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***Streptococcus mutans* serotypes and Collagen-Binding Proteins Cnm/Cbm in Children with Caries analyzed by PCR**

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SUMMARY

Streptococcus mutans, a primary bacterium associated with dental caries, has four known clinical serotypes (*c*, *e*, *f*, and *k*). Some serotypes, presence of multiple serotypes and strains with collagen-binding proteins (CBP, Cnm and Cbm) have been linked with systemic disease. Evaluation of *S. mutans* serotype distribution and caries association is needed in the United States. The purpose of this study was to evaluate the prevalence of *S. mutans* serotypes from two cohorts of African American children in rural Alabama using 3 sample types (saliva, plaque, and individual *S. mutans* isolates) by PCR detection for association with caries. Detection of CBP was also performed by PCR. In total, 129 children were evaluated and overall prevalence of serotypes were: serotype *c*(98%), *e*(26%), *f*(7%), and *k*(52%). Serotype *c* was statistically associated with higher caries scores in older children ($p<0.001$) and serotype *k* was statistically more likely in females ($p=0.004$). Fourteen percent of children had CBP. Thirteen *S. mutans* isolates from 5 children tested positive for both CBP. This study is the first to report on prevalence of *S. mutans* serotypes in a US population using the PCR-based approach. The frequency of serotype *k* in this study is the highest reported in any population, illustrating the need for further study to determine prevalence of this clinically relevant serotype in the US. This is the first study to report *S. mutans* isolates with both Cnm and Cbm in the same strain, and further analysis is need to determine the clinical significance of these strains.

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CONFLICTS OF INTERESTS

The authors have no conflicts of interest to declare.

SUPPORTING INFORMATION

Additional Supporting information may be found online in the supporting information tab for this article.

Keywords

Streptococcus mutans; serotypes; collagen-binding proteins; dental caries; PCR

1 | INTRODUCTION

Streptococcus mutans, one of the primary bacteria associated with dental caries, has four known clinical serotypes *c*, *e*, *f* and *k*.^{1–3} The most common serotypes are serotype *c* and *e* comprising approximately 70% and 20% of *S. mutans* strains isolated from the oral cavity, respectively, using traditional plate culture methodology and immunodiffusion assays.^{4,5} Serotypes *f* and *k* are considered minor oral serotypes since these are recovered less frequently from oral samples and typically make up less than 5% of *S. mutans* strains each.^{4,6} Serotypes are differentiated based on difference in the glucose side chains of the rhamnose-glucose polysaccharide (RGP) on the cell wall. Serotype *c* (α 1,2 linkage), serotype *e* (β 1,2 linkage) and serotype *f* (α 1,3 linkage) have unique glucose side chain linkages, whereas serotype *k* is characterized by the lack of glucosidic residues.^{3,7,8}

All four serotypes were originally described using immunodiffusion method.^{2,3} Studies have already summarized the prevalence of *S. mutans* serotypes by this approach and other traditional approaches.^{4,9} More recently, PCR based identification approaches have been developed and utilized for serotyping based on the variability in genes associated with the RGP synthesis. Shibata *et al.* established a multiplex PCR approach for serotypes *c*, *e*, and *f* based on variations in the sequences of *rgpH*, ORF3e and ORF2f respectively, of the RGP operon.¹⁰ Later, after the discovery and characterization of serotype *k*, Nakano *et al.*, developed a PCR approach for identification of serotype *k* based on variations in the *rgpF* gene as compared with serotype *c* strains.¹¹ Studies have been conducted to determine the distribution or prevalence of *S. mutans* serotypes using these PCR based methods in Central America, India, Thailand and Japan, but data on the prevalence of *S. mutans* serotypes using the PCR approach within the United States is limited to our previous study using random *S. mutans* isolates.^{5,6,9,12–15} Furthermore, summary analysis across different populations would be beneficial.

One of the key advantages of using the PCR based detection system is the ability to detect multiple serotypes within a single saliva or plaque sample without the need for culturing the bacteria. Previous studies have suggested that individuals typically harbor a single serotype of *S. mutans*.^{6,10,13} However, individuals with multiple *S. mutans* serotypes have been reported.^{3,6,9,10} It has been suggested that individuals with multiple serotypes are at higher caries risk,^{10,16} while others report a lower caries risk for individuals with multiple serotypes.⁹ Cardiovascular disease has been linked with multiple *S. mutans* serotypes, in particular serotypes *e* and *k*, as well as strains with undetermined serotype(s).¹⁴

