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ANALYSIS OF SALIVARY ORAL BACTERIA ASSOCIATED WITH SEVERE EARLY CHILDHOOD CARIES: A CROSS-SECTIONAL STUDY

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ABSTRACT

Background: Severe early childhood caries is a rapidly progressing form of dental caries that affects young children and is strongly associated with microbial factors. Understanding the salivary microbial profile in children with S-ECC is critical for developing targeted preventive and therapeutic strategies. **Objectives:** This study aims to analyze the presence of S. mutans, S. sobrinus and S. wiggsiae in saliva samples obtained from children with severe-ECC. **Materials and Methods:** This cross-sectional study included children aged 3 to 5 years diagnosed with S-ECC, from January 2020 to May 2020. Ethical approval was obtained from Marmara University Clinical Research Ethical Committee and written informed consent was obtained from parents. Oral examinations were conducted under WHO criteria (1997), recording dmft and ICDAS II scores alongside demographic and dietary habits. Unstimulated saliva samples were collected using a saliva ejector and stored for analysis. q-PCR was performed to detect saliva S. mutans, S. wiggsiae, and S. sobrinus using species-specific 16S rRNA primers. **Results:** A total of 54 children (40.7% female, 59.3% male) with a mean age of 4.2 ± 0.8 years and a mean dmft score of 13.2 ± 3.2 participated in the study. All children were classified within the ICDAS code 5–6 group. Saliva samples from 52 children were analyzed via qPCR, revealing S. mutans in 96.2%, S. wiggsiae in 67.3%, and S. sobrinus in 26.9%. Co-detection rates were 66.0% for S. mutans and S. wiggsiae, 26.9% for S. mutans and S. sobrinus, and 9.6% for S. sobrinus and S. wiggsiae. No significant associations were found between S. mutans levels and the duration of bottle feeding or breastfeeding (p=0.207 and p=0.184, respectively). **Conclusions:** This study suggests that S. mutans and S. wiggsiae are prevalent in children with S-ECC, but feeding practices showed no significant impact on the quantity of S. mutans.

KEYWORDS: SECC, saliva, S. mutans, S. wiggsiae, and S. sobrinus.

INTRODUCTION

Early childhood caries (ECC) is defined as the occurrence of one or more decayed (cavitated or noncavitated lesions), missing (due to caries), or filled tooth surfaces in any primary tooth of a child aged 71 months or younger. For children under the age of 3, the presence of any smooth surface caries is considered indicative of severe early childhood caries (S-ECC) [1]. The terms early childhood caries (ECC) and severe ECC (S-ECC) have been used for nearly 13 years to describe the caries status in children under the age of 6. ECC is a chronic, transmissible infectious disease with a multifactorial and complex etiology. Factors contributing to its development include prolonged bottle-feeding with sugar-containing liquids, breastfeeding on demand or while falling asleep, and nursing beyond the recommended weaning age. Additional factors linked to ECC encompass genetic predisposition, parental education, and various nutritional, environmental, socioeconomic, and parenting style influences [2].

ECC is a rapidly advancing process of tooth demineralization caused by the interaction of cariogenic diets, a susceptible host, and oral microbiota in children under 6 years of age [3]. According to the ecological plaque hypothesis, a low pH oral environment promotes the proliferation of acidogenic and aciduric microbiota. Streptococcus mutans is a microorganism frequently isolated from dental plaque [4, 5]. It is not only aciduric and acidogenic but also exhibits the ability to adhere to and colonize tooth surfaces [4]. S. mutans can generate acid from carbohydrates and withstand low pH environments. Previous research has demonstrated a strong association between S. mutans and ECC, making it a key microbial parameter for assessing children's caries risk [4, 6].

Scardovia wiggsiae is an anaerobic Gram-positive bacillus. In vitro studies indicate that the growth and acid tolerance of S. wiggsiae are comparable to those of S. mutans. Additionally, it is a potent acid producer, with acidogenic capacity equal to or exceeding that of S. mutans [7]. A research has identified a strong association between S. wiggsiae and severe early childhood caries (S-ECC), suggesting that S. wiggsiae may serve as a significant primary pathogen in dental caries [7]. Some studies have observed that in S. mutans-negative samples, S. wiggsiae was present, indicating its potential role as a secondary aggressor involved in caries progression during later stages of the disease when S. mutans is no longer the predominant pathogenic species [7]. Furthermore, the combination of S. wiggsiae, S. sobrinus and S. mutans has been associated with caries, highlighting its potential utility in caries risk assessment [7-9]. This cross-sectional study was undertaken with the objective to quantitatively analyse the salivary S. mutans, S. sobrinus and S. wiggsiae from 3- to 5-year-old children with S-ECC.

