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DETECTION OF SCARDOVIA GENUS FROM ORAL SAMPLES IN CHILDREN USING REAL-TIME QUANTITATIVE POLYMERASE CHAIN REACTION

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A THESIS

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DENTISTRY

ABSTRACT

Streptococcus mutans (Sm) and *Streptococcus sobrinus (Ss)*, are considered the major bacterium associated with dental caries. However, recent findings identify Scardovia species *(*Sc*)* is associated with early childhood caries, with or without the presence of *Sm* or *Ss*. This IRB approved study quantified the presence of *Sm* and Sc in saliva by real-time quantitative PCR (qPCR) in relation to caries. Saliva samples were collected from 72 preschool children in a high-caries risk community in rural Alabama. SYBR Green-based qPCR using extracted DNA and primers specific for *Sm, Ss*, Sc and total bacteria determined the copy number (CN/ml). Twenty two subjects (30.6%) were in caries-free group (DMFS/dmfs=0), whereas 50 (69.4%) subjects in caries-experience group (DMFS/dmfs>0). The detection limited for quantification was determined to be 1000 CN/ml. Sc was detected in 72.8% in the caries-free group and 80% of subjects in the caries-experience group. *Sm* was ubiquitous (approached 100%) in both groups. None of the samples had quantitatable levels of *Ss.* Generally, the

level of *Sm* was much higher than Sc. Although the caries-experience group had higher mean ratio of *Sm*/total bacteria and Sc/total bacteria than children in caries-free group, the only significant difference between the two groups was found in the mean ratio of Sc/total bacteria (p=0.022). No statistical difference was found in detection frequency between caries-experience and caries-free group in the three different bacterial combination groups (Sc negative+ *Sm* negative, Sc negative+ *Sm* positive, Sc positive+ *Sm* positive). The overall results indicated that Sc was highly detected in caries-experience group and the ratio of Sc/total bacteria was significantly associated with caries history. Although the presence of *Sm*, Sc or the combination of both bacteria didn't show correlation to caries, other factors (e.g., diet & dietary practices, ethnicity, oral hygiene factors, socioeconomic influences, host factors and general health conditions, medications) must also be considered since caries is a multifactorial disease.

Keywords: real-time quantitative PCR, *S. mutans*, Scardovia, dental caries

DEDICATION

This thesis is dedicated to my family; my mother, Ping-ping Miao; my brother: Timothy Liu and my father, Siou-shing Liou. Thank you for always being there to support me and encourage me to fulfill my dream to study aboard. It is also dedicated to my boyfriend; Chih-liang Tien. Thank you for always being with me when things are difficult. Thank you all for the supports and encouragement that I got from you all. I couldn't finish this without your help and support.

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LIST OF ABBREVATIONS

CN	Copy number
Cq	Quantification cycle
DMFS	Decay, missing, filled surface score in permanent teeth
dmfs	Decay, missing, filled surface score in primary teeth
DMFT	Decay, missing, filled tooth score in permanent teeth
dmft	Decay, missing, filled tooth score in primary teeth
DNA	Deoxyribonucleic acid
gtf	Glucosyltransferases
ECC	Early childhood caries
HIV	Human immunodeficiency virus
MS	Mutans streptococci
NHANES	National Health and Nutrition Examination Survey
PCR	Polymerase chain reaction
qPCR	Real-time quantitative polymerase chain reaction
RTF	Reduced transport fluid
Sc	Scardovia
S.D.	Standard deviation

S-ECC	Severer early childhood caries
Sm	*Streptococcus mutans*
Ss	*Streptococcus sobrinus*
Sw	*Scardovia wiggsiae*
TE buffer	Tris-EDTA buffer
Tm	Melting temperature
UAB	the University of Alabama at Birmingham
US	United States

INTRODUCTION

Dental caries is the most common chronic disease that affects children in the United States (US)[1, 2]. According to the data from National Health and Nutrition Examination Survey (NHANES) 1988–1994 and 1999–2004, dental caries is increasing among children aged 2–5 years[1]. The prevalence of caries was found to be 24% in children 2-5 years old in 1988-1994, and increased to 28% in 1999-2004[1]. The increasing rate between 1988–1994 and 1999–2004 was seen in numbers of restored dental surfaces (not untreated caries). This finding indicates that the increase in caries under 6 years old may be due to receiving more dental treatment[3].

According to guidelines from the American Association of Pediatric Dentistry, children are classified as having severe early childhood caries (S-ECC) if they present with more than 1 cavitated, missing (due to caries), or filled smooth surface in primary maxillary anterior teeth at age 3 to 5 or with a dmfs (decay, missing, filled surface) score of more than 4 at age 3, more than 5 at age 4, or more than 6 at age 5[4]. For children under three years old, any caries on smooth tooth surface is also classified as S-ECC. The impact of S-ECC in the United States cannot be underestimated. It not only affects the quality of life for the

children, but also may affect their families because of financial, educational, and social costs[5,6,7]. Untreated dental caries can result in a range of negative outcomes, from chronic pain to severe infection, threatening the child's life. Dental pain can also cause long-term problems for children with eating, speaking, and school attendance/performance. Treatment for S-ECC often requires full mouth rehabilitation with extensive treatment. For example, a 1996 study in Northern California[8] showed the cost of dental treatment for dmft (decay, missing, filled tooth) score 2-5 was $408 to $1725 for dmft score 16-20. According to the study, many patients failed to finish treatment because of the cost, and prices for these procedures are undoubtedly higher now. Due to uncooperative behavior of children at a young age, general anesthesia or deep sedation may be required for dental treatment, resulting in even higher costs. For example, one study from Louisiana reported that the cost for dental treatment in the hospital operating room was as much as fifteen times more than treatment that did not use general anesthesia[9]. In order to treat and prevent dental caries effectively, it is important to know the etiology of caries, including the bacteria that are associated with this infectious disease.

There are three main hypotheses for the cause of dental caries: the nonspecific plaque hypothesis, the specific plaque hypothesis, and the ecological plaque

hypothesis[10-12]. The nonspecific plaque hypothesis suggests that plaque, the accumulation of bacteria, is the cause of dental caries and that there is no bacteriological difference between plaque from a carious lesion and a non-carious lesion. However, the composition of plaque can vary from site to site. The specific plaque hypothesis states that the accumulation of certain bacteria, such as *Streptococcus mutans*, causes caries. The third hypothesis, the ecological plaque hypothesis, proposes that caries can be explained as a result of dynamic changes in the environment of dental plaque. Excess sugar intake results in acids production, then leads to the changes of pH value in the environment of plaque. This new environment encourages the growth of acidogenic and aciduric species and causes a shift in the balance of the microflora and resulting demineralization in tooth enamel. Most recent research focuses on this ecological theory to determine the diversity and complexity of the microbiota of dental caries biofilm.