The clinical significance of *S. mutans* in systemic disease may be related to two collagen-binding proteins that have been described for *S. mutans*; Cnm and Cbm encoded by the genes *cnm* and *cbm*, respectively.^{17,18} It has been suggested that *S. mutans* serotype *f* and *k* having either of these two proteins are more invasive to human endothelial cells and that serotype *k* may interfere with platelet aggregation leading to hemorrhagic stroke.^{18–20}

Patients with Cnm+ *S. mutans* strains isolated from the oral cavity were significantly more likely to have microbleeds, which are predictive risk factors for hemorrhagic and ischemic stroke.²¹

The purpose of this study was to determine the prevalence of *S. mutans* serotypes in children and infants using three sample types: whole saliva, dental plaque, and individual bacterial isolates, by a real-time PCR approach to determine possible associations between caries and serotype prevalence. The presence of collagen binding proteins Cnm and Cbm among *S. mutans* isolates from children and infants were also assessed.

2 | MATERIALS AND METHODS

2.1 | Study Population and Initial Exam

The University of Alabama at Birmingham (UAB) Institutional Review Board approved this study (#IRB-060328001). Parents of participating subjects gave informed consent, while children either gave assent for participation or was waived due to age. Oral samples were collected from two cohorts of children as part of a longitudinal epidemiological study of *S. mutans* in Uniontown, Alabama, USA, using methods previously reported and described briefly in the next section.^{22,23} Samples for Cohort 1 (CH1, N= 61) were pre-adolescent age (approximate age 11-12 years). Samples for Cohort 2 (CH2, N = 68) were younger children (approximate age 5-6 years). These two sample groups were selected since these were the most recently extracted and thus stored the shortest period (best sample integrity). All children were African-American from a low socioeconomic background and non-fluoridated water community.

Dental examinations were performed using three calibrated examiners (kappa scores for intra- and inter-examiner reliability were previously reported) as part of the larger epidemiological study using previously described methods.^{24,25} Caries scores were recorded using decayed, missing, filled surfaces (dmfs+DMFS, primary and permanent dentition, respectively) in accordance with the World Health Organization criteria.²⁶

2.2 | Sample selection

Three oral sample types were available for analysis including plaque, whole saliva, and 7-10 individual *S. mutans* bacterial isolates. For individual bacterial isolates, up to 10 presumptive *S. mutans* colonies (when available) were selected based on morphological appearance on Gold's Media (Mitis Salivarius, Becton Dickinson, Sparks, MD, USA; supplemented with bacitracin and sucrose).²⁷ When possible, individual isolates chosen for analysis were selected from the same individual and tooth as the plaque sample. In rare cases, when plaque DNA was unavailable, DNA from saliva or tongue isolates was analyzed. In addition to colony morphology, individual isolates were previously confirmed as *S. mutans* using *gfbB* sequence specific primers as part of the larger epidemiological study.^{22,28} Individual bacterial isolates were frozen, re-isolated on Todd Hewitt Agar and a single colony inoculated into 5 ml of Todd Hewitt Broth grown anaerobically at 37°C for 18-24 hours.

Individual tooth plaque samples were collected from all surfaces of a permanent molar tooth and from primary molars for CH1 and CH2, respectively using a sterile toothpick, then

stored in 1 mL of reduced transport medium.²⁹ Approximately 5 mL of expectorated whole stimulated saliva was collected from CH1 children after chewing paraffin. For CH2, all oral surfaces were swabbed and transferred to reduced transport media as the saliva sample. Each saliva sample was diluted 1:10 by transferring 0.5 mL of saliva into 4.5 mL of reduced transport medium. Samples were stored on ice and transported 100 miles to the UAB School of Dentistry for plating within 24 hours. Plaque and saliva DNA extractions were performed within 48 hours. Individual isolates were stored at -80°C until DNA extraction.

2.3 | DNA Isolation

DNA was extracted as previously described from 1 ml aliquots of plaque and whole saliva samples using The MasterPure™ Gram Positive DNA Purification Kit (Epicentre Biotechnologies, Madison, WI, USA) according to the manufacturer's instructions.³⁰ Individual bacterial isolates sample preparation and DNA extraction were performed as previously described.²² Briefly, an overnight (18-24 hours) 5 ml pure culture was centrifuged at 3,700 rpm for 10 minutes. Supernatant was discarded and remaining pellet washed with 1 ml phosphate buffered saline prior to extraction using the UltraClean Microbial DNA Isolation Kit (MoBio, Carlsbad, CA, USA) according to the manufacturers' instructions with the following modification to improve recovery of DNA from clinical *S. mutans*. A 5-minute incubation at 80°C was performed prior to beginning extraction and the bead beating time was extended to 10 minutes. The bead-beating step was performed using a Vortex Genie with adapter on medium high speed that generates minimal heat. DNA for each of the three sample types was stored at -20°C until PCR analysis was performed. Previous analysis of these samples using 16S and total Streptococci specific primers confirmed that extractions used for this study were successful.

