*c, e, f,* and *k*) have been associated with the development of dental caries. In *S. mutans*, carbohydrate antigens that are specific to a serotype are confined to the cell wall.[7] Studies have been intended to amplify specific variant regions of rhamnose-glucose polymer (*rgp*) operon,[8] which encodes for enzymes that catalyze the formation of serotype-specific *rgp*'s in the cell wall and is used to classify the four serotypes of *S. mutans*: *C*, *e*, *f,* and *k*.[9] These antigens of *S. mutans* are composed of α1,2- and α1,3-linked rhamnose backbone with glucose α1,2-linked in serotype *c* isolates, β1,2- and α1,3-in serotypes *e* and *f,* respectively.[10] Serotype *k* strains have a unique "untypeable" phenotype in terms of known serotype antibodies because they lack glucose side chains linked to their rhamnose backbones. Serotype *k* strains seem to have been derived principally from serotype *c* rather than serotype *e* and *f*.[11] Recent studies have shown a relationship between serotypes of *S. mutans* and infective endocarditis (IE), an inflammatory condition of endothelial cells of the heart. A study in Japan on IE has found that the most common causative microorganisms are streptococci (approximately 50%).[12] *S. mutans* serotypes have certainly been found in the heart valves and blood samples of patients with IE.[13] These findings highlight the clinical significance of elucidating the different serotypes of *S. mutans* 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 groups.[8,9] Results of the same have been reported elsewhere.[14] Since the findings of studies on serotypes *e*, *f*, k, are in contrary to those that have been observed earlier, researchers predicted that it could be due to a shift in *S. mutans* serotype in subjects from region to region and also ecological shift of microbes observed from childhood to adulthood, thus making it necessary to study *S. mutans* serotypes in adults. Studies on the detection and prevalence of *S. mutans* serotypes in adults are lacking. The recent advances in molecular techniques have permitted prompt identification of targeted bacterial species in specimens, with significantly enhanced specificity and sensitivity. Hence, this study aims to determine the association of serotypes of *S. mutans* and its overall proportions in adults with and without dental caries using molecular techniques.

MATERIALS AND METHODS

Patient selection

A total of 150 individuals who visited the outpatient department of the institute for routine oral examination and dental treatment were enrolled. The subjects were equally divided into two groups of 75 each, where Group 1 included individuals with decayed, missing, filled teeth (DMFT) >5 as caries active (CA) group and Group 2 included individuals DMFT <1 as caries-free (CF) group. Caries is considered the predominant oral health cause of disability-adjusted life years (DALY's) among people ≤35 years of age, whereas extensive tooth loss was prevalent and an important contributor to DALY's among middle-aged and older adults.[15] Hence, we chose to conduct a study on subjects with the age range of 18–35 years. Individuals undergoing orthodontic treatment, suffering from periodontitis, with physical/mental disabilities, on antibiotic therapy within the previous 3 months, soft-tissue pathologies and chronic habits such as smoking, betel nut and tobacco chewing, and alcohol intake were excluded from the study. Based on the study observation done by Punitha *et al*., we considered subjects who consume three meals a day and those who did not consume any junk food/snacks in between the meals.[16] The medical records of these patients were reviewed, and information concerning gender and age was noted. Written informed consent was obtained from all the subjects who were enrolled in the study. Ethical clearance was obtained from the institute's ethical review board (The number of the certificate was 2015-16/1121).

Oral microbial ecology is diverse and site specific. Hence, in the present experimental study, plaque samples from site-specific areas such as buccal surfaces of the maxillary molar and lingual surfaces of mandibular molars were used because these surfaces are bathed in fresh saliva from the openings of adjacent salivary ducts which causes removal of debris and allows the presence of plaque.[17]

Sample collection

Plaque samples of 150 subjects were collected using sterile universal curettes (4R/4L). Supragingival plaque samples were collected from CA and CF groups. The samples were then transferred into an Eppendorf tube containing Tris- ethylenediaminetetraacetic acid (EDTA) (TE) buffer under sterile conditions and sent to the central research laboratory of the institute immediately for processing. Deoxyribonucleic acid (DNA) extraction was done by

modified proteinase K method and stored at −80°C until PCR analysis was carried out.