Streptococcus mutans and Dental Caries

Mutans streptococci (MS) including *Streptococcus mutans* and *streptococcus sobrinus* play an important role in the initiation and development of dental caries [13, 14, 15, 16]. In Hughes' study[17], the *S. mutans* and *S. sobrinus* levels are associated with S-ECC via culture method. Other studies also show similar

association by molecular analysis[18, 19]. Several publications demonstrate that the quantity of *S. mutans* obtained from clinical samples has high association with dental caries prevalence[20,21,22,23]. The unique feature that makes *S. mutans* so important in caries development and progression is its acidogenic and aciduric properties. It can produce acid to demineralize enamel tooth surfaces which make them is susceptible to surface breakdown and caries. Moreover, *S. mutans* not only survives, but actually thrives in the low pH environment resulting from the acidic environment produced. *S. mutans* can adhere to the enamel salivary pellicle coated by saliva[24]. Glucosyltransferases (GTFs) enable *S. mutans* to metabolize sucrose and produce a sticky, extracellular, dextran-based polysaccharide that provide a further and stronger adhesion to tooth surface[25]. This is due to the sticky nature of these polysaccharides, that facilitates the adherence of *S. mutans* to the tooth and resist its detachment by normal forces like mastication, swallowing or chewing[26]. *S. mutans* not only metabolize sucrose but also utilize other sugars, such as glucose, fructose, lactose via glycolysis which produce lactic acid as the end product[27].

Other Bacteria Associated with Caries

Although *S. mutans* and *S. sobrinus* are considered the major bacterium associated with dental caries, recent findings conflict with the concept that proposes *S. mutans'* uniquely prominent role[27, 28]. It has been reported that some individuals with dental caries did not possess high levels of *S. mutans.* From one study by Aas[28], 10-15% of caries-active subjects do not have detectable levels of *S. mutans.* Thus, the presence of the *S. mutans* does not necessarily indicate caries activity. The authors[27] also point out that 10 to 20 % of subjects with severe caries may not have detectable *S. mutans,* but rather exhibit other acid-producing bacteria species.

Several analyses[28, 29] report that *S. mutans* commonly occurs in low proportion compared to the total count of microbiota biofilm. These reports also show that there may be other bacteria associated with dental caries. Becker[29] and colleagues, in a 2002 analysis, compared the microbial content from children with early childhood caries (ECC) to caries-free children using molecular identification methods. They concluded that other bacteria, such as *Actinomyces gerencseriae* and other *Actinomyces spp*., may be associated with the initiation of caries. They also found that *Bifidobacterium* may be dominant in deep caries. Using cultural and molecular techniques, Munson[30] and his colleagues isolated *S. mutans*,

Propionibacterium sp., *Lactobacillus gasseri/ johnsonii*, *Lactobacillus rhamnosus*, and *Olsenella profuse* from adults with carious lesions. They indicated there are some new species found in carious lesions and concluded that *S. mutans* is not the main bacteria causing caries.

Scardovia wiggsiae and Dental Caries

One possible explanation for the findings that caries is not always associated with *S. mutans* is that there may be other important cariogenic bacteria that are responsible for the pathogenicity of caries. With the advancement of molecular techniques, recent findings have demonstrated a newly identified/named bacteria; *Scardovia wiggsiae,* which has been associated with ECC without the presence of *S. mutans* [29, 31, 32]. This new species was previously classified as an unidentified *Bifidobacterium species*.

Scardovia wiggsiae, was named in honor of Lois Wiggs, an American microbiologist, for her contributions to anaerobic microbiology. Like *S. mutans*, *S. wiggsiae* bacteria is acidogenic and aciduric. It is gram-positive, anaerobic (facultative), non-spore-forming and non-motile bacilli. *S. wiggsiae* is saccharolytic and produce acetic and lactic acids as end products of fermentation[33]. An animal study with *S. wiggsiae* concluded that *S. wiggsiae* has the cariogenic traits of

acidogenicity and acid tolerance, and it is associated with caries. However, as a single species it was not cariogenic in the animal model system as tested[34].

Becker and his colleagues[29] reported that *S. wiggsiae* is associated with advanced dentinal caries in young children, and also can be found in white spot lesions. *S. wiggsiae* is also reported to be found in advanced dentinal caries in adults[6]. In 2009, Mantzourani[35] isolated *S. wiggsiae* from occlusal carious lesions from adults and children. Tanner[31] showed that a combination of *S. wiggsiae* and *S. mutans* is highly associated with S-ECC via sequence-based analysis of the 16S rRNA from culture isolates. Tanner also found that *S. wiggsiae* was associated with S-ECC in the absence of *S. mutans* in their preliminary study[31]. The possible explanation is that *S. wiggsiae* is involved in caries when it extends into dentin[31]. Later Tanner developed traditional PCR method to detect *S. wiggsiae* to supplement their previous findings related to caries[36]. They found that *S. wiggsiae* was more strongly association with S-ECC than *S. mutans*. These findings suggested the need for further studying the relation of high caries-risk groups with this species.

Real-time Quantitative Polymerase Chain reaction

There are various ways to detect bacteria in oral samples. Traditional methods to identify bacteria are based on phenotypic identification using gram staining, culture and biochemical methods. However, these methods have the limitation that this method can only be used only for organisms that can be cultivated in vitro. Molecular techniques have proven beneficial in overcoming some limitations of traditional phenotypic procedures for the detection and characterization of bacterial phenotypes[21].

Real-time polymerase chain reaction (qPCR) is used to amplify and quantify a targeted DNA using species or genus specific primer sets. The difference between qPCR reaction and traditional PCR is that the qPCR amplified DNA is detected as the reaction progresses in "real time". The amplification products in qPCR are measured using a fluorescent label. There are several methodologies for florescent label (i.e., TaqMan, Molecular Beacons, SYBR Green). The most economic method is SYBR Green. The amount of DNA is measured after each cycle via SYBR Green dye which bind to any double-stranded DNA such that increasing fluorescent signal results which is proportional to the number of PCR product molecules (amplicons) generated. In traditional PCR, detection and quantification of the amplified sequence are performed at the end of the reaction

after the last PCR cycle via electrophoresis and image analysis.

qPCR is used to rapidly detect and quantify bacterial from samples[37]. Studies show that qPCR is more sensitive in detecting bacteria than traditional culture methods[19, 38] as well as conventional PCR[39]. qPCR does not limit the detection to live bacteria. Therefore, it overcomes the inability of detecting nonviable bacteria when using the culture method. Samples for qPCR can be handled easily and remain stable over long time periods, especially upon freezing. This advantage can be a great benefit for handling large numbers of samples in epidemiologic studies[40].

Studies using qPCR for Detection and Quantification of Scardovia

Several studies have used qPCR for detecting and quantifying MS from oral samples[19,18, 20, 21, 41]. However, to date there is only one study[42] that reports using qPCR to detect *S. wiggsiae* from clinical samples. Tanner[42] et. al., found an association of *S. wiggsiae* with white spot lesion in orthodontic patients.

According to the literature, *S. wiggsiae* may be present in oral samples and may contribute to the etiology of dental caries. The purpose of this study is to use qPCR to detect and quantify MS and *S. wiggsiae* in oral samples from high-caries-risk children in Alabama. We hypothesize that participants with caries

and low level of *S. mutans* will have a higher proportion of *S. wiggsiae*. To test our hypothesis, we isolated DNA samples from high caries-risk children in Uniontown, Alabama, a rural high caries risk community. Species-specific qPCR was used to detect *S. wiggsiae, S. mutans and S. sobrinus.* The findings are important to dental research and treatment because successful evaluation of the presence of *S. wiggsiae* may be a useful caries-risk assessment tool.

SPECIFIC AIMS

1. To develop a sensitive and specific assay using qPCR for Scardovia genus detection and quantification from clinical samples.
2. To study the correlation between the level of Scardovia genus in clinical samples and caries prevalence.
3. To compare the level of Scardovia and MS in clinical samples relative to correlation with caries prevalence.