2.4 | PCR Analysis

A listing of all primers used in this study can be found in Table S1. For plaque and saliva samples, conventional real-time PCR was performed using an IQ5 Thermal Cycler and Software (Bio-Rad Hercules, CA) using Fermentas Maxima SYBR Green/Rox qPCR Master Mix (2x) (Thermo Scientific, Lafayette, CO) in a 25 μL reaction with 2 μL of DNA template. Plaque and whole saliva samples PCR amplifications were performed for each serotype individually using the primers and conditions published, except cycles were increased from 30 cycles to 35 cycles to maximize detection.^{10,11} Both 2% agarose and 8% acrylamide gels were used to visualize amplicons.

PCR analysis for the individual bacterial isolates was performed using a multiplex PCR with the four previously published primer sets for each of the four serotypes. Confirmation of positives was performed using conventional PCR approach. Conditions used included a hot start, in accordance with the manufacturer's guidelines, of 95°C for 10 mins followed by 35 cycles of 95°C for 20 sec, 60°C for 30 sec, and 72°C for 45 sec. Melt curve data was obtained using a $60-95^{\circ}\text{C}$ temperature gradient with 0.5°C intervals. Control *S. mutans* prototype strains used for PCR analysis of plaque, whole saliva, and individual bacterial isolates were UA159 (serotype c), LM7 (serotype e), OMZ-175 (serotype f), and FT1 (serotype k). All PCR amplifications were performed in duplicate.

PCR analysis for collagen-binding proteins was performed on individual bacterial isolates using a multiplex approach conditions described above with previously published primers.³¹ Control *S. mutans* prototype strains used were OMZ-175 (*cnm* positive), YT1 (*cbm* positive) and UA159 (*cnm* and *cbm* negative). Since 13 strains presented with both *cnm*⁺ and *cbm*⁺, amplicons could not be reliably differentiated using the BioRad IQ5 software (single peak), but were visualized using 2% agarose and 8% acrylamide gels. A subset of isolates identified as amplifying both *cnm* and *cbm* (N = 2) by multiplex were next amplified separately then sequenced using Sanger sequencing through the UAB Heflin Genomics laboratory to confirm the presence of both *cnm* and *cbm* in single isolates by comparison of isolate sequence with NCBI sequences for Cnm and Cbm aligned using CLC DNA Workbench v5.7.1. Resulting consensus sequences for each strain were subject to NCBI blast alignments (performed Nov 20, 2018).

2.5 | Statistical Analysis

Statistical comparisons of frequency of serotype detections among plaque, saliva and individual isolates sample types were performed for each serotype individually using the extended McNemar's test. The comparisons were performed for children in CH1 alone, CH2 alone and both CH1 and CH2 combined. Saliva was selected as the reference sample type since saliva has been used in other PCR based serotyping publications as listed in Table 1 and saliva was considered to have a more standardized volume.

Discriminatory power, concordance and congruence were evaluated between sample types by Simpson's index of diversity (SID), Adjusted Rand, and Adjusted Wallace coefficients calculated using the Comparing Partitions software (<http://www.comparingpartitions.info>).^{32–36}

The statistical relationships between the presence/detection of each individual serotype (covariate) or CBP and dmfs/DMFS (outcome was defined as presence of decay ever during follow-up; yes/no, and severity of decay; maximum dmfs/DMFS count during any visit) were assessed using Chi square and negative binomial modeling, respectively in each cohort individually and combined. Also the relationship between sex and the presence of a particular serotype was assessed using Chi square. All analyses were conducted using SAS 9.4 (SAS Institute Inc., Cary, NC, USA) with the significance level set at $p < 0.05$. Furthermore, analysis for association for presence of single or multiple serotypes with dmfs/DMFS (yes/no, count) was also performed using Chi square and negative binomial modeling for count outcome (dmfs/DMFS counts).