MATERIALS AND METHODS

Ethical approval was obtained from the Marmara University, School of Dentistry Clinical Research Ethical Committee with number of 2019-283 and from the Republic of Turkey Ministry of Health, Turkish Medicine and Medical Devices Agency with under number 20-AKD-167.

A cross-sectional study included children aged 3 to 5 years from January 2020 to May 2020 who presented for their first dental examination at the Department of Pediatric Dentistry clinic, Faculty of Dentistry, Marmara University. The study was performed in accordance with the Declaration of Helsinki (1964) and written informed consent was obtained from parents of the children after explaining the study in detail. The inclusion criteria required participants to be systemically healthy, cooperative, and willing to participate in the study. All participants met the diagnosis protocol of American Academy of Pediatric Dentistry (AAPD) 2018–2019 [1], which defines S-ECC as the presence of more than four

decayed teeth in 3-year-olds, more than five in 4-year-olds, and more than six in 5-year-olds. The exclusion criteria included children with systemic diseases, visually detectable enamel or dentin hypoplasia, antibiotic use within 30 days prior to sample collection, a history of fluoride treatment within the month preceding the study, as well as noncooperative or immunosuppressed children.

The sample size was calculated by G Power 3.1 with α=0.05 and power of 95%. The calculated total sample size was 45 and to account for possible 20% drop-out, at least of 54 children needed to be recruited.

Oral and dental health examinations of the children were conducted according to the criteria of the World Health Organization (WHO, 1997) under reflector light on a dental chair, using a hand mirror and a community periodontal index probe by a single trained and calibrated examiner. The children's caries index values, demographic information, and responses to questions regarding their early childhood feeding habits were recorded. The teeth of all children participating in this study has been examined by using both decayed, missing, and filled teeth (dmft) score [10] and International Caries Detection and Assessment System (ICDAS II) criteria [11].

Saliva sampling

Saliva samples were collected from the children who met the AAPD criteria for a diagnosis of S-ECC and agreed to participate in the study. Unstimulated saliva samples were obtained in the morning by a trained pediatric dentist using a saliva ejector, following a method developed by Kuşcu ÖÖ [12] and were collected into sterile tubes provided by the laboratory. The collected saliva samples (1 mL) were labeled and stored for subsequent PCR analysis.

Quantitative real-time PCR (qPCR)

The presence of S. mutans, S. wiggsiae, and S. sobrinus in the collected saliva samples was analyzed using qPCR with species-specific 16S rRNA primers.

DNA extraction

Saliva samples collected from the patients were transferred to sterile 1.5 mL microcentrifuge tubes and stored at -80°C until DNA isolation. For DNA isolation, the saliva samples were first homogenized using a vortex and then centrifuged at 5,000g for 10 minutes. The supernatant was removed, and the remaining pellet was resuspended in 100 µL of TE buffer. DNA isolation was carried out using the E.Z.N.A. Bacterial DNA Kit (Omega Biotek) according to the manufacturer's protocol. Finally, the purified DNA sample was eluted in 100 µL of elution buffer. The concentration and purity of the isolated DNA samples were determined using a microvolume spectrophotometer (NanoDrop 2000). Absorbance values at 230 nm, 260 nm, and 280 nm were measured, and the 260/280 and 260/230 ratios were calculated and recorded. The isolated DNA samples were stored at -20°C until further qPCR analysis.

qPCR analysis

qPCR was applied to determine the relative quantities of Streptococcus mutans, Streptococcus sobrinus, and Scardovia wiggsiae microorganisms in the isolated DNA samples. The forward and reverse primers used for the detection of each target microorganism in the qPCR experiments are provided in Table 1.

Table 1 *(Primers and amplification protocols used for PCR analysis.)*

Bacteria	Primer sequences (5'→3')	Amplicon length (bp)	Reference
S mutans	F: GGTCAGGAAAGTCTGGAGTAAAAAGGCTA	282	Corpet et al. 1988
	R: GCGGTAGCTCCGGCACTAAGCC		
S sobrinus	F: CGGACTTGCTCCAGTGTTACTAA	546	Corpet et al. 1988
	R: GCCTTTAACTTCAGACTTAC		
S wiggsiae	F: GTGGACTTTATGAATAAGC	146	Tanner et al. 2011
	R: CTACCGTTAAGCAGTAAG		

In the study, the qPCR reaction mixture was prepared as shown in Table 2, and the PCR conditions, as depicted in Table 3, were applied using the Light Cycler 480 (Roche Life Science, Germany) device at Artı Biotechnology Laboratory (Istanbul / Türkiye).