MATERIAL AND METHOD

Study Population

The sample population was from a low socioeconomic, high caries risk community in Perry County, Alabama. Subjects are part of an ongoing longitudinal epidemiological study in this rural community. The subjects were pre-school children who had no regular access to dental care in the county. Oral samples from 72 children were included in this study. Children included in this study were free of systemic diseases, i.e., genetic and birth defects, kidney disorders, hepatitis, cancer, bleeding disorders, endocrinal disorders, epilepsy, immunodeficiencies, heart condition, heart murmur, bone disorders, HIV positive, or immunosuppressant drugs users. This study was approved by the University of Alabama at Birmingham (UAB) Institutional Review Board. Parents of participating subjects were provided with and signed informed consent and signed wavier of assent (i.e., for the children due to age) for sample collection, oral examination and questionnaire completion. The questionnaire included survey of medical history, diet and nutrition practices, oral hygiene and dental history.

Oral Examination

Oral examination by trained and calibrated examiners was performed using light source, compressed air source or 2x2 gauze, mirror and explorer (limited for confirmatory use only); no radiographs were taken. Dental status was diagnosed and scored as decayed, missing, filled surface score (DMFS/dmfs index) according to WHO criteria[43].

Sample Collection

Saliva samples were collected from each child for qPCR analysis using sterile cotton swab placing in the mouth and rubbing along checks, tongue and teeth until saturated with saliva, then placed into 4.5 mL reduced transport fluid[44]. These saliva samples were estimated to be diluted approximately 1:70 (determined by pilot study of weight of saliva absorbed by cotton swabs). After collection, all samples were stored on ice while being transported approximately 100 miles to UAB School of Dentistry. The samples were processed within 24-36 hours of collection for bacterial DNA extraction.

Isolation of DNA from Oral Samples

One milliliter of oral sample was used for DNA extraction and purification with MasterPure™ Gram Positive DNA Purification Kit (Epicentre Biotechnologies, Madison, WI, USA) according to the manufacturer's instruction. After isolation, the DNA was re-suspended in 35 µl of Tris-EDTA Buffer (TE Buffer). Therefore, this volume resulted in a 28.5-fold concentration of the DNA sample (i.e, from 1 ml sample).

PCR Primers

The extracted DNA from clinical samples were used as a template for qPCR using species-specific primers for *S. mutans*, *S. sobrinus*, Scardovia and total bacteria (Table 1).

Table 1: Primers used in the study.

Species	Sequence	Amplicon size (bp)	Target	Source or reference
Scardovia genus	Scar448-Foward: 5'-GTGGACTTTATGAATAAGC-3' Scar619-Reverse: 5'-CTACCGTTAAGCAGTAAG-3'	146 bp	16S rRNA	Tanner, 2011[36]
S. mutans	*Sm*ut3368-Foward: 5'-GCCTACAGCTCAGAGATGCTATTCT-3' *Sm*ut3481-Reverse: 5'-GCC ATACACCACTCATGAATTGA-3'	114 bp	gtfB	Yoshida, 2003[45]
S. sobrinus	Ssob287-F: 5'-TTCAAAGCCAAGACCAAGCTAGT-3' Ssob374-R: 5'-CCAGCCTGAGATTCAGCTTGT-3'	88 bp	gtfT	Yoshida, 2003[45]
Universal primer	Forward: 5-TCCTACGGGAGGCAGCAGT-3 Reverse: 5-GGACTACCAGGGTATCTAATCCTGTT-3	466 bp	16S rRNA	Nadkarni, 2002[46]

The primers for *S. mutans* and *S. sobrinus* were specifically targeted for gtfB and gtfT, respectively as published by Yoshida[45]. Scardovia primers were specifically targeted for the 16S rRNA as reported by Tanner[36]. Using these primers, *Scardovia inopinata* produces a slightly different amplicon than *S. wiggsiae*. Although this protocol for qPCR generated two different values in melting temperature, the melting temperature of *S. inopinata* was less than 1^{o}C higher than *S. wiggsiae.* Therefore, the primers were not sufficient to differentiate the two species by qPCR. The protocol developed therefore, focused on identifying Scardovia genus. The universal primer set targeted the 16sRNA and

was used to determine the total number of bacteria presented in samples. The bacterial specificity has been established by Nadkarni[46] with no amplification with human DNA sample. The specificity of all these primer sequences for *S. mutans*, *S. sobrinus,* Scardovia and Universal primers was confirmed through Basic Local Alignment Search Tool (BLAST) nucleotide analysis.

Real-time Quantitative PCR

Using the genomic size of both *S. mutans* and *S. wiggsiae*, the single cell DNA weight in 1 molecule (m) can be calculated by using the following formula[47] m= n (bp)×660 (g/mole)/6 $\times 10^{23}$ (molecules/mole). Therefore, the total DNA weight in 10^9 cell was then calculated for *S. wiggsiae* (C1A_55, Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH) extracted DNA. The concentration of DNA from $OD_{260/280}$ was used to construct a standard curve for each qPCR run to estimate the “copy number” per milliliter (Figure 1). In this regard, the standards were 10-fold dilutions from 10^7 to 10^3 cells in Tris-EDTA buffer from MasterPure Gram Positive DNA Purification Kit (Epicentre Biotechnologies) and used for standard curve construction of *S. wiggsiae.* Similarly*, S. mutans* UA 159 genomic DNA was used to construct standards by 10-fold dilutions from 10^9 to 10^3 cells in Tris-EDTA buffer.

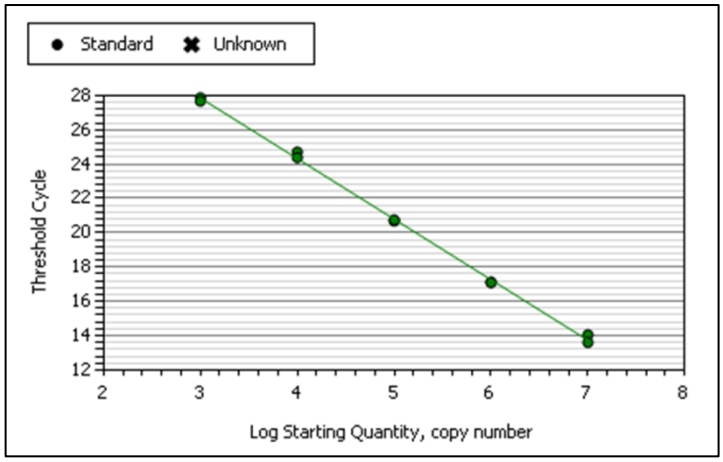


Figure 1: Standard curve of *S. wiggsiae.*

Standard curve of a ten-fold dilution series of known template concentrations of *S. wiggsiae* (10^7CN/ml to 10^3 CN/ml) for determining the quantity of Scardovia in each oral sample.

PCR amplification was performed in a total reaction mixture volume of 12.5 µl. The reaction mixtures contained 6.25 µl Maxima® SYBR Green/Fluorescein qPCR Master Mix (Fermentas, USA), 0.75 µl of forward and reverse primer, 3 µl of nuclease-free water and 2.5 µl of purified DNA standard or “unknown” obtained from oral sample. An iQ5 Real-Time PCR Detection System (Bio-Rad) was used to perform the following cycling parameters for *S. mutans*, *S. sobrinus* and universal primers: samples were amplified by pre-heating (95°C, 10 min), then denaturation (95°C, 15 sec), annealing (60°C, 30 sec), and elongation (72°C, 30 sec) for 40 cycles. A final melting curve program was used the following cycling

parameters: 60ºC for 30 seconds, 5ºC temperature changes to the end temperature of 95ºC. For Scardovia genus, the cycling parameters the same except for the annealing temperature which was set at 51ºC. The iQ5 Optical System Software generates quantitative cycle (Cq) values and analyzes the melting point data resulting in a cycle threshold (Ct) value, which is proportional to the DNA copy number.