3 | RESULTS

3.1 | Prevalence of Serotypes

A total of 129 children (CH1, n = 61; CH2, n = 68) were evaluated for prevalence of serotypes using three sample types: plaque (N = 129), saliva (N = 128), and individual bacterial isolates (N = 1,247 isolates from 127 children). All children evaluated were positive for *S. mutans* serotypes except Child #197. Saliva and plaque were both used to determine if results were comparable. Individual isolates were added to allow for

comparison with previously published studies. Prevalence of *S. mutans* serotypes by sample type and overall total for the current study are listed in Table 1. Note the overall values for the present study in Table 1 are derived from a positive result from any of the 3 sample types and is not an average of the 3 sample types since a child may be positive with one sample type but not another. The results were also compared with other published PCR-based serotyping analysis across different populations. The overall prevalence data from the current study was generated from the combined serotype profile from the three sample types evaluated. The combined prevalence and prevalence by age group (i.e., cohort) of each serotype are listed in Table S2. Statistical comparisons were performed for children with all 3 sample types (N = 126). High prevalence levels are reported for all serotypes, most notably serotype *k* (52%), when compared with the overall mean and median of other PCR based studies evaluated. Results for the current study indicate serotypes *e* and *k* were higher in the younger children, whereas serotype *f* and *k* were elevated in the older children.

Table 2 lists the number of children by cohort with multiple serotypes. Overall 59.7% of children presented with multiple serotypes, the most frequently observed combination was serotypes *c* and *k*. Single serotype *c* (N = 50) was detected most often whereas the other individual serotypes occurred rarely as single serotypes (serotypes *e* and *f*; N = 1 each). Although more children were analyzed in CH2, older children (CH1) had two more multiple serotype (i.e., *c*, *f* and *e*, *k*) not found for the younger children (CH2). Younger children were observed to have more single serotypes (CH2 had 29 vs. CH1 with 23). The prevalence of single and multiple serotypes are compared with other published studies in Table S3. Overall, the difference between single and multiple serotypes was not significant (data not shown).

Comparisons of serotype detection between sample types are reported in Table S4. Simpsons Index of Diversity (SID) was higher for saliva and plaque samples indicating either sample of these sample types can comparably discriminate between serotypes. Congruence as measured by Adjusted Rand was very low between all sample types. This low agreement is further supported by the Adjusted Wallace values, which indicate that, at best, a serotype predicted with saliva has about a 34% probability of predicting the serotype of individual isolates sampling but only a 13% probability of predicting serotypes identified by plaque sampling.

Table S5 provides the detection frequency of plaque and individual isolates as compared with saliva as a reference. Overall, serotypes *c*, *e*, and *k* were statistically significantly more likely to be detected in saliva for individual isolates (i.e., from a give subject) and serotype *c* ($p = 0.002$) for plaque samples. For the older children in CH1, serotypes were statistically significantly more likely to be detected in saliva than plaque for all 4 serotypes. In CH2, saliva was significantly more likely to detect serotype *c* than either plaque ($p = 0.016$) or individual isolates ($p = 0.001$). Insufficient numbers of serotype *f* were available for analysis with individual isolates.

3.2 | Serotypes and Caries

The mean caries score (dmfs/DMFS) was 19.82 (range 0-90) and the sex distribution was 73 males/56 females. The relationship of caries with serotype can be found in Table 3. Initially,

significant findings were found between *S. mutans* serotype *c* and dmfs/DMFS (count) for CH1 (IRR=21.5, p-value < 0.001). The serotype *c* primer was found to also amplify serotype *k* and subsequent analysis including only children with confirmed serotype *c* by individual isolates (N = 59) remained significant for dmfs/DMFS counts for CH1 (IRR=20.7, p-value < 0.001). No other significant associations between serotype (*e*, *f*, or *k*) and caries (yes/no) or dmfs/DMFS counts were observed. Overall, the likelihood of having serotype *k* was statistically significantly greater among females as compared to males with p-value = 0.004 (OR=2.79, 95% CI=1.35-5.75) (Table 4). No association was observed between having multiple serotypes and caries presence or count (data not shown).

3.3 | Prevalence of Collagen-binding Proteins and Caries

The genes encoding collagen-binding proteins Cnm and Cbm were detected among *S. mutans* isolates in this study by SYBR green real-time PCR and are shown in Table 5. Overall 14.2% of children were either *cnm*⁺ or *cbm*⁺. The gene encoding *cnm* was detected most commonly in children in CH1 (11.4%), whereas children in CH2 (13.6%) had more *cbm*⁺ strains. Both *cnm*⁺ (2.8%) and *cbm*⁺ (2.5%) strains in this population are frequently found in serotype *c* isolates, although serotype *e* and *k* were also reported. Serotype *e* presented the highest prevalence for *cnm*⁺ strains (3.4%) whereas *cbm*⁺ strains were more often serotype *k* (7.0%).

The relationship between caries and CBP was also assessed using caries (yes/no) and severity for 126 children. No significant associations were observed for CBP and caries (Table 3). Additional analysis of individual CBP was also not significant (data not shown).