Deoxyribonucleic acid extraction

DNA extraction was carried out by spinning the plaque sample pellet at 7435 g force for 5 min at room temperature. Pelleted cells were resuspended in 500 µL fresh TE buffer centrifuged for 3–4 min. The procedure was repeated 3–4 times with fresh TE buffer. After discarding supernatant, 50 µL lysis buffer I (1M Tris buffer; Triton X-100; and 0.5M EDTA) was added and vortexed for 5 min. To this, 50 µL lysis buffer II and 10 µL proteinase-K (100 µg/mL) were added, and mixture was vortexed vigorously. The tube was kept in the water bath at 60°C for 2 h, then in boiling water bath for 10 min. The extracted DNA was stored at −80°C until used for PCR analysis.

Polymerase chain reaction analysis

Identification of *S. mutans* and serotype *k* was carried out by conventional PCR method. Identification of serotypes *c, e,* and *f* was carried out using multiplex PCR using specific primers [Table 1]. *S. mutans* was identified by species-specific primers based on the 16S rRNA gene sequences. 25 µL of premix containing 12.5 µL of AMPLIQON RED 2X Mastermix (Tris-HCL pH 8.5, $[NH_4]_2SO_4$, 3 mM $MgCl_2$, 0.2% Tween 20; 0.4 mM of each dNTP; 0.2 units/µL AmpliqonTaq DNA Polymerase; Inert red dye and stabilizer), 10 pmole/µL of forward primer and 10 pmole/µL of reverse primer was mixed with 3 µL of template DNA. Vortexing was done, and tubes were placed in a thermocycler, and amplification conditions were set (initial denaturation: 95°C, 5 min; Annealing: 56°C, 30 s; Extension: 72°C, 1 min; Final extension: 72°C for 5 min). Thirty-five cycles of amplification were employed for *S. mutans*-specific PCR. The control strain used for *S. mutans* identification was ATCC No 25175.

The PCR thermal cycling conditions for multiplex reaction (Serotype *c, e,* and *f*) were initial denaturation at 95°C for 5 min, a total of 30 PCR cycles were performed consisting 30 s of denaturation at 95°C, 30 s of annealing at 50°C, and 1 min of extension at 72°C. Final extension was carried out at 72°C for 5 min followed by storage at 4°C.

For serotype *k*, the PCR conditions were similar to that of conventional PCR carried out for amplification of *S. mutans* except for the annealing temperature which was maintained at 60°C for 30 s. Amplified products of serotypes were detected by agarose gel electrophoresis and bands were compared with DNA ladder.[8,9] Results were tabulated, and statistical analysis was done using SPSS software version 14.0 version, SPSS Inc., Chicago, IL, (USA). The statistical significance of the data was evaluated by Fisher's exact test.

RESULTS

The study includes a total of 150 subjects of 54 males and 96 females with mean age of 26 years in CA and 21.8 years in CF. The mean DMFT was 7.7 for CA group. Gel electrophoresis analysis of the PCR amplified samples showed *S. mutans*-specific band [Figure 1] in 66.7% (50/75) of CA and 42.7% (32/75) of CF groups, and the difference was statistically insignificant.

Gel analysis of the PCR amplification reaction showed serotype-specific DNA band with respective sets of primers

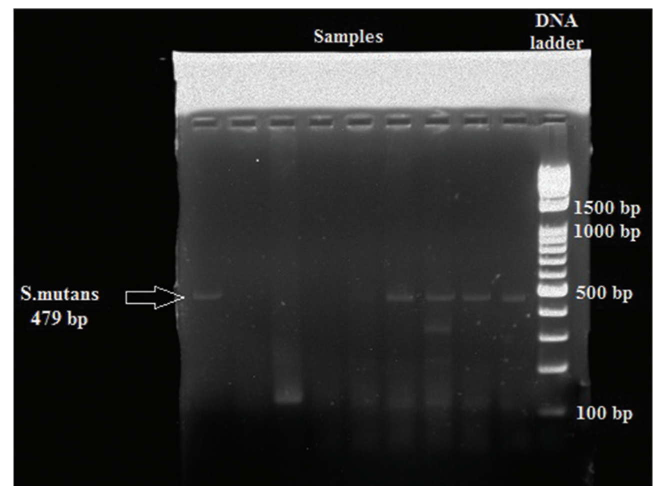

Figure 1: Polymerase chain reaction - generated deoxyribonucleic acid bands in gel electrophoresis. Arrow shows clinical samples positive for *Streptococcus mutans* (479 bp band). DNA: Deoxyribonucleic acid, *S. mutans*: *Streptococcus mutans*