Statistics

The levels of *S. mutans*, *S. sobrinus* and Scardovia from duplicate clinical samples was obtained by the average of results from interpolation of the standard curve (DNA extracted from prototype strains) and multiplied by the dilution factor to determine copy number per milliliter of sample. The total bacterial counts were determined using the universal primers targeting on 16S rRNA gene in qPCR assay. The ratio of *S. mutans*, *S. sobrinus* and Scardovia per total bacterial count was calculated for normalization in order to make comparisons between and among samples.

Spearman correlations were used to evaluate the correlation between the bacterial level, bacterial ratio and caries prevalence (DMFS/dmfs index)[48]. T-test was used to compare the mean bacterial level, mean bacterial ratio and caries

prevalence (DMFS/dmfs index) between disease categories[48]. Difference in detection frequency between disease categories was tested by Chi-square[48]. ANOVA was used to evaluate the difference between three different combination of bacteria (Sc negative+ *Sm* negative, Sc negative+ *Sm* positive, Sc positive+ *Sm* positive) in the mean bacterial level, mean bacterial ratio and caries prevalence (DMFS/dmfs index)[48]. The level of significance was set at $p < 0.05$. All analysis was done using SAS V9.2 (SAS Institute, Cary, NC, USA).

RESULTS

Seventy-two pre-school children, average age of 4.6 years (age range 3.2- 5.8 years) participated in the study. Thirty-two subjects were males and 40 were females. The study population was comprised of 100% African American. One saliva sample was collected from each subject to use for qPCR.

Detection Frequency and Bacterial Level of All Samples

The detection threshold of this qPCR was determined to be 1000 CN/ml based on the last dilution of the standard curve for qPCR with good linear reproducibility. *S. mutans* was detected from 97.2% of all samples (70/72) while Scardovia was found in 77.8% of samples (56/72). The mean CN/ml of samples were: *S. mutans* ($3.79x10^7$), *S. sobrinus* ($3.40x10^3$) and Scardovia ($6.48x10^6$). Because *S. sobrinus* was only detected in 7 samples at levels below quantifiable limits, *S. sobrinus* was not included in any of the analyses (there were no associations noted with caries prevalence when *S. sobrinus* was detected). The mean ratio of *S. mutans, S. sobrinus* and Scardovia to total bacteria were 1.31%, 0.00%, 0.18%, respectively (Table 2). The average DMFS/dmfs index of the participants was 15.21 (DMFS/dmfs index range 0-90, S.D.=19.37).

Table 2: Mean, standard deviation, median of *Sm*, *Ss* and Sc from all subjects.

	Sm	*Ss*	Sc	Total bacteria count	*Sm*/total bacteria	*Ss*/total bacteria	Sc/total bacteria
Mean* (CN/ml)	$3.79x10^7$	$3.40x10^3$	$6.48x10^6$	$2.41x10^{10}$	1.31%	0.00%	0.18%
S.D. (CN/ml)	$1.22x10^8$	$1.04x10^4$	$1.66x10^7$	$3.32x10^{10}$	4.33%	0.00%	0.55%
Median (CN/ml)	$1.46x10^6$	0	$8.07x10^5$	$9.18x10^9$	0.02%	0.00%	0.01%

* Mean values were determined from samples that were quantitated (i.e., the denominator was the number of samples with *Sm*, *Ss*, or Sc).

Bacterial Level and its Correlation to Caries

There was a positive relationship between the mean Scardovia and DMFS/dmfs index (r=0.245, p=0.038) by Spearman correlation analysis (Table 3). However, no significant correlations were found between the level of *S. mutans* to DMFS/dmfs index, ratio of *S. mutans*/total bacteria to DMFS/dmfs index (r=0.003, p=0.979), the ratio of Scardovia/total bacteria to DMFS/dmfs index (r=0.119, p=0.319), nor the combination of *S. mutans* and Scardovia to DMFS/dmfs index (r=0.029, p=0.811) (Table 3).

Table 3: Spearman correlation value between bacterial level and DMFS/dmfs index.

	Sm mean	Sc mean	*Sm*/ total bacteria	Sc/ total bacteria	*Sm*+Sc/ total bacteria
Spearman Correlation Coefficient to DMFS/dmfs	0.026	0.245	0.003	0.119	0.029
P-value	0.828	0.038*	0.979	0.319	0.811

* Correlation is significant at the 0.05 level (2-tailed).

Detection Frequency in Caries-experience and Caries-free Group

The seventy-two children were divided into caries-free group (DMFS/dmfs=0, n=22, 30.6%) and caries-experience group (DMFS/dmfs>0, n=50, 69.4%). There was no statistical difference ($p>0.05$) between the two groups in age, sex or body mass index. The average of decayed, missing, and filled surfaces were 0.7, 19.5, 1.7, respectively. In the caries-experience group, *S. mutans* was detected in 96% (48/50) of the samples, while Scardovia was found in 80% (40/50). In the caries-free group, Scardovia and *S. mutans* was found in 72.8% (16/22) and 100% (22/22), respectively. No statistical difference ($p>0.05$) was found in the detection frequency of *S. mutans,* Scardovia, *S. mutans*+Scardovia between caries-experience and caries-free group (Figure 2).

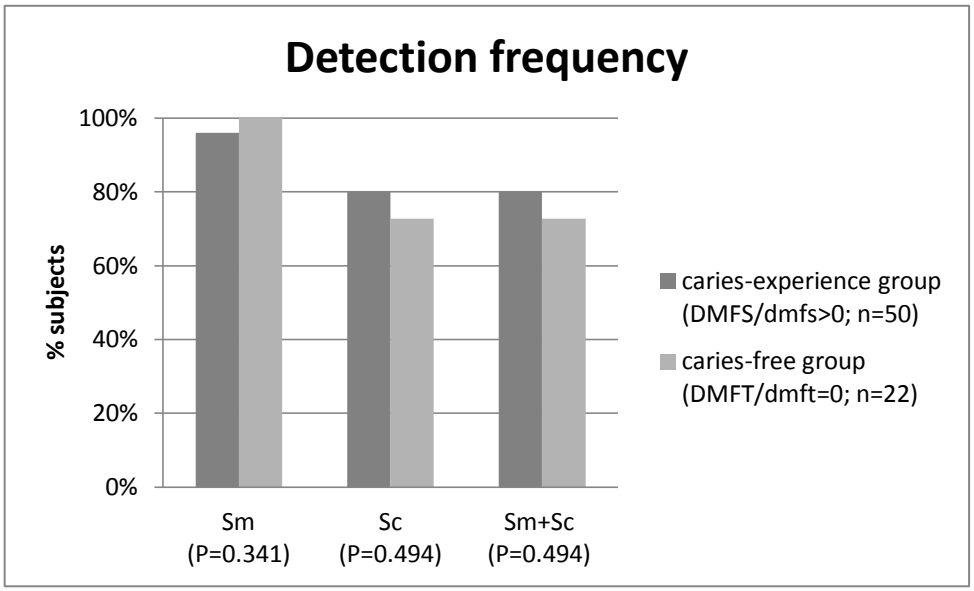


Figure 2: Detection frequency of *Sm*, Sc, *Sm*+Sc in caries-experience and caries-free group.