A total of 5 children had *cnm/cbm*⁺ *S. mutans* isolates and these double band samples were found in multiple isolates analyzed (N = 13). Sanger sequencing confirmed for a subset of *cnm/cbm*⁺ isolates that the amplicons from the single strains were consistent with NCBI Cnm and Cbm sequences corresponding to the partial gene amplicons generated. NCBI Blast alignments resulted in 100% identity matches for *cbm* and 99-100% identity for *cnm* in these strains (Table S6). Interestingly, detection by SYBR green indicated very low peaks and bands were faintly visible on agarose or acrylamide gels. Furthermore, one band (either *cnm* or *cbm*) typically demonstrated a greater intensity as compare with the other. Sample contamination was ruled out with repetition and sequence analysis.

4 | DISCUSSION

4.1 | Overview

Serotype *k* is typically reported as representing less than 5% of *S. mutans* isolates.^{3,6,13,18} Our laboratory previously reported the first detection of serotype *k* in a United States population at a notably high prevalence of 17.5% from a random selection of *S. mutans* isolates.¹⁵ These findings suggested that the prevalence of serotype *k* in this population would be higher if biofilms (i.e., plaque) and saliva were evaluated instead of individual isolates. In the present study, the prevalence of *S. mutans* serotypes among young children (5-6 years) and pre-adolescents (11-12 years) in a high-caries risk, localized population in rural Perry County, Alabama was evaluated for 3 available samples types and assessed for

association with caries. The prevalence of genes for collagen binding proteins Cnm and Cbm and their association with caries were also assessed. The children in this study population have increased dental caries prevalence (65.8% by age 4) and typically higher *S. mutans* counts.²⁵

4.2 | Serotype Prevalence

The overall prevalence of *S. mutans* serotype *k* was remarkably higher than typically reported and represents the highest reported to date (Table 1). The prevalence of serotype *k* was equally represented between the two cohorts indicating age was not a factor in the detection of this serotype (Table S1). The next highest prevalence level (44.6%, Table 1) of serotype *k* was reported in India.⁹ The higher prevalence in the present study is likely due to the inclusion of additional sample types. Even when the prevalence of serotype *k* from saliva alone is considered separate from other sample types, the overall prevalence of serotype *k* remained the second highest reported at 35.2%.

The present study reports a higher prevalence for serotype *e* in the younger cohort (30.9%) than in the older cohort and this finding is similar to levels reported for Thai, Japanese and Finnish isolates (Tables 1 and S1).^{6,14} Detection of serotype *f* was elevated in the pre-adolescents cohort (CH1, 8.2%) as compared to the younger cohort (CH2, 5.9%) and was higher than typically reported by others (2.1-6%) but lower than the India study (18.5%).^{5,6,9,12-14}

Multiple serotypes were reported for 60% of the children in the current study (Tables 2 and S3). This finding is contrary to other published studies, which reported that the majority of subjects have only a single serotype (Table S3). Having multiple serotypes has been reportedly associated with having increased caries,^{10,16} however, in the present study no significant relationship between having multiple serotypes and caries was observed. Nonetheless, the high prevalence children with multiple serotypes may be of clinical concern since the next highest report of multiple serotypes was in heart specimens (Table S3) from a study in which the presence of multiple serotypes was associated with cardiovascular diseases.¹⁴

A unique aspect of this study is the use of three different sample types. As the data suggests, using only a single sample type tends to underreport the diversity of observed serotypes (Tables S4 and S5). Many studies typically used individual bacterial isolates isolated from selective media. The current data illustrates the bias of using only individual bacterial isolates to determine the serotype profile for a subject. PCR-based molecular typing methods allow for more sensitivity and detection of multiple serotypes in a single sample. The Simpson's Index of diversity suggest that using saliva or plaque will yield more serotype configurations and are more comparable than using individual isolates. Based on the data, it appears that using both sample types to evaluate prevalence of serotypes is most accurate, but if only one sample type is to be used, then the current data suggest that whole saliva may yield more results.