Table 1: Strain specific primers

Strain	Specific Primer
S. mutans (amplicon size 479 base pair)[18]	Forward primer: 5′-TCGCGAAAAAGATAAACAAAC A-3′ Reverse primer: 5′-GCC CCTTCACAGTTG GTTAG-3′
Serotype *c* (amplicon size 727 bp)[9]	Forward primer: 5′-CGGAGTGCTTTTTACAAGTGCTGG-3′ Reverse primer: 5′-AACCACGGCCAGCAAACCCTTTAT-3′
Serotype *e* (amplicon size 517 bp)[9]	Forward primer: 5′-CCTGCTTTTCAAGTACCTTTCGCC-3′ Reverse primer: 5′-CTGCTTGCCAAGCCCTACTAGAAA-3′
Serotype *f* (amplicon size 316 bp)[9]	Forward primer: 5′-CCCACAATTGGCTTCAAGAGGAGA-3′ Reverse primer: 5′-TGCGAAACCATAAGCATAGCGAGG-3′
Serotype *k* (amplicon size 293 bp)[8]	Forward primer: 5′-ATTCCCGCCGTTGGACCATTCC-3′ Reverse primer: 5′-CCAATGTGATTCATCCCATCAC-3′

S. mutans: Streptococcus mutans

for c, *e*, *f* [Figure 2], and *k* [Figure 3] in both CA and CF groups, which were confirmed by their relative band size of migration with reference to respective positive controls. The number of samples that tested positive for either one of the serotype c, *e*, *f*, and *k* in the CA group was 26, 15, 12, and 11, respectively, whereas the same in the CF group was 10, 8, 5, and 12, respectively [Table 2]. CA group has serotype *c* in high proportion, whereas serotype *k* is in higher proportion in the CF group. There was a nonsignificant trend toward a positive association between caries and cumulative percent prevalence of single serotypes (*c*, *e*, *f*, and *k*). About 4% of CA and 6.3% of CF samples were not typeable for any of the three serotypes and hence were classified as untypeable serotypes.

DISCUSSION

S. mutans is observed in the greatest number of samples from subjects with caries, and levels were considerably related to the presence or absence of caries.[4,19] Although *S. mutans* is one of the many cariogenic organisms for causing dental caries, it has a crucial role in the initiation of caries, and four different serotypes of *S. mutans* have been identified and have been implicated in dental caries.[17] A study conducted by Nakano *et al*. points out serotype *c* as the ancestral strain of *S. mutans*, and the other serotypes have branched from the serotype *c* unceasingly in the course of evolution hitherto.[20] Apart from dental caries, these serotypes of *S. mutans* have shown a predominant role in IE.[21] It is also of particular importance to understand the microbial etiology of the initiation of caries to interrupt the process before irreversible damage occurs to a tooth using preventive interventions such as probiotics or vaccines.[22] It is a known fact that major serotypes in CA children are serotypes *c* and *e*.[23-25] The prevalence of major serotypes in adult carious lesions is not well known. Considering the ecological shift in oral microbiota from children to adults, there could also be a shift in the prevalence of serotypes of *S. mutans* from children to adults. This makes it necessary to identify and redefine the serotypes of *S. mutans* as it is a causal agent for the initiation of dental caries. Hence, this study was ventured to identify *S. mutans*, and its serotypes and their association in CA and CF subjects among the adult population.

A higher prevalence of *S. mutans* positivity was observed in the CA group (66.7%) than the CF group (42.7%). A similar study conducted in plaque samples by Hirasawa *et al*. in the Japanese population with an age group of 6–28 years showed higher levels of positivity (95.7%) in the CA group

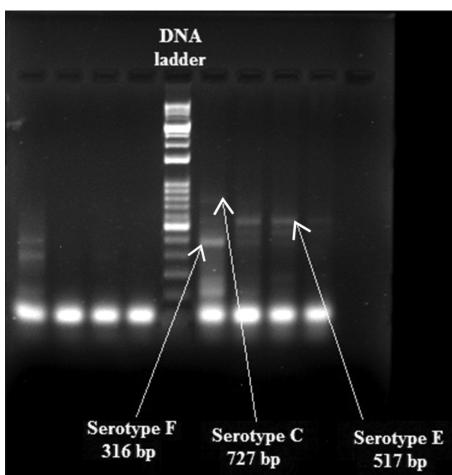

Figure 2: Polymerase chain reaction - generated deoxyribonucleic acid bands in gel electrophoresis. Arrow shows clinical samples positive for serotype *c, e, f* (727 bp, 517 bp, and 316 bp bands, respectively). DNA: Deoxyribonucleic acid