Bacterial Level in Caries-experience and Caries-free Group

The average DMFS/dmfs index for the children in caries-experience group was 21.9. The mean CN/ml in caries group were: *S. mutans* ($4.59x10^7$) and Scardovia ($7.74x10^6$). The mean ratio of *S. mutans,* Scardovia*,* both *S. mutans* and Scardovia to total bacteria were 1.36%, 0.25%, 1.61%, respectively (Table 4). The mean CN/ml in caries-free group were: *S. mutans* ($1.98x10^7$) and Scardovia ($3.62x10^6$). The mean ratio of *S. mutan,* Scardovia and both *S. mutans* and Scardovia to total bacteria were 1.22%, 0.03%, 1.25%, respectively (Table 4).

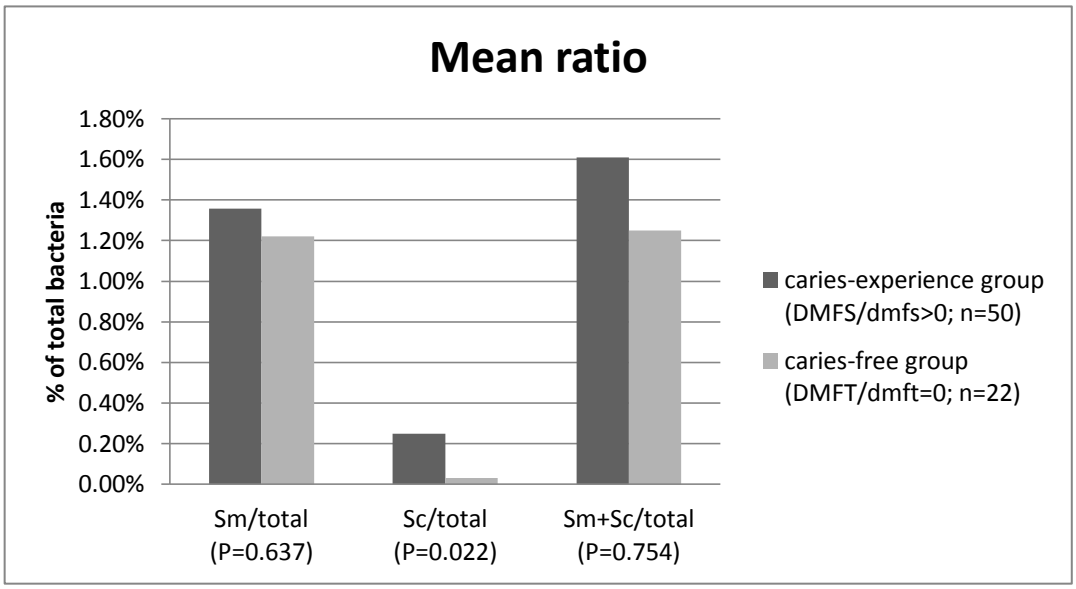
Table 4: Bacterial level in caries-experience and caries-free group and t-test analysis.

	Sm mean (CN/ml)	Sc mean (CN/ml)	*Sm*/ total bacteria	Sc/ total bacteria	Sc+*Sm*/ total bacteria
Caries-free group (n=22)	$1.98x10^7$	$3.62x10^6$	1.22%	0.03%	1.25%
Caries-experience group, (n=50)	$4.59x10^7$	$7.74x10^6$	1.36%	0.25%	1.61%
T-test	0.409	0.335	0.904	0.022 *	0.754

* The level of significance was set at $p < 0.05$ by t-test analysis

When comparing children according to caries status, children in caries-experience group had higher level of *S. mutans*, Scardovia and higher ratio of *S. mutans*/total bacteria, Scardovia/total bacteria than children in caries-free group (DMFS/dmfs=0). However, statistical differences between two groups were found only in the mean ratio of Scardovia/total bacteria (p=0.022) (Table 4 and Figure 3).

Figure 3: Mean ratio of bacteria in caries-experience and caries-free group.



Children with active caries surfaces (decayed surfaces>0, n=12) had a higher mean ratio of Scardovia/total bacteria, but lower mean ratio of *S. mutans*/total bacteria than the children in caries-free group (DMFS/dmfs=0, n=22). However, no statistical difference was found between two groups (Table 5).

Table 5: Bacterial level in caries-free group and active caries group with t-test analysis.

	Sm mean (CN/ml)	Sc mean (CN/ml)	*Sm*/ total bacteria	Sc/ total bacteria	Sc+*Sm*/ total bacteria
caries-free group, DMFS/dmfs=0 (n=22)	$1.98x10^7$	$3.62x10^6$	1.22%	0.03%	1.25%
Active caries group, decayed surface>0 (n=12)	$1.52\ x10^7$	$3.99\ x10^6$	0.72%	0.22%	0.94%
P value*	0.791	0.894	0.644	0.377	0.287

* The level of significance was set at $p < 0.05$ by t-test analysis

None of the children in the caries-experience group (n=50) was found to harbor detectable Scardovia without the presence of *S. mutans*. However, 6.0% (3/50) of subjects in the caries-experience group had low level of *S. mutans* and high level of Scardovia. In our study, only 4.0% (2/50) of subjects in the caries-experience group were void of detectable *S. mutans* and Scardovia, while 80% (40/50) had both *S. mutans* and Scardovia detected. 16% (8/50) had only *S. mutans* alone. When both both *S. mutans* and Scardovia were present, the level of both bacteria and DMFS/dmfs index tended to increase. However, there was no statistical difference between three different combination of bacteria (negative Sc and *Sm*, negative Sc and positve *Sm*, positive Sc and *Sm)* by ANOVA (Table 6).

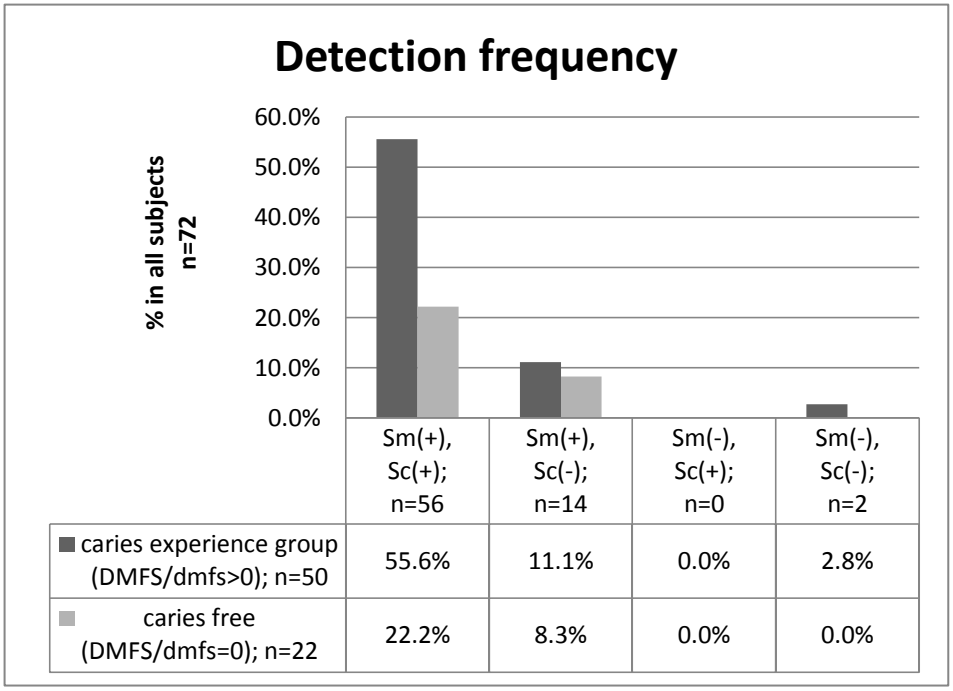
Table 6: Bacterial ratio and DMFS/dmfs score in caries-experience group with Sc and/or *Sm* with ANOVA analysis.