4.3 | Serotypes and Caries

Most studies investigating serotypes report only on *S. mutans* serotype distribution/prevalence. Few studies have looked at the relationship of specific serotypes with caries presence or severity.^{12,16,37} In this study, only serotype *c* was found to be significantly associated with higher dmfs/DMFS scores (ORR 21.5, p-value <0.001) for children in CH1. This finding is consistent with results reported by Seki, et al. who found serotype *c* was significantly associated with predicting caries development among 3-4 year old Japanese children, although a significant difference was not observed for the younger cohort, CH2, in the current study.¹⁶ The current study's association of serotype *c* is in contrast to findings reported by Carletto-Korber and colleagues, who reported that serotype *c* was significantly lower (p-value <0.05) in the higher caries score group among 6-8 year olds in Argentina.¹²

Using the multiplex approach, a number of the individual bacterial isolates in this study were observed to have two amplicons band that are consistent with the molecular weight of serotypes *c* (727 bp) and *k* (294 bp). Samples were confirmed using conventional PCR. Sequencing and repeated rep-PCR ruled out contamination. This is likely because the primers for the identification of serotypes *c*, *e*, and *f* were designed separately from serotype *k*.^{10,11} In the methods for primers design for serotype *k*, Nakano et al. stated that the *rgpH* gene had no significant difference between serotypes *c* and *k* in by sequence analysis, but serotype *c* primers were previously designed to *rgpH*.¹¹ This has not been reported before since serotypes *c*, *e*, and *f* are typically amplified separately from *k* in other PCR based studies. All individual bacterial isolates that tested positive with two bands by multiplex PCR in this study were assigned as serotype *k*. It should be noted that the primers for serotype *k* are specific and the lack of serotype *c* primer specificity has no impact on the serotype *k* prevalence reported in this study since multiplexing was only performed on individual isolates where only one serotype was expected. However, the prevalence of serotype *c* in this, and other published studies using PCR-based analyses, may be overestimated. In this study, to account for this possible overestimation a follow-up analysis was performed for only those children with serotype *c* using the data from the individual isolates and the findings remained significant for serotype *c* and CH1 dmfs/DMFS counts. A future direction will include the design of new primers for serotype *c*.

The finding that females in this study are significantly more likely to have serotype *k* was unexpected (Table 4). This finding was also significant in the Cohort 2 (younger children) but not in Cohort 1 (older children). Others have suggested that serotypes *e* and *k* are more often associated with cardiovascular events.¹⁴ In adults, typically males are at greater risk of infective endocarditis than females; whereas in pediatric infective endocarditis cases, the difference between sexes is not significant.³⁸⁻⁴⁰ However, there remains a need for more studies investigating the distribution of *S. mutans* serotypes and collagen-binding proteins obtained from cardiovascular specimens.

When using plaque or whole saliva as the DNA template, detection of some serotypes were possibly reduced as compared between each other and with the individual isolates for 3 reasons. First, *S. mutans* constitutes only a small portion of the whole saliva or plaque samples (1 ml used) as compared with isolated, pure culture strains (5 ml). Second, the DNA extraction methods used were different since these samples were processed at different times

during the course of the larger epidemiological study. Finally, difference between sample types may have been observed because of longer storage time for some DNA templates.

4.4 | Collagen-binding Proteins

Current literature suggests the prevalence of clinical *S. mutans* isolates that are *cnm+* is estimated to be approximately 10-20% and typically found to be serotype *f* or *k*.^{41,42} The prevalence of *cbm+* strains is estimated to be 2% and *cbm+* strains are most often found to be serotype *k*.¹⁸ The overall prevalence for *cnm+* and *cbm+* in the present study were 7% and 11%, respectively. Most of the *cnm+* and *cbm+* (79.7%, 55/69) strains were serotype *c*, while 18.8% (13/69) of strains were serotype *k* (Table 5). When considering the overall prevalence of strains that were *cnm+* and *cbm+*, this study supports the proposal that *cbm+* strains are most prevalent among serotype *k* strains, although at a lower proportion of only 7% in this study versus the 20% reported by Nomura *et al.*⁴¹ In the current study *cnm+* strains were most prevalent among serotype *c* (2.8%) and *e* (3.4%). Furthermore, no serotype *f* strains were identified from the individual bacterial isolates. Therefore, all *cbm+* strains in this study were either serotype *c* or *k*. It is noteworthy that all serotype *k* strains with CBP were *cbm+* strains, of which one was a *cnm+/cbm+* strain. Thus all serotype *k* were *cbm+*, but not all *cbm+* were serotype *k*. The increased prevalence of *cbm+* reported in this population may be of clinical significance, since *cbm+* strains have previously been reported to have significantly greater collagen-binding ability than *cnm+* strains.¹⁸

In contrast to recent publications that indicate a link between individuals with *cnm+* *S. mutans* and caries severity,^{43–46} no significant associations were observed in the current study between collagen-binding proteins and presence or severity of dental caries (Table 3). However, this study used only individual isolates and relatively few isolates were positive for collagen-binding proteins, thus the small sample size may account the lack of significance between CBP and caries in this study.