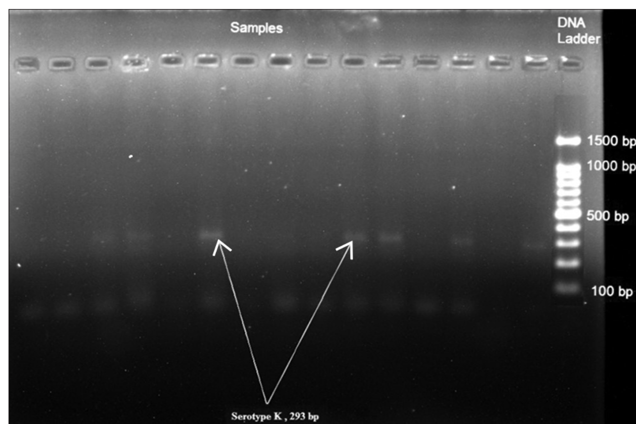

Figure 3: Polymerase chain reaction - generated deoxyribonucleic acid bands in gel electrophoresis. Arrow shows clinical samples positive for Serotype *k* (293 bp band). DNA: Deoxyribonucleic acid

Table 2: Proportions of *Streptococcus mutans* serotypes and its association in caries-active and caries-free groups

Variables	Overall positive cases, *n* (%)	CA (*n*=75), *n* (%)	CF (*n*=75), *n* (%)	Fisher's exact test
S. mutans positive	82 (54.7)	50 (66.7)	32 (42.7)	*P*=0.05; NS
Serotype *c*†	36 (43.9)	26 (52)	10 (31.3)	*P*=0.073; NS
Serotype *e*†	23 (28)	15 (30)	8 (25)	*P*=0.802; NS
Serotype *f*†	17 (20.7)	12 (24)	5 (15.6)	*P*=0.415; NS
Serotype *k*†	23 (28)	11 (22)	12 (37.5)	*P*=0.140; NS
Untypable†	4 (4.9)	2 (4)	2 (6.3)	*P*=0.641; NS
Single serotype‡	59 (75.6)	34 (70.8)	25 (83.3)	*P*=0.282; NS
Multiple serotypes‡	19 (24.4)	14 (29.2)	5 (16.7)	

†Percentages calculated on 82 *S. mutans* positive samples, ‡Percentages calculated on 78 serotype positive samples excluding 4 samples which are untypable. $P<0.05$ was considered statistically significant. NS: Nonsignificant, CA: Caries active, CF: Caries free, *S. mutans: Streptococcus mutans*

and lower levels of *S. mutans* (58.3%) in CF group using PCR. Our study results of *S. mutans* in the CF group were similar to the results found in saliva samples of Rao and Austin study conducted in a specific Indian population where they identified 43.5% positivity in the CF group.[17] Keene *et al*. in plaque samples of naval officers showed higher proportions of *S. mutans* in ascending order of Orlando, Saudi Arabia, San Diego, and Hawaii.[26] Higher levels of *S. mutans* were observed in studies conducted in Vietnam (100%) and Finland (62%) by Hölttä *et al*.[27] attributing ethnic groups as one of the factors.

PCR conditions for serotypes *c*, *e,* and *f* were similar. Hence, multiplex PCR was carried out to identify serotypes *c*, *e,* and *f*. PCR condition was different for serotype *k*. Thus, conventional PCR was carried out to identify serotype *k*. Serotypes *c*, *e,* and *f* reveal proportions of 52%, 30%, and 24%, respectively, in the CA group which was corroborative with the findings of studies conducted by Keene *et al*. and Hölttä *et al*. in plaque samples. In the CF group, proportions of serotypes *c, e,* and *f* group were 31.3%, 25%, and 15.6%, respectively. These results were in contrast with a study conducted by Lapirattanakul *et al*. on plaque samples of the Thailand population with an age range of 13–62 years (*c* – 70% and *f* – 4.4%) using immunodiffusion technique.[21] These variations in serotypes provide an insight how microbial ecology differs with the factors associated with dental caries even within a species.