Caries-experience group (DMFS/dmfs>0)	N	*Sm*/total bacteira	Sc/total bacteria	DMFS/dmfs
Sm -, Sc -	2	0.00%	0.00%	3.00
Sm +, Sc -,	8	0.09%	0.00%	19.38
Sm +, Sc +	40	1.68%	0.31%	23.35
Sm-, Sc+	0			
P value*		0.859	0.752	0.273

* The level of significance was set at $p < 0.05$ by ANOVA analysis

Further, no statistical difference was found in detection frequency between caries-experience and caries-free group in the three subgroups (Sc negative+*Sm* negative, Sc negative+ *Sm* positive, Sc positive+*Sm* positive) (Figure 4). In the caries-experience group , there was a higher porportion (55% of all subjects) that had both *S. mutans* and Scardovia. However, there were only two subjects in the group that neither species was detected (Figure 4).

Figure 4: *Sm* and/or Sc combinations in the caries-experience and caries-free groups.



DISCUSSION

Saliva samples were collected from 72 pre-school children with high caries risk to detect and quantify *S. mutans*, *S. sobrinus* and Scardovia and its correlation to caries activity. In this study, most subjects were found to have *S. mutans* and Scardovia present in saliva samples. Scardovia was detected in 80% of subjects in the caries-experience group (DMFS/dmfs>0) and 72.8% in the caries-free group (DMFS/dmfs=0). According to Tanner's study in 2011[31], *S. wiggsiae* was detected in over 50% of the children using cultural method. In a later study, *S. wiggsiae* was found in 36.6 - 60% by qPCR in the study of orthodontic patients with white spot lesions[42]. In this study, the detection rate of Scardovia was higher than was reported by others[31, 42]. Since our primers detected Scardovia species, not just *S. wiggsiae,* we may have detected other Scardovia species in our samples to account for the higher positive rate of detection. Alternatively, differences in this high risk population compared to Tanner's cohorts could account for the observation differences.

None of our subjects had quantifiable *S. sobrinus*. The differences in *S. sobrinus* detection among the present study and other studies[18, 39, 49, 50] might be due to different ethnicity of sample population. However, since some studies

reported *S. sobrinus* in African populations[39], the lower level of *S. sobrinus* found in this study may be also due to geographical location of sample population.

This study took advantage of the ability to quantify oral samples with qPCR. We were able to quantify each bacteria to determine the correlation to dental caries data. Although the mean ratio of *S. mutans* (1.31%) is 7 times higher than the ratio of Scardovia (0.18%), when comparing between caries-experience and caries-free group, the ratio of Scardovia to total bacteria was the only variable that was significantly different (p=0.022) among the groups. This finding is similar to those in Tanner's 2012 study[42]. No statistical difference was found in other comparsions, including the consideration of *S. mutans* and Scardovia together (i.e., no added association was found when compared with caries status). This finding contradicts those reported in Tanner's [36] study where they found that the combination of *S. mutans* with *S. wiggsiae* was more strongly associated with S-ECC than when evaluated individually. The difference may be due to characteristics of the caries experience (i.e., most caries was treated) in our study population compared to Tanner's study since their observations were before caries was treated.

Several studies using qPCR for detection and quantification of MS from oral samples have reported a high association between MS findings to caries

prevalence [19,18, 20, 41, 39, 50]. However, in the present study we did not find a correlation between the level or the presence of *S. mutans* (nor *S. sobrinus*) to caries status. This finding, is similar to a study reported by Vieira[51]. They found that DMFT scores of adults didn't correlate with *S. mutans* colonization measured by real-time PCR assay. Neither was an association between the level of *S. mutans* and caries experience found in children in Acevedo's study[52].

Although the average DMFS/dmfs index (DMFS/dmfs=21.9) in the caries-experience group from this study was high, the index was influenced mostly by the number of the presence of filled surfaces (mean=1.7) and missing surfaces (mean=19.5). There were only 12 subjects (24%) in caries-experience group (n=50) that had active caries (mean decayed surface=0.7). *S. mutans* is considered to be associated with the initiation of caries. It was presumed that the level of *S. mutans* would increase when new carious lesions were developing. However, the extent of dental caries when cavitated lesions were detected in the present study may be too late to detect a significant association between *S. mutans* and caries prevalence. Further, the 76% (38/50) of this cohort in the caries-experience group were with filled or missing tooth surfaces and therefore may not be associated with the initiation of caries pathogenesis.

We defined the DMFS/dmfs=0 group to be "caries-free". Even though cavitated

lesions were not observed, our data with relatively high *S. mutans* in this group may indicate we missed some caries activity (i.e., in the initial stages). This could also reflect a lack of sensitivity in caries disease detection due to lack of radiographs.

In this study, 2 subjects in the caries-experience group had no detectable *S. mutans* or Scardovia. Of these two subjects, one had active caries lesions while the other had only history of "filled" tooth surfaces. Therefore, of the subjects with active caries, only 1 of 12 (8.3%) didn't have detectable *S. mutans.* This finding may reflect what other studies have found with caries but no detectable *S. mutans*[27, 28]. However, the negative finding in *S. mutans* may be a sampling error because of the small number of subjects.

In contrast to other studies, none of our subjects with caries had detectable Scardovia without the presence of *S. mutans*. Further in this study, we found that only 6.0% (3/50) of subjects in the caries-experience group that had low level of *S. mutans* and high level of Scardovia. Therefore, although *S. mutans* was not significantly associated with caries, it is not possible to separate the presence with the significant association with Scardovia.

The universal primers used in this study were to serve as the internal control for amount of DNA obtained from each saliva sample. We used saliva rather than

plaque samples for this study to predict the bacterial component in oral cavity. Our experience with plaque samples was that the amount of plaque collected was too variable and therefore not as reliable in assessing the quantity of oral bacteria counts, even when normalized for total bacteria using universal primers. Further, saliva was chosen because it is more representative of the entire oral cavity rather than the selection of individual teeth[53]. Additionally, it has been demonstrated that saliva, being continuously in contact with all teeth, better reflects the colonization of MS on all the dentition[54]. In other studies, the bacterial counts from saliva samples are considered as a reflection of bacterial load from dental biofilm of individual's oral cavity[18, 55, 56]. The levels of MS in saliva have been shown to be a mean of predicting caries activity[40, 51]. It is anticipated that the use of saliva samples in our study provided a generalized picture of the microbiological load of different bacteria species, especially because the method of collection included rubbing the sterile q-tips over the teeth.