Table 2 *(qPCR reaction mixture.)*

The content	1 reaction (total volume of 25 µL.)
dH_2O	7 ul
GoTaq MasterMix (2X)	12,5 ul
Primer F (2,5 mM)	1,25 ul
Primer R (2,5 mM)	1,25 ul
DNA	3 ul

Table 3 *(The steps of qPCR.)*

	Program	Number of cycles
Enzyme activation and initial denaturation	95°C for 2 min	1
Denaturation	95°C for 15 sec	40
Annealing and extension	60°C for 60 sec	

Melting curve analysis	60-95°C	1

Following the qPCR analysis, Ct (cycle threshold) values for each sample were obtained using the relative quantification analysis module of the device software and were used for quantification calculations. The positivity of the samples was also verified through melting curve analysis. For relative comparisons of the obtained Ct values, the average Ct value of the positive samples for each target microorganism was calculated, and all sample Ct values were normalized to this average using the 2−ΔCT method to determine fold-change ratios. Subsequently, the fold-change ratios were further normalized based on the initial volume of DNA isolation, as most samples used 600 µL of saliva; however, some samples had different starting volumes due to insufficient material. Using the fold-change ratios obtained for each sample, comparisons were performed across groups and sampling times.

Statistical Analysis

Statistical analysis was carried out using Statistical Package for the Social Sciences (SPSS) 23 software (SPSS Inc., Chicago, IL, USA). The values were represented as number (%) and mean ± standard deviation (SD), with descriptive statistics presented as mean ± SD. In the study, a one-way ANOVA test was used to evaluate whether there were significant differences in the assessed microorganisms based on different feeding types across various time intervals. Level of statistical significance (p value) was set as <0.05.

RESULTS

The families of 80 children diagnosed with S-ECC and meeting the inclusion criteria were informed about the research, and 54 parents consented to participate. Among the participating children, 22 (40.7%) were female and 32 (59.3%) were male, with mean ages of 4 ± 0.82 years and 4.3 ± 0.77 years, respectively. The mean age of children was 4,2 ⍰ 0,8. The mean dmft score of the children was calculated as 13.2 ± 3.2, and all children were classified within the ICDAS code 5–6 group.

Following the intraoral examination, saliva samples were collected from the children. However, only the saliva samples of 52 children were included in the qPCR analysis. In this analysis, S. mutans was found to be positive in 50 children (96.2%), S. wiggsiae in 35 children (67.3%), and S. sobrinus in 14 children (26.9%). Table 4 evaluates the co-positivity rates of microorganisms identified through the q-PCR analysis. According to the q-PCR results, saliva samples collected from children with S-ECC showed co-detection of S. mutans and S. sobrinus in 14 samples (26.9%), S. mutans and S. wiggsiae in 33 samples (66.0%), and S. sobrinus and S. wiggsiae in 5 samples (9.6%) (Table 4).

Table 4 *(Evaluation of co-positivity rates of microorganisms in q-PCR analysis.)*

Microorganisms		q-PCR value n=52
S.mutans + S.sobrinus	+	14 (26,9%)

S.mutans + S.wiggsiae	+	33 (66,0%)
S.sobrinus + S.wiggsiae	+	5 (9,6%)

When the qPCR data were separately evaluated for S. mutans and S. wiggsiae, it was found that in 1 child (2%), S. mutans was negative while S. wiggsiae was positive; in 15 children (30%), S. mutans was positive while S. wiggsiae was negative; and in 33 children (66%), both S. mutans and S. wiggsiae were positive. (Table 5)

Table 5 *(Evaluation of the isolation rates of S. mutans and S. wiggsiae in the q-PCR analysis.)*

S.wiggsiae	S.mutans	Total (n, %)
+	-	1 (%2,0)
-	+	15 (%30,0)
+	+	33 (%66,0)
-	-	1 (%2)

Table 6 evaluates the relationship between S. mutans and feeding practices. According to the results, there were no statistically significant differences in the mean S. mutans values based on the duration of bottle feeding or breastfeeding (p=0.207 and p=0.184, respectively).