It has been shown that collagen-binding proteins encoded by the *cnm* and *cbm* genes found in *S. mutans* can have invasive qualities, thus allowing strains with these proteins to adhere to and invade human endothelial cells possibly leading to serious health concerns, such as infective endocarditis, cerebral microbleeds, and hemorrhagic stroke.^{4,19,21,47,48} Nomura and colleagues stated that the structure of Cbm is similar to Cnm and speculated that one of the one of these proteins could be a precursor to the other.¹⁸ In the present study a total of 13 isolates from 5 children demonstrated positive amplification for both genes encoding *cnm* and *cbm* within the same pure cultured isolate. To our knowledge, this is the first report of *S. mutans* strains containing both Cnm and Cbm collagen-binding proteins. These findings support that these genes are independent of each another, rather than one being a precursor to the other as previously suggested by Nomura *et al.*¹⁸ Strains analyzed were pure cultures and DNA was of known purity and concentration, thus it was surprising that amplification peaks and band intensities varied within an isolate. This may be due to lower primer binding affinity. Further study is needed to determine if these strains have increased collagen binding, platelet aggregation or cell invasive attributes.

4.5 | Conclusions

This study is the first to report on the prevalence of *S. mutans* serotypes in a United States localized population of African American children using the PCR based serotype identification approach. The notably high prevalence of *S. mutans* serotype *k* reported herein is the highest reported to date at 52%, highlighting the need for further investigation into this *S. mutans* serotype *k* and its association with systemic disease. Additional studies determining the frequency of *S. mutans* serotype *k* from oral and cardiovascular samples in other populations within the United States are needed. The use of three different samples types in this study suggests that using a single sample type for PCR analysis or using individual isolates from traditional culture methodology underreports serotypes. This study is the first to report *S. mutans* strains containing both collagen-binding proteins Cbm and Cnm in the same strain and further study is need to determine to the clinical significance of these strains.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Table 1.
Comparison of serotypes detected using three sample types analyzed in the present study with published studies using PCR approach.

Population	Sample Type	Number of Subjects	Subjects with Serotype Detected (%)				
			Serotype <i>c</i>	Serotype <i>e</i>	Serotype <i>f</i>	Serotype <i>k</i>	Serotype <i>l</i>
United States (Alabama, Present Study)	Saliva	128	78.9	17.2	4.7	35.2	
	Plaque	129	63.6	13.2	3.9	29.5	
	Isolates	127	89.8	3.9	0.0	22.8	
Overall		129	97.7	26.4	7.0	51.9	
Argentina ¹²	Isolates (S)	133	37.6	22.6	6.0	6.4	
South India ⁹	Saliva ^a	65	na	24.6	18.5	44.6	
Columbia ¹³	Isolates (S)	47	10.6	0.0	2.1	4.3	
Mexico ⁵	Isolates (S)	83	50.6	28.9	3.6	16.9	
Thailand ⁶	Isolates (S)	50	76.0	32.0	6.0	4.0	
Japan (81), Finland (12) ¹⁴	Saliva	192	76.0	36.5	4.2	4.5	
Japan ¹⁶	Isolates (S, P)	410	42.7	7.1	1.2	na	
Japan ¹⁰	Isolates (S, P)	214	83.2	13.1	1.9	na	
Mean from other studies			53.8	20.6	5.4	13.4	
Median from other studies			50.6	23.6	3.9	5.5	

Highest reported percentages for each serotype are in bold. For the present study saliva and plaque were analyzed by SYBR Green PCR; Individual isolates were analyzed using multiplex PCR. na = not analyzed, S = saliva, P = plaque

^a unstimulated saliva

Table 2. Children with single versus multiple *Streptococcus mutans* serotypes by age group detected by three sample types combined.

Cohort 1			Cohort 2			Overall					
Single Serotypes	Multiple Serotypes		Single Serotypes	Multiple Serotypes		Single Serotypes	Multiple Serotypes				
<i>c</i> only	23	<i>c, e</i>	6	<i>c</i> only	27	<i>c, e</i>	4	<i>c</i> only	50	<i>c, e</i>	10
<i>e</i> only	0	<i>c, f</i>	1	<i>e</i> only	1	<i>c, k</i>	18	<i>e</i> only	1	<i>c, f</i>	1
<i>f</i> only	0	<i>c, k</i>	22	<i>f</i> only	0	<i>c, e, k</i>	13	<i>f</i> only	0	<i>c, k</i>	40
<i>k</i> only	0	<i>e, k</i>	1	<i>k</i> only	1	<i>c, f, k</i>	1	<i>k</i> only	1	<i>e, k</i>	3
		<i>c, e, k</i>	4			<i>c, e, f, k</i>	3			<i>c, e, k</i>	15
		<i>c, f, k</i>	2							<i>c, f, k</i>	3
		<i>c, e, f, k</i>	2							<i>c, e, f, k</i>	5
Total Number of children			61				68				129
Total Children with multiple serotypes			38				39				77
% of Children with multiple serotypes			62.3%				57.4%				59.7%

Approximate ages were 11-12 years and 5-6 years for Cohort 1 and Cohort 2, respectively.