Newer research has pointed out that serotype *k* strains may possibly be dispersed worldwide with a varied frequency in the oral cavity. It is speculated that serotype *k* strains have lost the antigens or determinants that are recognized by host innate and adaptive immune defenses. The presence or absence of these surface antigens associated with virulence are unique features in these strains.[28] Nakano and Ooshima learned the phenotypic origin of "serotype *k*" of *S. mutans* as genetic dysfunction of serotype *c* or *f* strains. It is also conceivable that all *S. mutans* strains may have the potential to become serotype *k* in future.[20] The present study revealed a higher prevalence of serotype *k* in the CF group than in the CA group. In our study, serotype *k* was in a higher proportion in CF group than in CA group. This observation is in contrast with the study conducted by Rao *et al*. where serotype *k* was higher in CA (27.1%) than in CF (23.5%). Many studies, however, have reported the occurrence of serotype *c* with the highest prevalence that ranged from 8% to 100%,[17,27] followed by serotypes *e*, *f,* and *k* in the range of 1%–32%,[13,29] 1.9%–32%,[8,9] and 2%–27%,[17,18] respectively. Additional studies focusing on serotype *k* of *S. mutans* strains are needed, which may clarify the association of these strains with dental caries.

Preliminary studies indicated serotypes *c* and *e* as frequently identified combinations.[27] An increase in newer technical modalities noted higher combinations of serotypes. In the present study, single serotypes were seen in 70.8% in the CA group and 83.3% in the CF group. Multiple serotypes [Table 3] were seen in higher proportions in the CA group (29.2%) than in the CF group (16.7%) in the present study. These results are in contrast to the study conducted on saliva samples by Rao *et al*. in schoolchildren. The most common multiple serotype combination was *c* and *e* in literature,[17] but our study revealed *c* and *f* and *c* and *k* as the most common multiple serotypes. These results imply an association between higher proportions of multiple serotypes and dental caries and difference in proportions among children and adults. The probable reason for this variation can be attributed to diversity in sample collection method, geographic location, and sample size.[17]

This study identified two untypeable serotypes separately in both CA and CF groups. Similar results were found (5%) in a study conducted by Hölttä *et al*. in plaque samples of children.[27] Further studies have to be carried out to understand these serotypes. There are only a few studies in the literature where they tried to identify the association of *S. mutans* in CA and CF groups among adult population.

Most of the previous studies reported *S. mutans* serotypes using biochemical, immunodiffusion, or immunofluorescence techniques to determine them. *S. mutans* serotype-specific PCR is a relatively recent advance, which was invented and used effectively by Shibata *et al*.[17] and Nakano *et al*.[17,20,28] PCR technique was employed in this study as it is highly sensitive and is more effective in detecting the presence of serotypes relative to immunostaining and is capable of detecting *S. mutans* and its serotypes from 1 to 10 pg of template DNA[22] and can be used to screen a large number of samples in epidemiological studies. Hence, in the present study, the identification of a nonsignificant trend toward positive association between caries and independent serotypes, especially that of *c* and *k* may be expected to reach a significant trend when longitudinal studies with a larger sample size are analyzed, which will be the future scope.

CONCLUSIONS

This study gives an insight into the various serotypes of *S. mutans* and their associations in CA and CF adult

Table 3: Combinations of multiple serotypes in *Streptococcus mutans*-positive cases

Variables	CA (*n*=75)	CF (*n*=75)
e and *f*	2	1
c and *f*	4	0
c and *e* and *k*	2	0
c and *k*	4	1
e and *k*	1	1
f and *k*	1	1
c and *e*	0	1

CA: Caries active, CF: Caries free

populations. Results were not statistically significant and can be attributed to a smaller sample size. The finding of this study showed variation in the serotypes between CA and CF groups. Suggesting that, it is not only the identification of the organism but also the recognition of serotype is also important. The clinical implication of this study would be, helping us understand the particular serotype which involved in the formation of dental caries. Furthermore, the knowledge of the higher prevalence of which serotypes is known to cause IE, which will help us prevent the IE at early stages. Studying these different serotypes will guide us in future for the development of specific vaccines, to target the specific serotype. So as to clinically prevent the formation of dental caries and systemic complications that might arise (ex: IE). Most of the previous studies are conducted in the younger age or mixed age groups, but this study done on adults showed different results compared to other studies done. This is mainly due to the transition of microflora from younger to adult groups. Hence, there is a need to conduct longitudinal studies to see the variations in prevalence rates of specific serotypes from childhood to adulthood.

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Conflicts of interest

There are no conflicts of interest.

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