Limitation

Our qPCR assay was limited in detecting Scardovia genus instead of being specific to *S. wiggsiae*. *Scardovia inopinata* is another potential human Scardovia species and would produce a different sized amplicon than *S. wiggsiae*. Although

this protocol for qPCR would generate two different values in melting temperature, the melting temperature of *S. inopinata* was less than 1^{o}C higher than *S. wiggsiae.* The Scardovia primers used herein were not specifically capable of differentiating the two species.

The population in our study was limited in African Americans from a small geographic rural community, which was uniform but nonetheless the unique bacteria profile in this cohort (i.e., high level of *S. mutans*) may not be representative of other populations.

More definitive discrimination of bacterial profiles related to caries activity may have resulted with a larger sample population especially if active caries could be better differentiated and assessed (i.e., with aid of radiographs) prior to restoration/extraction of carious teeth.

CONCLUSION

Dental caries is the most prevalent infectious disease in children. Although caries can be prevented by fluoride, sealants, optimal nutrition & dietary practices, and other measures, in the US this disease affects 28 % of children from aged 2-5 years. The cause of caries is multifactorial, dietobacterial and therefore complex. In this study, most subjects had *S. mutans* and Scardovia present from saliva samples. The level of *S. mutans* was much higher than Scardovia; however, no association was found between *S. mutans* and caries experience. The ratio of Scardovia/total bacteria was significantly higher in the caries-experience group. With *S. mutans* and Scardovia together, no clear added association was seen with caries experience.

Since caries is a multifactorial and a complex disease, other factors (i.e., diet, ethic race, social economic class) should be considered. Further, the overall profile of microbiota (i.e., more than focusing on the MS and Scardovia) may provide more relevant information related to the bacterial contribution to a caries risk assessment.

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APPENDIX

IRB APPROVAL FORMS

Project Revision/Amendment Form

Form version: October 28, 2010

In MS Word, click in the white boxes and type your text; double-click checkboxes to check/uncheck.

- **Federal regulations require IRB approval before implementing proposed changes. See Section 14 of the IRB Guidebook for Investigators for additional information.**
- **Change means any change, in content or form, to the protocol, consent form, or any supportive materials (such as the Investigator's Brochure, questionnaires, surveys, advertisements, etc.). See Item 4 for more examples.**

1. Today's Date	06/11/2012

2. Principal Investigator (PI)

Name (with degree)	Noel K. Childers, DDS, MS, PhD	**Blazer ID**	nkc
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Contact person who should receive copies of IRB correspondence (Optional)

Name	Tonya Wiley	**E-Mail**	tnett@uab.edu
Phone	934-6211	**Fax Number**	934-7013
Office Address (if different from PI)			

3. UAB IRB Protocol Identification

3.a. Protocol Number F060328001

3.b. Protocol Title Epidemiology of Dental Caries and Immunity in Children (Alabama)

3.c. Current Status of Protocol—Check ONE box at left; provide numbers and dates where applicable

- ☐ **Study has not yet begun** — **No participants, data, or specimens have been entered.**
- ☒ **In progress, open to accrual** — **Number of participants, data, or specimens entered:** 925
- ☐ **Enrollment temporarily suspended by sponsor**
- ☐ **Closed to accrual, but procedures continue as defined in the protocol (therapy, intervention, follow-up visits, etc.)**

  Date closed: **Number of participants receiving interventions:**

  Number of participants in long-term follow-up only:
- ☐ **Closed to accrual, and only data analysis continues**

  Date closed: **Total number of participants entered:**

4. Types of Change

Check all types of change that apply, and describe the changes in Item 5.c. or 5.d. as applicable. To help avoid delay in IRB review, please ensure that you provide the required materials and/or information for each type of change checked.

- ☐ **Protocol revision (change in the IRB-approved protocol)**
  In Item 5.c., if applicable, provide sponsor's protocol version number, amendment number, update number, etc.
- ☐ **Protocol amendment (addition to the IRB-approved protocol)**
  In Item 5.c., if applicable, provide funding application document from sponsor, as well as sponsor's protocol version number, amendment number, update number, etc.
- ☒ **Add or remove personnel**
  In Item 5.c., include name, title/degree, department/division, institutional affiliation, and role(s) in research, and address whether new personnel have any conflict of interest. See "Change in Principal Investigator" in the IRB Guidebook if the principal investigator is being changed.
  - ☐ **Add graduate student(s) or postdoctoral fellow(s) working toward thesis, dissertation, or publication**
    In Item 5.c., (a) identify these individuals by name; (b) provide the working title of the thesis, dissertation, or publication; and (c) indicate whether or not the student's analysis differs in any way from the purpose of the research described in the IRB-approved HSP (e.g., a secondary analysis of data obtained under this HSP).
- ☐ **Change in source of funding; change or add funding**
  In Item 5.c., describe the change or addition in detail, include the applicable OGCA tracking number(s), and provide a copy of the application as funded (or as submitted to the sponsor if pending). Note that some changes in funding may require a new IRB application.

☐ **Add or remove performance sites**
In Item 5.c., identify the site and location, and describe the research-related procedures performed there. If adding site(s), attach notification of permission or IRB approval to perform research there. Also include copy of subcontract, if applicable. If this protocol includes acting as the Coordinating Center for a study, attach IRB approval from any non-UAB site added.

☐ **Add or change a genetic component or storage of samples and/or data component—this could include data submissions for Genome-Wide Association Studies (GWAS)**
To assist you in revising or preparing your submission, please see the IRB Guidebook for Investigators or call the IRB office at 934-3789.

☐ **Suspend, re-open, or permanently close protocol to accrual of individuals, data, or samples (IRB approval to remain active)**
In Item 5.c., indicate the action, provide applicable dates and reasons for action; attach supporting documentation.

☐ **Report being forwarded to IRB (e.g., DSMB, sponsor or other monitor)**
In Item 5.c., include date and source of report, summarize findings, and indicate any recommendations.

☐ **Revise or amend consent, assent form(s)**
Complete Item 5.d.

☐ **Addendum (new) consent form**
Complete Item 5.d.

☐ **Add or revise recruitment materials**
Complete Item 5.d.

☐ **Other (e.g., investigator brochure)**
Indicate the type of change in the space below, and provide details in Item 5.c. or 5.d. as applicable.
Include a copy of all affected documents, with revisions highlighted as applicable.

5. Description and Rationale

In Item 5.a. and 5.b, check Yes or No and see instructions for Yes responses.
In Item 5.c. and 5.d, describe—and explain the reason for—the change(s) noted in Item 4.

☐ Yes ☒ No **5.a. Are any of the participants enrolled as normal, healthy controls?**
If yes, describe in detail in Item 5.c. how this change will affect those participants.

☐ Yes ☒ No **5.b. Does the change affect subject participation, such as procedures, risks, costs, location of services, etc.?**
If yes, FAP-designated units complete a FAP submission and send to fap@uab.edu. Identify the FAP-designated unit in Item 5.c.
For more details on the UAB FAP, see www.uab.edu/cto.

5.c. Protocol Changes: In the space below, briefly describe—and explain the reason for—all change(s) to the protocol.

We are adding the following personnel: Kelsey Jordan, Devon Cooper, Leanne Bowman, Vanessa Freitas, Jung-yi Liu, Carter Thomas, Priya Gulati and Marina A. Perez. Current IRB training records for all are attached. Records are already on file for Marina A. Perez. ✓SIRB

5.d. Consent and Recruitment Changes: In the space below,
(a) describe all changes to IRB-approved forms or recruitment materials and the reasons for them;
(b) describe the reasons for the addition of any materials (e.g., addendum consent, recruitment); and
(c) indicate either how and when you will reconsent enrolled participants or why reconsenting is not necessary (not applicable for recruitment materials).