Table 3.

Overall bivariate results of the associations between dental caries (yes/no, count) or CBP (yes/no, count) and each of serotype.

Modeling caries as a dichotomous variable using Chi square and Fisher exact tests					
Variable	Level	Dental Caries		OR (95% CI)	p-value
		Yes	No		
Presence of serotype c	Yes	104	20	5.20 (0.31-86.6)	>0.05 ^a
	No	1	1		
Presence of serotype e	Yes	27	6	0.86 (0.3-2.5)	>0.05 ^b
	No	78	15		
Presence of serotype f	Yes	9	0	0.82 (0.8-0.9) ^c	>0.05 ^a
	No	96	21		
Presence of serotype k	Yes	55	11	1.00 (0.4-2.6)	>0.05 ^b
	No	50	10		
Presence of CBP	Yes	16	1	1.80 (0.4-8.5)	>0.05 ^a
	No	89	20		

Modeling caries as a count variable using negative binomial modeling		
Variable	Estimate (SE)	p-value
Presence of serotype c	2.60 (0.9)	>0.05 ^d
Presence of serotype e	-0.37 (0.26)	>0.05
Presence of serotype f	0.15 (0.45)	>0.05
Presence of serotype k	-0.14 (0.23)	>0.05
Presence of CBP	0.22 (0.33)	>0.05

^a Fisher exact test.

^b Chi square.

^c Relative Risk estimate was used because OR could not be calculated.

^d In analysis by cohort, for Cohort 1 (older children) serotype c and dmfs/DMFS (count) was significant (IRR=21.5, p-value < 0.001).

Table 4.

Bivariate results of the associations between sex and serotype.

Variable	Level	Sex		OR (95% CI)	p-value
		Male	Female		
Presence of serotype c	Yes	70	54	0.77 (0.1-12.6)	>0.05 ^a
	No	1	1		
Presence of serotype e	Yes	15	18	1.82 (0.8-4.0)	>0.05 ^b
	No	56	37		
Presence of serotype f	Yes	6	3	0.63 (0.2-2.6)	>0.05 ^a
	No	65	52		
Presence of serotype k	Yes	29	37	2.98 (1.4-6.2)	0.004 ^{b, c}
	No	42	18		

^a Fisher exact test.^b Chi square.^c In analysis by cohort, for Cohort 2 (younger children) females were significantly more likely to have serotype *k* (OR =6.07, 95% CI= 2.00-18.37, p-value < 0.001)

Table 5.

Prevalence of *cnm+* or *cbm+* for children and strains as detected by multiplex PCR using individual bacterial isolates.

Prevalence of <i>cnm+</i> and <i>cbm+</i> strains among children						
	Cohort 1		Cohort 2		Overall	
	Children (%)	No. of isolates	No. of isolates	Children (%)	Total Children (%)	No. of isolates
Total Children	61	619	66	628	127	1,247
Children with <i>cnm+</i>	7 (11.4)	26	2 (3.0)	5	9 (7.1)	31
Children with <i>cbm+</i>	5 (8.2)	10	9 (13.6)	29	14 (11.0)	39
Children with CBP	9 (14.7)	27	9 (13.6)	29	18 (14.2)	56
Children with <i>cnm+</i> / <i>cbm+</i> in same isolate	3 (4.9)	8	2 (3.0)	5	5 (3.9)	13
Children with CBP from serotype c	7 (11.5)	25	7 (10.6)	18	14 (11.0)	43
Children with CBP from serotype e	1 (1.6)	1	-	-	1 (<0.1)	1
Children CBP from serotype k	1 (1.6)	1	3 (4.5)	11	4 (3.1)	12
Prevalence of <i>cnm+</i> and <i>cbm+</i> among strains						
Serotype	<i>cnm+</i> (%)	<i>cbm+</i> (%)				
<i>c</i>	29/1046 (2.8)	26/1046 (2.5)				
<i>e</i>	1/29 (3.4)	-				
<i>f</i>	-	-				
<i>k</i>	1/171 (0.6)	12/171 (7.0)				
Total	31/1247 (2.5)	38/1247 (3.0)				

Note: Children may have both *cnm+* and *cbm+* as individual isolates or together in the same isolate. CBP: collagen-binding proteins, referring to either *cnm+* or *cbm+*.