Table 6 *(Evaluation of the relationship between S. mutans and feeding type and duration.)*

	Bottle Feeding Duration				Breastfeeding Duration			
	0-6 months	6-12 months	12-24 months	24+ months	0-6 months	6-12 months	12-24 months	24+ months
S.mutans mean ± SD	7,89 ± 9,8	1,57 ± 2,5	6,27 ± 11,8	1,94 ± 2,2	4,85 ± 0,2	1,79 ± 1,5	3,92 ± 4,4	12,85 ± 18,8
p	0,207				0,184			

One-way ANOVA test, p<0,05 statistical significance

DISCUSSION

Dental caries is the third most common disease worldwide, following heart disease and cancer. S-ECC is a destructive form of dental decay prevalent among infants and preschool children, ranking among the most significant dental issues in childhood. The clinical manifestations of ECC include pain, acute and chronic abscesses, fever, and swelling of the lips or cheeks. It can lead to chewing difficulties, malnutrition, gastrointestinal problems, speech issues, and loss of self-esteem. Notably, growth retardation in terms of height and weight has also been reported [13]. S-ECC is a global public health issue that requires complex treatment procedures and incurs high costs. It has been suggested that the prevention of S-ECC is positively correlated with the acceptance of an etiopathogenesis involving cariogenic bacteria [14]. Untreated primary teeth impact not only general health but also quality of life associated with oral health [15]. This study aims to investigate the microorganisms involved in the etiology of S-ECC and evaluate their relationship with the duration of bottle-feeding and breastfeeding. The definition of ECC includes children aged 71 months or younger [1]. For this study, children aged 3–5 years who presented to our clinic were included. Children under the age of three were excluded due to the difficulty of conducting intraoral examinations and potential cooperation issues during the saliva sample collection phase. To determine the prevalence of dental caries, the World Health Organization (WHO) recommends the use of the DMFT (Decayed, Missing, Filled Teeth) index [10]. In this study, the DMFT index was utilized to evaluate the number of carious lesions in children. However, the DMFT index only identifies cavitated lesions. In recent years, researchers have emphasized the importance and necessity of identifying both active and inactive non-cavitated lesions, which led to the development of the ICDAS) [16]. ICDAS enables the detection and assessment of dental caries from the earliest stages of initiation to the most advanced and destructive stages reaching the pulp. For coronal caries, ICDAS codes range from 0 to 6, depending on the severity of the lesion [11]. In this study, in addition to the DMFT index, the ICDAS II criteria were used to determine the severity of carious lesions. Children meeting the ECC definition with a dmft score and classified as code (5–6) according to ICDAS II criteria were included in the study.

Studies evaluating the microbiota of S-ECC have reported that saliva, plaque, or swab samples are analyzed in children using various methods [17, 18]. In our study, saliva samples were examined to maintain standardization and ensure the evaluation of equal amounts of samples from each child. Klinke et al. collected saliva samples by soaking sterile cotton-tipped applicators placed under the tongue and on the dorsal surface of the tongue, which were then directly transferred to culture media [19]. Colombo et al. collected unstimulated saliva by having participants spit directly into 50 mL sterile Falcon tubes over 5–10 minutes [15]. Zhan et al. suggested that saliva samples could be collected using cotton swabs [20]. Collecting saliva samples from children can be challenging due to their developmental stage, which may include incomplete development of spitting reflexes and difficulties with cooperation, making it harder to obtain sufficient samples. In our study, due to the young age of the participants, unstimulated saliva samples were collected using a method previously described in the literature to facilitate the collection of the required amount in a shorter time [12]. Saliva was collected using a saliva ejector and transferred to sterile Falcon tubes provided by the laboratory.

Most microbiological clinical studies related to ECC have routinely focused on S. mutans and lactobacilli, which are commonly detected using selective culture-based methods. However, it has long been known that the microbiota of caries-related biofilms contains a wide variety of microorganisms, including species such as Actinomyces, Fusobacterium, Scardovia, Bifidobacterium, Atopobium, Prevotella, Veillonella, and Candida [21]. Due to S. wiggsiae being a newly isolated species in ECC and the limited

studies available in the literature, our study also investigated the presence of S. wiggsiae in saliva samples, alongside S. mutans and S. sobrinus.

In many studies investigating the etiology of ECC, the results have shown that the levels of Mutans Streptococci (MS) are a significant risk factor for ECC [22]. MS are cariogenic microorganisms that metabolize carbohydrates to produce organic acids, survive in low pH environments, and increase adhesion to tooth surfaces by producing extracellular polysaccharides [23]. S. mutans and S. sobrinus are the acidogenic group of MS and are the most commonly isolated biofilm bacteria associated with the initiation and progression of dental caries in humans [22]. Studies have noted that although Mitis Salivarius or Mitis Salivarius Bacitracin agar are ideal selective media for S. mutans, isolating S. sobrinus on Mitis Salivarius Bacitracin agar is challenging [24]. In our study, species-specific q-PCR was used for the isolation and quantitative monitoring of both species.