Also, indicate the number of forms changed or added. For new forms, provide 1 copy. For revised documents, provide 3 copies:
- **a copy of the currently approved document (showing the IRB approval stamp, if applicable)**
- **a revised copy highlighting all proposed changes with "tracked" changes**
- **a revised copy for the IRB approval stamp.**

Signature of Principal Investigator Mark K Childs Date 6/11/12

FOR IRB USE ONLY

☐ Received & Noted ☒ Approved Expedited* ☐ To Convened IRB

Leslie Cooper, CIP — Signature (Chair, Vice-Chair, Designee)

6-13-12 — Date

DOLA 9-28-11

Change to Expedited Category Y / N / (NA)

*No change to IRB's previous determination of approval criteria at 45 CFR 46.111 or 21 CFR 56.111

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Page 3 of 3

OMB No. 0990-0263
Approved for use through 3/31/2015

Protection of Human Subjects
Assurance Identification/IRB Certification/Declaration of Exemption
(Common Rule)

Policy: Research activities involving human subjects may not be conducted or supported by the Departments and Agencies adopting the Common Rule (56FR28003, June 18, 1991) unless the activities are exempt from or approved in accordance with the Common Rule. See section 101(b) of the Common Rule for exemptions. Institutions submitting applications or proposals for support must submit certification of appropriate Institutional Review Board (IRB) review and approval to the Department or Agency in accordance with the Common Rule. Institutions must have an assurance of compliance that applies to the research to be conducted and should submit certification of IRB review and approval with each application or proposal unless otherwise advised by the Department or Agency.

1. Request Type	2. Type of Mechanism	3. Name of Federal Department or Agency and, if known, Application or Proposal Identification No.
[ ] ORIGINAL [X] CONTINUATION [ ] EXEMPTION	[ ] GRANT [X] CONTRACT [ ] FELLOWSHIP [ ] COOPERATIVE AGREEMENT [ ] OTHER:_____	

4. Title of Application or Activity	5. Name of Principal Investigator, Program Director, Fellow, or Other
Epidemiology of Dental Caries and Immunity in Children (Alabama)	CHILDERS, NOEL

6. Assurance Status of this Project *(Respond to one of the following)*

[X] This Assurance, on file with Department of Health and Human Services, covers this activity:
Assurance Identification No. FWA00005960, the expiration date 01/24/2017 IRB Registration No. IRB00000726

[ ] This Assurance, on file with *(agency/dept)*_____, covers this activity.
Assurance No._____, the expiration date_____ IRB Registration/Identification No._____*(if applicable)*

[ ] No assurance has been filed for this institution. This institution declares that it will provide an Assurance and Certification of IRB review and approval upon request.

[ ] Exemption Status: Human subjects are involved, but this activity qualifies for exemption under Section 101(b), paragraph_____.

7. Certification of IRB Review (Respond to one of the following IF you have an Assurance on file)

[X] This activity has been reviewed and approved by the IRB in accordance with the Common Rule and any other governing regulations.
by: [X] Full IRB Review on (date of IRB meeting) 8/29/2012 or [ ] Expedited Review on (date)_____
[ ] If less than one year approval, provide expiration date _____

[ ] This activity contains multiple projects, some of which have not been reviewed. The IRB has granted approval on condition that all projects covered by the Common Rule will be reviewed and approved before they are initiated and that appropriate further certification will be submitted.

8. Comments
Protocol subject to Annual continuing review.

Title F060328001
Epidemiology of Dental Caries and Immunity in Children (Alabama)

IRB Approval Issued: 09-12-12

9. The official signing below certifies that the information provided above is correct and that, as required, future reviews will be performed until study closure and certification will be provided.	10. Name and Address of Institution
11. Phone No. *(with area code)* (205) 934-3789 12. Fax No. *(with area code)* (205) 934-1301 13. Email: irb@uab.edu	University of Alabama at Birmingham 701 20th Street South Birmingham, AL 35294
14. Name of Official Ferdinand Urthaler, M.D.	15. Title Chairman, IRB
16. Signature Ferdinand Urthaler, MD/db	17. Date 09-12-12

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Protection of Human Subjects
Assurance Identification/IRB Certification/Declaration of Exemption
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Policy: Research activities involving human subjects may not be conducted or supported by the Departments and Agencies adopting the Common Rule (56FR28003, June 18, 1991) unless the activities are exempt from or approved in accordance with the Common Rule. See section 101(b) of the Common Rule for exemptions. Institutions submitting applications or proposals for support must submit certification of appropriate Institutional Review Board (IRB) review and approval to the Department or Agency in accordance with the Common Rule. Institutions must have an assurance of compliance that applies to the research to be conducted and should submit certification of IRB review and approval with each application or proposal unless otherwise advised by the Department or Agency.

1. Request Type	2. Type of Mechanism	3. Name of Federal Department or Agency and, if known, Application or Proposal Identification No.
[ ] ORIGINAL [X] CONTINUATION [ ] EXEMPTION	[ ] GRANT [ ] CONTRACT [ ] FELLOWSHIP [ ] COOPERATIVE AGREEMENT [ ] OTHER:_____	
4. Title of Application or Activity Epidemiology of Dental Caries and Immunity in Children (Alabama)		5. Name of Principal Investigator, Program Director, Fellow, or Other CHILDERS, NOEL

6. Assurance Status of this Project *(Respond to one of the following)*

[X] This Assurance, on file with Department of Health and Human Services, covers this activity:
Assurance Identification No. FWA00005960 , the expiration date 01/24/2017 IRB Registration No. IRB00000726

[ ] This Assurance, on file with *(agency/dept)*_____, covers this activity.
Assurance No._____, the expiration date_____ IRB Registration/Identification No._____*(if applicable)*

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[ ] Exemption Status: Human subjects are involved, but this activity qualifies for exemption under Section 101(b), paragraph_____.

7. Certification of IRB Review (Respond to one of the following IF you have an Assurance on file)

[X] This activity has been reviewed and approved by the IRB in accordance with the Common Rule and any other governing regulations.
by: [X] Full IRB Review on (date of IRB meeting) 8/7/2013 or [ ] Expedited Review on (date) _____
[ ] If less than one year approval, provide expiration date _____

[ ] This activity contains multiple projects, some of which have not been reviewed. The IRB has granted approval on condition that all projects covered by the Common Rule will be reviewed and approved before they are initiated and that appropriate further certification will be submitted.

8. Comments	Title F060328001
Protocol subject to Annual continuing review. HIPAA Waiver Approved?: No Partial HIPAA Waiver Approved?: No IRB Approval Issued: 1/21/14	Epidemiology of Dental Caries and Immunity in Children (Alabama) IRB Approval No Longer Valid On: August 7, 2014

9. The official signing below certifies that the information provided above is correct and that, as required, future reviews will be performed until study closure and certification will be provided.	10. Name and Address of Institution University of Alabama at Birmingham 701 20th Street South Birmingham, AL 35294
11. Phone No. *(with area code)* (205) 934-3789 12. Fax No. *(with area code)* (205) 934-1301 13. Email: irb@uab.edu	
14. Name of Official Albert Oberman, M.D., MPH	15. Title Vice Chair, IRB
16. Signature Albert Oberman, MD, MPH/MD	17. Date 1/21/14

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