In a study conducted by Unsal et al. on children with and without caries, they investigated the microbial diversity in the saliva samples of children with ECC using species-specific PCR. They found that 70% of children with ECC had S. mutans and 7.5% had S. sobrinus [25]. In our study, saliva samples from children with ECC revealed S. mutans in 96.2% of cases and S. sobrinus in 26.9%. Additionally, Okada et al. found that children who had both S. mutans and S. sobrinus had a higher incidence of caries compared to those with only S. mutans [24]. Unlike Okada et al., in our study, S. mutans and S. sobrinus were isolated together in 26.9% of the children. It is believed that the differences in these rates may be due to the different patient populations assessed.

S. wiggsiae, a member of the Bifidobacteriaceae family, was recently identified and is considered an important part of the microbial complex associated with caries. Tanner et al. found a highly significant relationship between ECC and S. mutans, S. wiggsiae, Veillonella parvula, Streptococcus cristatus, Fusobacterium nucleatum, and Actinomyces gerencseriae. They also reported that the presence of S. wiggsiae was strongly correlated with ECC, regardless of the presence or absence of S. mutans [6]. Studies have shown a relationship between the presence of caries and the high prevalence of Bifidobacteriaceae (Bifidobacteria and Scardovia). The study by Tantikalchan and Mitrakul quantitatively analyzed the presence of Bifidobacterium, S. wiggsiae, and S. mutans in plaque samples from Thai children aged 2–5 years, comparing S-ECC and caries-free groups, and found higher levels of S. wiggsiae and S. mutans in the S-ECC group [18]. Kanasi et al. reported a strong association between S. mutans and Bifidobacterium species with ECC, while Kaur et al. demonstrated that high Bifidobacterium levels in saliva were associated with caries activity in children [26, 27]. In another study by Tanner et al., PCR analysis revealed that the relationship between S. wiggsiae and ECC was stronger than that of S. mutans, in contrast to culture-based data [28]. In Chanda et al.'s study, S. wiggsiae was isolated in significantly higher levels from the saliva samples of children with ECC and severe ECC compared to caries-free children. Moreover, the levels of S. wiggsiae showed a positive correlation with the dmf-t scores, with higher correlation observed in children with severe ECC compared to those with ECC [29]. Tanner et al. also reported a highly significant association between the combination of S. mutans and S. wiggsiae and ECC [28]. In our study, S. wiggsiae was isolated in 67.3% of the saliva samples collected from children with ECC, using q-PCR. Furthermore, it was isolated together with S. mutans in 66% of the cases and with S. sobrinus in 9.6%. In only one sample, S. mutans was negative while S. wiggsiae was isolated, and in 30% of the samples, S. mutans was positive while S. wiggsiae was negative. In line with Tanner et al. findings, our study also shows a high co-isolation rate of S. mutans and S. wiggsiae.

Present study, evaluated the relationship between S. mutans levels and feeding practices. The results indicated that there were no statistically significant differences in the mean S. mutans values based on

the duration of bottle feeding or breastfeeding. This finding contrasts with the results of Bullappa et al. study which explored the same relationship but found a statistically significant correlation between S. mutans count and caries experience in both mothers and children. Bullappa et al. demonstrated a moderate but statistically significant negative correlation between the number of decayed, missing, and filled teeth (DMFT) in mothers and the number of decayed, extracted, and filled teeth (deft) in children with high S. mutans counts. Furthermore, Bullappa et al. found no significant difference in the dental caries experience among children who were exclusively breastfed, exclusively bottle-fed, or both breast- and bottle-fed [30]. These findings align with our study in that no clear link was found between the type of feeding method and S. mutans levels in children. Present findings indicate that other factors, such as oral hygiene practices, diet, or socioeconomic status, might play more significant roles in S. mutans proliferation and subsequent caries development in young children. Thus, further studies with larger sample sizes and a more detailed examination of these factors are necessary to fully understand the complex interactions influencing ECC development.

CONCLUSION

The results of this present study suggest that while S. mutans and S. wiggsiae are commonly found in children with ECC, feeding practices do not appear to have a significant impact on the microbial composition in this study.

Availability of Data and Materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Ethics Approval and Consent to Participate

Ethical approval was obtained from the Marmara University, School of Dentistry Clinical Research Ethical Committee with number of 2019-283 and from the Republic of Turkey Ministry of Health, Turkish Medicine and Medical Devices Agency with under number 20-AKD-167.

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Conflict of Interest

The authors declare no conflict of interest.

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